APPLICATION NOTE

Profile and Recover Polyfunctional T Cells Using Multiplexed Cytokine Secretion Assays

APPLICATION SPOTLIGHT

- Collect physiologically relevant data by simultaneously profiling multiple cytokines secreted from an individual, live T cell
- Recover live T cells for further characterization to link cytokine profiles with gene expression
- Multiplex with other functional assays to comprehensively classify T cell subpopulations

INTRODUCTION

T lymphocytes play a central role in the adaptive immune system and are the major mediator of anti-tumor immunity. They are highly heterogeneous in both phenotype and functional profile and are classified based on cell-surface markers, like CD4 and CD8, and the type of cytokines that they produce when the T cell is activated. T cells are also classified as either monofunctional T cells that produce one cytokine in response to antigen, or polyfunctional T cells that secrete multiple cytokines to more effectively respond to antigen[1]. With the emergence of immuno-oncology as a treatment path for several types of cancer, profiling T cells by the pattern of cytokine secretion and linking that profile to T cell function is important since polyfunctional T cells are considered the most potent effector T cells against tumors[2]. Therefore, understanding the underlying mechanisms of polyfunctional T cell differentiation and function is likely critical for the development of effective therapeutics. However, most existing methods for analyzing T cell cytokine secretion at the single cell level are destructive, as live cells cannot be recovered following analysis, preventing studies that correlate cytokine secretion with other functional parameters like cytotoxicity and gene expression.

In this application note, we share two use cases to demonstrate how the Multiplex Cytokine Assay in the Opto™ Cell Therapy Development 1.0 workflow, running on the Lightning™ system, can identify individual T cells that secrete up to three cytokines and upregulate activation markers. Berkeley Lights' Multiplex Cytokine Assay screens more than one thousand single T cells in a single experiment and is customizable for maximum flexibility. Measure the secretion of many combinations of three cytokines in response to antigenic stimulation by physiologically relevant T cells or chemical stimulants. This assay can be multiplexed with other functional assays in the workflow and live cells can be recovered for genomic analyses to investigate the underlying mechanisms driving T cell functional diversity.

MATERIAL AND METHODS

Generating and stimulating anti-CD19 CAR T cells

We cultured healthy donor peripheral blood mononuclear CD3+ T cells (StemCell Technologies) in T cell media containing Advanced RMPI 1640, 1X Glutamax, 10% human serum, and 50 µM β-Mercaptoethanol (Thermo Fisher Scientific), supplemented with 25 ng/mL IL-7 and 10 ng/mL IL-15 (R&D Systems, Bio-Techne). Cells were activated for 3 days with anti-CD3/CD28 Dynal beads (Thermo Fisher Scientific) at a ratio of 1 bead:1 cell. To generate CAR cells, we used an anti-CD19 chimeric antigen receptor (CAR) lentivirus expressing the scFv of anti-CD19 antibody linked to a CD28 transmembrane domain/ endodomain, and CD137 (4-1BB) and CD3-zeta signaling domains (Creative Biolabs) for viral transduction. The lymphoblast-like cells derived from Burkitt's lymphoma (Raji) (ATCC® CCL-86™) expressing the CD19 antigen (CD19-WT Raji) were used as target cells. CD19-null Raji cells generated in-house using the CRISPR-Cas9 system were used as a negative control (CD19-KO Raji).

Isolating and stimulating Th17 cells

Th17 cells were enriched from healthy donor peripheral blood mononuclear cells using EasySep™ Human Memory CD4+ T cell Enrichment and EasySep™ Human Th17 Cell Enrichment kits for magnetic bead isolation (StemCell Technologies). Isolated T cells were cultured in T cell media containing IL-7 and IL-15.

Anti-CD19 CAR T cell experiments on the Lightning instrument

We loaded OptoSelect™ chips with cytokine capture beads specific for IFNγ (B3), TNF-α (B7), and IL-2 (A5) (Biolegend). The beads were differentiated by size and internal fluorescence intensity in the CY5 channel. Each bead type was imported and loaded separately and sequentially, starting with the bead with the lowest internal fluorescence (IFNγ), followed by the bead with intermediate fluorescence (IL-2), and finally the bead with the highest internal fluorescence (TNF-α). Individual T cells were then loaded into the NanoPen™ chamber followed by CD19-WT Raji cells. A subset of the chip was loaded with CD19-KO Raji cells before the chip was perfused with T cell media overnight. Cytokines captured by the beads were detected by incubating with a cocktail of biotinylated antibodies followed by PE-conjugated streptavidin (Biolegend). CD137 expression on T cells was detected using a Brilliant Violet™ 421-conjugated antibody. The data generated were analyzed using Cell Analysis Suite software.

