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Literature deep dive:

High-resolution spectral flow compared to conventional flow: what are developers finding?

Ramji Srinivasan
Teiko CEO

Problem

"If a scientist is interested only in T cells, as that is the main modality of the drug, why would he or she do the extra work to analyze the rest of the immune state?"

- Paraphrased from an immunotherapy developer

Literature tour

Three examples

Paper	# Markers on Panel	Sample Type	# Patients	Stage
Design, optimisation and standardisation of a high-dimensional spectral flow cytometry workflow assessing T-cell immunophenotype in patients with melanoma - PMC	27, 20	PBMC	15 melanoma, 16 healthy	Pre-treatment
Combination of pembrolizumab and pelareorep promotes anti-tumour immunity in advanced pancreatic adenocarcinoma (PDAC) | British Journal of Cancer	20	PBMC	13 pancreatic adenocarcinoma	Phase 2
PSCA-CAR T cell therapy in metastatic castration-resistant prostate cancer: a phase 1 trial | Nature Medicine	25	PBMC	14 prostate cancer	Phase 1

Melanoma: Simplified Study Design

15 melanoma patients
16 healthy controls

27 marker resting T-cell panel

20 marker activated panel

Van Zelm, et al 2023

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Target marker	Fluorochrome
Ki67	BUV395
Viability	Live/Dead blue
CD45RA	BUV496
CD19	BUV563
ICOS	BUV661
CD56	BUV737
CD3	BUV805
TIGIT	BV421
CD57	Pacific Blue
CD16	BV510
CXCR5	BV605
CCR7	BV750
PD-1	BV786
TIM-3	BB515
CD8	Spark Blue 550
CD45	PerCP
TCRγδ	PerCP-Vio700
Tox	PE
CD4	cFluorYG584
CD95	PE-Dazzle594
Tbet	PE-Cy5
CD25	PE-Fire 700
EOMES	PE-Cy7
KLRG1	APC
IRF4	AF647
CD127	R718
CD39	APC-Fire750

27 marker Resting Panel

Target marker	Fluorochrome
TNFα	BUV395
Viability	Live/Dead blue
CD45RA	BUV496
CD19	BUV563
CD3	BUV805
IL-10	BV421
Granzyme B	BV510
IFNγ	BV605
CCR7	BV750
CTLA-4	BV786
IL-2	AF488
CD8	Spark Blue 550
CD45	PerCP
TCRγδ	PerCP-Vio700
CD4	cFluorYG584
CD95	PE-Dazzle594
CD25	PE-Fire 700
IL-17A	PE-Cy7
IL-4	APC
CD127	R718

20 marker Activated Panel

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Results

Statistically significant findings (and those ruled-out)

- ⬆ activated γδT cells in melanoma patients compared to controls.

- ⬇ IL-17A (P = 0.027) and TNFα (P = 0.047) in melanoma patient γδT cells.

- ⬆ expression of CD57 (P = 0.041) and EOMES (P = 0.033) in melanoma patient γδT cells.

What if?

Marker	Fluorochrome
CD3	BUV805
CD4	BV605
CD8	Spark Blue 550
CD45	BUV496
CCR7	BV750
TCRgd	PerCP-Vio700
CD19	BUV563
Viability Dye	BUV395

8 marker truncated panel

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Marker	Fluorochrome	
CD3	BUV805	✅
CD4	BV605	
CD8	Spark Blue 550	
CD45	BUV496	✅
CCR7	BV750	
TCRgd	PerCP-Vio700	✅
CD19	BUV563	✅
Viability Dye	BUV395	✅

Supplementary table 2: Panel 1 (resting) population definitions.

