APPLICATION NOTE

Perform Multi-omic Analysis to Directly Link T Cell Polyfunctionality to Gene Expression

APPLICATION SPOTLIGHT

- Simultaneously measure the secretion of multiple cytokines to identify polyfunctional T cells
- Perform multi-omic analysis of differential gene expression and polyfunctionality of live T cells to gain insight into T cell biology
- Explore the genetic drivers of the most promising T cells to develop more effective therapeutics

INTRODUCTION

Polyfunctional T cells, which produce multiple cytokines, are considered the most potent anti-tumor effector T cells[1]. Understanding the underlying mechanisms of polyfunctional T cell differentiation and function is critical for developing effective therapeutics. However, T cells are extremely heterogeneous, in both phenotype and function, and have variable responses to stimulation. This makes predicting specific T cell responses extremely difficult.

To identify polyfunctional T cells within a heterogeneous sample, scientists must first be able to characterize cytokine secretion at the single-cell level. However, most existing single-cell methods, such as ELISPOT, are destructive end point assays and live T cells cannot be recovered after analysis. Such methods prevent the correlation of cytokine secretion with other functional parameters like cytotoxicity or gene expression.

This application note demonstrates how the Opto™ Cell Therapy Development workflow makes it possible to identify drivers of T cell polyfunctionality by characterizing the function of single T cells and linking this phenotypic data to the gene expression profiles of those same cells. First, the Multiplex Cytokine Assay was used to characterize the secretion of three cytokines from individual T cells. Then, the OptoSeq™ Single Cell 3' mRNA kit was used to perform single cell RNAseq on those same cells. This multi-omic workflow is the only commercially available workflow that enables phenotype and gene expression to be linked using the same, live T cells.

Berkeley Lights PrimeSeq™ software facilitates this analysis on the Berkeley Lights Platform by correlating phenotypic data collected on the Beacon® optofluidic system to RNAseq data, enabling you to easily identify the genes that contribute to T cell polyfunctionality. These data can be used to identify T cells for therapeutic development and engineer T cells with greater clinical efficacy.

MATERIALS AND METHODS

Generating and stimulating T cells

To generate melanoma antigen-specific T cells, we isolated naïve, CD8+ T cells from HLA-A2:01 donor peripheral blood mononuclear cells and stimulated them with autologous dendritic cells (DCs) that had been pulsed with

an antigenic peptide. We cultured T cells with DCs for two weeks, and then sorted tetramer positive cells. Sorted T cells were expanded for 13 days on irradiated feeder cells and then were cryopreserved. We thawed cells one day before the experiment and incubated them overnight 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).

Characterizing polyfunctionality

In order to identify polyfunctional T cells, we performed the Multiplex Cytokine Assay according to its protocol (BLI Protocol Manual MAN-08113). We loaded individual NanoPen™ chambers on OptoSelect™ chips with three different cytokine capture beads conjugated to antibodies specific to cytokines of interest (IFNγ, TNF-α, and IL-2) (Figure 1). Fluorescence intensity in the Cy5 channel differentiated the cytokine beads. Each bead type was imported and loaded separately and sequentially, starting with the bead with the highest internal fluorescence (IFNγ), followed by the bead with intermediate fluorescence (IL-2), and finally the bead with the lowest internal fluorescence (TNF-α).

To stimulate cells overnight, we loaded 1,500–2,500 antigen-enriched T cells per chip and used anti-CD3/anti-CD28 beads. During overnight incubation, secreted cytokines bound to the capture beads and we detected them using a cocktail of biotinylated antibodies followed by PE-conjugated streptavidin.

Profiling single-cell gene expression

We used the OptoSeq Single Cell 3' mRNA kit to assess the gene expression of individual T cells of interest. We loaded a single mRNA capture bead into each chamber containing a T cell and the OptoSeq kit generated barcoded cDNA. Cells lysed on-chip and barcoded beads containing a poly T tail captured mRNA released from the T cells (Figure 1). Then, captured mRNA was reverse transcribed into cDNA. We exported barcoded beads with cDNA into a 96-well plate in batches of up to 120 unique beads per well. Up to 24 bead batch exports can be performed.

