Development and Validation of an Assay to Assess Functional T Cell Responses Using Intracellular Cytokine Staining and Flow Cytometry in Global Clinical Trials

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Background

Intracellular cytokine staining combined with multiparametric flow cytometry (ICS-FC) is a powerful approach for detecting immune responses with single cell resolution. Clinically, ICS-FC has been used to detect antigen-specific responses to vaccines (e.g SARS-CoV-2) and evaluate cytokine release syndrome (e.g for cell and gene therapy trials)[1,2], mainly in small studies. Standardized, high throughput ICS-FC workflows are needed to bring this method to large, global clinical trial settings.

Objective

Develop and validate a robust, high throughput-compatible ICS-FC assay for global clinical trials.

Methods

FC panel was developed with following markers: CD45, CD3, CD4, and CD8 for general T cell lineage; CXCR3 and CCR4 for Tc1/Th1 and Tc2/Th2 subset identification; IFNγ, IL-2, and TNFα as representative Tc1/Th1 cytokines; IL-4 as a representative Tc2/Th2 cytokine; and IL-6 as a "drop-in" marker of interest. Phorbol myristate acetate (PMA, Calbiochem) and ionomycin (Iono, Calbiochem) stimulated peripheral blood mononuclear cells (PBMC) were used for workflow development and assay validation. PBMC were isolated from healthy donor whole blood collected in sodium citrate CPT tubes according to the manufacturer's protocol. PBMC were stimulated with PMA/Iono or with medium alone ("mock") and incubated for 6 hours at 37°C. Protein transport inhibitors (brefeldin-A and monensin) were added for the last 4 hours to preserve cytokines for intracellular detection. After stimulation, P/I and mock PBMC were harvested, stained for extracellular markers for 30 min at room temperature (RT), then fixed using Cytofix (BD Biosciences) for 15 min at 4°C. After washing, fixed cells were stored overnight at 4°C. The next day, cells were permeabilized using permeabilization solution 2 (BD Biosciences) for 10 min at RT, then stained for intracellular markers for 30 min at RT. Stained samples were acquired using a BD FACSLyric cytometer and data were analyzed using FCS Express (IVD version 7).

Results Summary

- P/I stimulation produced strong pan-T cell cytokine responses that were robustly detected by our panel.
- Repeatability and reproducibility criteria were met for all major reportables, including relative % of cytokine-positive cell and median fluorescence intensity (MFI).
- Adjustment of panel design, staining workflow, instrument settings, and gating strategy were necessary to obtain precise detection, especially for less abundant populations (e.g IL-4+ cells)
- Protocol could be modified to increase efficiency by removing overnight incubation step and combining staining for certain markers.

Conclusions

We validated a robust T cell immune response testing platform fit for large-scale clinical trials. This assay can be customized for multiple applications including testing for SarS-CoV2 and influenza vaccine responses, as well as investigating cytokine release syndrome.

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Results

Detector (laser)	FL1 (405)	FL2 (405)	FL3 (405)	FL4 (405)	FL5 (405)	FL6 (488)	FL7 (488)	FL8 (488)	FL9 (488)	FL10 (640)	FL11 (640)	FL12 (640)
Fluorochrome (Filter)	BV421 (448/45)	eFluor506 (528/45)	BV605 (606/36)	BV711 (715/50)	BV786 (755LP)	AF488 (527/32)	PE (586/42)	BB700 (700/54)	PE-Cy7 (783/56)	APC (660/10)	AF700 (720/30)	APC-Cy7 (783/56)
Tube 1 (FMX, Stimulated)		Viability	CD45	CD4	CD8						CD3	
Tube 2 (Full Stain, Unstimulated)	IL-2	Viability	CD45	CD4	CD8	TNFα	IL-6	IL-4	CD194	CD183	CD3	IFNγ
Tube 3 (Full Stain, Stimulated)	IL-2	Viability	CD45	CD4	CD8	TNFα	IL-6	IL-4	CD194	CD183	CD3	IFNγ

Figure 1. ICS-FC Assay Panel Configuration

Marker and fluorochrome pairings for panel, as well as tube setup for assay, are shown above. Each marker is mapped to the corresponding instrument detector and laser line in the top two rows, while markers for each tube are shown in the bottom three rows. Tube 1 (FMX) was used to verify T cell subpopulation gating, while tube 2 (unstimulated cells) was used as an additional control to verify cytokine gating.

