Application Note

Immunotherapy Research

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Ex Vivo Phenotyping and Potency Monitoring of CD19 CAR T Cells

Using a combined flow cytometry and impedance-based real-time cell analysis workflow

Authors

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Abstract

The development of cell-mediated immunotherapies has revolutionized cancer research as well as the study of the immune system. One of the most promising types of cell therapy involves the genetic engineering of novel chimeric antigen receptor (CAR) T cells to target cancer cells. To efficiently determine optimal CAR construction, researchers must develop a robust screening protocol to identify the ideal parent T cell populations and evaluate their cell killing potency. Here, we describe a workflow using a combination of the Agilent xCELLigence RTCA analyzer and Agilent NovoCyte Quanteon flow cytometer to thoroughly evaluate and characterize CAR T cells.

Introduction

Advancements in immunotherapy have altered the available treatments for cancer, using the specific ability of the immune system to recognize and kill cancer cells. A novel class of immunotherapy, CAR T cells, involves genetically engineering T cells to target a tumor antigen. Currently, adoptive T cell therapies are used for the treatment of B cell malignancies. However, significant challenges remain in the application to many cancers, including the treatment of solid tumors, side effects such as cytokine release syndrome, and long development timelines. The ideal universal receptor structure for highly potent CAR T cells is undergoing continuous improvements, with new generations of CAR structures being developed to maximize T cell longevity and cytotoxicity. More recent versions of CAR structures include costimulatory molecules and signaling molecules that help with better T cell function and persistence *in vivo*. The development of novel CAR T cells requires fast and in-depth evaluation of their potency to ensure efficacy and the identification of any nonspecific effects such as antigen-independent signaling.

Unlike other cytolytic endpoint assays, the Agilent xCELLigence RTCA continuously monitors CAR T cell cytolytic activity in real time over multiple days. To determine the quality of the CAR T cells under investigation, orthogonal Agilent NovoCyte flow cytometry assays can be performed to evaluate T cell activation, differentiation, and exhaustion. Here, we combined impedance-based real time cell analysis (RTCA) and flow cytometry workflow for *ex vivo* cytolytic potency monitoring of CD19-specific CAR T cells (CART19). We also examined phenotypic and functional responses to antigen exposure over time. The potency evaluation and characterization of CAR T cells were performed in several ways:

- CAR expression and T cell phenotyping
- Cytolytic potency by an RTCA cytotoxicity assay
- Cytokine production in response to antigen with a flow cytometry multiplex cytokine detection assay
- Characterization of CAR T cell state following antigen-specific activation.

This powerful workflow can be used to easily measure the cytolytic capacity of CAR T cells in conjunction with an in-depth analysis of T cell cytokine production, cell differentiation, and activation state.

Experimental

Effector and target cell culture and characterization

HEK-293 cells were maintained at 37 °C/5% CO_2 in MEM/EBSS (HyClone, catalog number SH30024.01) supplemented with 10% FBS (Gibco, catalog number 16050-122). These cells were engineered to express CD19 using lentiviral transduction. Expression of CD19 on the surface of HEK-293 cells was verified by flow cytometry with an Agilent anti-CD19 PE antibody (part number 8920007).

CD19-specific CAR T cells were constructed using PBMCs from a healthy donor and cultured for nine days with anti-CD3/anti-CD28 beads with the addition of 200 IU/mL of IL-2 in the same growth media as target cells.

CAR expression on T cells

CAR expression was analyzed in T cell populations using Agilent anti-CD3 FITC (part number 8931016), anti-CD4 PE-Cy5 (Biolegend 300510), Agilent anti-CD8 PE-Cy7 (part number 8931024), Agilent anti-CD19 APC (part number 8930007), anti-CD16 APC-Cy7 (BD, 561726), and anti-CD56 BV605 (Biolegend 318334) antibodies, and a CARTEST-19 kit provided from the CAR T manufacturer. The CAR T kit consisted of a CD19 antigen fused to a Fc tag, followed by a secondary anti-FC PE antibody. 1×10^6 CAR T cells were stained with live/dead AViD stain followed by incubation of cells with Fc block buffer (D-PBS containing 10% heat-inactivated human serum, 0.5% BSA, and 0.5% heat-inactivated FBS). Following this, cells were stained with the primary CAR T antibody for one hour on ice, then excess antibody was removed with the addition of 4% bovine serum albumin followed by centrifugation. Cells were then resuspended in antibody cocktail with anti-CD3, anti-CD4, anti-CD8, and secondary CAR T cell antibody for 30 minutes at 4 °C. Following incubation, cells were washed with the addition of 1% BSA and resuspended for flow cytometry acquisition. All analyses were performed on an Agilent NovoCyte Quanteon.