|	Population name	Phenotype definition
	All populations gated from live lymphocytes:	CD45$^+$ SSC^{low} Live/Dead Blue$^-$
1	B cells	CD3$^-$ CD19$^+$ ✅ ✅
2	NK cells	CD3$^-$ CD19$^-$ CD16/CD56$^+$
3	T cells	CD3$^+$ CD19$^-$
4	└ TCRγδ$^+$	CD3$^+$ CD19$^-$ TCRγδ$^+$
7	└ TCRαβ$^+$	CD3$^+$ CD19$^-$ TCRγδ$^-$ ✅ ✅ ✅
10	└ CD4$^-$ CD8$^-$	CD3$^+$ CD19$^-$ TCRγδ$^-$ CD4$^-$ CD8$^-$
11	└ CD4 T cells	CD3$^+$ CD19$^-$ TCRγδ$^-$ CD4$^+$ CD8$^-$
12	└ Treg	CD3$^+$ CD19$^-$ TCRγδ$^-$ CD4$^+$ CD25$^+$ CD127$^{-/lo}$
13	└ NOT Treg	CD3$^+$ CD19$^-$ TCRγδ$^-$ CD4$^+$ CD25$^{+/lo}$ CD127$^{+/-}$
14	└ Naive/stem-like	CD3$^+$ CD19$^-$ TCRγδ$^-$ CD4$^+$ CD25$^{+/lo}$ CD127$^{+/-}$ CD45RA$^+$ CCR7$^+$
15	└ Tnaive	CD3$^+$ CD19$^-$ TCRγδ$^-$ CD4$^+$ CD25$^{+/lo}$ CD127$^{+/-}$ CD45RA$^+$ CCR7$^+$ CD95$^-$
16	└ Tscm	CD3$^+$ CD19$^-$ TCRγδ$^-$ CD4$^+$ CD25$^{+/lo}$ CD127$^{+/-}$ CD45RA$^+$ CCR7$^+$ CD95$^+$
17	└ Tcm	CD3$^+$ CD19$^-$ TCRγδ$^-$ CD4$^+$ CD25$^{+/lo}$ CD127$^{+/-}$ CD45RA$^-$ CCR7$^+$
18	└ TemRO	CD3$^+$ CD19$^-$ TCRγδ$^-$ CD4$^+$ CD25$^{+/lo}$ CD127$^{+/-}$ CD45RA$^-$ CCR7$^-$
19	└ TemRA	CD3$^+$ CD19$^-$ TCRγδ$^-$ CD4$^+$ CD25$^{+/lo}$ CD127$^{+/-}$ CD45RA$^+$ CCR7$^-$
20	└ Tfh	CD3$^+$ CD19$^-$ TCRγδ$^-$ CD4$^+$ CD25$^{+/lo}$ CD127$^{+/-}$ CD45RA$^-$ CXCR5$^+$
21	└ CD8 T cells	CD3$^+$ CD19$^-$ TCRγδ$^-$ CD8$^+$
22	└ Naive/stem-like	CD3$^+$ CD19$^-$ TCRγδ$^-$ CD8$^+$ CD45RA$^+$ CCR7$^+$
23	└ Tnaive	CD3$^+$ CD19$^-$ TCRγδ$^-$ CD8$^+$ CD25$^{+/lo}$ CD127$^{+/-}$ CD45RA$^+$ CCR7$^+$ CD95$^-$
24	└ Tscm	CD3$^+$ CD19$^-$ TCRγδ$^-$ CD8$^+$ CD25$^{+/lo}$ CD127$^{+/-}$ CD45RA$^+$ CCR7$^+$ CD95$^+$
25	└ Tcm	CD3$^+$ CD19$^-$ TCRγδ$^-$ CD8$^+$ CD45RA$^-$ CCR7$^+$
26	└ TemRO	CD3$^+$ CD19$^-$ TCRγδ$^-$ CD8$^+$ CD45RA$^-$ CCR7$^-$
27	└ TemRA	CD3$^+$ CD19$^-$ TCRγδ$^-$ CD8$^+$ CD45RA$^+$ CCR7$^-$

Minimum 5 markers consumed in gating γδ T cells

Applying the truncated panel

15 melanoma patients
16 healthy controls

27 marker resting T-cell panel

8 marker truncated panel

20 marker activated panel

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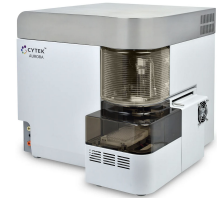
# Missed or incomplete understanding of the immune response

- ⬆ activated γδT cells in melanoma patients compared to controls. ⚠️

- ⬇️ IL-17A (P = 0.027) and TNFα (P = 0.047) in melanoma patient γδT cells. ❌

- ⬆ expression of CD57 (P = 0.041) and EOMES (P = 0.033) in melanoma patient γδT cells. ❌

