Effects of Resting Cryopreserved PBMCs on Cytokine Production Assessed by Flow Cytometry–Based ICS Assay

Lifeng Tian, Daniel Marad, Kristen Belusko, John Kolman
QPS, Newark, Delaware, USA

CONTACT INFORMATION: lifeng.tian@qps.com

M1530-07-41

aaps PharmSci 360

PURPOSE

Monitoring cellular immune responses during drug development and clinical trials is critical for assessing the safety and efficacy of therapeutic interventions. In our previous study, we evaluated the effects of resting cryopreserved PBMCs for different time frames on IFN-γ secretion by ELISpot assay and suggested that overnight resting (18–22 hours) improves IFN-γ detection by ELISpot assay. In this presentation, we assessed whether resting should also be performed for the flow cytometry–based ICS assay.

METHOD(S)

Cryopreserved PBMC samples derived from 8 individual healthy human donors were obtained from BioIVT and stored in a vapor-phase liquid nitrogen tank until use. An overview of the experimental workflow is shown in **Table 1**. Briefly, PBMC samples were thawed in a 37 °C water bath at a predefined time, washed twice with RPMI 1640 complete medium containing 10% CultraPure FBS, and resuspended in an appropriate volume of medium. Cell counts and viability were determined using an imaging-based cell counter (Cellaca MX) with AO/PI staining. The resting procedure followed Kutscher's publication [1] with minor modifications. Cell counts were performed after each resting period, if applicable, followed by sample allocation (1 × 10^6 cells per well) for cell stimulation at a predefined time. Each sample was stimulated with mock control and CEF peptide pool in the presence of the transport inhibitor monensin for 22-24 hours. After stimulation, ICS flow cytometry panels were performed to assess IFN-γ, TNF-α, and IL-2 production in different subsets. Panel information is provided in **Table 2**.

Table 1: An Overview of the Experimental Workflow

	Day 0			Day 1				Day 2
	Assays		Procedure	Assays		Procedure		Assays
	Thawing	cell counting (Viability)	Resting	Thawing	cell counting (Viability)	Resting	Stimulation	ICS
18hr Resting	16:00pm		17:00pm	10:00am			11:00am	8:00am
22hr Resting			17:00pm	14:00pm			15:00pm	14:00pm
2hr Resting				8:00am		9:00am	11:00am	8:00am
6hr Resting						9:00am	15:00pm	14:00pm
0hr Resting				10:00am		11:00am	11:00am	8:00am

Table 2: Panel Information

Panel	Description	Clone	Purpose
12-color panel	LD Zombie NIR	NA	Viability
12-color panel	CD45 Spark UV387	HI30	Leukocyte marker
12-color panel	CD4 Spark Plus UV395	SK3	CD4 T cell marker
12-color panel	CD56 BUV563	NCAM16.2	NK cell marker
12-color panel	CD8 BUV805	SK1	CD8 T cell marker
12-color panel	CD3 SB436	SK7	pan T cell marker
12-color panel	CD16 Pacific Blue	3G8	NK, monocyte marker
12-color panel	CD19 RB545	SJ25C1	B cell marker
12-color panel	CD14 AF647	M5E2	Monocyte marker
12-color panel	*TNFa BV650*	Mab11	Cytokine
12-color panel	*IL-2 RB705*	MQ1-17H12	Cytokine
12-color panel	*IFNg PE*	B27	Cytokine
19-color panel	CD127 BV421	HIL-7R-M21	Treg cell marker
19-color panel	CD25 PE Cy5.5	BC96	Treg cell marker
19-color panel	TCR γ/δ RB613	B1	γδ T cell marker
19-color panel	CD161 BV480	DX12	MAIT, iNKT cell marker
19-color panel	TCR Va24-Ja18 BUV661	6B11	iNKT cell marker
19-color panel	TCR Va7.2 BV711	3C10	MAIT cell marker
19-color panel	CD183 (CXCR3) PE Cy7	G025H7	Chemokine receptor

RESULT(S)

