APPLICATION NOTE

Droplet-based single-cell analysis reveals hidden CD4+ and CD8+ CAR T cell cytotoxicity

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Summary

- Single-cell resolution: The Xdrop® assay with StarBright™ Dye-conjugated antibodies enables precise profiling of CD8+ and CD4+ CAR T cells.
- Notably, ~2% of CD4+ CAR T cells exhibit a striking cytotoxicity in addition to ~12% of CD8+ cells.
- Unlike bulk assays, this single-cell assay in droplets enables in-depth functional characterization of CAR T cells and CAR cassette construct designs.

Introduction

Evaluating cytotoxicity is critical for developing T cell–based therapies, as it drives therapeutic efficacy and informs clinical translation. Conventional bulk assays measure only average responses, masking the heterogeneity within cell populations. To capture each cell's full functionality and phenotype, reliable single-cell analysis methods are needed.

Droplet-based single-cell cytotoxicity assays have revealed pronounced heterogeneity in the cytotoxic activity of individual Chimeric Antigen Receptor (CAR) T cells or tumor-infiltrating lymphocytes against target cells[1,2]. Extending the analysis to CD4+ and CD8+ subsets would enable the functional contributions of each population to be evaluated independently. While the cytotoxic function of CD8+ T cells is well established, the cytotoxicity of CD4+ CAR T cell subsets and their contribution to anti-tumor immunity is only emerging[3, 4].

Here, we apply Samplix' Xdrop® platform combined with Bio-Rad StarBright Dye fluorescently tagged antibodies to unveil the cytotoxicity of CD4+ and CD8+ CAR T cell subsets. As expected, only a subset of CD8+ CAR T cells killed their co-encapsulated target cell. Strikingly, we also found a smaller subset of CD4+ CAR T cells (~2%) that both secreted granzyme B (GzmB) and killed the target cell. These findings illustrate the potential of Xdrop single-cell cytotoxicity assays to deliver critical insights in therapeutic CD4+ and CD8+ T cells in cancer and immunotherapy.

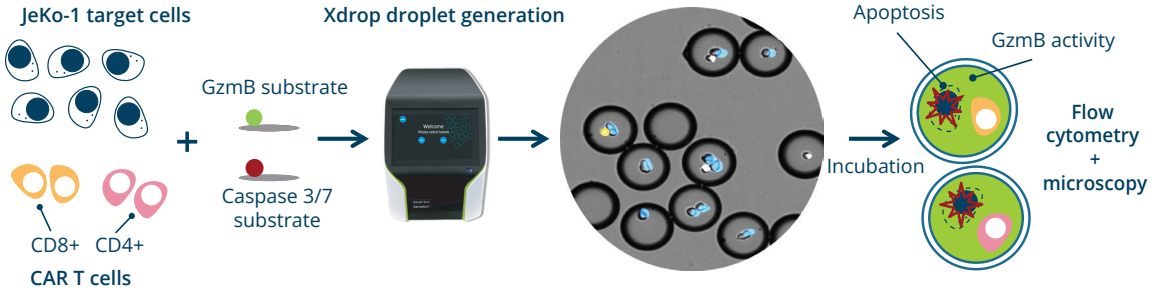

Figure 1. Workflow for single-cell assay of CD4+ and CD8+ T cell cytotoxicity. Target cells (JeKo-1) are tracked by an intracellular stain and CAR T cells labeled with anti-CD4 and anti-CD8 StarBright Dye-conjugated antibodies. Using Xdrop, CAR T cells or untransduced T cells (UTDs) are co-encapsulated with target cells (JeKo-1) in double-emulsion (DE50) droplets together with peptide substrates for detection of caspase 3/7 and GzmB activity. The assay occurs within the droplets during 6.5 hours of incubation. Secreted GzmB activity is revealed by green fluorescence emission after GzmB cleaves the substrate. Caspase 3/7 activity is indicated by a red DNA intercalating dye released by substrate cleavage. After incubation, DE50 droplets containing cells can be analyzed by microscopy and/or flow cytometry.

