# FUNCTIONAL VALIDATION OF CAR-T CELLS

The ultimate function of a CAR-T cell in cancer immunotherapy is to kill the tumor cells. Once activated, cytotoxic T cells trigger the apoptosis of the target cells, although it is suspected that other death mechanisms also take place since cell death can still happen when the tumor cell apoptotic pathway is disabled. Mechanistically, activation of the T cell is initiated by the binding of the antibody ScFv portion of the CAR to the antigen present on the surface of the tumor cells. The intracellular signaling portion of CAR activates several signaling pathways, culminating in activation of transcription factors such as NFAT (Nuclear Factor of Activated T Cell), increased gene transcription and release of death ligands FasL (Fas Ligand) and TRAIL (TNF-related apoptosis-inducing ligand). These bind to specific death receptors present on the tumor cells to activate programmed cell death. In addition, the granule exocytosis pathway of the T cell is engaged, leading to the outpouring of pore-forming protein perforin and serine proteases of the granzyme family into the immunological synapse, directly contributing to the lysis of the tumor cells (reviewed in [1]).

Apart from sending death signals, T cells produce TNF-α (Tumor Necrosis Factor-α) and IFN-γ (Interferon-γ) upon activation, *in vitro* and *in vivo*, suggesting that both TNF-α and IFN-γ release may be a prerequisite to their function. IFN-γ is a cytokine primarily secreted by activated T cells and natural killer (NK) cells; it plays an important role in innate and adaptive immunity. In the clinic, patients infused with anti-CD19 CAR-T cells displayed increases in serum levels of IFN-γ and TNF. Peripheral blood T cells from these patients also produced TNF and IFN-γ *ex vivo*, in a CD19-dependent fashion, indicating that the
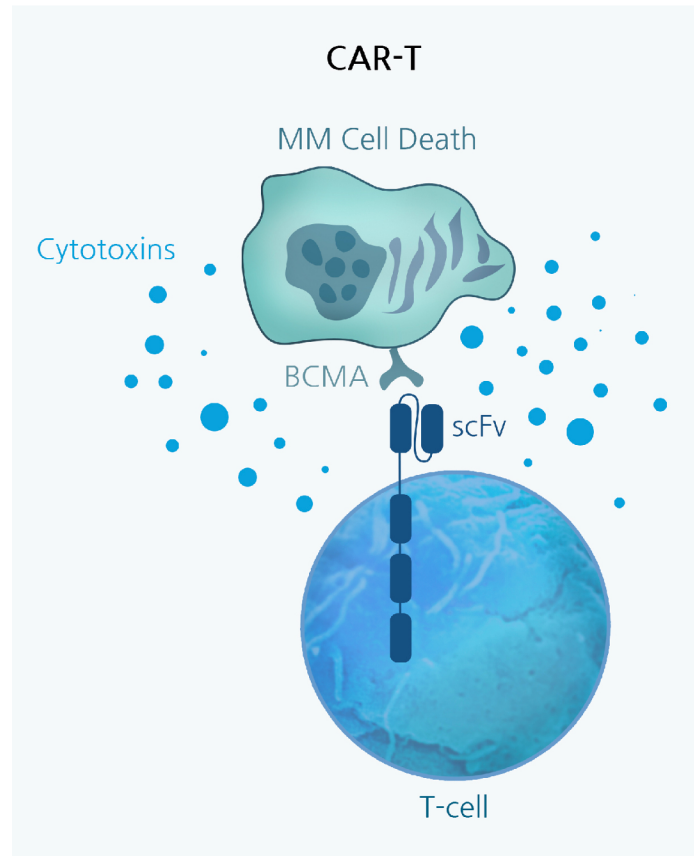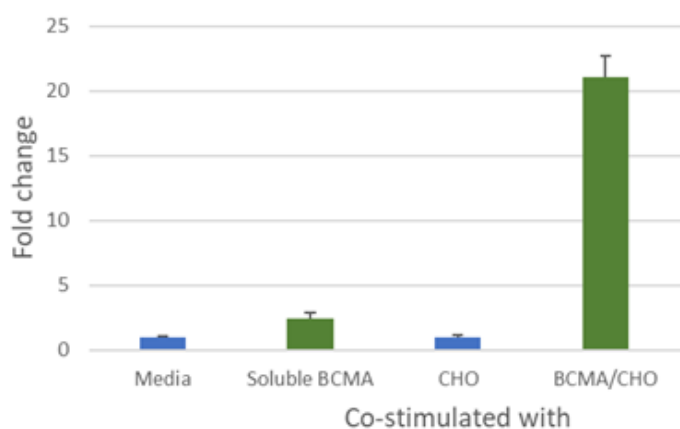


CAR-T cells were the cause of this cytokine release [2].

