EVALUATION OF CAR EXPRESSION AND ANTIGEN BINDING

Chimeric Antigen Receptors (CAR) are transmembrane receptors engineered to redirect the function of cytotoxic T cells. A CAR construct contains an extracellular antibody fragment ScFv (single-chain fragment variable) that recognizes a chosen antigen independently of HLA (Human Leukocyte Antigen), a transmembrane domain and a cytoplasmic signaling module typically containing a CD3ζ domain and two co-activator/signaling modules. Target antigens are rationally chosen based on a selective expression pattern and high level of expression in tumor cells. Ideally, the target is expressed only in tumor cells, although in practice very few targets display such selectivity. The target must also be expressed on the surface of the tumor cell to be accessible for T cell recognition.

CD19 and BCMA (B Cell Maturation Antigen) are well validated targets expressed in B lymphoma and multiple myeloma cells, respectively, which were used to generate the first series of FDA-approved CAR-T cell therapies. These have shown remarkable efficacy, although relapse to treatment occurs frequently owing to antigen escape. This is likely due to the selection of pre-existing antigen-negative tumor cells or the emergence of new cell populations. Further research is needed to identify new targets that may be used in bi-specific targeting or in sequential lines of treatment to decrease the likelihood of antigen escape. Ongoing research also aims at targeting solid tumors, which have not been very amenable to CAR-T cell therapy so far.

Targets under investigation for the treatment of hematopoietic cancers include CD22, CD33, CD37, CD38, CD123 and CD30 [1]. The alternatively spliced variant of the EGFR (Epidermal Growth Factor Receptor) termed EGFRvIII, and the interleukin-13 receptor α2 are being investigated for the treatment of glioblastoma [2, 3]. Additional promising targets in solid tumors include HER2 for sarcoma and the follicle stimulating hormone receptor for ovarian cancer, among others [4].

Once an antigen has been chosen, engineering of the CAR construct is initiated by the screening and characterization of the extracellular antibody portion. High specificity for the target is required. It is also particularly important to demonstrate CAR expression in T cells as well as recognition of the target in intact cells.

CAR expression is often evaluated by flow cytometry using the labeled target protein or anti-Fab antibodies to detect the extracellular domain of the CAR construct. Purified recombinant proteins, labeled or unlabeled, containing either an N-terminal or a C-terminal tag are very desirable because they offer low-background, high-specificity staining options (Figure 1).