Characterization of CAR T cells and cytokine production

CAR T cells were analyzed before addition into the T cell cytolytic assay and at 40 and 88 hours after addition. Cells were stained with live/dead AViD stain followed by incubation of cells with Fc block buffer (D-PBS containing 10% heat-inactivated human serum, 0.5% BSA, and 0.5% heat-inactivated FBS). Excess stain was removed with the addition of PBS and the solution was centrifuged for five minutes at 300 g. The antibodies used are specified in Table 1, which provides information on antibody clone and fluorophore used. Cells were stained with the antibody cocktail for 30 minutes on ice. Following antibody incubation, cells were washed with the addition of 1% BSA and resuspended for flow cytometric analysis. FMO controls

were made by staining a sample with all antibodies except the one in which background was to be assessed.

Cytokine production bead-based multiplex assay

50 µL of cell supernatant was collected 24 hours after addition of T cells into the T cell cytolytic assay. IL-2, 4, 6, 10, 17A, IFN-γ, TNF-α, soluble Fas, soluble FasL, Granzyme A, Granzyme B, Perforin, and Granulysin in the cell supernatant were detected following the kit manufacturer's instructions, Human CD8/NK Panel, Biolegend, catalog number 740267.

CAR T cell cytolytic assay

The CAR T cell cytolytic assay was monitored on the Agilent xCELLigence MP, impedance measurements were taken every 15 minutes. The same growth medium and conditions were used as described for the target cells. After measuring background impedance using 50 µL of media/well, 10,000 target cells, in 100 µL of media, were added to each well. Proliferation was monitored for 23 hours, then 50 µL of media was removed and replaced with 50 µL of CD19 CAR T cells. Total numbers of T cells added differed to achieve E:T ratios of 0.06, 0.12, 0.25, 0.5, 1, 2, or 4. When using the impedance data, % cytolysis = [1 – Normalized $CI_{treatment}$/Normalized CI_{target} only] × 100

Results and discussion

Determination of T cell CAR expression and T cell state

Assessment of CAR expression and CAR T-cell phenotype is essential during CAR T discovery and for quality control during manufacturing. A CAR construct that targets CD19, a receptor expressed on B cell lymphoma cells was generated and used to transduce peripheral blood mononuclear cells (PBMCs). After generation, cells were expanded *ex vivo* and examined for the expression of CAR (Figure 1). *Ex vivo* expansion of PBMCs with anti-CD3 and anti-CD28 antibodies activates the T cell receptor and exclusively expands T cell populations. After nine days of culture, over 99% of live cells were CD3+ demonstrating that T cells were specifically expanded, and only small numbers of other cells were present. Approximately 50% of the total cells expressed chimeric antigen receptor (CAR), specifically 50% of CD4+ T cells and 46% of CD8 T cells, demonstrating successful stable transduction of the CAR construct.