After export, we amplified cDNA to prepare sequencing libraries and pooled it. We used the Illumina next-generation sequencing (NGS) platform to sequence cDNA. PrimeSeq software assigned unique optical barcodes to associated T cells in specific NanoPen chambers. This association enabled the on-chip phenotypic data to be linked to the barcoded cDNA and resulting sequences.

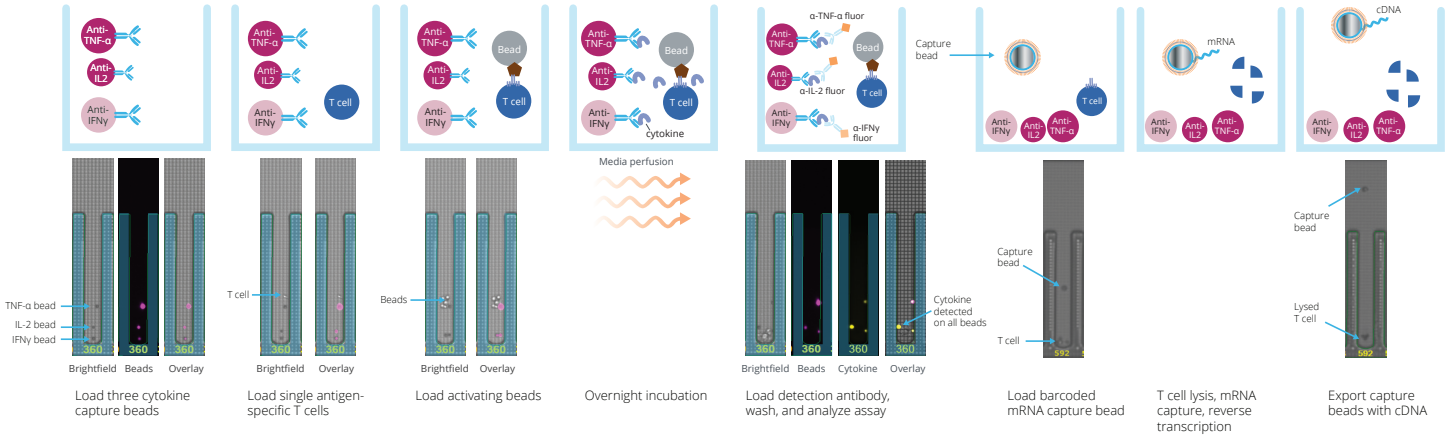

Figure 1. The Multiplex Cytokine Assay followed by the OptoSeq Single Cell 3' mRNA kit (mRNA capture and cDNA generation) and subsequent sequencing enable the linking of T cell polyfunctionality with gene expression profiles.

RESULTS AND DISCUSSION

Identifying polyfunctional T cells

Cytokine secretion by T cells plays an important role in mediating tumor cell killing, and therapeutic efficacy is associated with the presence of polyfunctional T cells[2,3]. Canonical cytokines produced by polyfunctional T cells include IFNγ, TNF-α, and IL-2. To that end, we performed the Multiplex Cytokine Assay to identify single CD8+ T cells that secreted some combination of these three cytokines (Figure 2). We loaded an OptoSelect chip with cytokine capture beads coated with antibodies against IFNγ, TNF-α, and IL-2, single CD8+ T cells, and activator beads and evaluated cytokine secretion to identify T cells producing one, two, or three cytokines in response to antigenic stimulation (Figure 1). This allowed us to identify 229 (32%) monofunctional T cells that only secreted one cytokine as well as 274 (38%) polyfunctional T cells that secreted two and 215 (30%) that secreted all three cytokines.

