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# Decreased intra-lymphocyte cytokines measurement in septic shock patients: A proof of concept study in whole blood

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ABSTRACT

Functional testing protocols are thought to be the gold standard for the exploration of the immune system. However, in terms of routine analysis, they present numerous drawbacks and consequently their use is mainly limited to research applications. In the clinical context of septic shock, characterized by marked lymphocyte alterations, a new approach for lymphocyte intracellular cytokine measurement in whole blood upon was evaluated in a proof-of-concept study. Following lymphocyte activation, simultaneous intracellular labeling of Interferon-γ (IFN-γ), Tumor Necrosis Factor-α (TNF-α), and Interleukin-2 (IL-2) was performed in $CD4^+$ and $CD8^+$ T cells (identified by surface marking). The analysis was carried out by flow cytometry (6 colors). Results obtained in septic patients (n = 22) were compared to those of healthy volunteers (n = 8). Independently of lymphopenia, there were significant differences between groups. In particular there was significant decrease in the production of IL-2 and TNF-α in septic patients, while the production of IFN-γ was not significantly altered. Polyfunctional results showed that patients presented with increased percentages of triple negative lymphocytes. In contrast, volunteers had higher proportions of triple positive cells. The approach could be performed in a robust and consistent way, taking 4.5 h to complete. Moreover, clear differences could be observed between clinical groups with this modified method. These characteristics illustrate the potential of this novel whole blood protocol for clinical applications. However, further research is required to determine the applicability compared to alternative test and to evaluate clinical performances in larger cohorts of patients.

## 1. Introduction

In clinical immunology laboratories, functional testing remains the gold standard because it directly measures the capacity of a cell population to respond to an immune challenge [1,2]. However, although providing excellent insights regarding pathophysiology, this approach remains barely usable for routine clinical monitoring due to several drawbacks (*e.g.,* long incubation time, lengthy cell purification procedures, cell permeabilization, several staining steps, and numerous wash cycles). As a consequence, these protocols remain difficult to be standardized and thus they do not easily fulfil criteria for certification and accreditation of clinical laboratories [3]. Thus, a major challenge is therefore to bring functional testing to routine clinical practice.

Among immune functional assays, measurement of intracellular cytokines by flow cytometry appears as one of the most helpful [4]. It is able to determine both cell functionality and phenotype, while enumerating them. For instance, it is frequently used to characterize immune responses to infectious diseases, assess pyrogenicity of solutions, and measure immunogenicity during clinical trials of vaccines [4–6]. However, this assay remains time-consuming (about 6–8 h for completion, including several technical steps) and is consequently not used in clinical monitoring except for rare conditions (*e.g.,* immune deficiencies, [1,7]).

We recently reported a novel approach to intracellular staining for flow cytometry that uses whole blood, and which is both rapid and robust [7]. Based on a similar approach, the aim herein was to assess simultaneous measurement of TNF-α, IFN-γ, and IL-2 in CD4 and CD8T cells in clinical samples. This proof of concept study was performed in the clinical context of septic shock in which T cells are believed to have an exhausted phenotype characterized by decreased cytokine production capacity [8].

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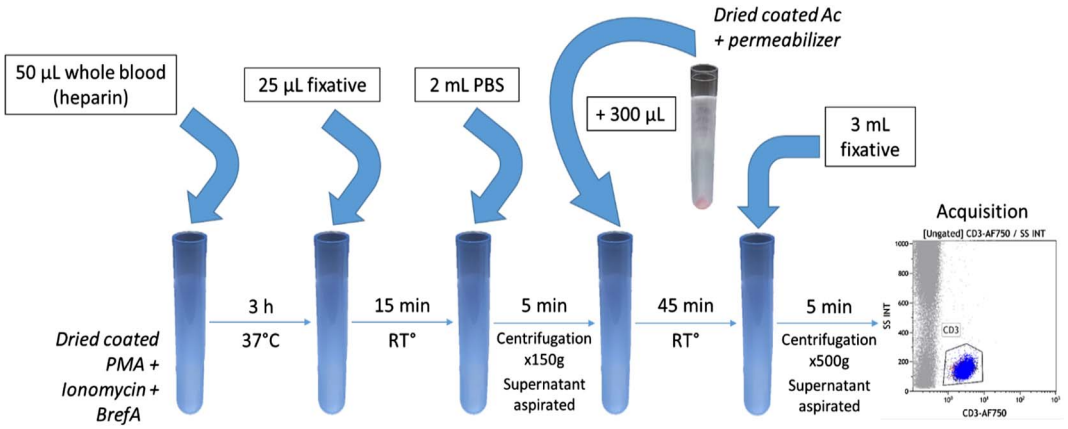


