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Short communication

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Variability of *ex-vivo* stimulated T-cells secretory profile in healthy subjects

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Abstract

Peripheral blood lymphocytes (PBL) are able to synthesize various cytokines that play key roles in the immune response and intercellular signaling. Since alterations in cytokine production and/or activity occur in many pathological processes, the study of cytokine synthetic capacity of PBL is a valuable tool for assessing the immune profile. In this paper, we aimed to investigate the variability of interleukin-2 (IL-2), tumor necrosis factor-alpha (TNF-α) and interferon gamma (IFN-γ) synthetic capacity of CD4+/CD8+ T-cells stimulated ex-vivo in healthy subjects, by means of a commercial intracellular cytokine staining (ICS) protocol. Peripheral blood mononuclear cells were isolated from 16 healthy subjects by Ficoll gradient centrifugation and activated ex-vivo with PMA/Ionomycin/Brefeldin-A for 4 hours. Activated PBL were surface-stained for CD3/CD4/CD8, fixed and permeabilized. ICS was performed using anti-human IL-2/TNF-α/IFN-γ and samples were analyzed on a BD-FACSAria-III flow cytometer. We recorded high post-isolation and post-activation mean viabilities: 82.1% and 82.4% respectively, p=0.84. Both CD4+/CD8+ subpopulations were found to partially produce each of the three cytokines, but in different proportions. On average, a significantly greater percentage of CD4+ cells was shown to produce IL-2 and TNF-α, compared with CD8+ cells (61.5%+/-5.8 vs. 25%+/-5.6 and 26.9%+/-11 vs. 7.5%+/-3.3 respectively, p<0.0001 for both). Contrarily, IFN-γ was produced by a higher proportion of CD8+ cells (8.4%+/-3.9 vs. 6.8%+/-3.2, p=0.01). These results show that the employed ICS protocol elicits a satisfactory and consistent cytokine response from PBL of healthy subjects. The collected data may be used to outline a preliminary reference range for future studies on both healthy/pathological subjects.

***Keywords**: peripheral blood lymphocytes, ex-vivo T-cells stimulation, cytokines, flow cytometry, reference intervals*

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Introduction

Immune Functional Assays (IFA) explore and quantify the response of the immune system to various stimuli and are especially helpful in determining an Individual Immune Profile (IIP). IFAs are generally used in research with only a few having found applicability in routine clinical practice so far. Currently, peripheral blood mononuclear cells (PBMC) are one of the most frequently employed biological specimens in IFA. PBMCs consist of different cell types that play important roles in the innate/adaptive immune system such as monocytes, natural killer cells, immature dendritic cells and T-cells, B-cells respectively. Lately, PBMCs have been increasingly regarded as a promising source of biomarkers as they can undergo immunophenotypic changes in many diseases (1-4). Thus, PBMCs represent a peripheral "keyhole" for researchers to monitor disease-specific gene expression and posttranslational modifications and can be used as a surrogate model of immune function (5,6).

PBMCs are able to synthesize and secrete various cytokines that can act as autocrine/paracrine/endocrine agents and play key roles in the immune response and intercellular signaling. Cytokines can be promptly produced and secreted in response to cellular activation. Alterations in cytokine production and/or activity occur in many pathological processes. Consequently, cytokine-production IFAs are a valuable and critical tool for assessing IIPs.

Due to the short half-life times of cytokines and naturally occurring cytokine inhibitors in human peripheral blood, measurement of serum cytokine levels may not accurately reflect the cytokine-producing potential of PBMCs (7,8). Alternatively, measurement of cytokine production by PBMCs stimulated *ex-vivo* with potent activators such as phorbol myristate acetate (PMA) (9), lipopolysaccharide (LPS), and phytohemagglutinin (PHA) (7) can be a reliable method for assessing immunocompetence. Also, the spontaneous *ex-vivo* release of cytokines may provide a measure of *in-vivo* activation (7).