Functional characterization is arguably one of the most important steps of the CAR-T cell development process. In addition to ensuring that the CAR is correctly activated, functional assays should demonstrate whether tumor cell death is achieved, since this is the ultimate purpose of the CAR-T cell. Experimentally, CAR-T cells are placed in culture with the target tumor cells expressing the antigen of interest, for example CD19 or BCMA. Cytokines released by the T cells in the culture medium are quantified, and tumor cell viability is assessed, for example using a reporter cell system.

## CAR NFAT-luciferase Reporter Jurkat Cells

The anti-CD19 and the anti-BCMA NFAT-luciferase Jurkat cells are used to optimize or test antigen-presenting cells, optimize co-culture assay conditions, and ensure that proper T cell activation is taking place in a particular assay.

A Firefly luciferase reporter, expressed under the control of a promoter containing an NFAT response element, monitors the activation of the CD3 signaling domain downstream of CAR, which in turn activates transcription factor NFAT when the cells are in contact with antigen-presenting cells. In addition to the conditional luciferase, the anti-BCMA CAR/NFAT-luciferase reporter Jurkat cell line stably expresses an anti-BCMA CAR construct. When these recombinant Jurkat cells are added to BCMA-presenting cells the NFAT transcription factor is activated and induces luciferase expression, which can be quantified as shown in Figure 1.
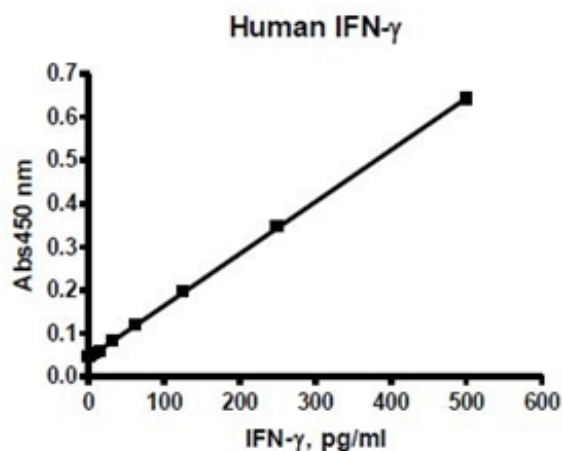
The Jurkat cells were not activated when cultivated alone or with control CHO cells not expressing BCMA. The addition of a soluble form of BCMA resulted in a 4-fold increase in luciferase activity, whereas the presence of BCMA-presenting CHO cells induced a 23-fold increase in luciferase activity.

## Interferon-γ ELISA

Since IFN-γ has been accepted as an indicator of T cell activation, measuring its release in response to target recognition is routinely performed as part of CAR-T cells functional validation. The IFN-γ Colorimetric ELISA Detection Kit quantifies human IFN-γ in cell culture medium.

*Assay principle:* An anti-IFN-γ capture antibody is coated on a 96-well plate. The test samples presumed to contain IFN-γ are incubated on the coated plate. The plate is washed, and an anti-IFN-γ detection antibody is added. After washing, the plate is treated with streptavidin-HRP followed by addition of a colorimetric HRP substrate. The assay is linear between at least 2 pg/ml and 500 pg/ml, as shown in Figure 2. This assay kit is useful to measure the production of IFN-γ by CAR-T cells cultivated together with antigen-presenting cells. If the CAR-T cells are properly activated, they will release cytokines in the culture medium, which can be sampled.

An example of IFN-γ measurement in a co-culture assay is shown in Figure 3.
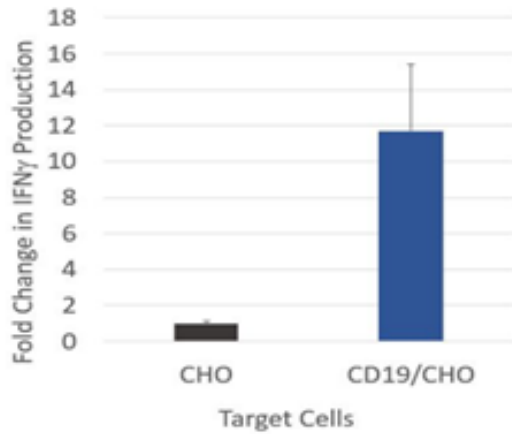
In this experiment, primary T cells from donors were stably transduced with an anti-CD19 CAR lentivirus. Twelve days later, the CAR-T cells were placed in culture with CD19-expressing CHO cells or with parental control CHO cells. The amount of secreted IFN-γ was measured using the Colorimetric Human IFN-γ ELISA Detection Kit. The anti-CD19 CAR-T cells cultivated with CD19-expressing CHO cells produced 12 times more IFN-γ than anti-CD19 CAR-T cells cultivated with control CHO cells, indicating that the T cells were properly activated by presentation of CD19 cells.
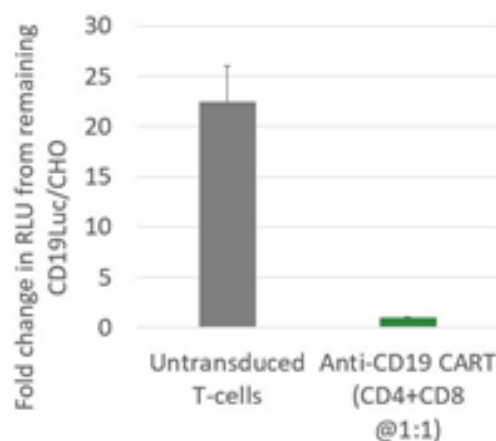
## CAR-T Cytotoxicity Assays