CD4/CD8 labeling with StarBright Dye-conjugated antibodies does not impair CAR T cell cytotoxicity

We used CD19-targeting CAR T cells generated by lentiviral transduction of T cells from a healthy donor followed by CAR-enrichment (~98% CAR+). Untransduced T cells (UTDs) from the same donor were used as negative controls to evaluate CAR-dependent cytotoxicity. CD19-expressing mantle cell lymphoma cells (JeKo-1) were used as target cells.

We verified that labeling with anti-CD4 nor anti-CD8 StarBright Dye-conjugated antibodies did not impact the assayed CAR T cells' functionality. StarBright Violet 760 and StarBright Violet 610 fluorescent antibodies were chosen for their unique excitation and emission spectra creating minimal overlap with the final assay panel. To track cell-types within droplets, JeKo-1 cells were stained with CellTrace™ Far Red (ThermoFisher Scientific) and T cells with CellTrace™ Violet (ThermoFisher Scientific). The T cells were then either labeled with anti-CD4 and anti-CD8 antibodies (Bio-Rad StarBright Violet 760, MCA1267SBV760 and StarBright Violet 610, MCA1226SBV610 respectively) or left unlabeled for comparison.

Using the Xdrop droplet generator and the Xdrop DE50 Cartridge (Samplix), T cells and JeKo-1 cells were co-encapsulated in Xdrop DE50 droplets together with assay medium (90% Complete RPMI medium, 10% OptiPrep™). In addition, both granzyme B (GzmB) peptide substrate (Samplix), a marker of cytotoxic activity and caspase 3/7 peptide substrate (Sartorius), a marker of apoptosis were included in the droplets to monitor cell killing. Cells were encapsulated at an effector:target ratio of 1:3. The droplets were incubated at 37°C in a 5% CO_2 incubator for 6.5 hours before flow cytometric analysis.

Figure 2A shows the flow cytometry gating strategy used to identify droplet populations and measure the secreted GzmB activity. GzmB (Figure 2B) and caspase 3/7 (Figure 2C) activity were identical both with and without labeling of CD4 and CD8 surface markers with Bio-Rad StarBright Dye-conjugated antibodies with no significant effect on target-induced or off-target anti-CD19 CAR T cell cytotoxicity (Figure 2).