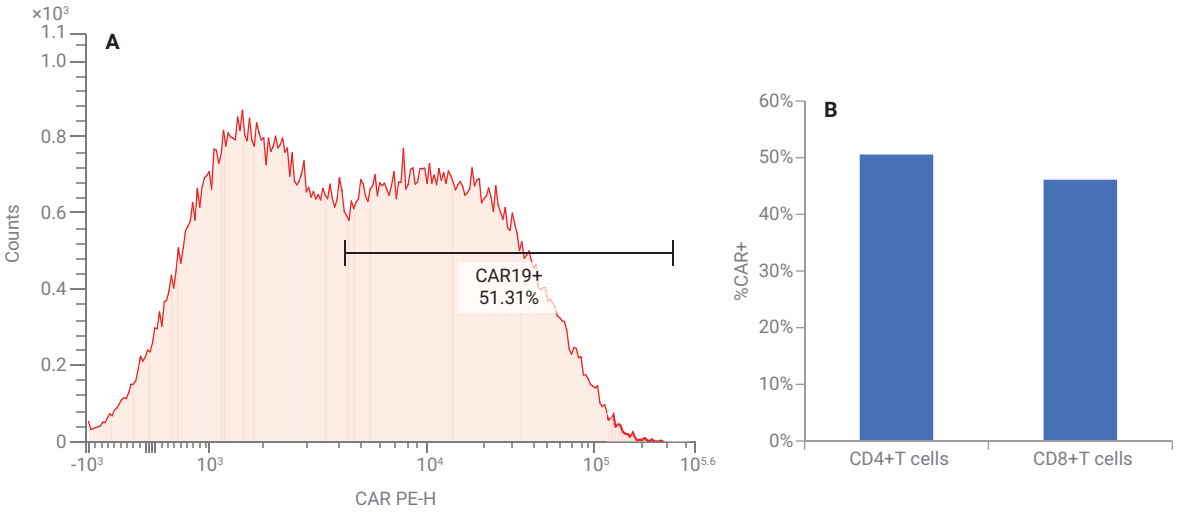

Figure 1. CAR expression and T cell phenotype of CART19 cells. CART19 cells were prepared by a collaborator for commercial use. Human PBMCs were transduced with a CD19 CAR construct or a mock vector and CAR19 expression was evaluated on T cell populations by flow cytometry. CAR T cells were stained with live/dead AViD stain, anti-CD3 FITC, anti-CD4 PE-Cy5, anti-CD8 PE-Cy7, anti-CD19 APC, anti-CD16 APC-Cy7, and anti-CD56 BV605 antibodies. The final CAR expression in total live cells was evaluated by staining with CD19 antigen with a FC tag and anti-FC PE antibody (A). Expression of CART19 in CD4+ and CD8+ subpopulations (B).

To further characterize the CART19 cells, a 12-color immunophenotyping flow cytometry panel was devised (Table 1) to examine T cell differentiation, activation, and exhaustion markers and provide insight into the status of CAR T cells. In this immunotherapy panel, after removal of dead cells and debris, T cells are identified by CD3 and divided into CD4 and CD8 T cell subsets (Figure 2A). The following differentiation states of T cells were identified using the expression patterns of CD45RA and CCR7; naïve-like (CCR7+CD45RA+), effector (CCR7-CD45RA+), effector memory (CCR7-CD45RA−), and central memory (CCR7+CD45RA−) T population. The naïve-like T cell population can be

Table 1. A 12-color flow cytometry panel was developed for analysis of differentiation, activation, and exhaustion of CART19 cells.

	Fluorochrome	Clone	Description
Dead cells	Aqua	AViD	Dead cells
CD3	BV570	UCHT1	T lineage
CD4	BV785	OKT4	
CD8	FITC	SK1	
CD45RA	BV650	HI100	Differentiation
CCR7	PE-Cy7	G043H7	
CD95	PE-Dazzle594	DX2	
CD25	BV421	M-A251	Activation
CD127	PE-Cy5	A019D5	
PD-1	APC	EH12.2H7	Exhaustion
TIM-3	PE	F38-2E2	
LAG-3	BV605	11C3C65	