The most relevant functional assay to assess CAR-T function is to measure cytotoxicity towards target tumor cells. This is best performed using co-culture assays in which the CAR-T cells are cultivated together with antigen-presenting tumor cells, and the number of live tumor cells is quantified. This can be made difficult by the presence of the T cells. A simple solution is to add a reporter into the tumor cells that allows detection of the tumor cells without interference from the T cells. Thus, target tumor cells are engineered to express eGFP (Green Fluorescent Protein), which can be quantified by fluorescence. Alternatively, tumor-specific expression of firefly luciferase allows sensitive and robust quantification of luciferase activity, which is directly proportional to the number of live cells present in the cell culture.
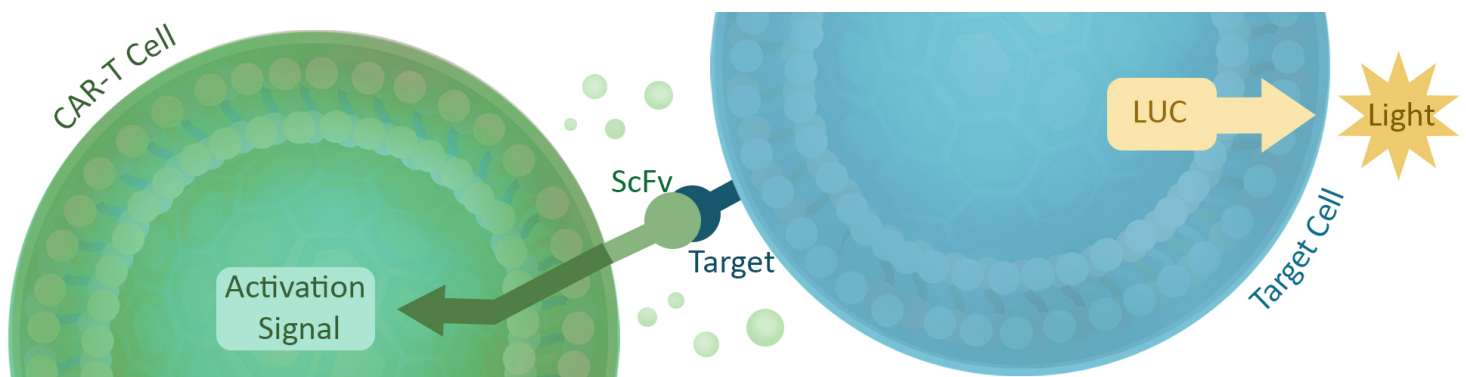


**Figure 4: Illustration of a co-culture assay using luciferase-positive target cells**

During the development of co-culture assays, a known CAR-T cell system is useful to optimize assay conditions and to use as positive control or internal control (standard) in subsequent experiments.

To assess CD19-directed activity of CAR-T cells expressing the anti-CD19 CAR construct, the CAR-T cells were added to a culture of CD19 Luciferase CHO cell line. As shown in Figure 5, presentation of CD19 by the CHO cells resulted in activation of the anti-CD19 CAR-T cells and killing of the CHO cells as indicated by the loss in luciferase activity. As expected, control T cells not expressing the CAR construct did not kill the CD19 CHO luciferase cells.



**Figure 5. Anti-CD19 cytotoxicity assay.** Primary T cells from donors were transduced with an anti-CD19 CAR lentivirus and expanded under conditions of antibiotic selection for twelve days. The resulting anti-CD19 CAR-T cells were cultivated with CD19-expressing CHO luciferase cells (#79714) at a 10 to 1 ratio for 16 hours. Non-transduced T cells were used as negative control. Luciferase activity was measured using the ONE-Step luciferase assay system (#60690).

**Bibliography**

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