APPLICATION NOTE

Multidimensional Analysis of T Cell Cytotoxicity and Serial Killing Using the Opto™ Cell Therapy Development Workflow

APPLICATION SPOTLIGHT

- Correlate antigen-specific target killing activity of single CAR T cells to other functional attributes like cytokine secretion and proliferation
- Identify serial killing behavior in single T cells
- Recover live T cells for further characterization to link target cell killing with gene expression

INTRODUCTION

Adoptive therapy with gene-modified T cells, such as CAR T cells, has shown significant efficacy as a therapy for patients with refractory pediatric B-cell acute lymphoblastic leukemia (B-ALL), adult B-ALL, and non-Hodgkin lymphoma[1]. However, clinical successes for solid tumors have been mixed for poorly understood reasons. Factors such as lack of CAR T cell persistence, immune suppression in the tumor microenvironment, and loss of antigen expression have been implicated. To develop more efficacious therapies, we need a better understanding of the critical aspects of CAR T cell biology that have remained unexplored largely due to technical limitations.

One of the signs of a successful therapeutic infusion is the presence of CAR T cells that kill multiple tumor cells, a phenomenon termed serial killing[1]. Anti-tumor activity by these cells is mediated by several mechanisms, including via the perforin and granzyme axis, the Fas and Fas ligand axis, as well as through cytokine release. Secretion of the inflammatory cytokine interferon gamma (IFNγ) following antigen-specific stimulation of CAR T cells is routinely used as a surrogate marker for effective anti-tumor immunity and serves as a standard lot release assay in CAR T clinical manufacturing. However, it remains unclear whether cytokine secretion is always linked to tumor killing activity and, therefore, which tumor-specific T cell functions should be optimized when developing new CAR T therapies.

Various techniques are currently used to assess tumor-specific T cell function, including flow cytometry, live-cell imaging, and chromium release to assess target cell killing and intracellular cytokine staining, ELISA, and ELISPOT to measure cytokine secretion. Most of these assays are destructive and do not allow for multidimensional analyses directly linking cytokine secretion to target cell killing. Until now, there has been no method that correlates these anti-tumor functions in single cells, which is important when facing the inherent heterogeneity of T cell populations.

In this application note we demonstrate how the Bruker Opto Cell Therapy Development workflow, run on the Lightning™ and Beacon® optofluidic systems, enables scientists to thoroughly characterize the anti-tumor activity of their T cells. We show how cytokine assays can be performed in combination with tumor killing assays, on thousands of individual T cells, to assess which cytokine secretion profiles correlate with efficient killing behavior. Once phenotypic assays have been run, live T cells can be recovered for genomic analysis, or further expanded on the Bruker Platform to assess their proliferative capacity.

MATERIALS AND METHODS

Generating and stimulating anti-CD19 CAR T cells

CD3+ T cells from healthy donor peripheral blood (STEMCELL Technologies) were cultured in T cell media containing Advanced RMPI 1640, 1X Glutamax, 10% human serum, and 50 µM β-Mercaptoethanol (Thermo Fisher Scientific) supplemented with 10 ng/mL IL-7 and 10 ng/mL IL-15 (R&D Systems). Cells were activated with anti-CD3/CD28 beads (Dynabeads, Thermo Fisher Scientific); viral transduction was performed with anti-CD19 chimeric antigen receptor (CAR) lentivirus expressing the scFv of an anti-CD19 antibody linked to a CD28 transmembrane domain/endodomain, and CD137 (4-1BB) and CD3-zeta signaling domains (Creative Biolabs). The vector also contained a truncated version of the epidermal growth factor receptor (EGFRt) coexpressed with the CAR, which can be used to identify CAR-expressing cells. Four days after transduction, cells were harvested and stained with a fluorescent antibody specific for EGFRt (R&D Systems). EGFRt+ cells were sorted on an Aria II flow cytometer (Becton Dickinson). Sorted T cells were expanded for 4 additional days and then cryopreserved.

