APPLICATION NOTE

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Characterizing the heterogeneity in CAR T cell cytotoxicity with Xdrop®

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Summary

- With single-cell assays in droplets, Xdrop reveals the heterogeneity of CAR T cells and highlights the intricate timing of CAR T cell cytotoxicity.
- In this study only about 1/5 of the analyzed CAR T cells kill their targets within 6 hours.
- At the same time point 1/3 of CAR T cells already secrete active granzyme B.
- This level of insight into cellular heterogeneity is unattainable with traditional bulk assays.

Introduction

Chimeric antigen receptor (CAR) T cell therapy represents one of the most advanced and successful immune cell therapies for cancer treatment with over 1600 studies registered at ClinicalTrials.gov[1]. Major breakthroughs have been achieved for treatment of hematological malignancies, and ongoing research aims to extend the application of CAR T cell therapy to the treatment of solid tumors[2].

Assessing CAR T cell-mediated cytotoxicity is required by the FDA in their industry guidelines on T cell product development[3]. This emphasizes the need to evaluate and balance cell efficacy and safety. Traditional immune cell-mediated cytotoxicity analysis are mainly done as bulk assays and do not provide information about the functional heterogeneity of the CAR T cell population. Consequently, it remains unclear whether all the CAR T cells in a batch can kill their targets or if only a fraction are potent.

Here, we applied an Xdrop droplet-based assay to provide insights into the cytotoxic heterogeneity of CAR T cell populations. This time-course study revealed the number of CAR T cells that secreted granzyme B (GzmB) upon encountering target cells and identified which CAR T cells were responsible for killing the target cells. Interestingly, only about one fifth of the CAR T cells killed their target cells within 6 hours of interaction.

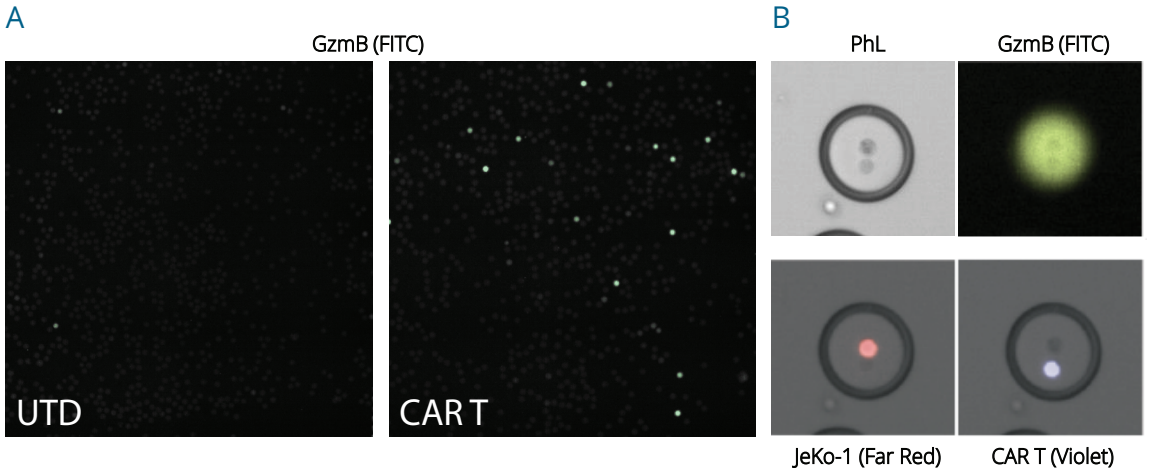


Figure 1. Microscopy images of droplets with encapsulated cells. A DE50 droplets with either un-transduced T cells (UTDs) (left) or CAR T cells (right) from the same donor. T cells were encapsulated with JeKo-1 target cells and incubated with the assay chemistry for 4 hours. An intense FITC signal (green) is observed in droplets with secreted GzmB activity. B A single DE50 droplet containing a GzmB-secreting CAR T cell and a JeKo-1 target cell. Top left: phase contrast image. Remaining images are pseudocolored fluorescence microscopy images overlaid on the phase contrast image to show the signal from secreted GzmB (green); CellTrace Violet-stained T cells (violet); CellTrace Far Red-stained JeKo-1 cells (red).