Linking phenotypic data to gene expression

Directly correlating polyfunctionality to specific gene expression profiles in single cells makes it possible to understand the underlying mechanisms of T cell diversity and therefore develop more effective therapeutics. To identify the genes that drove the different phenotypes identified by the Multiplex Cytokine Assay, we used the OptoSeq Single Cell 3' mRNA kit to genetically characterize the same T cells. Barcoded mRNA beads in this kit enable the linking of individual cells' mRNA expression patterns to their phenotypic data. Using the resulting data, we generated UMAP plots using PrimeSeq software to observe how cell phenotypes clustered based on their gene expression profiles (Figure 3).

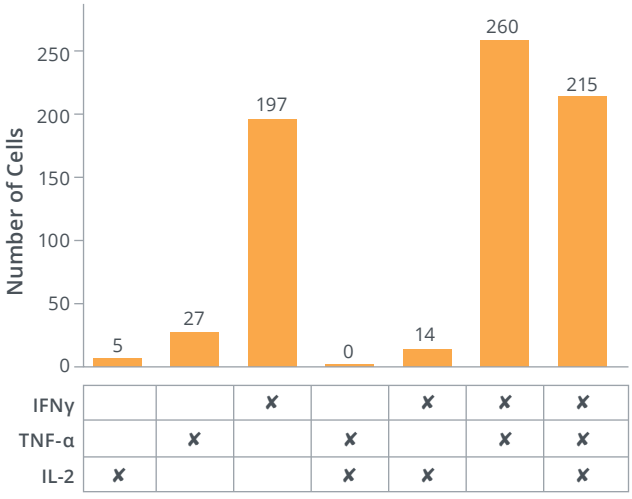

Figure 2. The Multiplex Cytokine Assay reveals the spectrum of monofunctional and polyfunctional CD8+ T cells. Quantification of cytokine secretion measured as the number of NanoPen chambers loaded with a single T cell and three cytokine capture beads that show a fluorescent signal in the PE channel, indicating the presence of cytokine on the surface of the bead.

When overlaying the phenotypic data onto the UMAP plots, two distinct populations were observed; the majority of the non-secreting and monofunctional cells formed one cluster (lower left) while the polyfunctional T cells formed another cluster (top right) (Figure 3 A-D). We also generated two UMAP plots of significantly upregulated and downregulated genes in these populations to reveal how their expression patterns correlate to polyfunctionality (Figure 3E, F). The GNLY gene was downregulated in polyfunctional cells. The FABP5 gene, however, was upregulated in polyfunctional T cells, linking it to the phenotype.

To explore the genetic drivers of polyfunctionality, we next compared the gene expression profiles of triple-secreting T cells and IFNγ-secreting T cells. To that end, we generated volcano and MA plots using PrimeSeq software (Figure 4).

These gene expression plots clearly indicate specific genes that significantly differed between the phenotypes. These genes and their pathways are the most probable drivers of polyfunctionality. In order to demonstrate the reproducibility of these data, we repeated the experiment and compared the gene expression patterns. We observed high correlation between the statistically significant genes from the replicate experiments (Figure 5).

Revealing the drivers of T cell polyfunctionality

In order to understand the genes that drive polyfunctionality, we tabulated the upregulated and downregulated genes identified in our differential expression analysis. Many of the genes that were expressed in polyfunctional T cells are known to regulate immune response (Table 1).