Intracellular cytokine staining (ICS) is a flow cytometry based IFA able to detect and to quantify the production of cytokines in previously stimulated PBMCs. Cells are activated *ex-vivo* and cytokines are retained within the cell by inhibiting protein transport. Cells are then fixed and permeabilized in order to allow intracellular cytokine staining. ICS is a powerful tool that can track cytokine synthesis at the single cell level, enabling an in-depth analysis of distinct cytokine-producing cell populations. ICS proved to be a very versatile and reliable method and is now widely used in research for the study of vaccines, antigen response, autoimmune disorders, cancer, etc. (10-29)

The aim of this study was to investigate the variability of interleukin-2 (IL-2), tumor necrosis factor-alpha (TNF-α) and interferon gamma (IFN-γ) synthetic capacity of *ex-vivo* stimulated CD4+/CD8+ lymphocyte subpopulations in healthy subjects.

Material and methods

The subjects were 16 healthy young students (8 women and 8 men) aged 21-26 years (mean age 24) who voluntarily gave written, informed consent for study participation. After an overnight fast, peripheral blood samples were collected from each subject using 9 mL NH Sodium Heparin vacutainer tubes (Greiner Bio-One, cat. No.455051) and processed within 2h. A complete blood count (CBC) was performed and C-reactive protein (hsCRP) plasma level was measured by nephelometry (BN ProSpec, Siemens) with white blood cells levels above 10×10^9/L and hsCRP above 3mg/L as exclusion criteria. Experimental procedures were performed in the Cellular and Humoral Immunology laboratories

of the Advanced Medical and Pharmaceutical Research Center (CCAMF – UMFST, Târgu Mureș, Romania). This study was approved by the Ethics Committee of the University of Medicine, Pharmacy, Science, and Technology of Târgu Mureș (UMFST) and by the Ethics Committee of the Emergency Clinical County Hospital of Târgu Mureș.

The main steps of the study are summarized in Figure 1A. PBMC were isolated by density gradient centrifugation using a high-yield protocol that was previously tested and implemented in our laboratory (30). PBMC were suspended in RPMI 1640 culture medium (Sigma Aldrich, cat. No. R8758) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich cat.No. F7524) and 1% Antibiotic Antimycotic Solution (Sigma Aldrich, cat.No. A5955). For activation and ICS, Human Intracellular Cytokine Staining Starter Kit (BD Pharmigen, cat.No.559302) was used. The Leukocyte Activation Cocktail (LAC) is a ready-to-use polyclonal cell activation mixture containing a phorbol ester (Phorbol 12-Myristate 13-Acetate, PMA), a calcium ionophore (Ionomycin, ION), and a protein transport inhibitor (BD GolgiPlug, containing Brefeldin A). PBMC were stimulated with LAC 0.83µL/10^6cells(BD recommendation: 2µL/1-2 x 10^6 cells) for 4h and then transferred to 96-well plates, 0.4 × 10^6 cells/well (Figure 1B).

Surface staining was performed prior to fixation using anti-human CD3-Alexa-Fluor700, CD4-PerCP-Cy5.5, and CD8-APC-Cy7 antibodies, 5µL each/10^6 cells. Lymphocytes were subtyped as Th (CD3+CD4+) and Tc (CD3+CD8+).

Fixation and permeabilization were performed by suspending the activated peripheral blood lymphocytes (PBL) in BD Cytofix/Cytoperm Buffer (BD Biosciences, cat.No. 554714) for 20 minutes. This buffer contains paraformaldehyde for the fixation of cellular proteins and preservation of cell morphology, along with saponin