Cytotoxicity, cytokine, and proliferation assays on the Beacon system

Cytokine capture beads specific for IFNγ were loaded into NanoPen™ chambers on OptoSelect™ chips. Then, single anti-CD19 CAR T cells and either CFSE+ CD19-expressing Raji cells (CD19 WT) or CD19-deficient Raji cells (CD19 KO) were loaded. Target cell killing was tracked by co-culturing cells while perfusing the chip with T cell media containing 5 µM NucView 530 Caspase-3 Substrate (Biotium); NanoPen chambers were imaged every 30 minutes in FITC and PE. We detected cytokine capture beads using a cocktail of biotinylated antibodies followed by PE-conjugated streptavidin (Biolegend). After an overnight incubation, the chips were transferred to the Culture Station™ system and perfused with T cell media for 5 days. Expanded T cells were detected using a PE-conjugated CD8 antibody (Biolegend). Data were analyzed using Assay Analyzer 2.1 and Image Analyzer 2.1 software.

RESULTS AND DISCUSSION

Correlating cytokine secretion and target cell killing activity in single T cells

Interferon gamma (IFNγ) supports effector responses of CD8+ cytotoxic T lymphocytes (CTLs) and is a surrogate marker for detecting T cells capable of killing target cells. On the population-level, studies have shown a strong correlation between T cell-mediated specific lysis of target cells and frequencies of IFNγ releasing-T cells[2]. To address whether both IFNγ secretion and cytotoxicity are also correlated in single cells, we combined a Cytokine Secretion Assay with a Tumor Cell Killing Assay on individual anti-CD19 CAR T cells on the Beacon system. Using CAS 2.1 and templates in the Bruker T Cell Cytotoxicity Protocol (Bruker MAN-08103), we loaded an OptoSelect chip with cytokine capture beads coated with antibodies against IFNγ. Single anti-CD19 CAR T cells were loaded into the same NanoPen chambers and incubated with CFSE-labeled Raji target cells (green) for approximately 16 hours. Fluorescent time-lapse imaging was performed during culture and apoptosis was monitored using a caspase-3 substrate that fluoresces when caspase-3 is activated (red). After overnight incubation, fluorescent antibodies were used to detect secreted IFNγ bound to capture beads. (**Figure 1**).

Using this experimental setup, we investigated the correlation between IFNγ secretion and tumor cell killing by single T cells. **Figure 2** highlights representative NanoPen chamber images from a single chip, in which anti-CD19 CAR T cells from the same bulk population were incubated with CD19-expressing Raji cells (green). Images from the first 5 hours of culture are shown. By detecting a fluorescent antibody that binds to IFNγ captured on the cytokine capture beads, we measured the amount of IFNγ secreted by the T cell during the entire incubation period.

This allowed us to identify fast-killing (**Figure 2A**) and slower-killing (**Figure 2B**) T cells. Unexpectedly, IFNγ secretion was not required for tumor cell killing: 8.3% of the single T cells observed across 3 OptoSelect chips killed tumor cells without IFNγ secretion; 8.8% killed tumor cells with IFNγ secretion; and 27.6% secreted IFNγ but did not kill tumor cells. The remaining 55% of cells did not secrete IFNγ nor did they kill tumor cells during the incubation period (**Figure 2C**).