Herein, we expanded our investigation into the flow cytometry-based ICS assay. First, PBMCs from three individual healthy donors were rested for 0, 2, 6, 18, or 22 hours, followed by stimulation. A 12-color ICS flow cytometry panel was then performed, and the gating strategy used for flow cytometry data analysis is illustrated in **Figure 1**. To investigate antigen-specific T-cell responses, the ratios of signal intensity and percentage expression between mock stimulation and CEF peptide pool stimulation were calculated for both CD8 and CD4 T cells across different resting periods. As shown in **Figure 2a**, either the ratio of signal intensity or percentage expression in CD8 T cells indicated that resting for more than 6 hours increased at least one of the cytokines (IFN-γ, TNFα, and IL-2). Representative flow cytometry figures are shown in **Figure 2b**. In contrast, this phenomenon was not observed in CD4 T cells (**Figure 2c and 2d**). Next, polyfunctional CD4 and CD8 T cells were assessed using Boolean gating for each combination of the three cytokines. As shown in **Figure 2e and 2f**, more polyfunctional CD8 T cells were observed after more than 6 hours of resting, particularly after 18 hours. To further confirm this observation, another five individual healthy donors were tested with and without 18 hours of resting. An expanded 19-color ICS flow cytometry panel was used. The gating strategy used for this 19-color flow cytometry data analysis is illustrated in **Figure 3**. The same data analysis strategy for monofunctional and polyfunctional CD8 and CD4 T cells was performed. As shown in **Figure 4a**, statistical analysis indicated that 18 hours of resting improved signal intensity, but not the percentage of cytokine production in CD8 T cells. As expected, both signal intensity and percentage expression of each cytokine did not increase in CD4 T cells. Polyfunctional CD8 T cells, but not CD4 T cells, were dramatically increased after 18 hours of resting (**Figure 4b**). To take advantage of the flow cytometry assay for the simultaneous detection of multiple immune subsets, cytokine expression was investigated in non-T cells (**Figure 5a**) to determine whether any responses during stimulation were induced by non-T cells, including monocytes, B cells, and NK cells. In addition, the $CD14^{-}CD19^{-}CD3^{-}CD56^{-}$ cell population, which should include DCs, was also assessed. Moreover, cytokine expression was investigated in some unconventional T cells, including TCRγδ T cells, iNKT cells, MAIT cells, and NKT-like cells (**Figure 5b**). These investigations suggest that some of the cytokine production was generated from non-antigen-specific T cells and that 18 hours of resting mitigated some of the non-specific T-cell responses and improved assay specificity.

Figure 1: Gating Strategy for 12-Color Panel

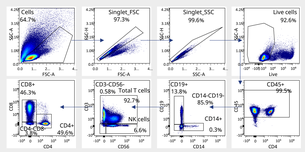


Figure 3: Gating Strategy for 19-Color Panel

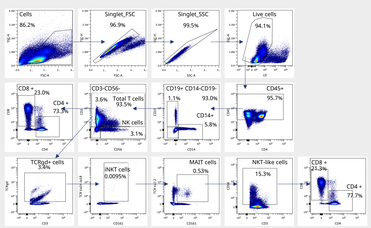


Figure 2a: The ratios between mock stimulation and CEF peptide pool on CD8+ T cells.

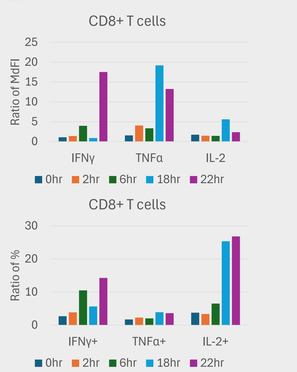


Figure 2b: Representative 2-D plots for CD8+ T cells.

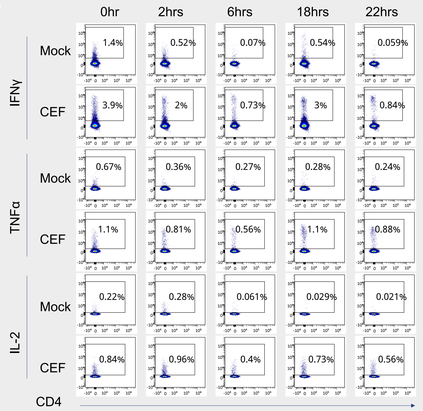


Figure 2c and 2d: The ratios between mock stimulation and CEF peptide pool on CD4+ T cells.