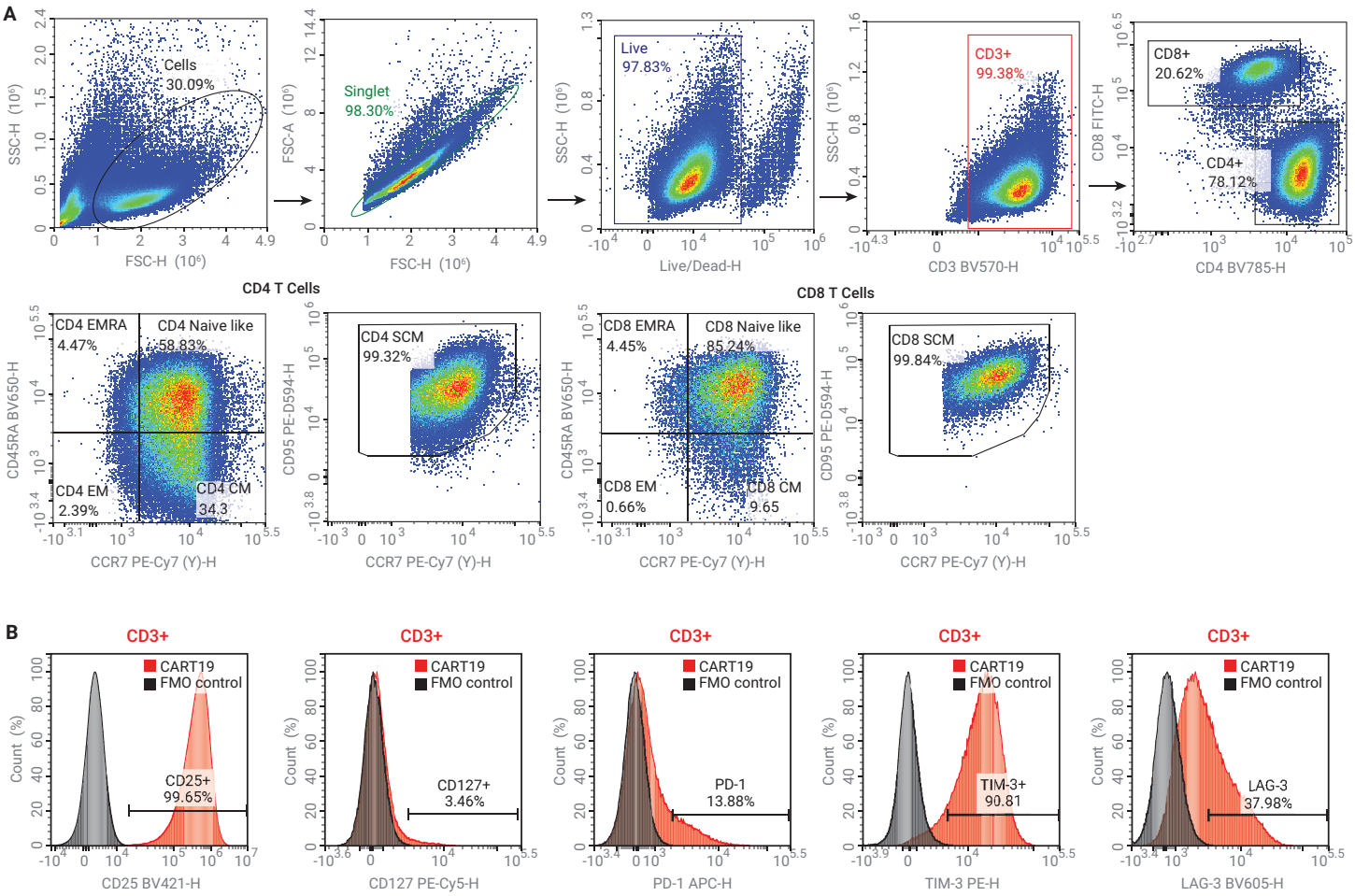

Figure 2. CART19 cell immunophenotyping before T cell cytolytic assay. CART19 cells stained with the 12-color immunophenotyping panel described in Table 1 (A). FMO controls are shown for CD25 BV421, CD127 PE-Cy5, PD-1 APC, TIM-3 PE, and LAG-3 BV605 (B).

