Novel Soluble Fc-Free Dimeric Proteins of CD19 and Immune Checkpoints for CAR-T Detection, Receptor/Ligand Binding and Antibody Characterization Assays

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Abstract

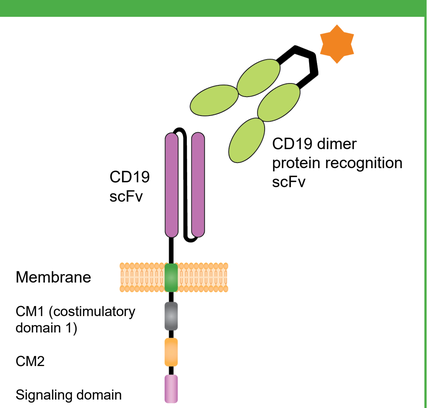
CD19-directed chimeric antigen receptor T (CAR-T) cell therapy has produced remarkable outcomes in patients with B-cell relapsed/refractory acute lymphoblastic leukemia (R/R-ALL) and B-cell non-Hodgkin lymphomas. Evaluating CAR expression on transfected T cells in vitro is essential to test the CAR construct design. Monitoring CAR-T cell kinetics in vivo is critical to elucidate the relationship between CAR-T cell expansion and persistence, as well as therapeutic responses and treatment-related toxicities. The assays rely on a recombinant human CD19 protein recognized by the single-chain variable fragment (scFv) of the CAR construct. CD19 is a type 1 integral membrane protein and the extracellular domain is the targeting region of CD19-specific scFv. In this study, we aimed to establish a robust, sensitive, and broadly applicable flow cytometry–based method for CD19 CAR-T cell detection. A novel soluble Fc-free CD19 cis-dimer protein with a His-Tag at C-terminus (CD19-dimer-His) was developed using the full-length extracellular domain (ECD). The CD19-dimer-His is designed to perform better CAR-T detection by increasing the binding avidity to scFv and reducing the aggregation of CD19-ECD monomer and the FcR binding noise caused by CD19-ECD Fc-fusion protein. CD19 CAR detection was performed using CD19-dimer-His followed by an APC-conjugated anti-His-Tag antibody by flow cytometry. The results demonstrated excellent sensitivity and specificity for CD19-CAR transfected human CD8 and CD4 T cells with EC50 <2ug/ml and no binding to un-transfected T cells. Validation of CAR detection using the CD19-dimer-His method showed a strong positive correlation with a surrogate marker. CD19-dimer-His also proved to be more cost-effective. CD19-dimer-His staining can be readily incorporated into multicolor flow cytometry panels, enabling detailed phenotypic characterization of CAR-positive subpopulations. Importantly, this approach is applicable across different anti-CD19 CAR products and sample types. Overall, our findings demonstrate that CD19-dimer-His based flow cytometry is a reliable, robust, and widely applicable method for detection of CD19 CAR-T cells. Many immune checkpoint proteins, as type 1 integral membrane proteins, have been reported to form dimers on cell membrane, including PD1, PD-L1, CTLA-4, CD28, CD80, TIGIT, CD155 and Nectin-4. Dimerization is important for their bioactivities. To better mimic the native dimer conformation and quaternary structure, we developed a panel of immune checkpoint proteins as soluble ECD Fc-free cis-dimer proteins. These dimer immune checkpoint proteins can bind to their specific antibodies very potently. More interestingly, these dimeric proteins dramatically enhanced the receptor ligand binding. For example, CD155 or Nectin-4 binding to their receptor TIGIT increased by >50 fold compared to the respective monomer proteins, as measured by ELISA and surface plasmon resonance (SPR). Additionally, very potent binding activities between dimeric CTLA-4 or CD28 and CD80, as well as dimeric PD1 and PD-L1 interactions were also demonstrated. These findings support that the engineered novel immune checkpoint cis-dimer proteins mimic native conformations and can be used to perform in vitro assays to study immune checkpoint receptor and ligand interactions, and antibody screening/characterization in immuno-oncology research and anti-tumor antibody discovery.

