Engineered Immune Checkpoint CD28 and CTLA-4 Homodimer Proteins Demonstrate Potent Binding Affinities to Their Ligand CD80

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Abstract

CD28 and CTLA-4 are immune checkpoint proteins on the T cell surface as homodimers and bind to the same ligands CD80 (B7-1) and CD86 (B7-2) on antigen presenting cells. However, CD28 and CTLA-4 play opposite roles in T cell activation. CD28 acts as a co-stimulatory receptor to promote T cell activation while CTLA-4 acts as a co-inhibitory receptor to suppress T cell activation. To better mimic the native dimer conformation and quaternary structure, we designed novel soluble CD28 and CTLA-4 cis-homodimer proteins with the ectodomain (ECD) fused to a dimer motif at the C-terminus, and the CD80-Fc dimer with the ECD fused with a dimeric Fc-Tag at the C-terminus. These dimeric proteins expressed/purified from HEK293T cells demonstrated potent binding to the specific antibodies. We further investigated the binding abilities of the CD28 homodimer and the CTLA-4 homodimer to the CD80 ligand. The results demonstrated that both CD28 and CTLA-4 dimeric proteins have high binding potencies to the CD80 homodimer as measured by ELISA, localized surface plasmon resonance (LSPR), and Bio-Layer Interferometry (BLI). AlphaFold 2 predicted that the CD28 and CTLA-4 ECD dimeric protein structures were well-superimposed with the known crystal structure from the RCSB Protein Data Bank. The findings support that the engineered novel CD28, CTLA-4 and CD80 dimeric proteins mimic native conformations and can be potentially used as advanced bioactive antigens for immuno-oncology research and drug discovery.

Materials and Methods

Recombinant Proteins: CD80 dimer Fc-tag (Conigen Bioscience, CSP-24033-04), CD28 dimer His-tag (Conigen Bioscience, CSP-24032), CTLA-4 dimer His-tag (Conigen Bioscience, CSP-24031). CD80 UniProt# P33681, CD28 UniProt# P10747, CTLA-4 UniProt# P16410
Antibody binding assays: CD80, CD28, or CTLA-4 specific antibody binding was measured by ELISA. CD28 and CTLA-4 dimer or monomer proteins were coated on 96-well microtiter plates at 2 μg/ml and detected by CD80, CD28, or CTLA-4 specific antibodies at a series of concentrations.
Ligand/Receptor binding assays: ELISA and Label-free kinetics assays were performed to evaluate CD28 or CTLA-4 dimer binding potencies to CD80.
ELISA: CD28 or CTLA-4 dimer proteins were coated on 96-well microtiter plates at 2 μg/ml and detected by CD80-Fc dimer at a series of concentrations.
LSPR: The dissociation constant (Kd) was determined using a Nicoya Alto localized surface plasmon resonance (LSPR) instrument. CD28 or CTLA-4 dimer was directly immobilized to a carboxyl cartridge and interacted with 5 serial dilutions of CD80 dimer in a titration (Single-cycle) binding kinetics assay.
BLI: The dissociation constant (Kd) was determined using a Gator Pilot BLI instrument. The CD80 dimer was immobilized on an anti-hFc probe and interacted with 7 serial dilutions of CD28 or CTLA-4 dimer in a binding kinetics assay.
Computational modeling: Structures were predicted using the ColabFold implementation of AlphaFold2. All predictions were run using PDB100 template mode, MMseqs2 (Uniref + environmental), AlphaFold2 multimer v3 model, and 12 recycles. Crystal structures (Xtal) were retrieved from the RCSB-PDB, and all alignments and visualizations were created with PyMOL3.

