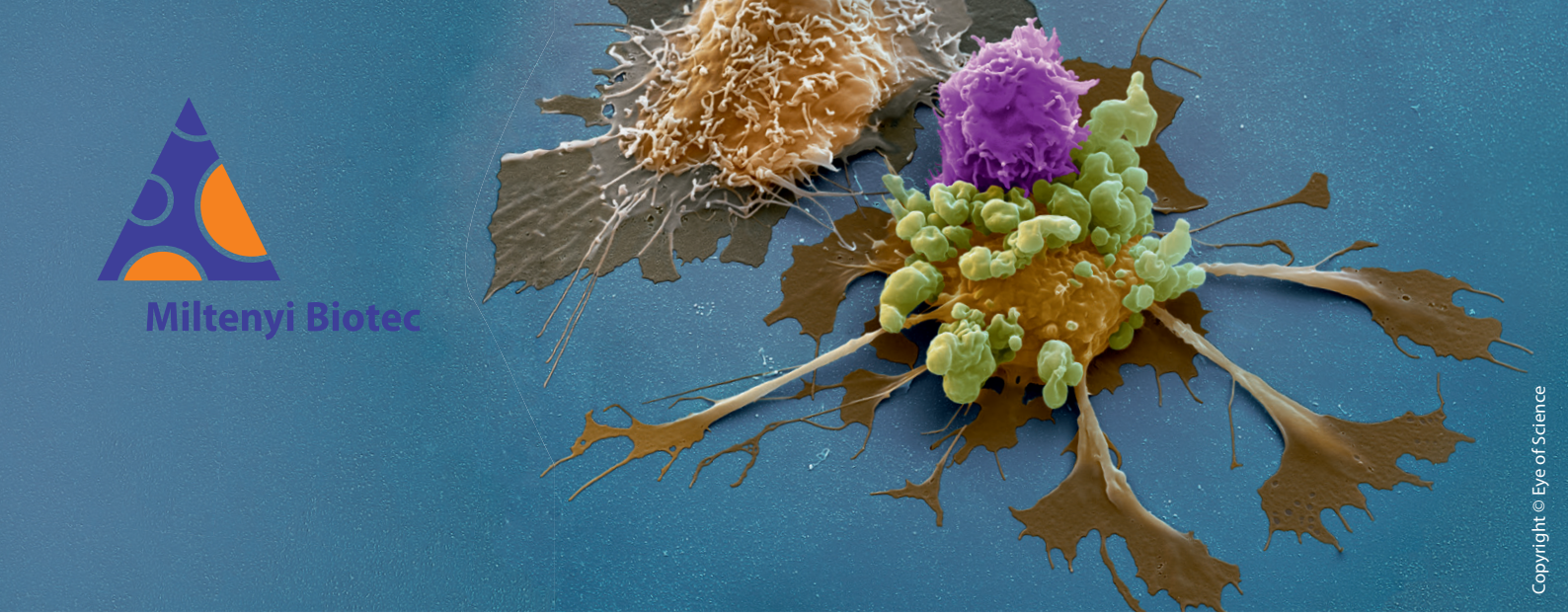

Maximize the efficiency of CAR T cell functionality assessment via flow cytometry

Introduction

In recent years, CAR T cell-mediated immunotherapy has shown clinical efficacy in the fight against cancer. Increased research efforts to address challenges such as poor efficacy and unwanted side effects are required to further advance the field. To investigate specificity, toxicity, and potency of CAR T cells many *in vitro* assays are used. Common *in vitro* assays for assessment of CAR T cells' potency include cytolytic activity, cytokine release, and CAR T cell expansion via cell counting, as well as phenotypic characteristics of CAR T cells (fig. 1).

However, there are several challenges associated with these assays that limit their use for large-scale screening, for example, loss of sample due to extensive washing steps, laborious planning, sedimentation of cells when acquiring large numbers of samples, low resolution, time-consuming manual steps of plate processing, as well as lack of absolute cell counting. Despite all efforts the results are often less reproducible and highly operator-dependent. Additional challenges come with the detection method, for example, use of radioactive substances for cytotoxicity measurement, complicated and time-consuming cytokine detection methods, etc. Flow cytometry has shown significant advantages in tackling these challenges.

To improve the quality of potency assay results, here we show how Miltenyi Biotec's flow cytometry solutions can be used for high-throughput and automated *in vitro* potency assays to screen for CAR T cells' antigen-specific killing, cytokine-secretion, and phenotype ability with the maximum number of readouts.