PDAC: Simplified Study Design

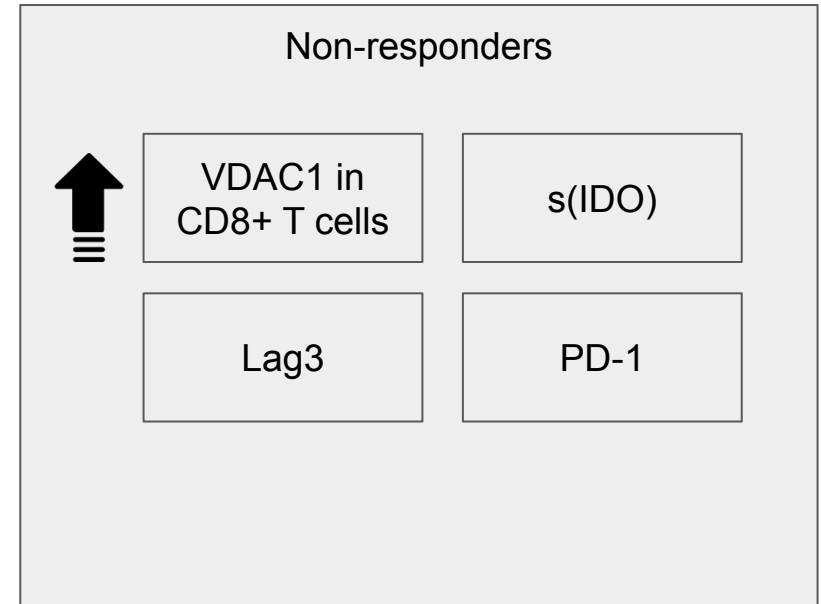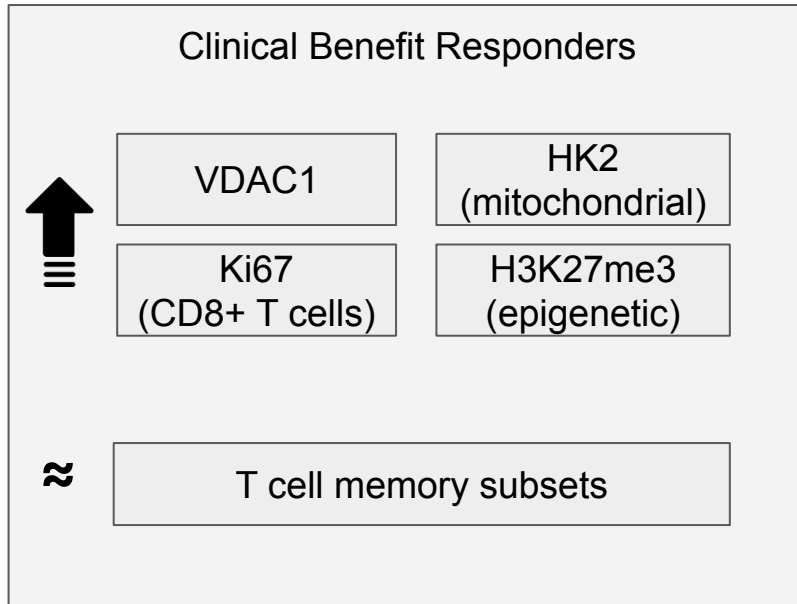13 pancreatic adenocarcinoma patients (6 non-responders)

21 marker panel

Zhang, et al 2023

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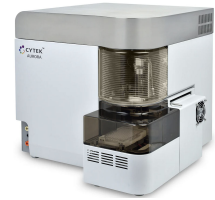
Target	Conjugate
Live and Dead	Zombie yellow
CD3	BV786
CD8	BV480
CD45RA	BV570
CCR7	BV650
CD25	BV510
HLA-DR	BV750
PD-1	BV711
CD4	PE/CY5
KLRG1	PE-CF594
CD49A	APC
CD19	APC/CY7
CD56	APC/CY7
CD28	Alexa Fluor 488
FOXP3	Pacific Blue
TOMM20	Alexa Fluor 405
VDAC1	PerCP5.5
KI67	PE/CY7
HK2	Alexa Fluor 680
GLUT1	Alexa Fluor 647
H3K27ME3	PE

21 marker panel

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Statistically significant findings (and those ruled-out)

Clinical Benefit Responders

↑≋
- VDAC1
- HK2 (mitochondrial)
- Ki67 (CD8+ T cells)
- H3K27me3 (epigenetic)

≈ T cell memory subsets

Non-responders

↑≋
- VDAC1 in CD8+ T cells
- s(IDO)
- Lag3
- PD-1

What if?