1. Conigen Novel Soluble Dimer Protein For Measuring CAR-T

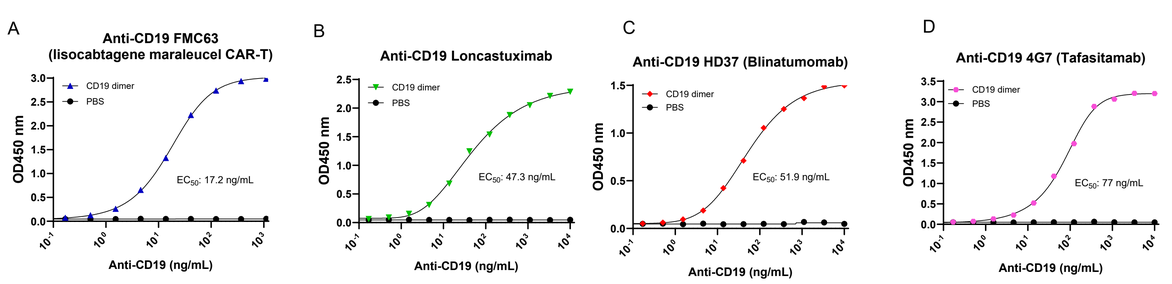
CAR-T cell therapy is a revolutionary new pillar in cancer treatment. There is an urgent need for CAR-T cell monitoring by CAR-T cell researchers, manufacturers, and clinician.

Challenge:
CAR-T-cells are often detected through tags added to the CAR construct. However, the tag detection methods often lack sensitivity and can not address CAR receptors recognition of its target.

Solution:
We developed a direct CD19 CAR-T cells detection method using novel Fc-free CD19 dimer protein reagents taking advantage of its high avidity to detect both high and low affinity CAR-T cells.

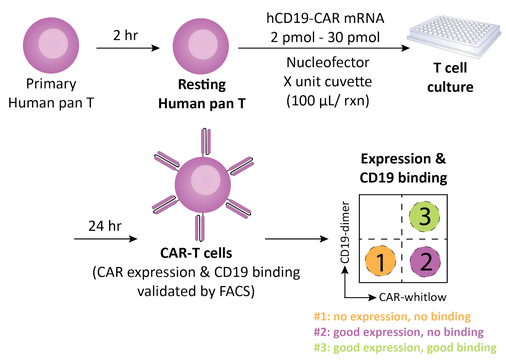


2. Fc-free CD19 Dimer Protein Preserves Natural Conformation, Potently Binding to CD19 Therapeutic Antibody Clones

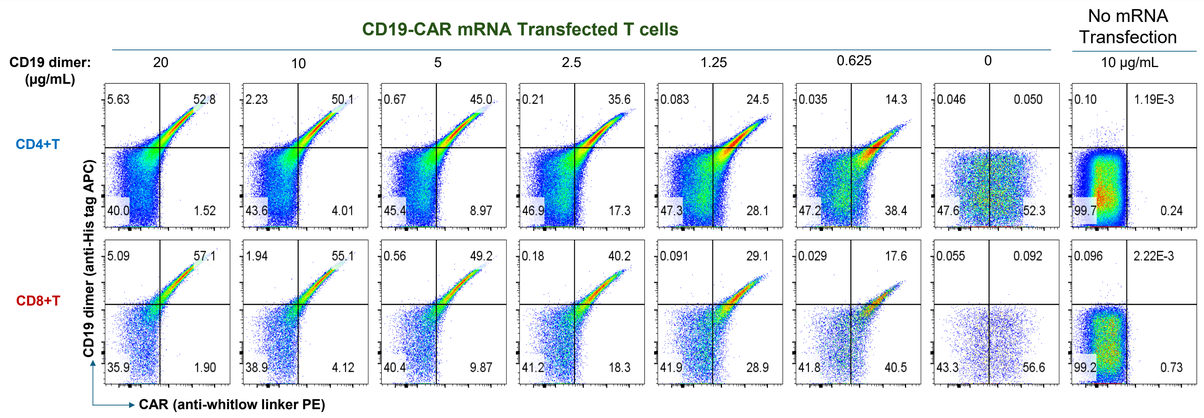


The Fc-free human CD19 dimer protein preserves the natural conformation. It can be potently and specifically recognized by FDA approved CD19 therapeutic antibody clones measured by ELISA. A. Lisocabtagene maraleucel CAR-T therapeutics, anti-CD19 clone FMC63; B. Loncastuximab antibody conjugate drug (ADC) anti-CD19 antibody clone; C. Blinatumomab bispecific therapeutic antibody, anti-CD19 clone HD37; D. Tafasitamab therapeutic monoclonal antibody clone 4G7.

3. CD19 Dimer Can Specifically and Potently Measure CD19-CAR mRNA–Transfected Human T Cells

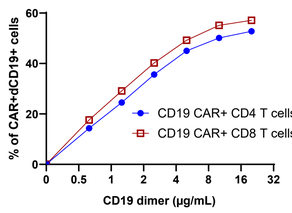


A. The workflow to measure CD19 CAR expression on mRNA transfection of human primary T cells.



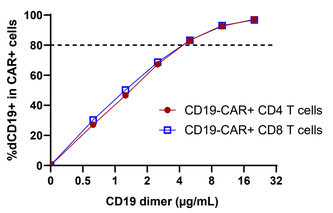
B. CD19-CAR+ CD4 and CD8 T cells transfected with FMC63 CD19 CAR mRNA (30 pmol) could be specifically and potently measured using CD19 dimer protein by flow cytometry.