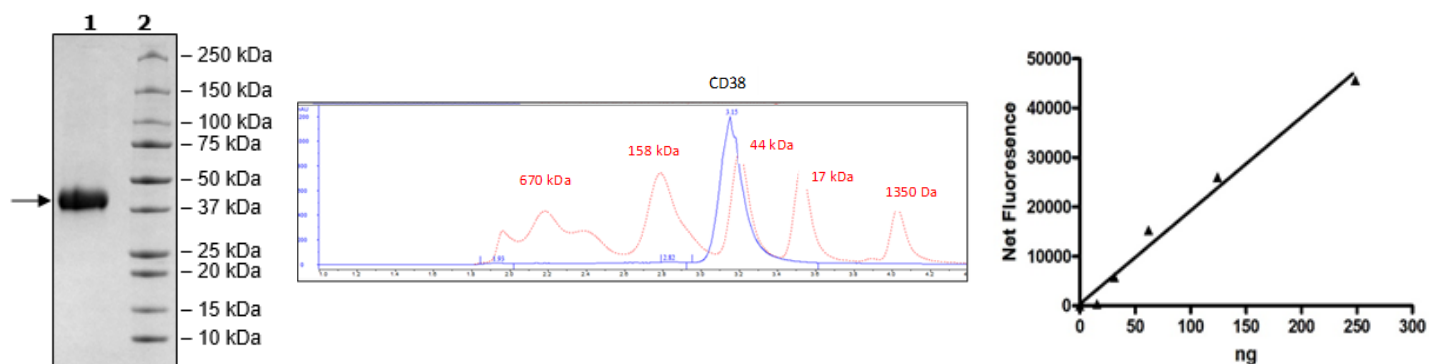Figure 1: protein binding to CAR
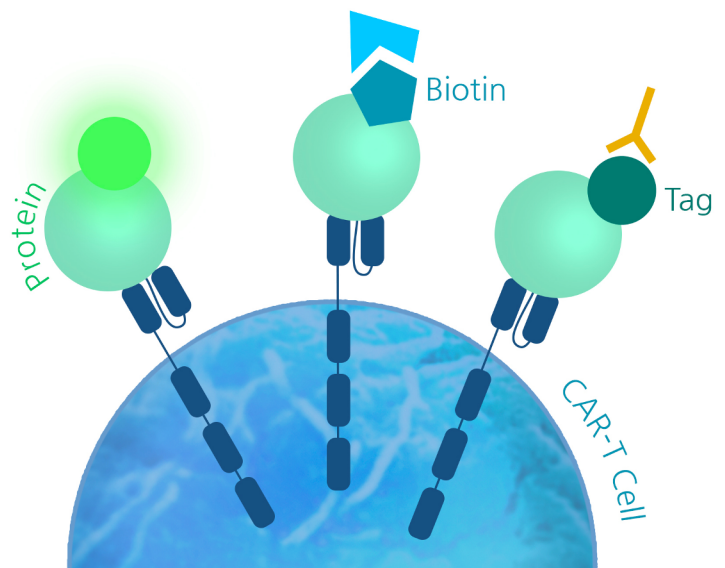
Figure 2: CD38, His-Tag (Human), HiP™(#71277). **Left panel:** 4-20% SDS-PAGE Coomassie staining. **Middle panel**: gel filtration trace of the purified protein. **Right panel:** a representative result of CD38 activity obtained using the fluorescent CD38 Inhibitor Screening Assay Kit, Cyclase Activity (#71275) with CD38 titrated from 66.3 ng/µl to 1 ng/µl.

For example (Figure 2), recombinant human CD38 containing a C-terminal His-Tag was affinity purified to obtain an active HiP™ protein, indicating a high purity protein (≥90%) and less than 10% aggregation as measured by gel filtration.

cells. Clonal stable CHO cell lines were engineered to constitutively express BCMA, as confirmed by flow cytometry (Figure 3). Each cell line was selected for low, medium or high levels of expression to mimic cancer cells displaying various BCMA expression levels.
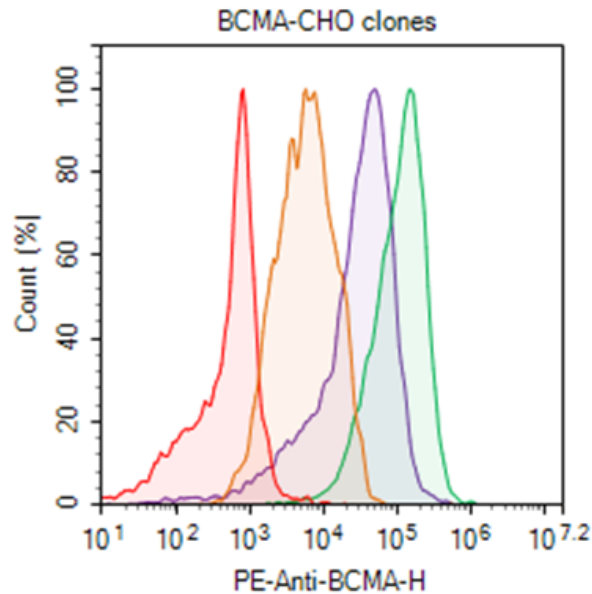
Antigen-expressing cell lines

Cells overexpressing an antigen of interest are useful for the screening or characterization of antibodies and CAR constructs in a biologically relevant model. In contrast to human primary cells, immortalized cell lines are easy to maintain and expand, do not have the variability of primary cells, and are well suited for some of the optimization steps involved in the successful design of a CAR-T cell. In addition, they do not carry the same risks of containing human pathogens inherent to the use of donated blood cells. Thus, recombinant cell lines simplify and standardize target recognition experiments.