Th17 experiments on the Lightning instrument

OptoSelect chips were loaded with cytokine capture beads specific for IFNγ (B2), IL-17A (B5), and IL-22 (B9) as described above. Single T cells were then loaded into the NanoPen chamber and the chip was perfused with a cocktail of phorbol 12-myristate 13-acetate (PMA) and ionomycin for 8 hours (eBioscience). Cytokines were detected and data analyzed as described above.

RESULTS AND DISCUSSIONS

Use case 1: Profiling polyfunctional CAR T cells

Chimeric antigen receptor T cells, also known as CAR T cells, are T cells that have been genetically engineered to express antigen receptors specific for tumor antigens. Inflammatory cytokine secretion by CAR T cells plays an important role in mediating tumor lysis, and the efficacy of anti-CD19 CAR T cell therapy is associated with the presence of polyfunctional T cells in the infusion product[3,4]. Canonical cytokines produced by polyfunctional CAR T cells include IFNγ, TNF-α, and IL-2.

We measured cytokine secretion from single anti-CD19 CAR T cells by first loading anti-IFNγ, anti-IL-2, and anti-TNF-α capture beads into individual NanoPen chambers (Figure 1A). The IFNγ and TNF-α capture beads were larger with low and high CY5 internal fluorescence, respectively and the IL-2 capture beads were smaller with intermediate internal fluorescence. Single T cells were then loaded into individual NanoPen chambers and stimulated overnight using CD19-WT Raji targeT cells. A subset of NanoPens were loaded with CD19-KO Raji