Marker	Conjugate
CD3	BV786
CD8	BV480
CD4	PE/CY5
CD45RA	BV570
CCR7	BV650
PD-1	BV711
HLA-DR	BV750

8 marker truncated panel

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Statistically significant findings (and those ruled-out)

Clinical Benefit Responders

⬆ ≣

VDAC1 ❌

HK2 (mitochondrial) ❌

Ki67 (CD8+ T cells) ❌

H3K27me3 (epigenetic) ❌

≈ T cell memory subsets ❌

Non-responders

⬆ ≣

VDAC1 in CD8+ T cells ❌

s(IDO) ❌

Lag3 ❌

PD-1 ⚠️

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Prostate Cancer, Phase 1: Simplified Study Design

14 prostate cancer patients
Before and after therapy
Response vs non-response
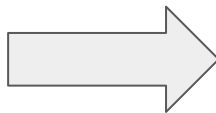
25 marker panel

<u>Priceman, et al 2024</u>

Marker	Fluorophore
CD3	Alexa Fluor 532
CD4	PerCP
CD8	BV570
CD45RA	BV650
CD45	Alexa Fluor 700
CD62L	BV711
CD19	V450
CD39	BV510
CD69	BV750
CD14	FITC
CX3CR1	PE-Cy7
LD	7AAD
CXCR3	PE
PD-1	BV421
CD28	APC-Cy7
LAG-3	APC
Tim3	PE-Cy5.5
CD16	BV786
CCR4	Alexa Fluor 647
CD27	PE-CF594
CCR7	BV605
CD45RO	BV480
CD25	PerCP-eFluor710
PD-L1	PE-Fire810
CD95	APC-Fire 810

25 marker panel

Statistically significant findings (and those ruled-out)

- ⬆ CX3CR1 in peripheral blood CAR T cells, correlated with response to immunotherapy with anti-PD-1 immune-checkpoint blockade.

- Few CX3CR1-positive T cells were observed in the product before infusion.

- ⬆ PD-1, LAG3 and TIM3 in CAR+ and endogenous non-CAR T cells.

What if?

Marker	Conjugate
CD3	Alexa Fluor 532
CD4	PerCP
CD8	BV570
CD45RA	BV650
CCR7	BV605
CD19	V450
PD-1	BV421
Viability	7AAD

8 marker truncated panel

Missed or incomplete understanding of the immune response

- ⬆ CX3CR1 in peripheral blood CAR T cells, correlated with response to immunotherapy with anti–PD-1 immune-checkpoint blockade.   ❌

- Few CX3CR1-positive T cells were observed in the product before infusion.   ❌

- ⬆ PD-1, LAG3 and TIM3 in CAR+ and endogenous non-CAR T cells.   ⚠️
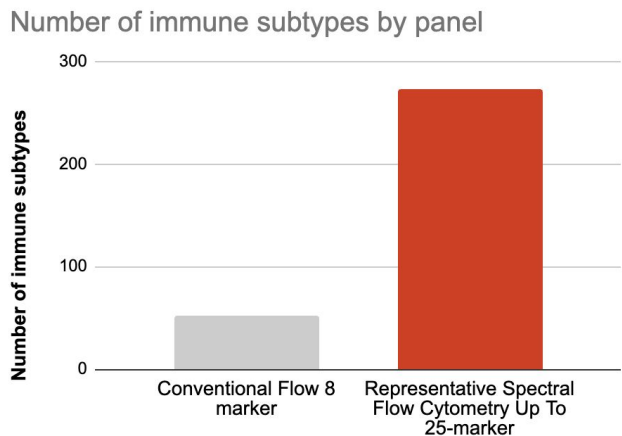
More like this

If you only used 8-marker conventional flow…

Number of immune subtypes by panel

Barber et al.

… but miss this

⬆PD-L1 on CD8+ T cells

…associated with

Clinical Response

biomarkers

Dizman et al.

… but miss this

⬆CXCL9 chemokines

…associated with

Clinical Response

biomarkers

Brown et al.

… but miss this

↓ CD28 on CAR+ T cells

…associated with

Clinical Response

biomarkers

In sum

"If a scientist is interested only in T cells, as that is the main modality of the drug, why would he or she do the extra work to analyze the rest of the immune state?"

- Paraphrased from an immunotherapy developer

"The immune system consists of more than one cell type."

- CSO, immunotherapy developer

Any examples we should cover?

Send us your ideas!

"Thus far, immune cell evaluations have been limited by conventional cytometry and relied on multiple panels to gain phenotyping depth…

…capturing the complexity of the immune response for biomarker screening and validation of previous findings requires robust and sensitive tools capable of simultaneously measuring a high number of parameters."

- Van Zelm, et al, Clinical & Translational Immunology, 2023