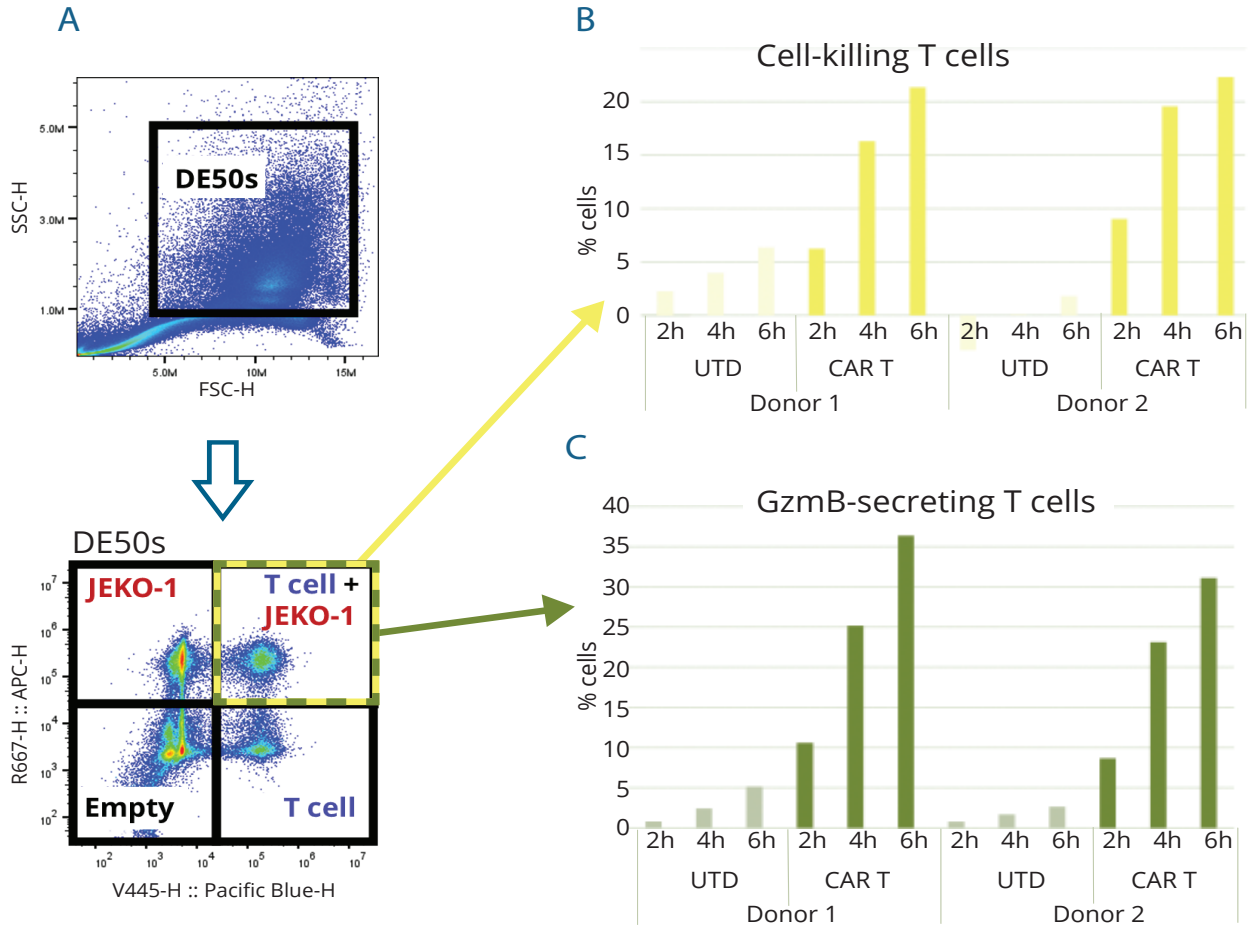


Figure 2. Quantification of CAR T cells with GzmB secretion and CAR T cell mediated cytotoxicity. A Top: Forward scatter (FSC-H) versus side scatter (SSC-H) density diagram identifies DE50 droplets. The events outside the gate represents oil droplets produced as a biproduct of double-emulsion droplets. Bottom: Gated DE50 droplets plotted based on the signal from the two cell stains: T cells stained with CellTrace Violet (Pacific Blue-H); JeKo-1 stained with CellTrace Far Red (APC-H). Four droplet populations are distinguishable: droplets containing both T cells and JeKo-1 cells (green and yellow square); droplets with JeKo-1 cells alone; droplets with T cells alone; and empty droplets. B Bar chart showing the percentage of cell-killing T cells in the droplets co-encapsulating a T cell and a target cell. Data from two donors are depicted. UTD: Un-transduced T cells. Normalized to the cell death in the droplets with T cells only and Jeko-1 cells only. C The percentage of GzmB-secreting T cells at each time point normalized to the percentage of live T cells.

Assay in droplets can distinguish activated and non-activated CAR T cells

CD19-targeting CAR T cells were generated by lentiviral transduction of T cells from two healthy donors while un-transduced T cells (UTDs) were retained as controls. CAR T cells and UTDs are collectively referred to as T cells. T cells were stained with CellTrace™ Violet (1:5,000 concentration) and malignant CD19-positive target cells (JeKo-1; Mantle cell lymphoma) were stained with CellTrace Far Red (1:5,000 concentration) to identify the cell type in the subsequent analysis.