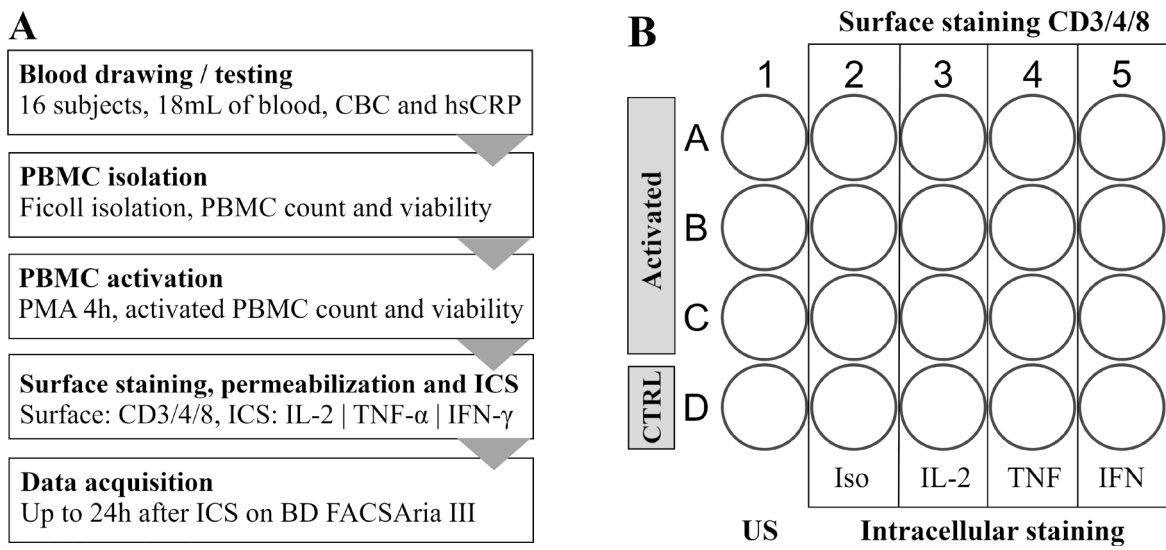

Fig. 1. A – schematic outline showing the main steps of the study; B – upper-left quadrant of a 96-well plate illustrating the staining template for one subject. PBMC were activated in triplicate (rows A-C) and compared to control, unstimulated cells (CTRL, row D). CBC – complete blood count; hsCRP – high-sensitivity C-reactive protein; PMA – Phorbol Myristate Acetate; US – unstained cells for negative staining control; Iso – isotype control wells.

which serves to permeabilize the cell membrane for subsequent staining of intracellular cytokines. Saponin-induced permeabilization is reversible, but maintaining cell membrane permeability throughout this phase of the protocol is crucial. Therefore, for the following steps, BD Perm/Wash Buffer (BD Biosciences, cat.No. 554723) was used in order to keep the cells permeabilized.

For the ICS step, we added PE-conjugated Isotype Control and PE-conjugated anti-cytokine antibodies (20µL/10^6 cells). All intermediary washing steps were performed according to the ICS kit instructions (31). We mention that each sample was activated, stained, and analyzed in triplicate. Moreover, in half of the subjects, we compared activated cells with control, unstimulated cells. Also, post-isolation and post-activation viabilities were tested in separate tubes by Annexin-V/PI double staining (FITC Annexin V Apoptosis Detection Kit I, BD Pharmingen cat.No. 556547). Lymphocytes were gated FSC/SSC and data were interpreted as follows: double negative for viability, Annexin-V single positive for early apoptosis and double positive for late apoptosis/necrosis (Figure 2).

Samples were analyzed within 24h on a FACSAria III cytometer with FACSDiva v8.0 Software (both BD Biosciences). Instrument characteristics and antibody specifications are shown in

Table 1. Parameter specifications and BD FACSAria III cytometer optics used for data acquisition. Window extension was set at 2µs, sheath pressure at 70 psi and a 70-micron nozzle was used.

*FITC-Annexin-V/PI viability double staining was performed in separate tubes. **Intracellular staining was performed in separate wells for isotype control and each cytokine (see Fig. 1B).

Parameter specifications				**Instrument optics**	
	Fluorochrome	**Isotype & Clone**	**BD Cat. No.**	**Filters LP and BP (nm)**	**LASER**
FSC	-	-	-	488/10	BLUE 488 nm
SSC	-	-	-		
CD4	PerCP-Cy5.5	Mouse IgG1,k & RPA-T4	560650	655 695/40	
-	PI*	-	556547 (kit)		
Isotype control	PE**	Mouse IgG1,k & MPOC-21. Rat IgG2b,k & A95-1. (Mixture)	559302 (kit)	556 585/42	
IL-2		Rat IgG2a,k & MQ1-17H12			
TNF-α		Mouse IgG1,k & Mab11			
IFN-γ		Mouse IgG1,k & B27			
Annexin-V*	FITC	-	556547 (kit)	502 530/30	
CD8	APC-Cy7	Mouse IgG1k & RPA-T8	557760	755 780/60	RED 633 nm
CD3	AlexaFluor700	Mouse IgG1k & UCHT1	557943	690 730/45	

Table 1. The flow cytometer was calibrated with BD setup and tracking beads (BD Biosciences, cat.No. 655050). For viability assessment, 10000 events were collected for each sample and cells were gated as shown in Figure 2. For the ICS cells, a minimum of 25000 events in the lymphocyte gate was collected for each sample and gating strategy was conducted as shown in Figure 3.