Detection of CD19 CAR expression in total CD4 and CD8 T cells.



C. Detection of CD19-CAR+ CD4 and CD8 T cells using CD19 dimer protein: EC50 1.589 µg/mL for CD4 T cells; :EC50 1.345 µg/mL for CD8 T cells.

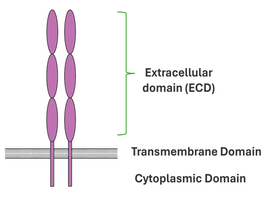
Detection of CD19 CAR in CAR expressing CD4 and CD8 T cells.



D. >80% of CD19-CAR+ CD4 and CD8 T cells could be measured by CD19 dimer when concentration ≥ 5 µg/mL.

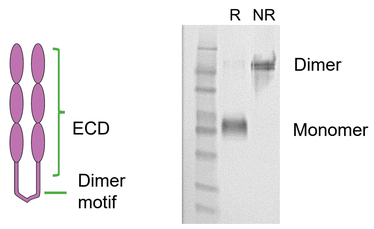
4. CD155 or Nectin-4 Homodimer Can Be Potently Recognized by Specific Antibody, Significantly Enhance Binding to TIGIT and Assay Window

CD155 and Nectin-4 form homodimers on cell surfaces



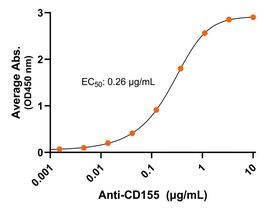
A. Schematic illustration of Nectin-4 or CD155 dimer on the cell surface. The extracellular, transmembrane and cytoplasmic domains are indicated.

Nectin-4 Cis-dimer design and expression example



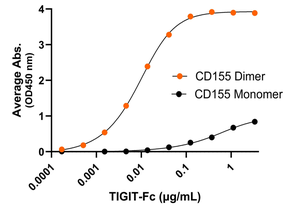
B. Schematic illustration of Nectin-4 Cis-dimer designed to express the extracellular domain fused with the dimeric motif (left). Purified Nectin-4 cis-dimer analysis using SDS-PAGE (right) under reduced (R) and non-reduced (NR) conditions

CD155-specific mAb binding to CD155 dimer



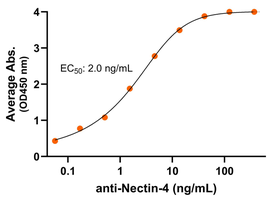
C. CD155 Fc-free dimer binding to CD155-specific monoclonal antibody, as detected by ELISA.

CD155 dimer enhanced binding to TIGIT



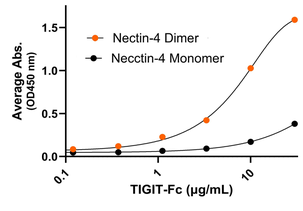
D. CD155 Fc-free dimer significantly enhanced the binding sensitivity and window to its receptor TIGIT-Fc dimer compared to CD155 monomer, as measured by ELISA.

Nectin-4-specific mAb binding to Nectin-4 dimer



E. Nectin-4 Fc-free dimer binding to Nectin-4 specific monoclonal antibody, as detected by ELISA.

Nectin-4 dimer enhanced binding to TIGIT



F. Nectin-4 Fc-free dimer significantly enhanced the binding sensitivity and window to its receptor TIGIT-Fc dimer compared to Nectin-4 monomer, as measured by ELISA.

Conclusions

- Novel Fc-free dimers of type 1 integral membrane protein ectodomain were designed to mimic the native dimer structures and increase the binding avidity.
- CD19 Fc-free homodimer shows strong binding to FDA approved therapeutic antibodies by ELISA.
- CD19 Fc-free dimer with His Tag can specifically and potently measure the CD19 CAR-T cells using flow cytometry, providing a reliable, robust, and widely applicable method for detection of CD19 CAR expressing cells.
- The CD155, Nectin-4 and CTLA-4 dimeric proteins expressed from HEK293T cells have high purity and can be potently recognized by specific antibodies.
- The CD155 dimer and Nectin-4 dimer show significantly increased binding potencies to their receptor TIGIT compared to CD155 monomer and Nectin-4 monomer.
- The CTLA-4 and CD28 dimer proteins have high binding potencies to their ligand CD80 as measured by ELISA and SPR.