Using Miltenyi Biotec's tools, it is possible to detect 12 different cytokines secreted by effector cells in a single assay, assess CAR potency by measurement of target cell killing, and fully characterize effector cells using recombinant antibodies. These assays are able to provide the maximum number of readouts quickly, saving time with automation and obtaining highly sensitive data with precision in order to ensure confidence when selecting and detecting functional CAR T cells.

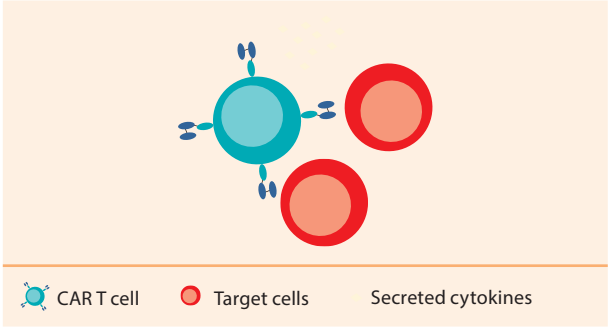

Figure 1: CAR T cells and target cells, interaction in a co-culture assay.

Materials and methods

CAR T cells were generated via the following protocol based on the Lock, D. 2017 study[1]. GFP-expressing target cells were co-cultured for four days with either mock T cells, adapter CAR T cells in the presence of an adapter molecule towards a given target, or adapter CAR T cells in the presence of a non-functional adapter molecule towards a given target, in 96-well plates at the ratio of 2.5 : 1 for effector : target cells. This four-day co-culture was repeated three times.

100 µL of culture supernatant was removed after one day of co-culture and collected in a fresh 96-well plate to assess cytokine concentration using the MACSPlex Cytokine 12 Kit, human. The cytokine assay was performed according to the kit manual. Cytokines were measured by MACSQuant® X Flow Cytometer and data were automatically analyzed using an Express Mode in the MACSQuantify™ Software.

Immunophenotyping of the cells was performed after the last co-culture experiment with an automated stain-no-wash protocol. Cells were automatically stained with a staining cocktail containing CD3 (VioGreen™), CD56 (APC), LNGFR (PE), CD27 (VioBlue®), and PD-1 (PE-Vio® 770), all of which are based on Miltenyi Biotec's revolutionary REAfinity™ Antibody Technology. 7-AAD Staining Solution was used to detect target cell killing. Stained cells were automatically acquired by MACSQuant X Flow Cytometer.

Phenotyping data were analyzed using FlowLogic Software. A summary of the experimental layout is shown in Figure 2.