further separated into stem cell-like memory T cells (CD95+) and true naïve T cells (CD95–). CAR T cells before addition to the cytolytic assay consisted of ~20% CD8 T cells and ~80% CD4 T cells. A substantial proportion of the cells were CCR7+CD45RA– central memory T cells (34% CD4+ cells, 9.38% CD8+ cells) and CCR7+CD45RA+CD95+ stem cell-like T cells (58% CD4+ cells and 85% CD8+ cells), demonstrating that most CAR T cells derived from PBMCs develop a memory-like phenotype in response to CD3/CD28 stimulation *in vitro*. Development of a memory like phenotype after *in vitro* expression has been demonstrated previously to be beneficial for CAR T cell persistence *in vivo*. Expression of T cell activation markers CD25 and CD127 as well as presence of co-inhibitory receptors PD-1, TIM-3, and LAG-3 provides an in-depth analysis of T cell status. After T cell activation and expansion, the CART19 cells express high levels of CD25 and express co-inhibitory receptors (exhaustion markers) PD-1, TIM-3, and LAG-3. While normally upregulated after T cell activation, sustained high expression of these co-inhibitory receptors can inhibit T cell cytolytic function. For efficient cancer immunotherapy, it is important to prevent CAR T cell exhaustion. Therefore, monitoring the expression of activation and exhaustion markers on novel CAR T candidates is essential for the development of new adoptive cell therapies. An in-depth investigation of CAR T cell candidates can be achieved, through the assessment of T cell phenotype and activation status, combined with functional assays, such as T cell cytolytic activity and cytokine production.

CD19 CAR T cell cytolytic activity measured by xCELLigence RTCA

The xCELLigence RTCA system uses a microplate with biosensors in the base of the wells to monitor cellular interaction with the plate surface. This cellular interaction is used as a measurement of cell concentration, adhesion, and morphology. The cell signal is recorded continuously throughout the assay at set intervals and represented as Cell Index, providing real-time analysis of the cells. Lymphocytes do not adhere to the bottom of the well and do not generate signal, therefore, in this assay, the Cell Index measurement is exclusively from the target cancer cells. As CAR T cells lyse target cancer cells, a decrease in the cell index is observed.

The cytolytic capacity of CART19 cells was measured using a T cell cytolytic assay. CART19 cells were cultured with CD19 expressing HEK-293 cells while real time measurements of T cell mediated cytolysis were taken with the xCELLigence instrument. One day before the addition of CART19 cells, HEK293 cells that ectopically expressed CD19 or controls were seeded. After the addition of CART19 cells, rapid cytolysis of HEK-293-CD19 cells occurs (Figures 3B to 3D) reaching more than 95% cytolysis within 24 hours post-CART19 addition (Figure 3C). There was almost no cytolysis from nonspecific CAR T cells, and it was only observed at very late time points of the coculture of effector and target cells (Figure 3D). The CART19 response was also dose-dependent, increased E:T ratios resulted in more rapid and a higher total percentage of cytolysis of target cells than lower E:T ratios. T cell cytolysis assays performed using xCELLigence RTCA allow rapid real-time assessment of CAR T function.

Upregulation of CD19 CAR T cell cytokine and cytolytic protein production

When CAR T cells are activated by the corresponding target cells, they release large amounts of cytokines important for T cell cytolytic activity and immune cell activation. CAR T adoptive transfer *in vivo* is marked by rapid elevation of several cytokines in serum including tumor necrosis factor-alpha (TNF-α), interferon γ (IFN-γ), interleukin 6 (IL-6), and interleukin-10 (IL-10). In addition to aiding in the immune cell response, an overabundance of cytokines can lead to cytokine release syndrome. It may be beneficial to monitor cytokine production in an *in vitro* model to better predict T cell efficacy and screen for an overexuberant cytokine response. Further characterization was performed by quantifying the amount of cytokine produced by CART19 while killing target cells. Cytokine levels were measured in the supernatant of CART19-HEK-293-CD19 cultures using a bead-based multiplex flow cytometric assay that measures multiple cytokines simultaneously. Higher E:T ratios of CART19 cells resulted in increased expression of cytokines IFNγ, TNFα, IL-2, and IL-10 as well as cytolytic proteins granzyme A, granzyme B, granulysin, perforin, and sFasL. Almost no cytokine production was observed from CART19 cultured with HEK-293 demonstrating that increased secretion of cytokines and cytolytic proteins was induced by CAR-dependent signaling.