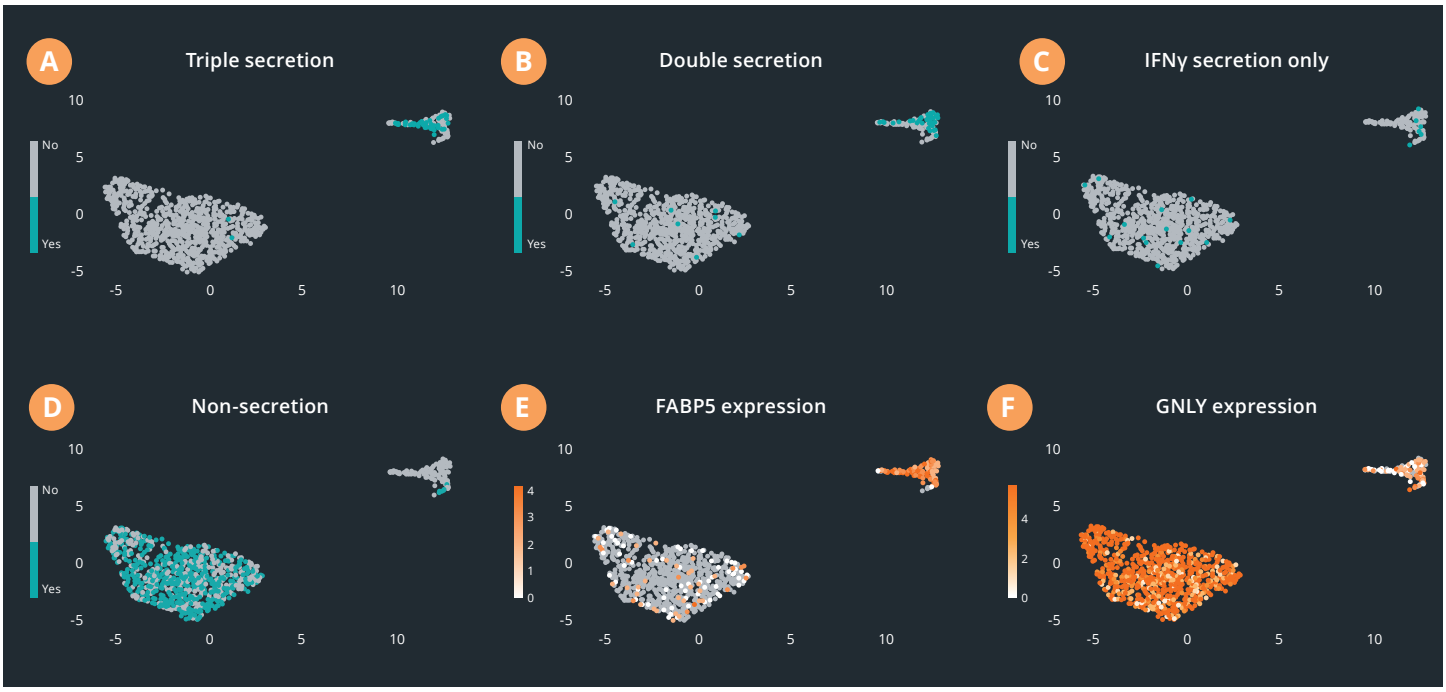

Figure 3. PrimeSeq software provides a powerful way to visually differentiate cell phenotype by gene expression. A and **B.** Polyfunctional T cells cluster to the top right. **C** and **D.** T cells secreting only INFγ as well as non-secreting T cells largely cluster in the lower left. **E.** The FABP5 gene is highly expressed in the polyfunctional T cells, suggesting it can be linked to this phenotype. **F.** The GNLY gene is highly expressed in the non- and IFNγ-secreting T cells and lowly expressed in the polyfunctional T cells, suggesting the gene is downregulated with polyfunctionality. One data point is a single cell.