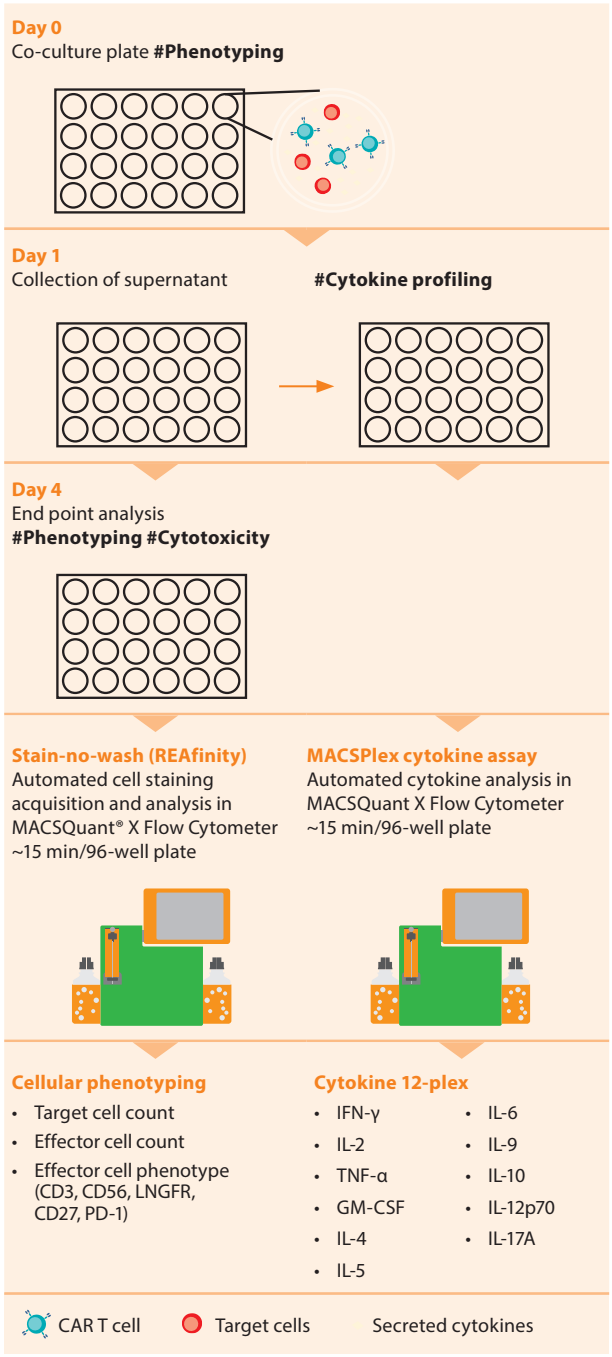

Figure 2: Schematic diagram showing experimental design.

Results

Cytokine release

Secreted cytokines were measured to verify the integrity of immune functions of the CAR T cells. Functional CAR T cells were able to release increased levels of cytokines, specifically IFN-γ, IL-4, TNF-α, and GM-CSF, one day after co-culture with the target cells, compared to mock T cells and T cells with non-functional CAR adapters (fig. 3).

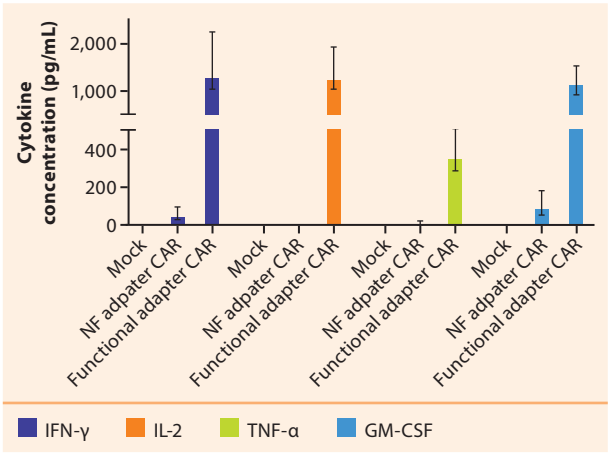

Figure 3: Cytokine release. Secreted cytokines upon co-culture were analyzed. Data depicts cytokine concentration measured in pg/mL of culture supernatant. NF = non-functional

Cytotoxicity assessment

To evaluate the potency of CAR T cells to specifically kill antigen-expressing target cells, both 7-AAD⁻ viable GFP⁺ target cells and GFP⁻ effector cells were counted. Upon day four of co-culture with CAR T cells in the presence of a functional adapter, significant reduction of viable GFP⁺ target cells was observed compared to mock and CAR T cells with non-functional adapters (fig. 4). In contrast, the co-culture experiment did not affect the viability of the effector cells, since the number of GFP⁻ effector cells remained similar in all tested conditions (fig. 4). The MACSQuant X can successfully provide absolute cell count information without the need for counting beads.

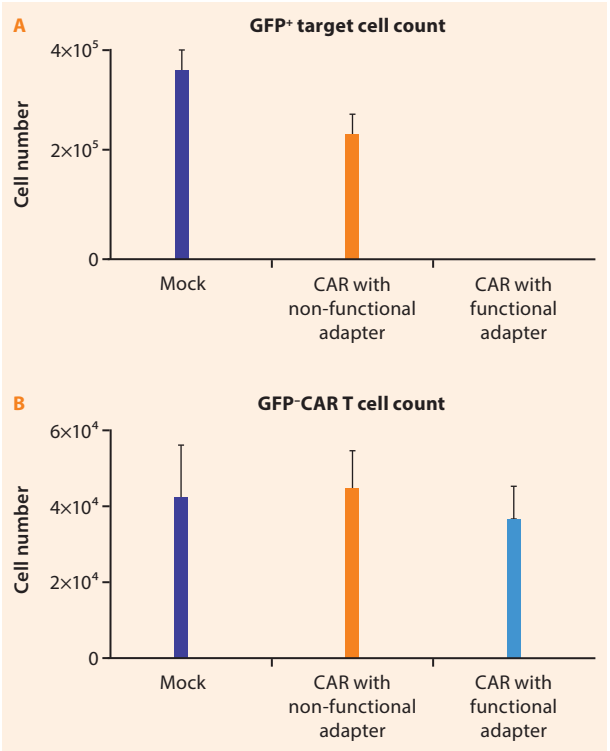

Figure 4: Cytotoxicity assessment. Cell viability was assessed by 7-AAD⁻GFP⁺ target cells (A) and GFP⁻ effector cells (B).