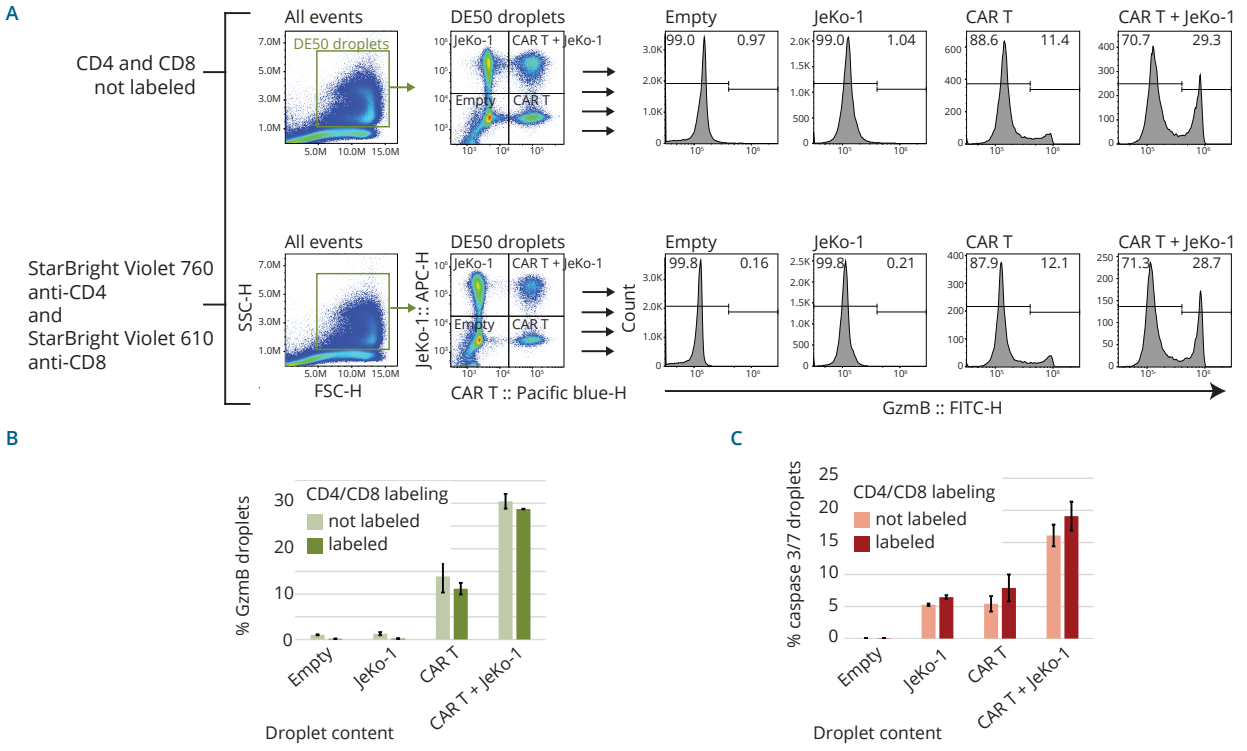

Figure 2. Anti-CD4/CD8 antibody labeling does not interfere with the CAR T cytotoxicity assay. A. Flow cytometry data without (top panel) or with (bottom panel) anti-CD4 and anti-CD8 antibody labeling. DE50 droplets were first identified in the scatter plot of all events. Next, a density plot of gated DE50 droplets was set up based on the signals from JeKo-1 cells (blue cell stain) and CAR T cells (red cell stain). The four droplet populations based on the droplet's cell content were: empty droplets with no encapsulated cells (lower left), JeKo-1 only (upper left), CAR T only (lower right), or both JeKo-1 and CAR T (upper right). The droplets with GzmB activity were quantified within each droplet population. The same analysis was applied for caspase 3/7 data. B. Percentage of droplets with GzmB activity, determined by flow cytometry as outlined in A. C. Percentage of droplets with caspase-3/7+ activity, determined by flow cytometry as outlined in A. All flow cytometric data were compensated based on a single-stain control–derived compensation matrix.