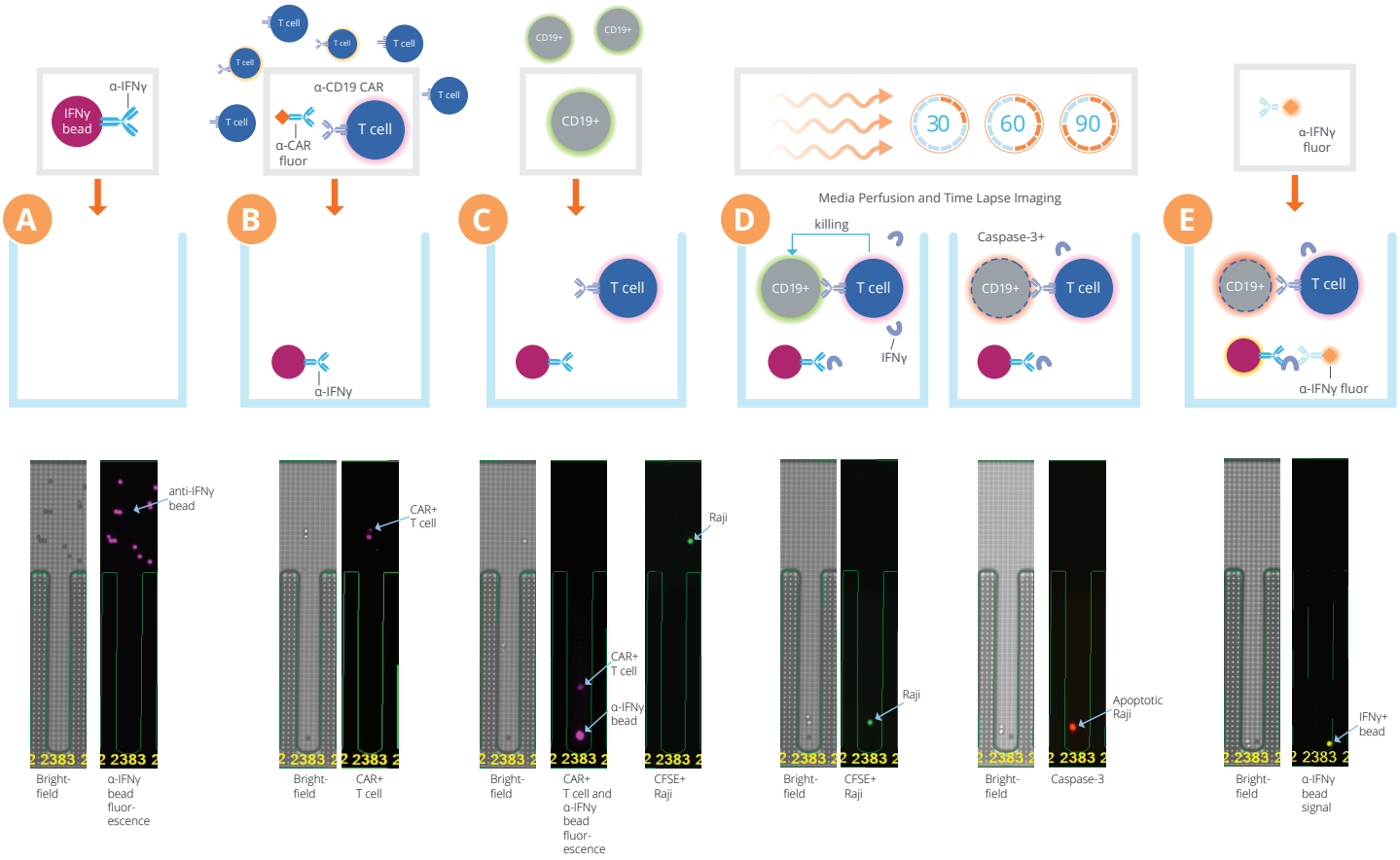

Figure 1. The Cytotoxicity Assay measures cytokine secretion and target cell killing in single CAR T cells. A. Images from representative NanoPen chambers show loading of anti-IFNγ capture beads, which are fluorescent in the Cy5 channel (magenta). **B.** Anti-CD19 CAR+ T cells are identified by fluorescence in the Cy5 channel (magenta) and are selectively loaded into NanoPen chambers. **C.** CFSE+ Raji cells are identified by fluorescence in the FITC channel (green) and selectively loaded into NanoPen chambers with T cells and cytokine capture beads. **D.** T cells and target cells are co-cultured and perfused with cell culture media containing a fluorescent reporter of caspase-3 activity. Fluorescent images are captured at intervals over 11–16 hours. Target cells which have activated caspase-3 are identified as PE+ (red) **E.** Chip is incubated with PE-conjugated α-IFNγ antibody (yellow). .