Flow cytometry plots and histograms were generated using FlowJo v10. In this study, all statistical processing was performed using Microsoft Excel, GraphPad Prism 6.0 or FACSDiva v8.0 and the significance threshold was set at 0.05. A Shapiro-Wilk normality test was applied to each data set. Paired t-Test was used for comparing viabilities and different parameters between cell populations. Only two-tailed p-values are reported.

Results

All data sets passed the Shapiro-Wilk normality test. Post-isolation and post-activation viabilities were tested in half of the subjects (n=8) using Annexin-V/PI double staining. The gating strategy for viability is presented in Figure 2A and 2B and PBMC viability is visually compared in Figure 2C. Mean viability values are presented in Table 2.

Surface staining and ICS were performed for all subjects (n=16) and cells were identified by flow cytometry using the gating strategy described in Figure 3. On average, singlets of CD3+ cells were 68% CD4+ (SD=5.7%) and 27% CD8+ (SD=4.8%), accounting for a mean CD4:CD8 ratio of 2.6 (range 1.7 to 3.9; SD=0.7).

No cytokines of interest were detectable in control, unstimulated cells. In PMA-activated samples, insignificant variability between triplicates was found, indicating high repeatability (>95%). Cytokine production in activated cells was compared between CD4+ and CD8+ lymphocyte subgroups and mean values are reported in Table 3. Both subpopulations were found to partially produce each of the three cytokines, but in different proportions. A significantly greater percentage of CD4+ cells was shown to produce IL-2 and TNF-α. On the contrary, a significantly higher proportion of CD8+ T-cells was found to produce IFN-γ.

Discussions

Isolation and activation of PBMC

Isolation of PBMC was performed using an already tested protocol for which we have previously reported high yields of 90% for 3mL blood samples (30). Nevertheless, our subsequent experience with PBMC density gradient isolation (this study included) showed us that processing blood volumes higher than 3mL will in fact significantly lower the cell yield.

Several concentrations of PMA/ION are reported in the literature for PBMC activation, ranging from 1 to 100ng/mL PMA (32-39) and from 0.4 to 1.4μM for ION (that is roughly 300-1000ng/mL) (32-36,38), depending on protocol requirements and personal preferences. While the exact PMA concentration values seem to be chosen arbitrarily (e.g. 1, 2.5, 5, 20, 25, 50 or 100ng/mL), most studies recommend that ION be added at a concentration of 1μM, that is 710ng/mL. Also, the incubation time may vary between protocols, depending on the desired downstream application of the activated cells. Short-term activation requires a minimum of 4-6h (32-38) and usually employs higher concentrations of PMA/ION, while longer activation times may reach up to 24-72h (33,37).

The concentrations of PMA, Ionomycin, and Brefeldin A are not reported in this paper because cells were activated using a validated cocktail provided by the ICS kit. However, as the LAC ingredients can also be purchased individually, BD makes recommendations on the