After staining, the T cells and JeKo-1 cells were separately resuspended in assay medium consisting of complete RPMI (RPMI1640 with 10% FBS and 1% Pen/Strep) + 10% OptiPrep™, supplemented with assay reagents: Propidium iodide (PI; 1 µg/ml) and FAM fluorophore-quenched GzmB peptide substrate (4 µM). The peptide substrate fluoresces when cleaved by secreted GzmB resulting in the accumulation of an FITC signal inside the droplets. PI is used to monitor cell death. For a detailed explanation of the workflow see Supplementary Figure 1.

The T cells and the target cells were co-encapsulation in 100 pl double-emulsion droplets (DE50 droplets) using Xdrop and the Xdrop DE50 cartridge as described in the Xdrop user manual[4]. Each T cell suspension (CAR T or UTD), was mixed with a JeKo-1 cell suspension immediately before encapsulation at an effector:target cell ratio of 1:3 (0.5 x 10^6 T cells and 1.5 x10^6 JeKo-1). Following encapsulation, each droplet production (CAR T+JeKo-1 and UTD+JeKo-1) was divided into three incubation tubes (for 2 hours, 4 hours, and 6 hours incubation). The cells were incubated inside the droplets at 37°C in 5% CO_2 and then analyzed by microscopy and flow cytometry at the indicated time points.

Figure 1A shows representative fluorescence microscopy images of double-emulsion droplets after 4 hours of incubation. The intensity of the green fluorescence signal (FITC) illustrates the level of secreted GzmB activity of CAR T cells or UTDs inside the DE50 droplets. Figure 1B presents phase contrast and fluorescence microscopy images of a single GzmB-positive droplet containing a CAR T cell and a JeKo-1 target cell.