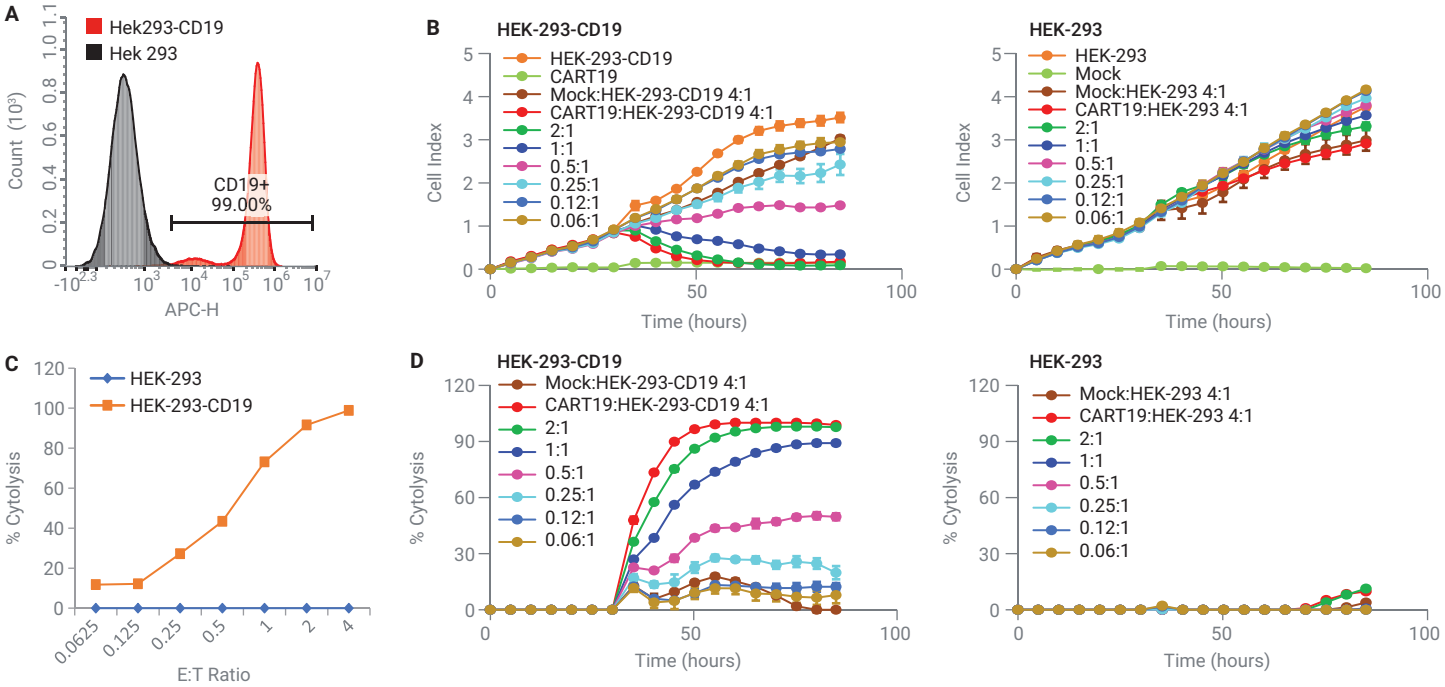

Figure 3. Evaluation of cytolytic activity of CART19 by the Agilent xCELLigence RTCA system. CD19 expression was evaluated on HEK-293 and HEK-293-CD19 cell lines by surface stain with anti-CD19 APC antibody (A). 10,000 HEK-293-CD19 and HEK293 cell were seeded in a 96-well E-Plate, and Cell Index (CI) measurements were taken every 15 minutes using an xCELLigence MP system. (B) After 24 hours, CART19 effector cells (left plot) or mock CAR T (right plot) were added at various E:T ratios ranging from 2:1 to 0.06:1. (D) The Cell Index plots are converted to % cytolysis by the xCELLigence Immunotherapy software. % Cytolysis was measured at 24 hours after CART19 addition with HEK-293 or HEK293-CD19 target cells (C).