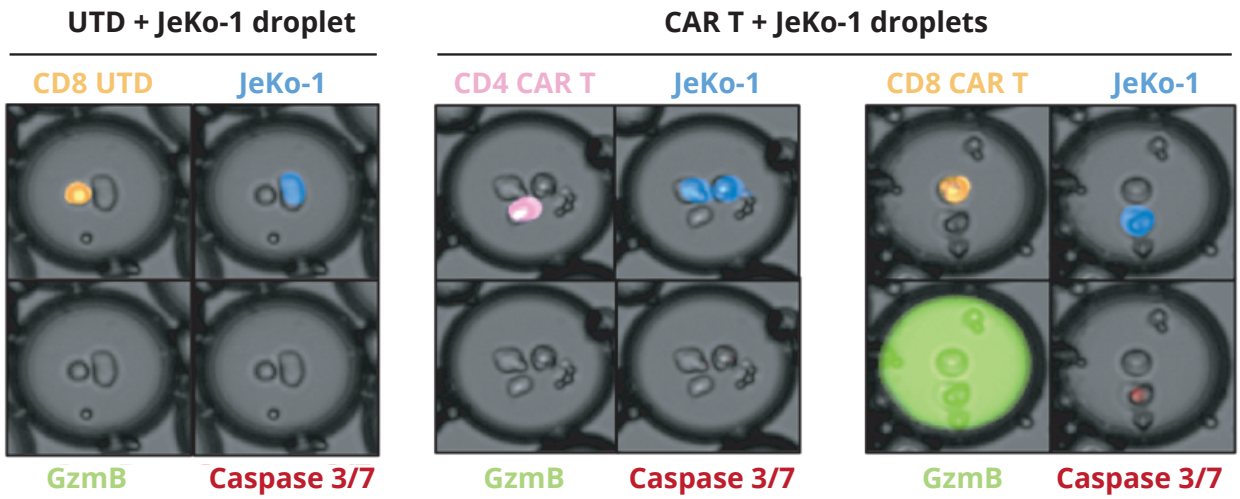

Figure 3. Target and effector cells co-encapsulated in double-emulsion droplets. Representative microscope images of DE50 droplets with one CAR T or untransduced (UTD) T cell encapsulated with one or more JeKo-1 target cells. Cells were encapsulated with the five-color assay to assess CD4+ and CD8+ T cell-mediated cytotoxicity. Droplets with both GzmB and caspase 3/7 activity were predominantly found in those containing JeKo-1 cells with CD8+ CAR T cells, and to a lesser extent in droplets containing JeKo-1 with CD4+ CAR T cells. No such activity was detected in droplets containing JeKo-1 with UTD T cells or CAR T cells alone. Images were acquired using the automated Xcyto®5 image cytometer (ChemoMetec).

Target and CAR T cell co-encapsulation in droplets enable functional analysis of CAR T cells

With no interference by CD4/CD8 labeling (Figure 2), we could construct a more advanced panel, which simultaneously measured GzmB and caspase 3/7 activities within the CD4+ and CD8+ CAR T cell populations (see the spectral profile of the panel in Figure 4B). To track cell content in the droplets, JeKo-1 target cells were stained with CellTrace™ Violet (ThermoFisher Scientific) while CAR T cells were stained with anti-CD4 StarBright Violet 760 and anti-CD8 StarBright Violet 610 antibodies (see Figure 1). The labeled CAR T cells – or UTD control cells from the same donor – and JeKo-1 target cells were co-encapsulated on the Xdrop droplet generator together with the assay reagents.

After incubation, the droplets were analyzed by automated imaging to evaluate the target-induced CAR-dependent T cell cytotoxicity. Figure 3 shows representative microscopy images from yhe Xcyto®5 image cytometer (ChemoMetec) of droplets with their cell content (JeKo-1, CD4+ / CD8+ T cells) as well as the functional markers for secreted GzmB and caspase 3/7 activity.

CD8+ as well as CD4+ cytotoxic subpopulations revealed

Flow cytometry, with its high throughput, enabled quantification of cytotoxic CD4+ and CD8+ CAR T cells (Figure 4). We verified that the fluorophores of the antibody panel had minimal spectral overlap by creating a spectral profile chart using the Bio-Rad spectraviewer tool (Figure 4B).

Approximately 20% of CD4+ CAR T cells secreted active GzmB when paired with Jeko-1 cells in droplets compared to ~2% in the absence of target cells. Since GzmB activity precedes CAR-T–mediated killing[5], this indicates that CD4+ CAR T cells may play a larger role in target-cell killing than previously thought. The corresponding proportions for CD8+ CAR T cells were ~39% with JeKo-1 cells versus ~8% without, reinforcing their dominant but complementary role in cytotoxic responses (Figure 4A).

Dual GzmB and caspase-3/7 activity was largely absent in droplets containing either UTDs with target cells or CAR T cells alone. In contrast, about 12% of CD8+ CAR T cells co-encapsulated with JeKo-1 cells were double-positive for GzmB and caspase 3/7, indicating active cytotoxicity. Interestingly, a smaller but notable fraction (~2%) of CD4+ CAR T cells showed similar dual activity under the same conditions (Figure 4C).

Conclusion

The Xdrop single-cell cytotoxicity assay, combined with Bio-Rad StarBright Dye-conjugated fluorescent antibodies, enables precise profiling of cytotoxicity within T cell subsets. It facilitates quantification of cytotoxic CD8+ CAR T cells and reveals a distinct cytotoxic CD4+ CAR T cell subpopulation. By resolving functional heterogeneity at single-cell resolution, this platform offers valuable insights into CAR T cell biology and supports the advancement of next-generation cell therapies.

For more information about Xdrop products and this note, contact Samplix at samplix.com/contact.