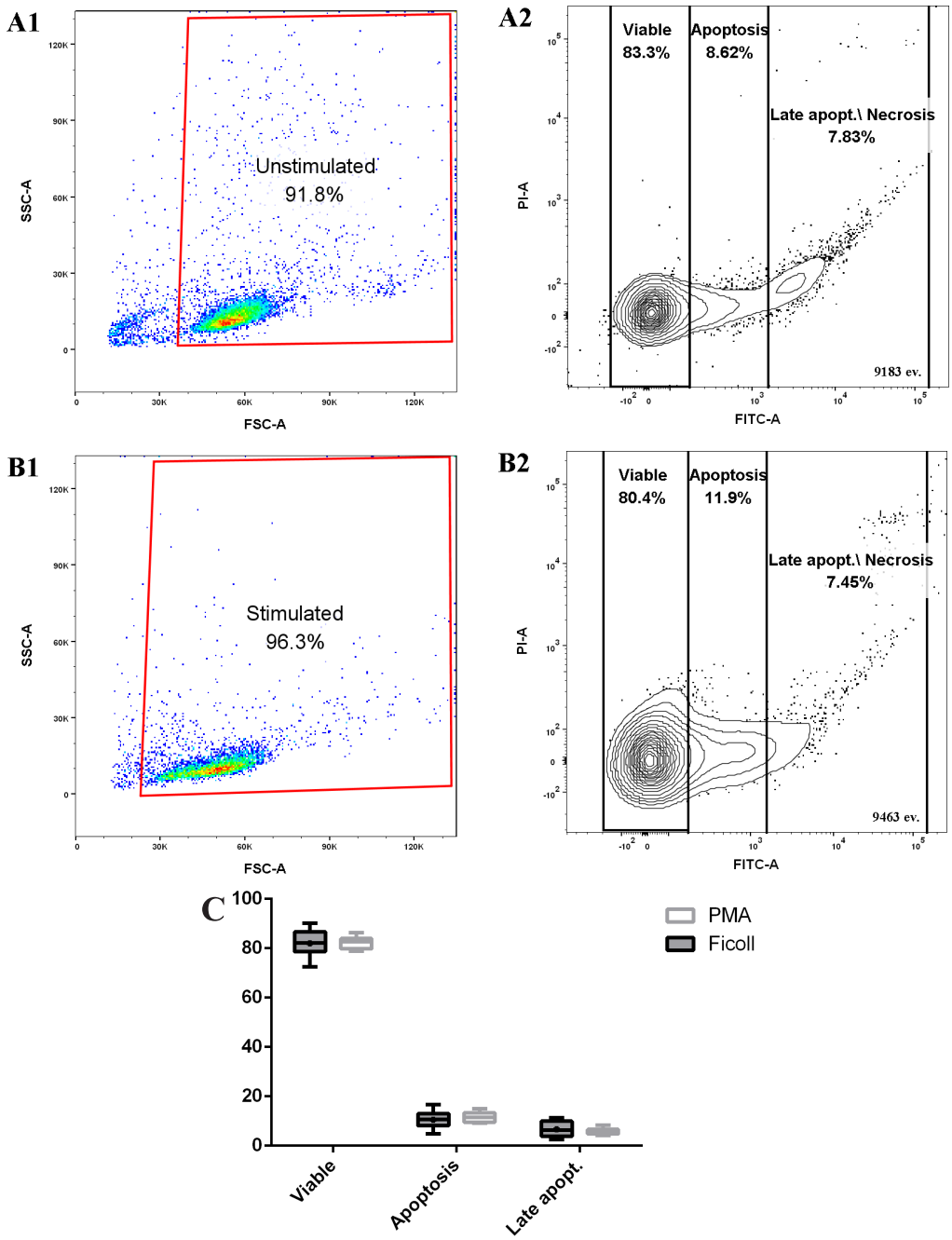

Fig. 2. Viability gating of non-debris events following isolation (A) and activation (B) of PBMC. A1, B1 – FSC/SSC pseudocolor plots (10000 events) for gating of all non-debris events. A2, B2 – contour plots (probability, 5%) for FITC-Annexin-V/Propidium Iodide gating of previously gated non-debris events: double negative events are considered viable, Annexin-V positive events are apoptotic and double positive events are considered late apoptotic or necrotic. C – Box and Whiskers plot showing viability comparison before (Ficoll) and after activation (PMA). Figures A and B were generated in FlowJo v10. Figure C was generated in GraphPad Prism 6.

Table 2. Post-isolation and post-activation viability of PBMC expressed as mean ± SD percentage of all non-debris events

	Viable (%)	Apoptosis (%)	Late apoptosis / necrosis (%)
Post-isolation	82.1 ± 5.5	10.5 ± 3.6	6.6 ± 3.2
Post-activation	82.4 ± 2.5	11.5 ± 2.1	5.9 ± 1.3
P value	0.84	0.35	0.6