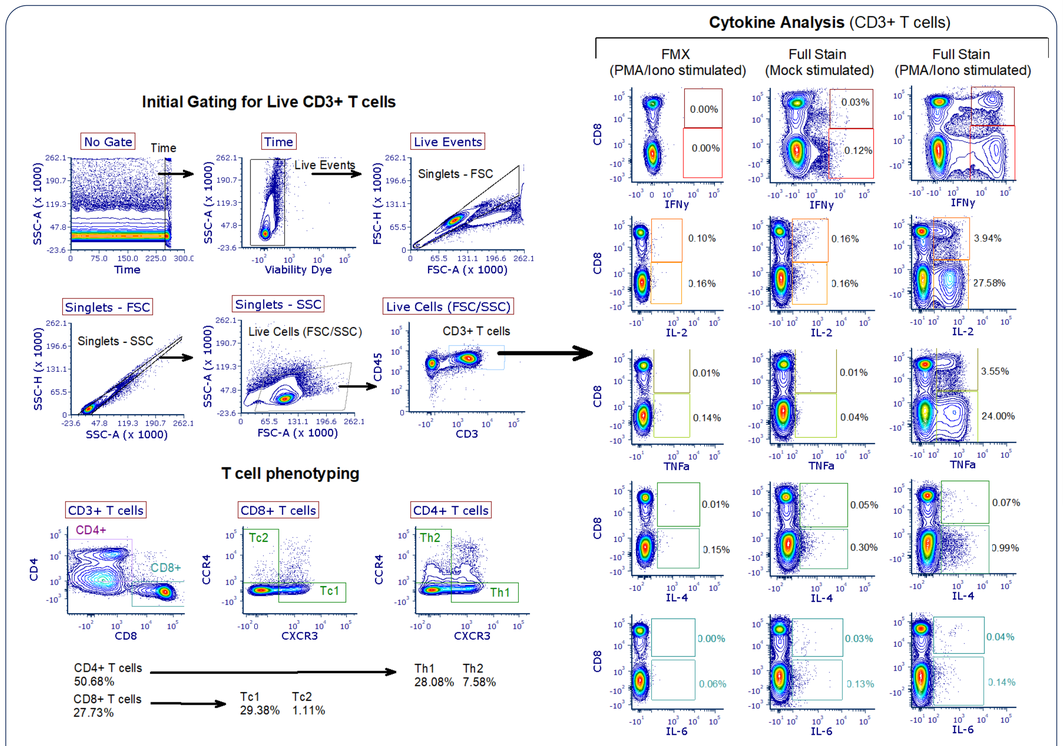

Figure 2. T cell phenotyping and cytokine profiling of healthy donor PBMC after PMA and ionomycin *in vitro* stimulation

PBMC were stimulated with PMA (1 mg/mL) and ionomycin (100 ng/mL) for 6 hours after an overnight resting period. Cells were stained for extracellular markers (excluding CD4), fixed, permeabilized, then stained for intracellular markers and CD4.

Example plots from a representative donor tested during the validation are shown. Initial gating and T cell phenotyping plots are taken from the full stain (stimulated) sample. CD4 T cell gating was adjusted to include CD4-dim cells, which appear due to receptor downregulation during stimulation. Cytokine-positive cell gating shown uses CD8 only for ease of visual comparison; actual reportable measurement was done from individual T cell subpopulations gated using corresponding markers (as shown in "T cell phenotyping" section).