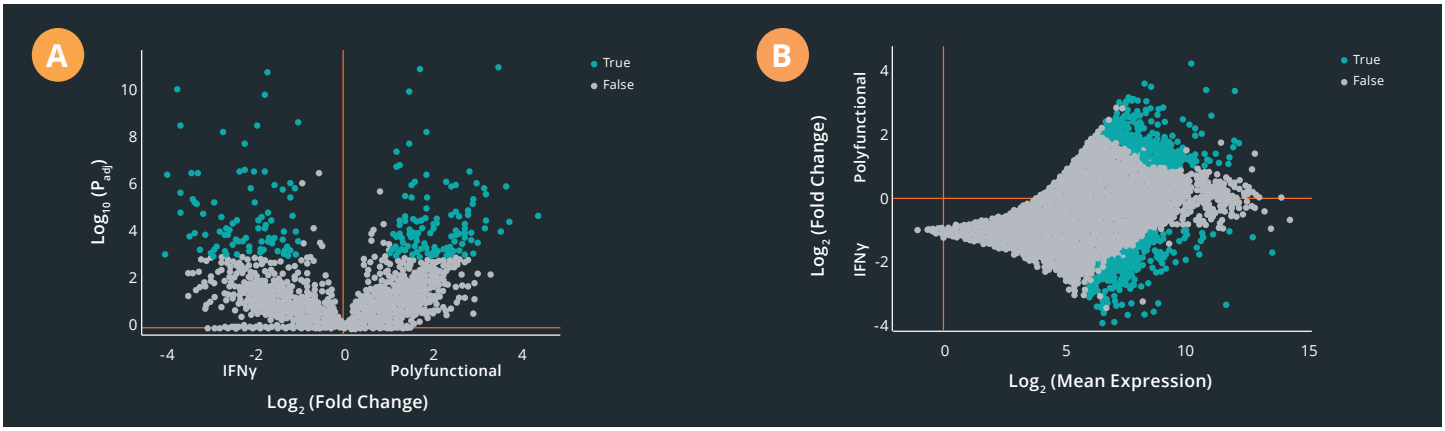

Figure 4. PrimeSeq software identifies potential drivers of polyfunctionality. A. A volcano plot and **B,** MA plot generated in PrimeSeq software to compare gene expression in triple and single secretors clearly highlight a subset of significant genes linked to each phenotype. p<0.01. Each data point is a gene of interest.

Both upregulated and downregulated pathways provided clues into which genes were driving polyfunctionality. We observed several upregulated genes known to be involved in cytokine and chemokine signaling, metabolism, cell proliferation, and transcriptional regulation. Conversely, many of the downregulated genes are involved in cAMP signaling (S100A4, S100A6, and TPT1), a potent negative regulator pathway of T cell receptor-mediated activation[4]. Most importantly, several genes that were downregulated are related to cytoskeletal signaling and structure. Cytoskeletal adaptivity regulates T cell receptor signaling — these genes could be potential targets for modifying therapeutic intervention[5].