Results

CD28 and CD80

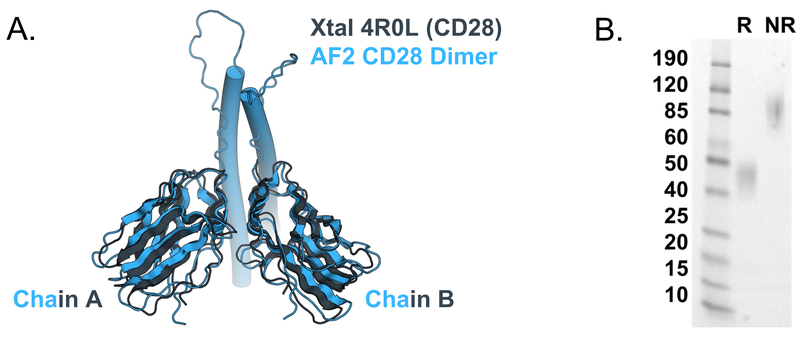

Fig. 1. CD28 protein dimer design and expression. The ectodomain of human CD28 was genetically fused to a proprietary linker and dimerization motif to promote soluble dimer formation. The recombinant protein was expressed in HEK293T cells and purified.
(A) Predicted structure of the recombinant CD28 dimer agrees with Xtal. (B) SDS-PAGE analysis of the purified CD28 dimer under non-reducing (NR) and reducing (R) conditions.

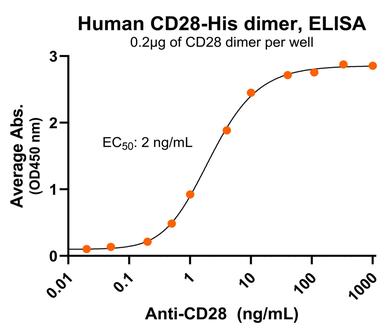

Fig. 2. CD28 protein dimer binding to CD28-specific monoclonal antibody, as detected by ELISA.

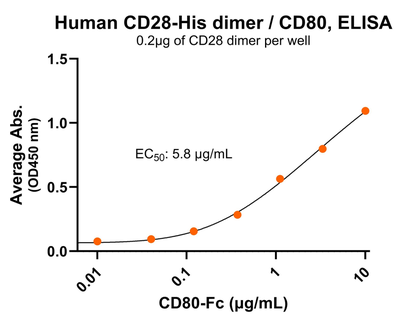

Fig. 3. CD28 protein dimer binding to the ligand CD80-Fc protein dimer, as detected by ELISA.

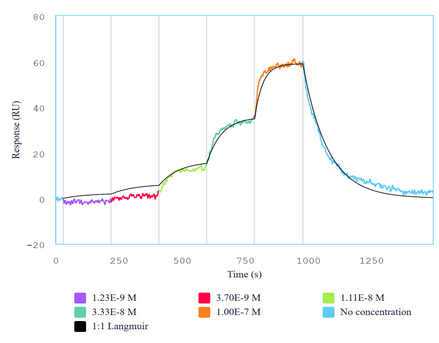

Fig. 4. CD28 protein dimer binding to the ligand CD80-Fc dimer, as measured by LSPR with binding Kd: **49 nM.**

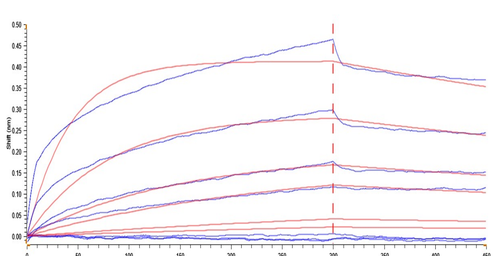

Fig. 5. CD28 protein dimer binding to the ligand CD80-Fc dimer, as measured by BLI with binding Kd: **49 nM.**