CD19 is present in B cell lymphocytes through almost all phases of B cell maturation. BCMA is a cell surface receptor preferentially expressed on mature B-lymphocytes. It is a highly attractive target for immunotherapy because of its low expression levels in non-malignant tissue and almost universal expression on multiple myeloma

Figure 3: BCMA-CHO Cell Lines (#79500). Expression of BCMA was validated by flow cytometry using PE-conjugated anti-BCMA antibody (Biolegend #357504). High, medium, and low expression shown in green, purple and brown, respectively. Parental CHO cells: red.

Of note, BCMA-overexpressing CHO cells were used to validate a new monoclonal antibody for CAR-T cell therapy in a study by Berahovich *et al.* entitled "CAR-T Cells Based on Novel BCMA Monoclonal Antibody Block Multiple Myeloma Cell Growth" [5].

Dual expression cell lines

To address the issue of resistance to therapy resulting from loss of antigen expression in the tumor cells, bi-functional antibodies and bi-functional CAR-T cells are being developed such as anti-CD20-CD19 or anti-CD20-BCMA bispecific CAR-T cells.

CD20 (MS4A1) is expressed on the surface of B cells and is present in more than 90% of patients with B cell lymphoma. This antigen has been a highly relevant target for immunotherapy since the 1997 approval of Rituximab (Rituxan), a chimeric monoclonal anti-CD20 antibody now classified by the World Health Organization as an "Essential Medicine". Additional monoclonal antibodies against CD20 have been approved or are being tested in clinical trials for the treatment of cancer as well as auto-immune diseases.

The BCMA / CD20 / Firefly Luciferase CHO Cell Line was developed specifically to study anti-BCMA/anti-CD20 bi-specific antibodies or bi-specific CAR-T cells. This cell line contains both human CD20 and human BCMA, in addition to the firefly luciferase reporter. The expression of BCMA and CD20 was validated by flow cytometry, while it was demonstrated that luciferase activity is directly proportional to the number of cells (Figure 4).

Tumor cell killing assays and cytokine production assays can be performed following co-culture of these antigen-presenting cells with BCMA/CD20-specific CAR-T cells.
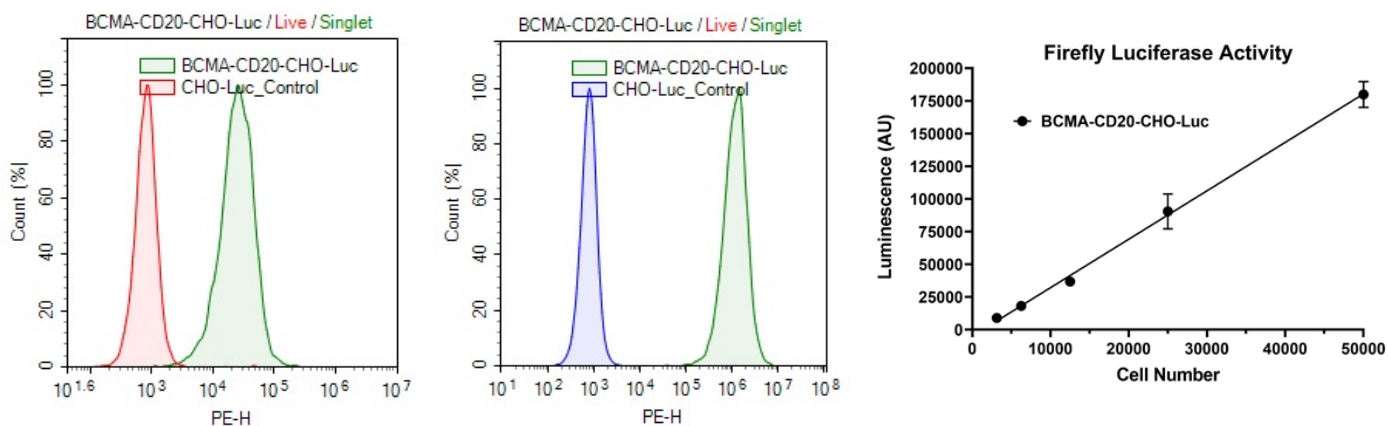