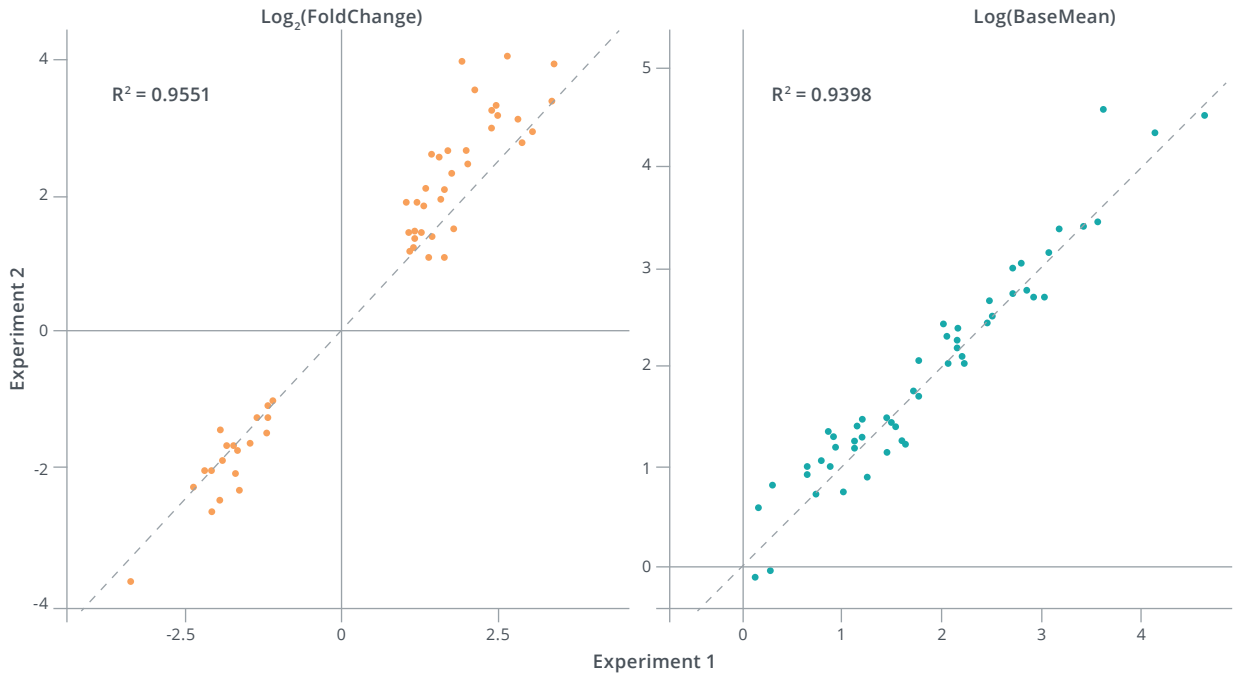

Figure 5. Running the Opto Cell Therapy Development workflow produces consistent results. Comparing results from two identical experiments revealed a high correlation of the statistically significant genes ($p<0.01$, $\log_2 FC \geq 1$).

UPREGULATED GENES IN POLYFUNCTIONAL T CELLS		DOWNREGULATED GENES IN POLYFUNCTIONAL T CELLS	
Gene	**Pathway / Function**	**Gene**	**Pathway / Function**
BOLA2B	Interleukin-12-mediated signaling pathway	AES	Innate immune system
CCL1	Chemokine super family	AHNAK	Phospholipase-C pathway
CCL13	Chemokine super family	CCSER2	Microtubule binding
DUSP4	Ret Signaling / TLR4 signaling	CD3D	T cell receptor signaling
DUT	Metabolism	CD3G	T cell receptor signaling
FABP5	Innate immune system	CYTIP	Family of immune cell regulators
H2AFZ	Epigenetic regulation	EMB	MAPK ERK pathway
HSPD1	Transcriptional regulation	**EVL**	**Cytoskeletal signaling**
IRF8	IFNγ signaling	FTH1	Innate immune system
JUNB	Cytokine signaling in immune system	GNLY	Innate immune system / allograft rejection
LINC00152	Cell proliferation	IKZF1	Dendritic cell development / innate lymphoid differentiation
MCM5	DNA replication / cell cycle	ITGA1	Focal adhesion / ERK signaling
MCM7	DNA replication / cell cycle	LAPTM5	Lysosome
MIR4435-1HG	lncRNA gene regulation	LCK	Ret signaling
NME1	Metabolism / translation control	MALAT1	Cell cycle regulation
PCNA	DNA repair	PTPRC	T cell receptor signaling / innate immune system
PKM	Metabolism	PTPRCAP	TNFα Signaling Pathway
PPIA	Innate immune system	S100A4	Ca, cAMP and Lipid signaling
RGS16	GPCR signaling / MAPK ERK signaling	S100A6	Ca, cAMP and Lipid signaling
RPS26	rRNA processing	SATB1	Chromatin regulation
SMS	Circadian gene expression	STK17B	NF-κB signaling
TYMS	Metabolism	**TPT1**	**Cytoskeletal signaling, Ca, cAMP and lipid signaling**
		VIM	**Cytokine signaling in immune system, ERK signaling**

Table 1. List of the genes exhibiting the highest significant difference in gene expression between polyfunctional T cells and monofunctional T cells. Examples of potential targets for modifying therapeutic response are highlighted.

CONCLUSION

Polyfunctional T cells are considered to have the greatest potential as highly effective therapeutics. The ability to study and better understand the genes and pathways that drive polyfunctionality may help to reveal mechanisms that contribute to T cell therapeutic efficacy and provide insights into how efficacy can be improved.

In this application note, we demonstrated how to use the Opto Cell Therapy Development workflow to reveal the genetic drivers of desired T cell phenotypes. Polyfunctional T cells can be identified using the Multiplex Cytokine Assay and polyfunctionality can be directly linked to associated gene expression profiles using the OptoSeq Single Cell 3' mRNA kit. This unique capability to directly link phenotype to gene expression provides new insight into T cell biology, immune responses, and how to design powerful T cell therapeutics.

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