Correlating cytokine secretion and serial killing activity in single T cells

The ability of tumor-specific T cells to sequentially lyse multiple target cells (serial killing) is likely necessary for tumor eradication and there is significant interest in understanding the mechanisms driving this behavior[1]. Current techniques make analyzing these mechanisms challenging, because they don't allow tumor killing activity of individual T cells to be measured concurrently with cytokine secretion. Using a similar experimental setup as described above, we were able to investigate both tumor cell serial killing and cytokine secretion in single anti-CD19 CAR T cells.

Individual T cells were incubated with IFNγ capture beads and multiple CFSE- labeled CD19-expressing Raji cells (Figure 3A). Fluorescent time-lapse imaging was performed during culture and caspase-3 activation was monitored (red). After 7 hours of co-culture, the tumor cells had divided, yielding 7 tumor cells that could be identified in the FITC fluorescent channel. During the next 4 hours of co-culture, we observed a T cell engaging in 4 killing events at the 474-, 510-, 582-, and 672-minute time points (Figure 3B). We also detected IFNγ secretion from the same T cell (Figure 3C). Finally, we transferred the chip to the Culture Station system and incubated the T cell with the remaining tumor cells for 4 additional days. During this time, the T cell proliferated into a large colony. By

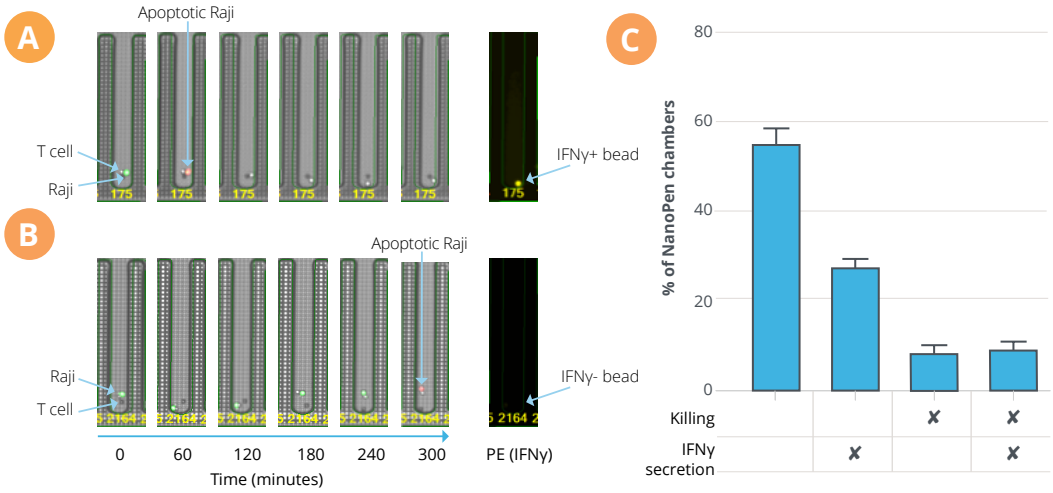

Figure 2. Directly linking cytokine secretion to tumor cell killing demonstrates that IFNγ secretion is not required for tumor cell killing. A. and **B.** Timelapse images from representative NanoPen chambers show T cells (unlabeled) interacting with target cells (green). Cells with activated caspase-3 fluoresce in PE (red). Beads that have captured IFNγ fluoresce in PE (yellow). Panel A highlights a NanoPen chamber in which tumor killing is associated with IFNγ secretion, while the NanoPen chamber in Panel B shows a cytotoxic T cell that does not secrete IFNγ. **C.** Quantification of cytotoxicity and cytokine secretion, measured as the percentage of NanoPen chambers loaded with a single T cell, Raji cells, and cytokine capture beads that exhibit IFNγ secretion and/or target killing. 8.8% of the T cells killed tumor cells and secreted IFNγ.

staining with an antibody that recognizes CAR+ T cells, we confirmed that the proliferating cells were CAR+, indicating that the observed T cell proliferation was antigen-specific (Figure 3D).