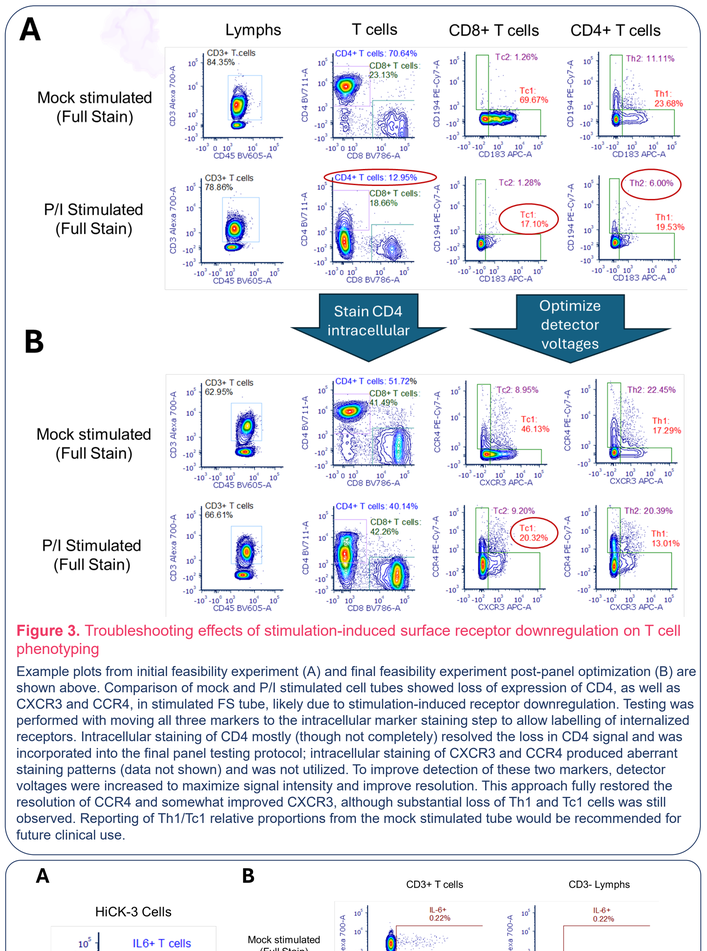

Figure 3. Troubleshooting effects of stimulation-induced surface receptor downregulation on T cell phenotyping

Example plots from initial feasibility experiment (A) and final feasibility experiment post-panel optimization (B) are shown above. Comparison of mock and P/I stimulated cell tubes showed loss of expression of CD4, as well as CXCR3 and CCR4, in stimulated FS tube, likely due to stimulation-induced receptor downregulation. Testing was performed with moving all three markers to the intracellular marker staining step to allow labelling of internalized receptors. Intracellular staining of CD4 mostly (though not completely) resolved the loss in CD4 signal and was incorporated into the final panel testing protocol; intracellular staining of CXCR3 and CCR4 produced aberrant staining patterns (data not shown) and was not utilized. To improve detection of these two markers, detector voltages were increased to maximize signal intensity and improve resolution. This approach fully restored the resolution of CCR4 and somewhat improved CXCR3, although substantial loss of Th1 and Tc1 cells was still observed. Reporting of Th1/Tc1 relative proportions from the mock stimulated tube would be recommended for future clinical use.

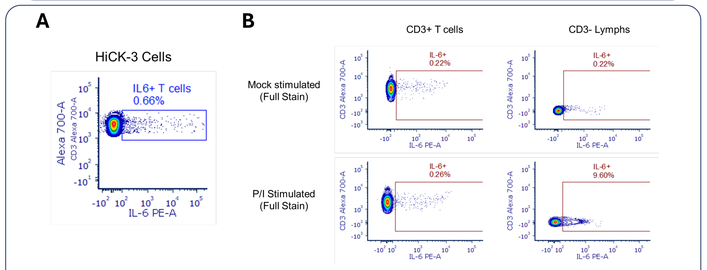

Figure 4. Detection of IL-6 expression in HiCK-3 cells and P/I-stimulated CD3- cells

Despite detecting little-to-no expression on conventional T cells, panel detects IL-6 expressed by commercially available HiCK-3 cells (A, BD Biosciences) and by CD3- lymphs in P/I stimulated samples (B, same sample as shown in Figure 3B). Monocytes were suspected as primary CD3- secretors of IL-6 but could not be confirmed due to lack of monocyte lineage markers.