CTLA-4 and CD80

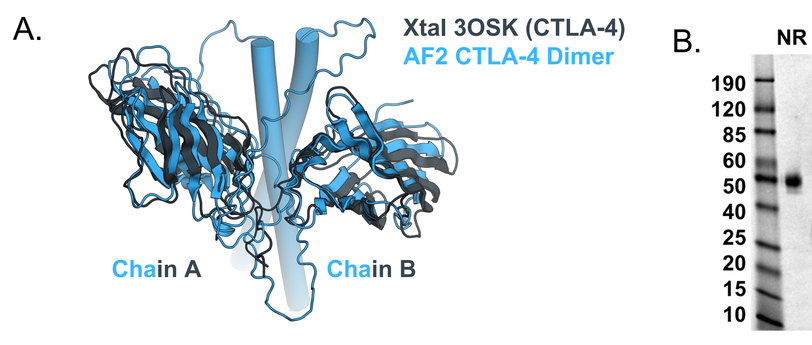

Fig. 6. CTLA-4 dimer protein design and expression. The ectodomain of human CTLA-4 was genetically fused to a proprietary linker and dimerization motif to promote soluble dimer formation. The recombinant protein was expressed in HEK293T cells and purified.
(A) Predicted structure of the recombinant CTLA-4 dimer agrees with Xtal. (B) SDS-PAGE analysis of the purified CTLA-4 dimer under non-reducing (NR) and reducing (R) conditions.

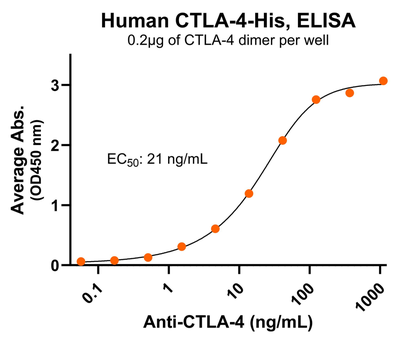

Fig. 7. CTLA-4 protein dimer binding to CTLA-4 specific monoclonal antibody, as detected by ELISA.

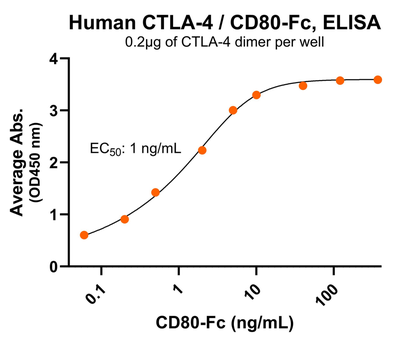

Fig. 8. CTLA-4 protein dimer binding to the ligand CD80-Fc protein dimer, as detected by ELISA.

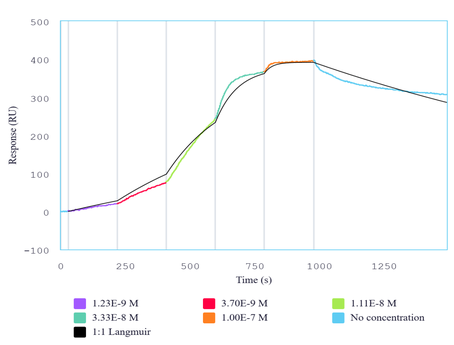

Fig. 9. CTLA-4 protein dimer binding to the ligand CD80-Fc dimer, as measured by LSPR with binding Kd: **2 nM.**

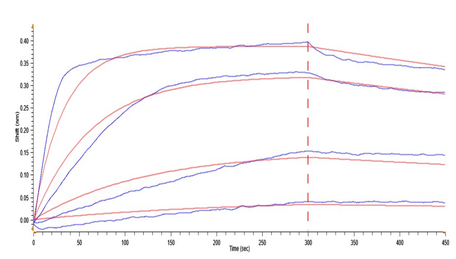

Fig. 10. CTLA-4 protein dimer binding to the ligand CD80-Fc dimer, as measured BLI with binding Kd: **4 nM.**

Conclusions

- The novel CD28 and CTLA-4 ectodomain homodimer proteins were designed to mimic the native dimer structures as predicted.
- The recombinant dimeric CD28, CTLA-4 and CD80 proteins expressed from HEK293T cells were purified with high purity and demonstrated specific potent binding to the respective antibodies.
- The CD28 homodimer and CTLA-4 homodimer proteins have high binding potencies to their ligand CD80 as measured by ELISA, LSPR, and BLI.
- These novel CD28, CTLA-4 and CD80 dimeric proteins can be used as antigens and immunogens for research and drug discovery.
- The high affinity and wider receptor/ligand binding windows can serve as more sensitive tools for therapeutic molecule evaluations.

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