Characterization of CD19 CAR T cell activation upon antigen stimulation

CAR T cells were also assessed at the end of the T cell cytolytic assay using the same 12-color immunophenotyping panel used to assess the CAR T cells before the cytolytic assay. This enabled the detection of any changes in the expression of T cell activation and exhaustion markers. Effector CART19 cells displayed a more activated T cell state at both 40 and 88 hours after T cell addition compared to control cocultures. CART19 cells that were cultured with CD19-HEK-293 cells showed increased FSC, higher expression of CD25, PD-1, TIM-3, and LAG-3 and downregulation of CD127. Combining CART 19 cytolysis measurements with flow cytometric assessment of T cell status and cytokine production may provide a comprehensive understanding of underlying mechanistic aspects of CAR T cell mediated killing.

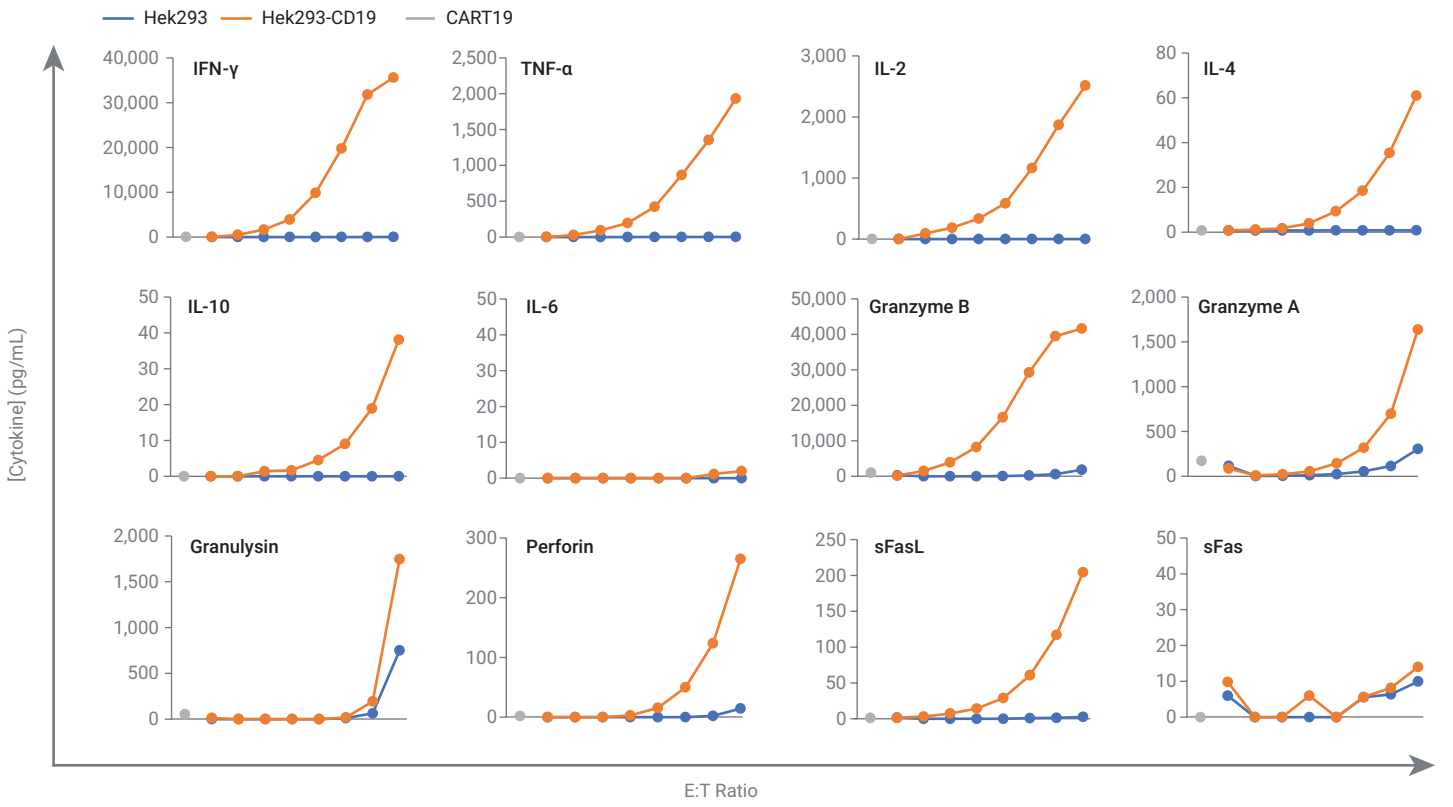

Figure 4. Cytokine production by CART19 after cocultured with HEK-293-CD19. CART19 cells were added at different E:T ratios 24 hours after HEK-293-CD19 or HEK-293 cells seeding. Supernatant was collected 18 hours later and the cytokine concentration was measured on the Agilent NovoCyte Quanteon flow cytometer using BioLegend LEGENDplex Human CD8/NK panel.