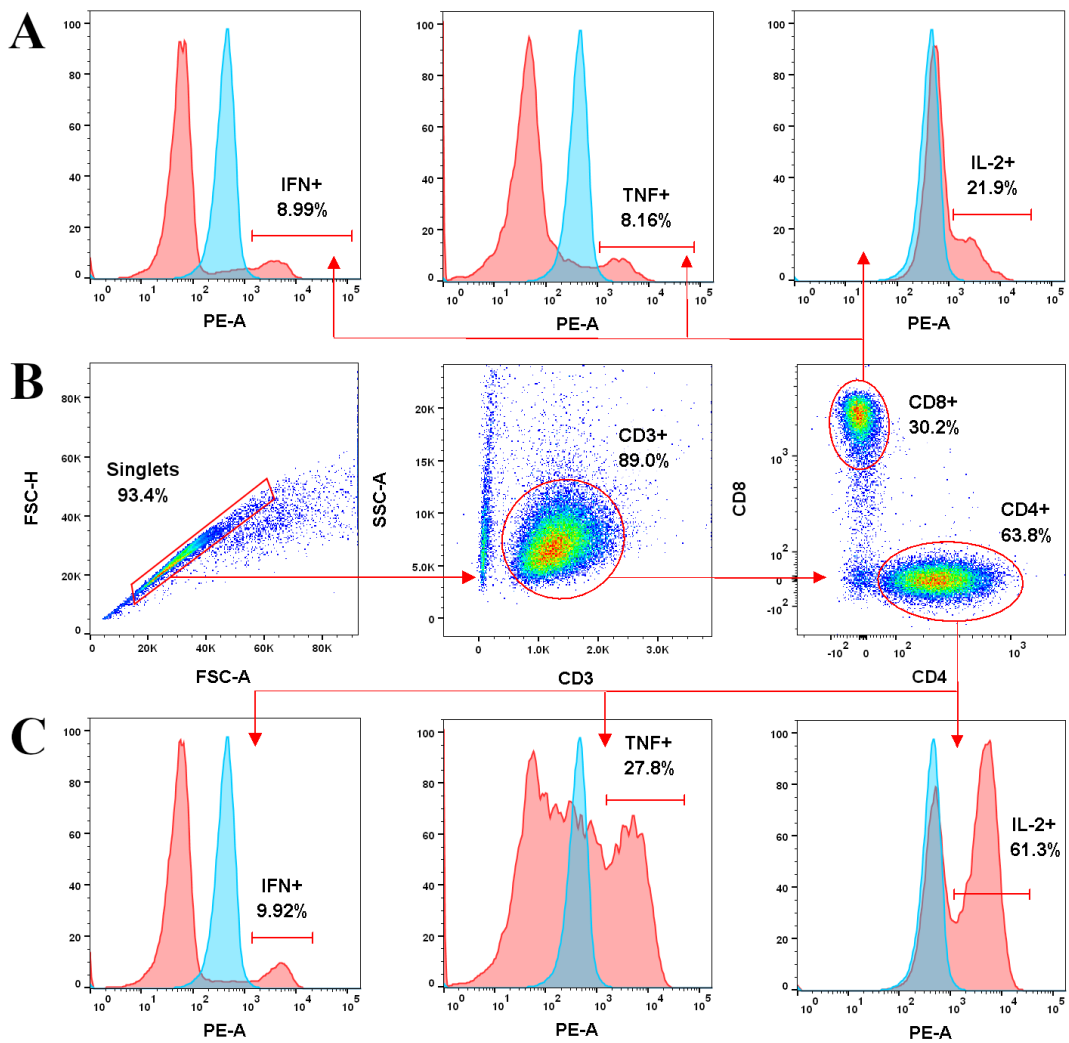

Fig. 3. Gating strategy for identification of cytokine-producing CD4+ and CD8+ cells. (Left to right) B – pseudocolor density plots: doublet discrimination, gating of CD3+ cells, gating of CD4+/CD8+ cells; A and C – histograms showing cytokine-producing cells (IFN-γ, TNF-α, IL-2) within the CD8+ and CD4+ populations, respectively. Surface staining was performed using anti-human CD3-AlexaFluor700, CD4-PerCP-Cy5.5 and CD8-APC-Cy7 antibodies. For intracellular staining, PE-conjugated isotype control and anti-cytokine antibodies were used. The blue population in histograms represents the isotype threshold. Plots and histograms were generated in FlowJo v10 and each plot displays a total of 25-30.000 events.

Table 3. Proportion of cytokine-producing cells expressed as mean ± SD percentage of CD4+ or CD8+ populations.