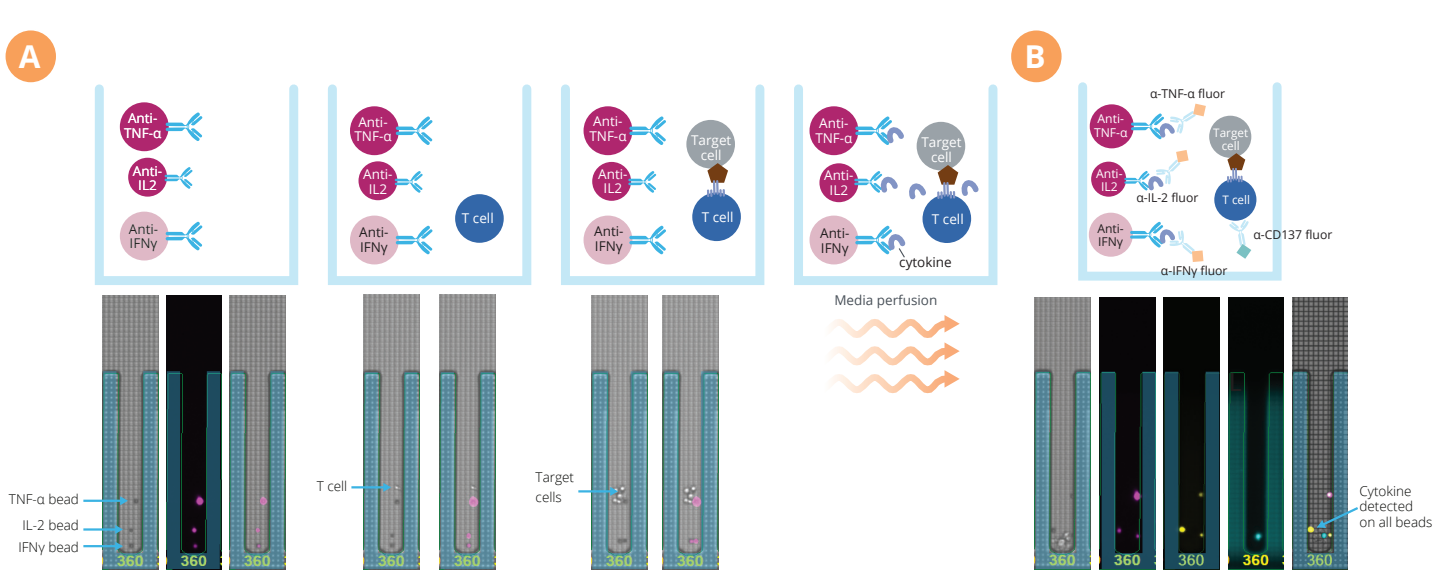

Figure 1. Multiplexed cytokine assay identifies single CAR T cells that secrete IFNg, IL-2 and TNF-α, and upregulate CD137.

cells to serve as a negative control. After the overnight incubation, we measured the cytokines bound by the capture beads by incubating with a cocktail of biotinylated antibodies followed by PE-conjugated streptavidin. CD137 expression on T cells was detected using a Brilliant Violet 421-conjugated antibody (Figure 1B).

The Multiplex Cytokine Assay identified single anti-CD19 CAR T cells that secrete different combinations of the three cytokines (Figure 2A). When the anti-CD19 CAR T cells were incubated with CD19-expressing target T cells, approximately 6% of the cells secreted only IFNγ, 3% of the cells secreted only TNF-α, and 1.5% of the cells secreted only IL-2. 11% of the cells secreted both IFNγ and TNF-α, 1% of the cells secreted IFNγ and IL-2, and 4% of the cells secreted TNF-α and IL-2. Finally, 10% of the cells secreted all three cytokines. Negligible cytokine secretion was detected in NanoPens with T cells incubated with CD19-null target T cells. Interestingly, only 20–40% of single cytokine-producing T cells upregulated CD137, while 60–90% of multicytokine-secreting T cells exhibited CD137 expression (Figure 2B).

Use case 2: Profiling Th17 cells

T helper 17 (Th17) cells are a subset of T helper cells that secrete IL-17, IFNγ, and IL-22 and have both pro- and anti-tumorigenic function. They can increase tumor progression by promoting angiogenesis and immunosuppressive mechanisms, and inhibit tumor progression by recruiting effector T cells and NK cells to tumors[5,6]. These disparate activities are driven by the combination of cytokines secreted by the Th17 cell subset. Understanding the mechanisms driving Th17 differentiation can provide insights into the role Th17 cells play in promoting or inhibiting tumorigenesis to aid in the design of more efficacious therapies.

We measured IFNγ, IL-17, and IL-22 secretion in individual Th17 cells by first loading anti-IFNγ, anti-IL-17, anti-IL-22 capture beads into individual NanoPen chambers (Figure 3A). The IFNγ B2 and IL-22 B7 capture beads were larger with low and high CY5 internal fluorescence, respectively and the IL-17 A5 capture beads were smaller with intermediate internal fluorescence. Single Th17 cells were loaded into individual NanoPen chambers and perfused for 8 hours with media containing PMA and ionomycin to stimulate cytokine secretion. NanoPen chambers loaded without any T cells served as negative