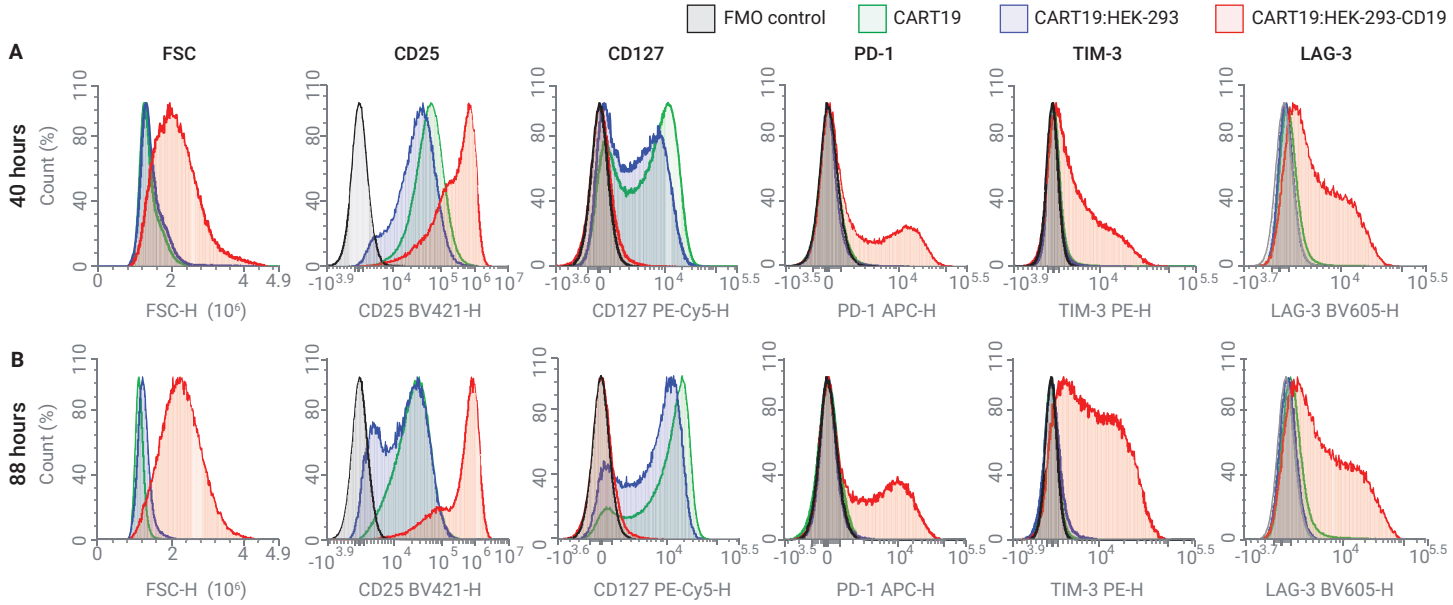

Figure 5. Characterization of CART19 cells after coculture with HEK-293-CD19. Cells were collected from CART and HEK-293 or HEK-293-CD19 cocultures after 40 hours (A) and 88 hours (B) at a 1:1 Effector:Target ratio. Cells were stained with the 12-color immunophenotyping panel described in Table 1. Expression of activation and exhaustion markers were compared.

Conclusion

The therapeutic potential of CAR T cell therapies has driven the development of these treatments, requiring rapid analysis of novel CAR T cell candidates. The combined Agilent xCELLigence RTCA and NovoCyte Quanteon flow cytometer cell analysis workflow provides researchers comprehensive and convenient results of cytolytic potency, cytokine secretion, CAR T characterization, and impurity analysis.

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RA.6848263889

Printed in the USA, October 27, 2020
5994-2377EN

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