REPEATABILITY

Reportables	Sample 1 Run 1 %CV	Sample 1 Run 2 %CV	Sample 2 Run 1 %CV	Sample 2 Run 2 %CV	Sample 3 Run 1 %CV	Sample 3 Run 2 %CV	Sample 4 Run 1 %CV	Sample 4 Run 2 %CV	Overall %CV
Lymphocytes (% Leukocytes)	0.64	0.54	0.68	0.61	0.42	0.29	0.20	0.36	**0.47**
T cells (% Lymphocytes)	1.61	0.47	1.19	2.22	0.72	0.93	0.10	0.46	**0.96**
CD4+ T cells (% T cells)	1.89	2.32	2.32	3.35	1.02	0.75	1.61	2.14	**1.92**
IL-2+ CD4+ T cells (% CD4+ T cells)	1.76	4.32	6.16	6.18	1.12	5.80	3.58	4.43	**4.17**
MFI IL-2 (CD4+ IL-2+ T cells)	4.56	1.87	2.19	4.24	1.87	2.06	4.48	3.79	**3.13**
IL-4+ CD4+ T cells (% CD4+ T cells)	1.10	5.58	23.76	7.77	13.84	22.84	20.65	6.19	**12.72**
MFI IL-4 (CD4+IL-4+ T cells)	0.99	4.57	4.24	5.72	6.70	5.16	1.87	3.86	**4.14**
IL-6+ CD4+ T cells (% CD4+ T cells)	0.00	17.32	17.32	17.32	21.65	**34.64**	21.65	0.00	**16.24**
MFI IL-6 (CD4+ IL-6+T cells)	7.65	5.75	19.05	15.68	5.12	6.11	13.42	6.49	**9.91**
IFNγ+CD4+ T cells (% CD4+ T cells)	4.18	6.25	11.98	6.24	7.04	11.75	4.88	3.91	**7.03**
MFI IFNγ (CD4+IFNγ+ T cells)	5.94	6.81	6.38	4.78	5.10	6.66	4.51	4.04	**5.53**
TNFα + CD4+ T cells (% CD4+ T cells)	7.39	7.29	13.12	4.34	8.19	12.45	7.96	6.06	**8.35**
MFI TNFα (CD4+ T cells)	9.25	7.25	8.42	8.73	11.11	7.96	7.78	5.66	**8.27**
CD8+ T cells (% T cells)	1.40	0.07	1.03	0.75	0.93	1.22	0.46	0.88	**0.84**
IL-2+ CD8+ T cells (% CD8+ T cells)	1.50	3.61	1.82	6.73	2.19	3.66	4.99	1.37	**3.23**
MFI IL-2 (CD8+ T cells)	3.54	2.22	3.24	1.72	1.16	1.25	3.06	0.30	**2.06**
IL-4+ CD8+ T cells (% CD8+ T cells)	3.50	5.35	**26.51**	6.68	**28.64**	**41.15**	**33.07**	14.29	**19.90**
MFI IL-4 (CD8+ T cells)	2.31	2.56	5.61	3.22	13.46	13.63	6.73	4.85	**6.55**
IL-6+ CD8+ T cells (% CD8+ T cells)	7.69	5.09	0.00	7.87	19.92	14.29	8.66	12.37	**9.49**
MFI IL-6 (CD8+ T cells)	6.41	19.97	21.31	11.71	**43.98**	11.68	11.35	8.55	**16.87**