Figure 4. Expression of CD20, BCMA and luciferase in the BCMA / CD20 / Firefly Luciferase CHO Cell Line (#78185). The cells were treated with PE-conjugated anti-human BCMA antibody (BioLegend, #357503; left panel) or PE-conjugated anti-human CD20 antibody (BioLegend, #302346; middle panel) and analyzed by flow cytometry to detect surface expression of the antigens. CHO luciferase cells were used as control. Cells were seeded in a 96-well plate at increasing densities (right panel). After four hours, luciferase activity was measured using the ONE-Step^TM luciferase assay system (#60690).

Case study

The expression of cell surface glycoprotein CD37 is restricted to cells of the immune system, with high expression observed in mature B cells and lowest expression in T cells and myeloid cells. It is also expressed in B cell non-Hodgkin Lymphomas, in chronic lymphocytic leukemia, and in some cutaneous and peripheral T cell lymphomas. Anti-CD37 CAR-T cells displayed antigen-specific activation, cytokine production and cytotoxic activity in models of B and T cell lymphomas *in vitro* and *in vivo*. They have substantial activity against lymphoid lineages without significant cytotoxicity against T cells. Anti-CD37 CAR-T cells therefore represent novel therapeutic agents for the treatment of CD37-expressing lymphoid malignancies.

In their recent publication entitled "Novel CD37, Humanized CD37 and Bi-Specific Humanized CD37-CD19 CAR-T Cells Specifically Target Lymphoma", V. Golubovskaya *et al*. used CD37-CHO cells for the validation of novel, humanized, bi-specific CD37-CD19 CAR-T cells, developed for the treatment of lymphoma [6]. A mouse monoclonal anti-CD37 antibody (clone 2B8D12F2D4) was generated with high binding affinity (KD = 1.6 nM). The antibody was able to bind recombinant CHO cells constitutively
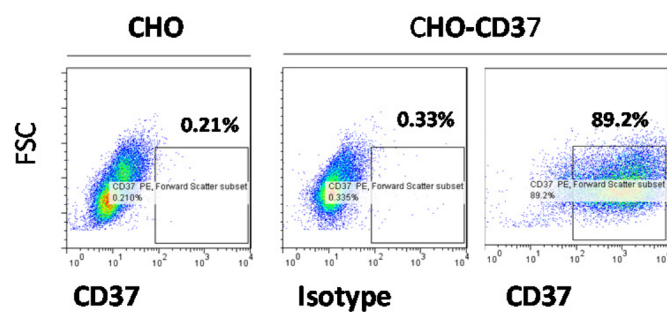
Figure 5: Anti-CD37 antibody binding to CD37-expressing cells. Recognition of human CD37 in a cellular context was assessed by flow cytometry using the new anti-CD37 antibody incubated with intact CHO-CD37 (#79607) or with control CHO cells. Figure reproduced in part and without modification under license CC BY 4.0. https://doi.org/10.3390/cancers13050981