**Fig. 1.** Schematic representation of the staining procedure.

## 2. Materials and methods

### 2.1. Study population

Twenty-two septic shock patients (according to the diagnostic criteria of the International Guidelines for Management of Severe Sepsis and Septic Shock [9]) admitted to the surgical ICU of E. Herriot Hospital (Lyon University hospitals) were enrolled in the study. This work is part of a comprehensive study of immune dysfunctions induced in ICUs (NCT02803346) which has been submitted to an ethics committee and approved by the institutional review board and registered with the French Ministry of Higher Education and Research (#DC-2008-509). Blood was collected in heparin coated tubes. Clinical and biological data were collected during the follow-up period (until 28 days)

### 2.2. Research reagents

Tubes (12 × 75 mm) containing a dry coating of a mixture of Phorbol 12-Myristate 13-Acetate (PMA) and Ionomycin to stimulate the production of cytokines by T cells, and Brefeldin A (BrefA) to block cytokine secretion via the Golgi apparatus. In addition, tubes (12 × 75 mm) containing a dry coating of conjugated antibodies formulated for this assay were also used: Fluorescein Isothiocyanate (FITC)-labeled anti-IFNγ (clone 45.15), Phycoerythrin (PE)-labeled anti-TNF-α (clone IPM2), PE-Cyanine 7 (PC7)-labeled anti-IL-2 (clone MQ1-17H12), Alexa Fluor 700 (A700)-labeled anti-CD8 (clone B9.11), Alexa Fluor 750 (A750)-labeled anti-CD3 (UCHT1) and Pacific Blue (PB)-labeled anti-CD4 (13B8.2). These reagents are custom-made, optimized for this study by Beckman Coulter Immunotech (Marseille, France).

### 2.3. Intracellular staining procedure

A schematic protocol representation is presented in Fig. 1. Briefly, 50 microliters of undiluted fresh whole blood (< 2 h after sampling) was directly added to the stimulation tube (PMA-Ionomycin-BrefA), or to an empty control tube, and incubated 3 h at 37 °C. Samples were then treated according to the regular PERFIX-no centrifuge (nc) procedure (Beckman Coulter, Brea, CA, US). Briefly, 25 µL of PERFIX-nc fixative reagent was added and the sample incubated for 15 min at room temperature in the dark, 2 mL PBS was then added and the tubes centrifuged at 150*g* for 5 min at 10 °C, the supernatant was aspirated, and 25 µL of FBS were added to the pellet. Samples were then concomitantly permeabilized and stained for 45 min at room temperature in the dark (dried antibodies were resuspended extemporaneously with 300 µL permeabilizing reagent). The cells were then washed with 3 mL PERFIX-nc final reagent. The pellet was finally resuspended by adding 500 µL of PERFIX-nc final reagent. The entire protocol needed approximately 4.5 h to be completed.

### 2.4. Flow cytometry data acquisition and analysis

Data acquisition was performed on a Navios Flow Cytometer (Beckman Coulter). Our instrument was daily calibrated with Flow-check Pro Fluorospheres (Beckman Coulter) to control optical alignment and fluidic system and Sphero Rainbow Calibration (8 peaks) beads (Spherotech, IL, USA) to ensure MFI stability overtime. T cells were first gated out from other cells on the basis of labelling with CD3. Within the $CD3^+$ T cell population, $CD4^+$ T cells and $CD8^+$ T cells were identified based on CD4/SSC and CD8/SSC dot-plots. Illustrative gating strategy is shown in Fig. 2. Intracellular TNF-α, IFN-γ, and IL-2 expressions were then measured on $CD4^+$ and $CD8^+$ T cell subpopulations. All results were expressed either as percentages of cytokine-positive T cells (% positive cells) among the total $CD4^+$ or $CD8^+$ T cell subpopulations (positivity threshold was defined based on non-stimulated values from healthy donors and set up at first decade), or as MFI of the entire T cell subpopulation (Fig. 2C). Polyfunctional analysis was performed with tree function of Kaluza software (Beckman Coulter).

### 2.5. Statistical analysis

Results (% positive cells and MFI) were expressed as individual values and medians ± IQR (interquartile range). Results for the three cytokines (TNF-α, IFN-γ, and IL-2) in both T cell subpopulations ($CD4^+$ and $CD8^+$) with or without PMA-Ionomycin challenge among healthy volunteers and septic shock patients were compared, using the non-parametric Mann-Whitney *U* test or Wilcoxon paired test. Correlations were investigated using the Spearman correlation test. Statistical analyses were performed with GraphPad Prism® software (version 5.0; GraphPad Software, La Jolla, CA, US). A p-value $< 0.05$ was considered as statistically significant.

## 3. Results

### 3.1. Patients and healthy volunteers

Twenty-two septic shock patients (sampled during first 48 h after diagnosis) and eight healthy controls were included. Five patients were sampled a second time at D3-4. At onset of shock, the median