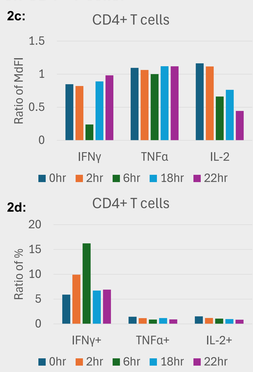


Figure 2e and 2f: Polyfunctional CD4 and CD8 T cells

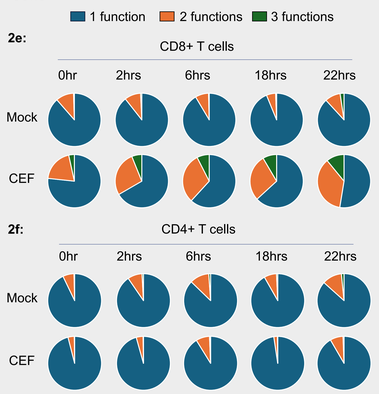


Figure 4a: The ratios between mock stimulation and CEF peptide pool on CD8+ T cells. * $p < 0.05$.

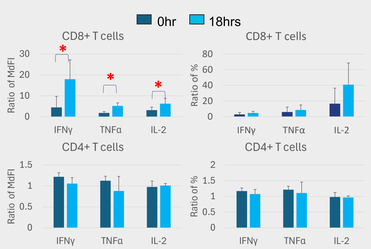


Figure 4b: Polyfunctional CD4 and CD8 T cells.

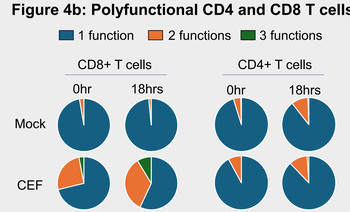
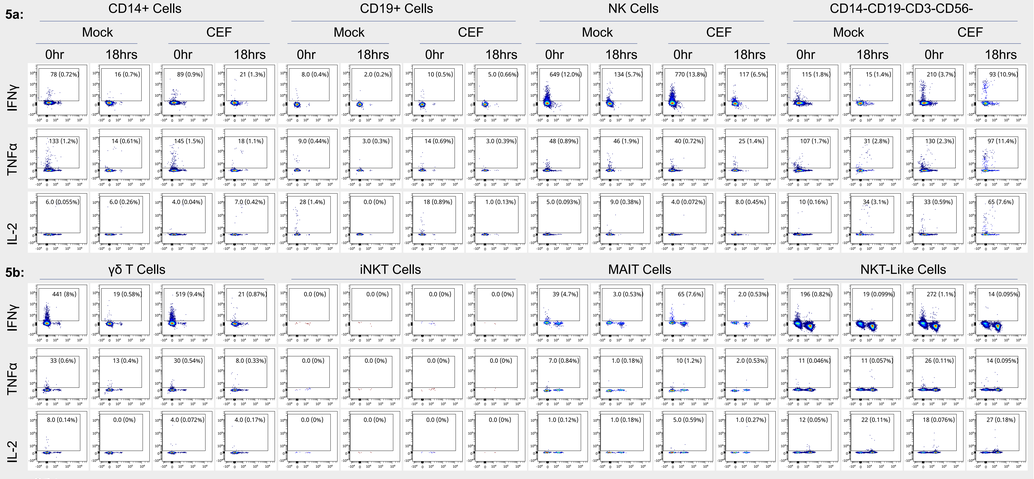


Figure 5a and 5b: Cytokine expression in non-T cells and unconventional T cells.



CONCLUSION(S)

In this study, we provided evidence to support resting cryopreserved PBMC samples for 18–22 hours prior to stimulation and the subsequent ICS flow cytometry assay, which improved the quantity and functional profile of antigen-specific T-cell responses. Further investigation with more samples and varied stimulation conditions is warranted to strengthen these findings.

REFERENCE(S)

[1] Kutscher S, Dembek CJ, Deckert S, Russo C, Körber N, Bogner JR, Geisler F, Umgelter A, Neuenhahn M, Albrecht J, Cosma A, Protzer U, Bauer T. Overnight resting of PBMC changes functional signatures of antigen specific T- cell responses: impact for immune monitoring within clinical trials. PLoS One. 2013 Oct 11;8(10):e76215.

QPS CUSTOM-BUILT RESEARCH