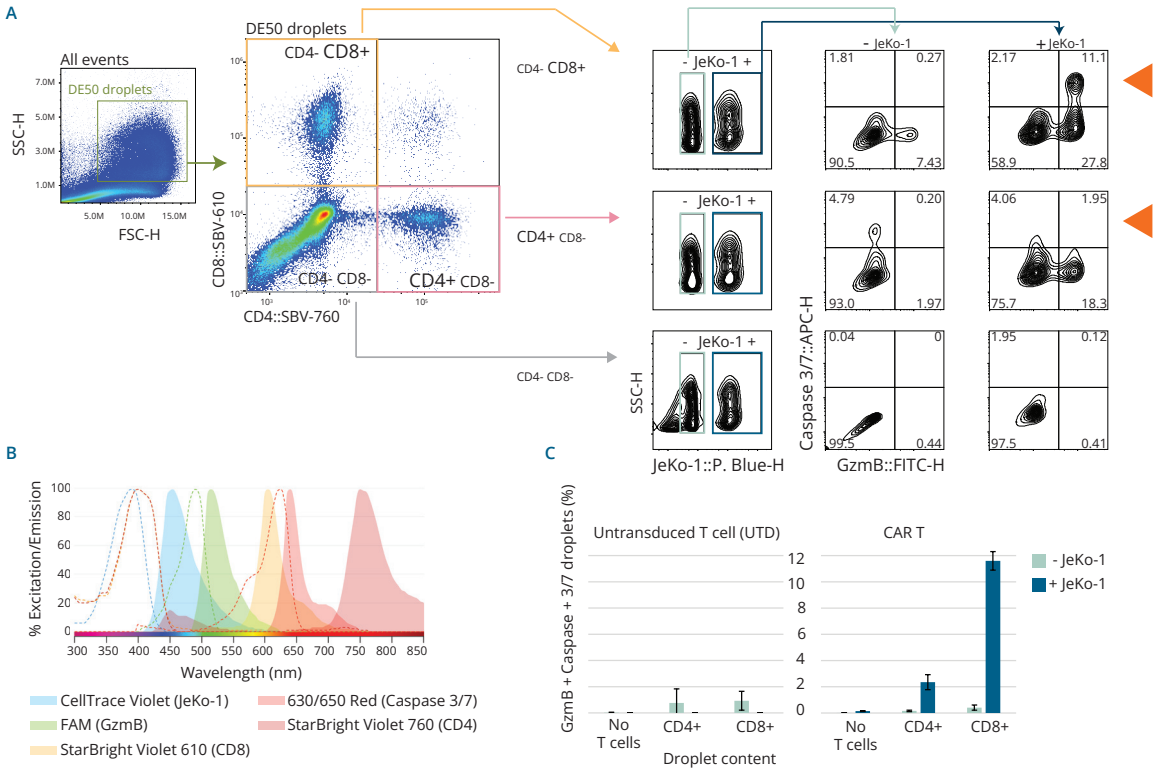

Figure 4. Quantifying cytotoxic potential of CD4+ and CD8+ populations in CAR T cells. A. DE50 droplets containing cells and assay reagents were analyzed by flow cytometry. From a scatter plot of all events, DE50 droplets were identified and gated. The DE50 droplets were further classified by staining with anti-CD4 StarBright Violet 760 and anti-CD8 StarBright Violet 610 antibodies into four groups: no CAR T cell (lower left), CD8+ CAR T cell (upper left), CD4+ CAR T cells (lower right) or droplets containing both CD4+ and CD8+ T cells (upper right). Three of these populations were then subdivided based on the presence (+) or absence (-) of JeKo-1 target cells. GzmB and caspase 3/7 activities within each droplet population were quantified to assess cytotoxic function. B. Spectral profile chart showing the excitation (dotted lines) and emission spectra of the fluorophores used in the antibody panel. C. Quantification of droplets with combined GzmB and caspase 3/7 activity. Left graph shows droplets containing untransduced T cell (UTD) with or without JeKo-1 target cells while the right graph shows droplets containing CAR T cells with or without JeKo-1 target cells. Droplets were incubated 6.5 hours before analysis. Flow cytometric data were compensated based on a single-stain control–derived compensation matrix.

References

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