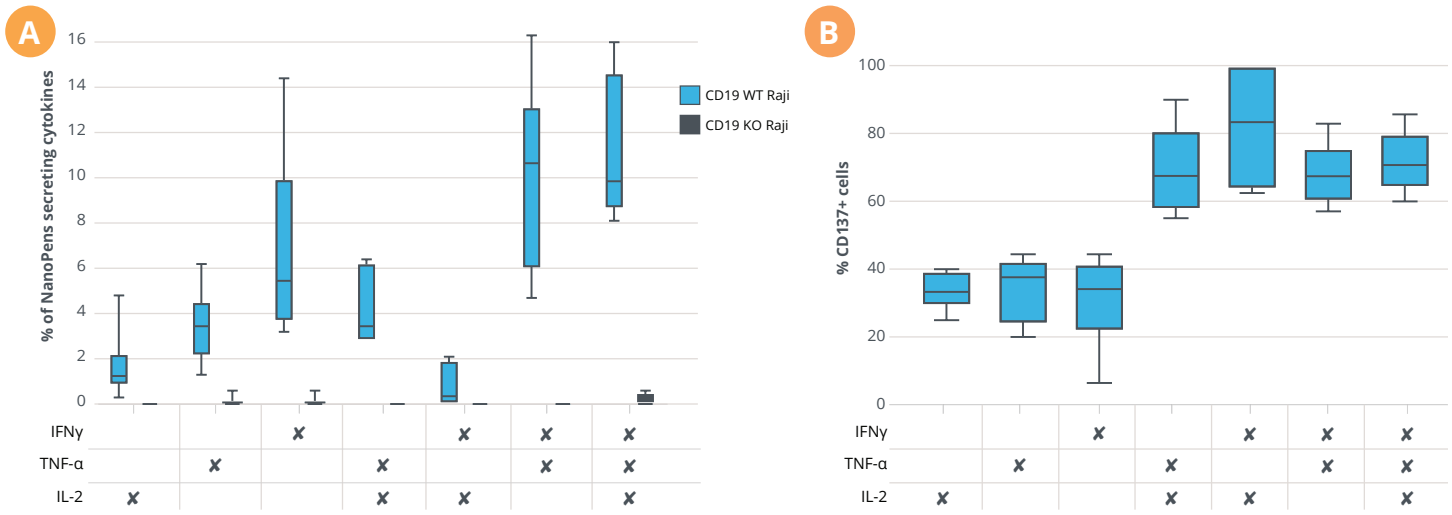

Figure 2. Quantitative cytokine profile generated using the multiplexed cytokine assay identifies subsets of monofunctional and polyfunctional CAR T cells along with CD137 upregulation. A. Quantification of cytokine secretion measured as the percentage of NanoPen chambers loaded with a single T cell, Raji cells and three cytokine capture beads that show a fluorescent signal in the PE channel, indicating the presence of cytokine on the surface of one or more beads. NanoPen chambers loaded with CD19-null cells serve as negative control. **B.** Quantification of CD137 expression on T cells measured as the percentage of cells secreting indicated cytokines that upregulate CD137.

control. The secreted cytokines were detected by incubating with a cocktail of biotinylated antibodies followed by PE-conjugated streptavidin (Figure 3B).

Populations of Th17 cells expressing different combinations of the cytokines screened were identified using the Multiplex Cytokine Assay (Figure 4). Roughly a third of the Th17 cells were monofunctional, as 19% of the Th17 cells secreted only IFNγ, 1% secreted only IL-17, and 11% of the cells secreted only IL-22. The remaining cells were polyfunctional as 30% of the cells secreted both IL-22 and IFNγ, 4% of the cells secreted IL-22 and IL-17, and 1% of the cells secreted IL-17 and IFNγ. The remaining 11% of cytokine-secreting cells secreted all three cytokines assayed.