Immunophenotyping of CAR T cells

At the end of the third round of four-day co-culture experiments, the CAR T cell phenotype was measured (fig. 5, gating strategy). Frequencies of CD56⁻ LNGFR⁺ cells were increased in co-culture experiment with functional adapters compared to mock control and with non-functional adapters (fig. 6).

LNGFR expression is a surrogate marker for successful transduction and detection of CAR T cells. Detection of LNGFR⁺ T cells can be used to determine frequencies as well as absolute numbers to investigate expansion of CAR T cells in course of repeated antigen encounters. CD27 and PD-1 were also highly expressed in the T cells with functional CAR adapters (fig. 6).

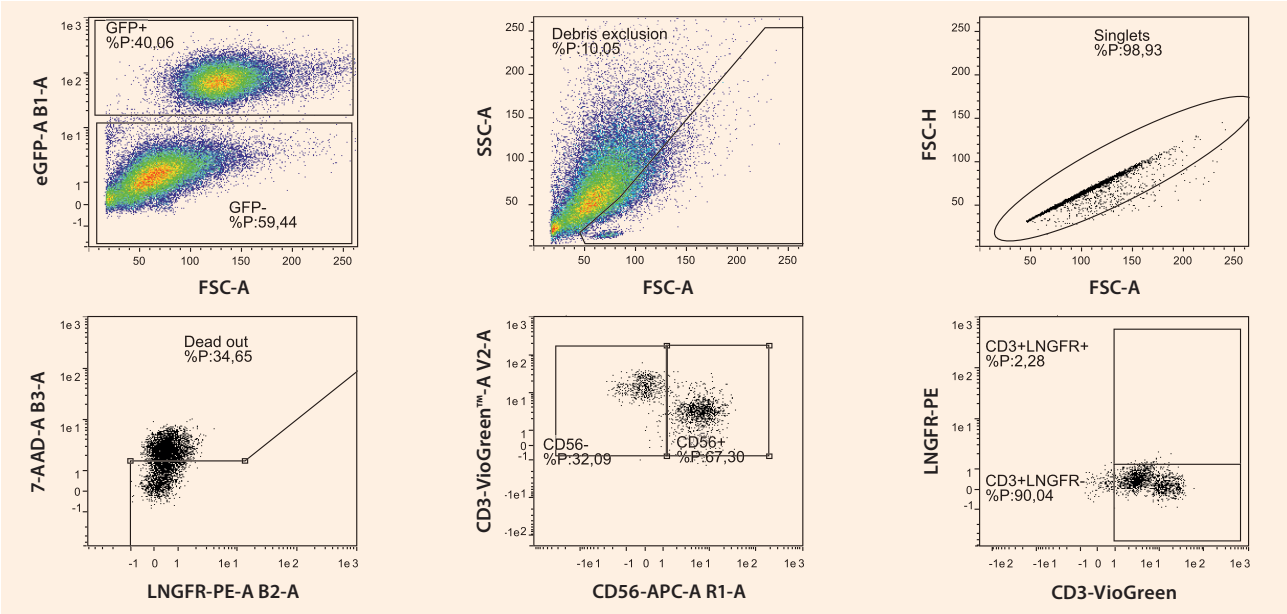

Figure 5: Gating strategy for immunophenotyping of CAR T cells. GFP⁻ CAR T cells were further analyzed to exclude debris and doubles based on their forward and side scatter properties. Dead cells were gated out based on 7-AAD⁻ gating. Finally, expression of different antigen doublets e.g. CD56, CD3, LNGFR, were monitored on the live cells with respective gating.