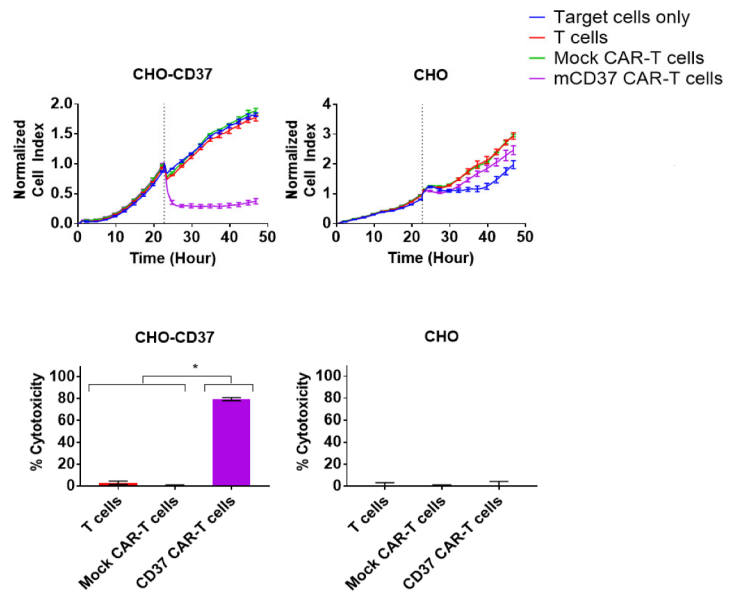expressing human CD37 but not control CHO cells, which do not express measurable levels of CD37 (Figure 5).

Figure 6: Real-time cytotoxicity assay. Adherent target cells (CHO-CD37 or CHO) were seeded into 96-well E-plates and monitored overnight with the impedance-based real-time cell analysis xCELLigence system (Acea Biosciences). Effector CAR-T cells or non-transduced T cells were added the next day. The cells in the E-plates were monitored in real-time for another 24–48 hours, and impedance was plotted over time. Cytotoxicity was calculated as (impedance of target cells without effector cells—impedance of target cells with effector cells) × 100/impedance of target cells without effector cells. Figure reproduced in part and without modification, under license CC BY 4.0. https://doi.org/10.3390/cancers13050981

The anti-CD37 CAR-T cells killed target cells CHO-CD37, but not the CD37-negative control cell line (Figure 6). Of note, these CAR-T cells also killed CD19-positive HeLa-CD19 cells but not parental HeLa cells (not shown). The authors went on to show that the bi-specific humanized CD37-CD19 CAR-T cells inhibited the growth of Raji xenograft tumors and increased survival in a mouse model [6].

Bibliography

1.   Guedan S, Ruella M, June CH. Emerging Cellular Therapies for Cancer. *Annu Rev Immunol*. (2019) **37**: 145-171. PMID: 30526160. https://pubmed.ncbi.nlm.nih.gov/30526160/

2.   O'Rourke DM, Nasrallah MP, Desai A, *et al.* A single dose of peripherally infused EGFRvIII-directed CAR T cells mediates antigen loss and induces adaptive resistance in patients with recurrent glioblastoma. *Sci Transl Med.* (2017) **9**: eaaa0984. PMID: 28724573. https://pubmed.ncbi.nlm.nih.gov/28724573/

3.   Brown CE, Alizadeh D, Starr R, *et al.* Regression of Glioblastoma after Chimeric Antigen Receptor T-Cell Therapy. *N Engl J Med*. (2016) **375**: 2561-2569. PMID: 28029927. https://pubmed.ncbi.nlm.nih.gov/28029927/

4.   Freitag F, Maucher M, Riester Z, Hudecek M. New targets and technologies for CAR-T cells. *Curr Opin Oncol.* (2020) **32**: 510-517. PMID: 32657796. https://pubmed.ncbi.nlm.nih.gov/32657796/

5.   Berahovich R, Zhou H, Xu S, Wei Y, Guan J, Guan J, Harto H, Fu S, Yang K, Zhu S, Li L, Wu L, Golubovskaya V. CAR-T Cells Based on Novel BCMA Monoclonal Antibody Block Multiple Myeloma Cell Growth. *Cancers,* Basel. (2018) **10**: 323. PMID: 30208593. https://pubmed.ncbi.nlm.nih.gov/30208593/

6.   Golubovskaya V, Zhou H, Li F, Valentine M, Sun J, Berahovich R, Xu S, Quintanilla M, Ma MC, Sienkiewicz J, Huang Y, Wu L. Novel CD37, Humanized CD37 and Bi-Specific Humanized CD37-CD19 CAR-T Cells Specifically Target Lymphoma. *Cancers,* Basel. (2021) **13**: 981. PMID: 33652767. https://doi.org/10.3390/cancers13050981