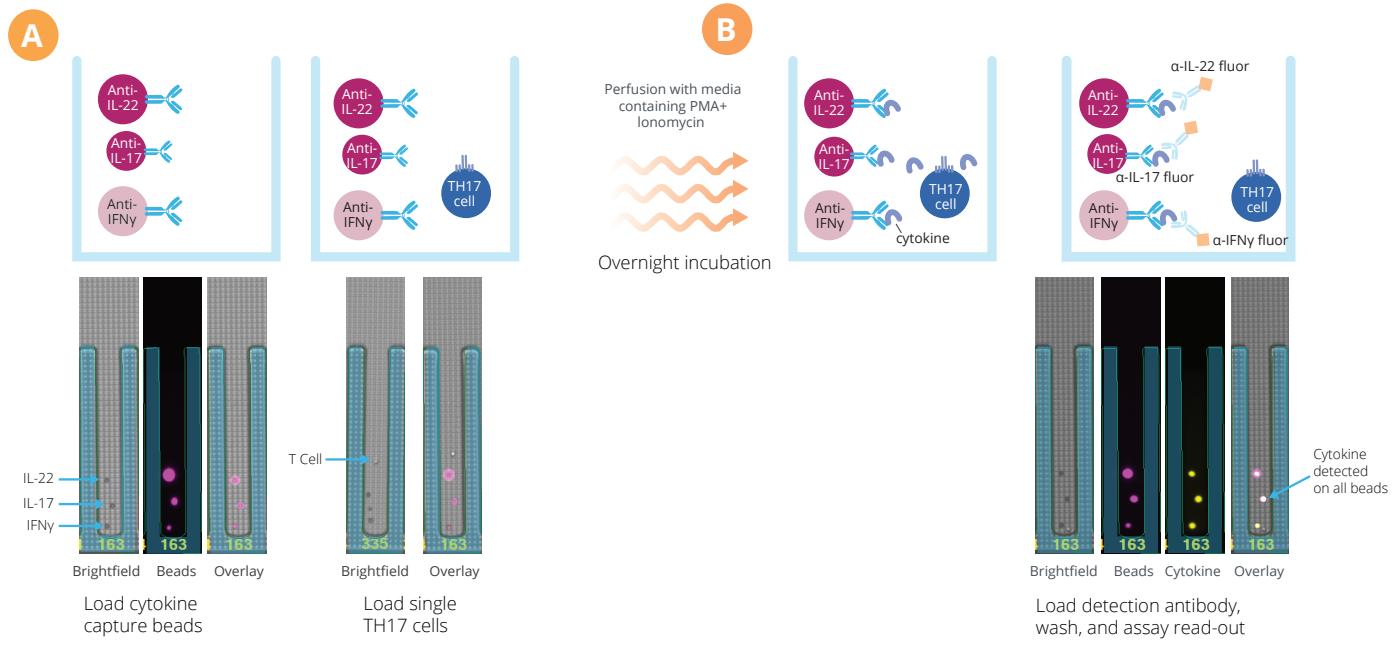

Figure 3. Multiplexed cytokine assay identifies single Th17 cells that secrete IL-22, IL-17, and IFNγ.

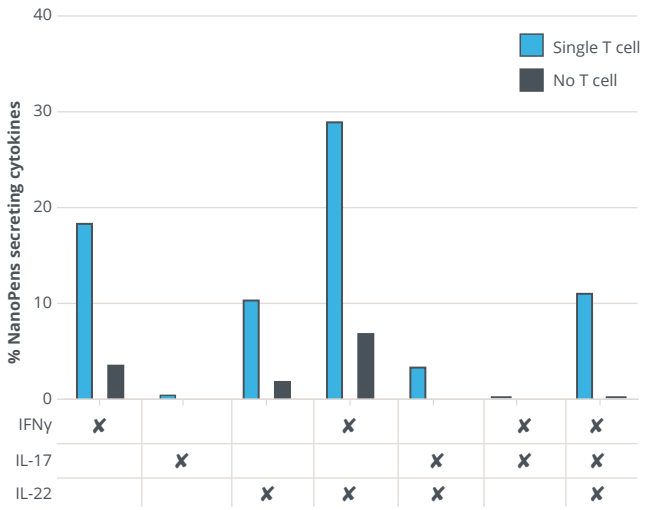

Figure 4. Quantitative cytokine profile generated using multiplexed cytokine assay identifies subsets of monofunctional and polyfunctional Th17 cells. Quantification of cytokine secretion measured as the percentage of NanoPen chambers loaded with a single T cell, and three cytokine capture beads that show a fluorescent signal in the PE channel, indicating the presence of cytokine on the surface of the bead. NanoPen chambers loaded with no T cell serve as negative control.

CONCLUSIONS

We present two different use cases that demonstrate how more than a thousand individual T cells can be simultaneously analyzed using the Multiplexed Cytokine Assay on the Lightning system to identify monofunctional and polyfunctional T cells and measure upregulation of activation markers. The flexibility of the assay lets users measure any combination of three cytokines secreted from the single T cell of their choice, which can be stimulated through cell-cell interactions or chemical stimulants like PMA and ionomycin. Importantly, this non-destructive process enables live T cell recovery for downstream analysis.

Polyfunctional T cells are critical mediators of antitumor immunity, yet there is still much to learn about how polyfunctional cytokine responses by individual cells contribute to an immune response. The Multiplex Cytokine Assay from Berkeley Lights can help elucidate this process by measuring the secretion of multiple cytokines from individual T cell subsets. This assay can also be combined with other functional assays in the Opto Cell Therapy Development 1.0 workflow and live cells can be recovered for genomic analyses focused on the underlying mechanisms driving T cell functional diversity. This ability to correlate multiple functional assays on single T cells to gene expression will be critical in developing therapies that promote anti-tumor T cell responses.

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