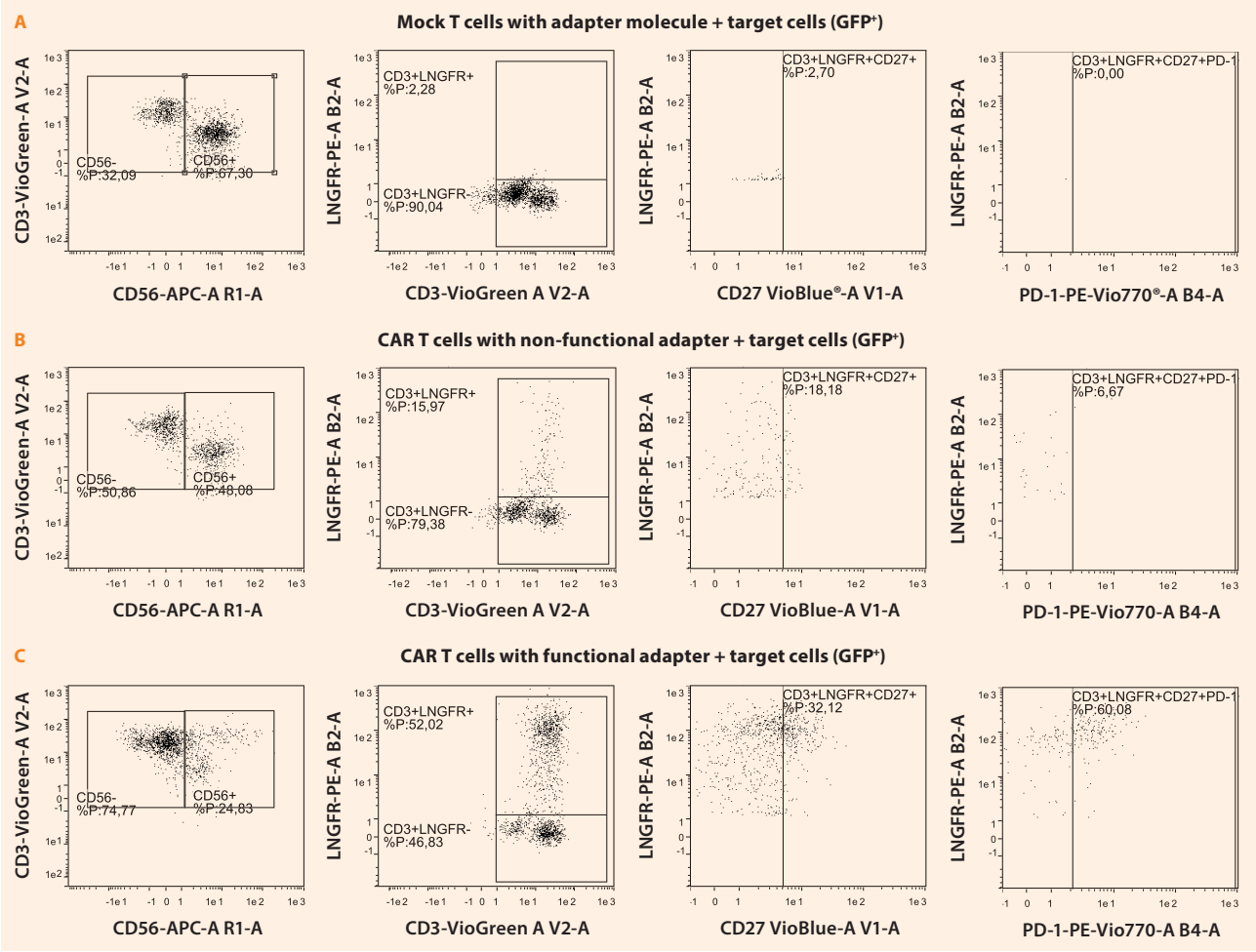

Figure 6: Phenotyping; Round 3, day 4 of co-culture.

Conclusion

This study demonstrates how flow cytometry can help to maximize the efficiency of CAR T cell functionality assessments. From cytotoxicity detection to cytokine measurement, our flow cytometry solution ensures reliable results in a high-throughput, automated manner. The automation-friendly MACSQuant® X Flow Cytometer analyzes a large number of cells with high sensitivity and provides absolute cell count information. The MACSPlex cytokine detection protocol allows easy-to-use and automated analysis of cytokine data. Finally, our recombinant antibodies allow highly reproducible phenotypic characterization data without the need for an FcR blocking step.

Antibody	Clone	Purpose	Fluoro-chrome	Detection channel (nm) (laser)
CD3	REA613	T cells	VioGreen™	525/50 (violet)
CD56	REA196	Activation marker	APC	655–730 (red)
LNGFR	REA844	Exhaustion marker	PE	585/40 (blue)
CD27	REA499	Exhaustion marker	VioBlue®	450/50 (violet)
PD-1	REA1165	Exhaustion marker	PE-Vio® 770	750 LP (blue)

Table 1: List of antibodies.

Product	Order no.
7-AAD Staining Solution	130-111-568
MACSQuant X	130-105-100
MACSPlex Cytokine 12 Kit, human	130-099-169
DAPI Staining Solution	130-111-570

Table 2: List of other products.

Reference

1. Lock; D. *et al,* (2017) Hum. Gen. Ther. "Automated Manufacturing of Potent CD20-Directed Chimeric Antigen Receptor T Cells for Clinical Use" (PMID: 28847167)

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