Active CAR T cells are identified by droplet flow cytometry

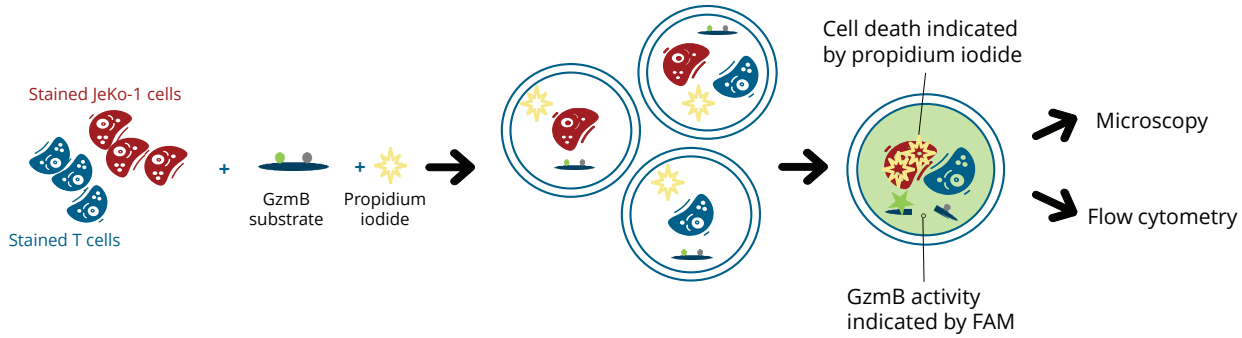
To quantify the CAR T cells with early GzmB secretion and cell killing activity, DE50 droplets with CAR T+JeKo-1 and UTD+JeKo-1 co-encapsulated cells were analyzed using a NovoCyte®Quanteon flow cytometer (Agilent® Technologies). By staining JeKo-1 target cells with CellTrace Far Red and T cells with CellTrace Violet we could identify three distinct cell-containing droplet populations: 1) with T cells alone, 2) with JeKo-1 cells alone, and 3) with T cells and JeKo-1 cells together (Figure 2A). We gated and analyzed the droplets co-encapsulating T cells with JeKo-1 cells for signals indicating cell death (PI, Figure 2B) and GzmB activity (FITC, Figure 2C). For reference, the droplets containing only T cells and only JeKo-1 cells were also analyzed to examine the background cell death in the two populations (data not shown).

As shown in Figure 2B and Figure 2C, CAR T cells from Donor 1 and 2 exhibited a time-dependent increase in both GzmB secretion and cell killing. After 6 hours of incubation, about 33% of the CAR T cells from the two donors had secreted GzmB and about 20% had killed the co-encapsulated target cell. For both donors, the percentage of CAR T cells with GzmB activity exceeded the percentage of killed target cells at each time point, consistent with expected sequence of events in GzmB mediated cytotoxicity[5]. UTDs showed limited GzmB and cell killing activity. The variation in killing and GzmB activity between the two donors highlights the necessity of individually assessing CAR T cell populations. Methods like Xdrop, which can distinguish single cell activity, are crucial for this evaluation.

Conclusion

Xdrop reveals the functional diversity both within and between cellular immunotherapies. Xdrop double-emulsion droplet assays with single-cell resolution, are rapid and compatible with flow cytometry analysis. We found that only about one-fifth of the examined CAR T cells can kill within 6 hours. Additionally, we can distinguish the differential timing of GzmB activity and cell killing between populations. This level of insight into cellular heterogeneity is not achievable with traditional bulk assays.

For more information about Xdrop products and applications, visit samplix.com.



Supplementary Figure 1. The workflow for a combined granzyme B (GzmB) and cell killing assay with single-cell resolution in droplets. Before encapsulation in double-emulsion droplets, the target and effector cells are separately stained. Using Xdrop and the Xdrop DE50 Cartridge, CAR T cells or un-transduced T cells (UTDs) are co-encapsulated with target cells (JeKo-1) in DE50 droplets together with complete RPMI, propidium iodide (PI), and FAM-labeled GzmB peptide substrate. The assay and incubation occur within the droplets. Secreted GzmB activity is revealed by green fluorescence emission after GzmB cleaves the substrate. Cell death is indicated by PI. After incubation, DE50 droplets can be analyzed by microscopy and/or flow cytometry.

SX-000793 - AN – ver. 01

References

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