IFNγ+ CD8+ T cells (% CD8+ T cells)	1.36	5.03	9.59	9.56	6.06	14.50	2.13	2.91	**6.39**
MFI IFNγ (CD8+ T cells)	8.95	7.87	5.29	5.85	10.07	6.73	6.95	5.41	**7.14**
TNFα + CD8+ T cells (% CD8+ T cells)	9.26	8.64	14.23	12.46	11.66	16.41	7.09	3.69	**10.43**
MFI TNFα (CD8+ T cells)	7.11	5.81	7.03	7.84	9.91	6.49	10.39	4.96	**7.44**
Th1 T cells (% T cells)	2.52	4.40	7.80	2.97	2.55	1.07	2.40	3.06	**3.35**
IL-2+ Th1 T cells (% T cells)	3.53	6.52	11.18	6.01	6.35	9.46	3.05	4.57	**6.33**
MFI IL-2 (Th1 T cells)	5.58	0.85	3.15	3.56	2.64	1.96	4.15	3.80	**3.21**
IFNγ+ Th1 T cells (% T cells)	3.54	6.36	13.27	7.91	7.18	11.13	3.78	4.30	**7.18**
MFI IFNγ+ (Th1 T cells)	7.16	7.67	6.03	8.04	7.08	6.57	6.76	4.29	**6.70**
TNFα+ Th1 T cells (% T cells)	7.90	8.05	18.23	8.46	10.36	11.86	5.87	5.09	**9.48**
MFI TNFα+ (Th1 T cells)	9.44	8.68	9.47	9.65	11.68	7.90	10.39	6.96	**9.27**
Tc1 T cells (% T cells)	1.50	3.17	7.57	5.64	5.43	8.26	1.66	2.17	**4.43**
IL-2+ Th1 T cells (% T cells)	2.87	7.28	3.65	6.98	5.26	9.75	5.51	3.53	**5.60**
MFI IL-2 (Th1 T cells)	2.99	1.77	1.43	1.62	1.72	1.49	3.56	1.06	**1.96**
IFNγ+ Th1 T cells (% T cells)	2.25	5.66	11.89	9.78	7.74	15.47	2.72	3.08	**7.32**
MFI IFNγ+ (Th1 T cells)	9.77	8.34	4.09	5.78	7.90	4.54	7.40	4.96	**6.60**
TNFα+ Th1 T cells (% T cells)	14.38	14.32	20.90	15.01	13.75	18.84	7.19	4.65	**13.63**
MFI TNFα+ (Th1 T cells)	7.45	7.60	7.67	7.01	12.50	5.99	10.46	5.36	**8.00**
Th2 T cells (% T cells)	1.09	2.53	3.87	0.44	2.67	2.43	4.55	2.35	**2.49**
IL-4+ Th2 T cells (% T cells)	0.88	4.95	11.89	6.98	0.00	11.46	12.85	4.76	**6.72**
MFI IL-4 (Th2 T cells)	5.77	5.93	2.49	6.33	3.10	5.77	6.08	4.48	**4.99**
Tc2 T cells (% T cells)	0.82	2.98	7.27	4.40	2.24	1.59	7.95	3.46	**3.84**
IL-4+ Tc2 T cells (% T cells)	2.04	6.26	4.06	10.30	17.32	17.32	**43.30**	0.00	**12.58**
MFI IL-4 (Tc2 T cells)	4.14	2.73	4.21	4.36	19.39	10.51	**28.48**	1.42	**9.41**