These data provide important insights into CAR T-tumor cell interactions that cannot be gleaned from other analytical approaches. By successfully combining analysis of serial killing, cytokine secretion, and proliferation on the same individual CAR T cells, we have defined a novel experimental setup by which new, improved CARs can be compared and may lead to therapeutics with increased efficacy.

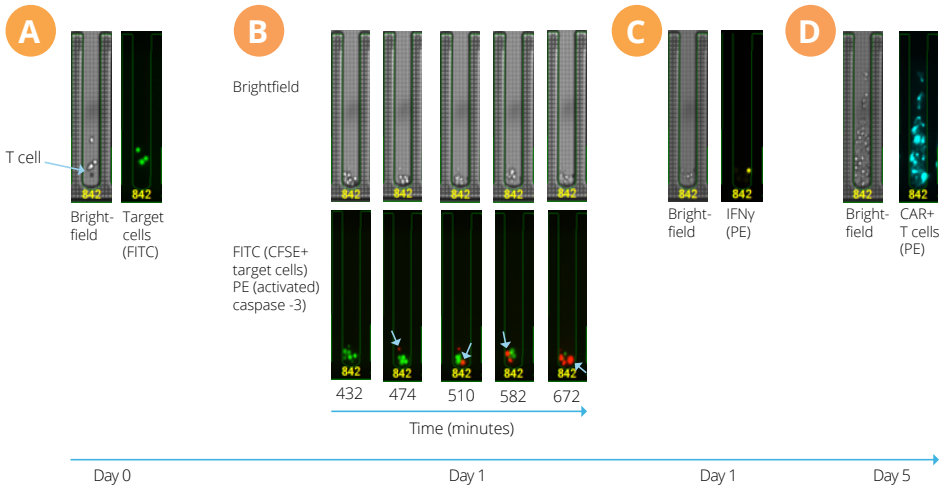

Figure 3. Multidimensional analysis of serial killing in single T cells. A. Images from a NanoPen chamber show a single T cell (unlabeled) at onset of incubation with target cells (green) **B.** Images from the same NanoPen chamber over time. Cells with activated caspase-3 fluoresce in PE (red). The arrows highlight serial killing events. **C.** Images from the same NanoPen chamber after 11 hours of incubation show that the single T cell has secreted IFNγ. The IFNγ-capture bead fluoresces in PE (yellow). **D.** After 5 days of culture, proliferating T cells are observed in brightfield and by staining with fluorescent anti-CAR antibody (blue). These images confirm that serial killing, IFNγ secretion and antigen-specific proliferation were observed from a single CAR T cell.

CONCLUSION

Modified T cells, like CAR T cells, have the potential to be mediators of powerful, improved immune responses for a list of diseases. To harness their true potential, we must understand the relationship between target cell killing and other critical functions of individual T cells. Multidimensional analysis of individual T cells has the greatest potential to provide the in-depth understanding of target cell killing needed to generate more efficacious therapies.

We have demonstrated that multidimensional analysis of individual T cells is possible using the Opto Cell Therapy Development workflow and the Bruker Platform. The ability to combine assays lets users interrogate antigen-specific serial killing activity of single CAR T cells and correlate this killing activity to other functional attributes, such as cytokine secretion and proliferation. The workflow is non-destructive, preserving T cells of interest for recovery for downstream analyses or expansion, providing insights that will be critical to developing more efficacious therapies.

REFERENCES

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