REPRODUCIBILITY

Reportables	Sample 1 %CV	Sample 2 %CV	Sample 3 %CV	Sample 4 %CV
Lymphocytes (% Leukocytes)	0.33	0.43	0.72	0.41
T cells (% Lymphocytes)	0.05	0.05	0.21	0.41
CD4+ T cells (% T cells)	1.78	11.42	15.74	9.07
IL-2+ CD4+ T cells (% CD4+ T cells)	0.89	20.00	**30.26**	13.15
MFI IL-2 (CD4+ IL-2+ T cells)	0.30	1.39	0.04	2.14
IL-4+ CD4+ T cells (% CD4+ T cells)	2.87	**28.51**	20.39	19.06
MFI IL-4 (CD4+IL-4+ T cells)	9.12	0.86	3.91	8.47
IL-6+ CD4+ T cells (% CD4+ T cells)	**28.28**	0.00	**32.64**	20.20
MFI IL-6 (CD4+ IL-6+T cells)	2.88	15.26	7.61	0.37
IFNγ+CD4+ T cells (% CD4+ T cells)	1.71	10.49	20.24	7.06
MFI IFNγ (CD4+IFNγ+ T cells)	12.02	1.55	10.63	8.11
TNFα + CD4+ T cells (% CD4+ T cells)	5.58	15.32	**27.08**	11.90
MFI TNFα (CD4+ T cells)	10.02	10.63	7.02	4.18
CD8+ T cells (% T cells)	0.18	2.34	0.39	1.44
IL-2+ CD8+ T cells (% CD8+ T cells)	3.44	1.99	0.86	1.34
MFI IL-2 (CD8+ T cells)	0.89	1.53	0.73	0.09
IL-4+ CD8+ T cells (% CD8+ T cells)	3.78	14.85	18.00	9.43
MFI IL-4 (CD8+ T cells)	8.99	3.81	6.73	**32.21**
IL-6+ CD8+ T cells (% CD8+ T cells)	9.69	6.15	6.43	24.96
MFI IL-6 (CD8+ T cells)	19.85	15.01	**25.99**	**34.51**
IFNγ+ CD8+ T cells (% CD8+ T cells)	3.83	6.60	0.76	13.86
MFI IFNγ (CD8+ T cells)	1.91	0.79	4.95	2.02
TNFα + CD8+ T cells (% CD8+ T cells)	9.50	10.05	2.09	18.41
MFI TNFα (CD8+ T cells)	6.67	8.93	3.56	3.52
Th1 T cells (% T cells)	4.00	10.21	17.33	8.43
IL-2+ Th1 T cells (% T cells)	2.95	12.73	24.38	11.08
MFI IL-2 (Th1 T cells)	0.44	0.11	0.51	1.96
IFNγ+ Th1 T cells (% T cells)	0.78	13.64	**25.20**	8.59
MFI IFNγ+ (Th1 T cells)	3.54	5.32	2.10	3.86
TNFα+ Th1 T cells (% T cells)	5.67	8.72	24.04	9.10
MFI TNFα+ (Th1 T cells)	12.72	12.83	7.75	5.04
Tc1 T cells (% T cells)	2.27	5.77	1.18	7.73
IL-2+ Th1 T cells (% T cells)	6.78	6.63	2.80	3.67
MFI IL-2 (Th1 T cells)	3.49	3.27	0.44	1.62
IFNγ+ Th1 T cells (% T cells)	4.77	7.51	0.80	14.29
MFI IFNγ+ (Th1 T cells)	0.76	2.50	3.44	1.51
TNFα+ Th1 T cells (% T cells)	14.84	15.45	4.06	20.15
MFI TNFα+ (Th1 T cells)	5.47	9.58	4.07	3.55
Th2 T cells (% T cells)	0.28	12.01	17.23	14.82
IL-4+ Th2 T cells (% T cells)	4.52	19.05	**28.28**	18.53
MFI IL-4 (Th2 T cells)	10.77	0.40	1.93	8.68
Tc2 T cells (% T cells)	4.63	0.85	5.74	3.63
IL-4+ Tc2 T cells (% T cells)	3.29	5.19	0.00	**28.28**
MFI IL-4 (Tc2 T cells)	10.29	1.91	2.31	23.50

Figure 5. Intracellular cytokine assay meets CLSI H62 guidelines for repeatability

Precision experiment was performed using PBMC derived from four individual healthy donors, which were divided into two sets (independent runs) and further split into three aliquots (independent replicates). All replicates across both runs were tested separately, including the resting and stimulation steps. Precision was assessed according to recommended CLSI H62 guidelines[3]. In brief, a coefficient of variation (CV) of ≤25% was used as the criterion for acceptance for all reportable values. Higher imprecision (≤35% CV) was accepted for values derived from rare populations or MFI values from dimly expressed antigens (such as IL-4 and IL-6). For repeatability, individual and overall CVs were calculated using the individual replicates from each run.

All primary reportables were within the acceptance criteria, except for several reportables from rare populations (red shading and right-to-left cross-hatching) and one reportable for IL-6 (red shading and left-to-right cross-hatching), which was barely expressed by T cells in these samples.

Above acceptance criteria	###
Rare populations (<100 events, and less than 5%)	###
MFI value from dimly expressed antigen	###

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