	IL-2 (%)	TNF (%)	IFN (%)
CD3+CD4+	61.5 ± 5.8	26.9 ± 11	6.8 ± 3.2
CD3+CD8+	25.0 ± 5.6	7.5 ± 3.3	8.4 ± 3.9
P value	< 0.0001	< 0.0001	0.01

optimal concentration of each ingredient for cellular activation purposes: PMA 5ng/mL, ionomycin 500ng/mL and BD GolgiPlug 1µL/mL of cell culture. Therefore, the LAC ingredients must have concentrations similar or identical to those recommended on the BD website (40). The ICS kit's manual (31) does not give the exact concentration of LAC needed for activation, but specifies that 10µL of LAC can activate $6 - 12 \times 10^6$ cells. It should be stated that, in this study, cells were activated using the lowest recommended LAC concentration ($10\mu L/12 \times 10^6$ cells, that is $0.83\mu L/10^6$ cells) which, given the results, seems to elicit a satisfactory cytokine response from T-cells.

Viability and cytokine synthesis

Using the same isolation and viability staining protocols, we have previously shown that the post-isolation viability of monocytes is low (17%), while lymphocytes have very high viabilities (86-91%) (30). However, in the present study, we report an average post-isolation viability of only 82%. The answer to this slight decrease in viability may come from the different gating strategy employed in this study. In the previous study, lymphocyte and monocyte populations were gated and analyzed individually (FSC/SSC). Here, we assessed the global viability of all non-debris events (Figure 2A1), that is, lymphocytes and monocytes altogether. Therefore, we expect the global post-isolation viability to be slightly lower than the real viability of the lymphocytes. Also, as PBMC viability assessment was not our main goal in this study, for practical reasons, the An-V/PI viability staining was only performed about half an hour after PBMC isolation. This delay between isolation and staining may have also negatively impacted overall cell viability.

Generally, monocytes account for about only 10% of the isolated PBMCs and we have previously shown that less than 20% of these monocytes are viable (30). Moreover, during the 4h activation period, most of the already few viable monocytes will either die or adhere to culture flasks. Therefore, as can be seen in Figure 2B1, the cell suspension recovered from the culture flasks following activation virtually contains no monocytes, only activated peripheral blood lymphocytes (PBL). However, Macian et al. showed that activation can induce cell death in obsolete effector T-cells (41). Consequently, not all PBL are expected to survive activation, especially if a potent activator is used, as is the case for PMA. Compared to other popular techniques, activation by PMA/ION was shown to induce the highest apoptosis rate (42) which is compensated by high proliferation rates (42) and abundant cytokine production (43). Therefore, PMA/ION induces a "survival of the fittest" type of activation which kills obsolete and susceptible cells while efficiently setting up the surviving cells for proliferation and secretion. In this study, the post-isolation and post-activation viabilities were very similar (see Table 2).

In our study, the mean CD4:CD8 ratio was 2.6, which is considered physiological in healthy subjects and is similar to other findings (43,44). Therefore, activation-induced cell death does not seem to (sensibly) affect one of these lymphocyte subtypes in particular (data not shown).

Although human lymphocytes are constantly stimulated *in-vivo* by various antigens, the activation status of the PBL in healthy subjects is considered a physiological baseline. In this study, the unstimulated PBMC served as a representative of this baseline to which PMA-stimulated cells could be compared. Both CD4+ and CD8+ stimulated T-cells were able to produce each of the three cytokines IL-2, TNF-α, and IFN-γ, but in different proportions. This observation is in accordance with the literature, as both of these lymphocyte subpopulations were shown to produce these cytokines (45-48). We found that CD4+ T-cells synthesize IL-2 and TNF-α in significantly higher proportions than CD8+ cells, while IFN-γ was produced by a significantly higher percentage of CD8+ T-cells. These results are also supported by the literature. It is well known that activated CD4+ T-cells are the main source of IL-2, being capable of consistently producing this cytokine (47). IL-2 production can be also detected in CD8+ T-cells, but it is not exemplary for this cell type. Upon initial activation, CD8+ T-cells produce IL-2 for a brief period of time, but then lose the capacity to produce this cytokine, therefore, during this phase of the immune response, CD8+ T-cells rely on extrinsic sources of IL-2 such as the activated CD4+ cells (45). Although macrophages are the main source of TNF-α (49), the production of this cytokine was also described for T-cells (46). A recent study, also aiming to establish a reference interval, reported the following proportions of activated cytokine-producing cells, compared with us: CD4+ 30% IL-2+ (vs. 61%), 25% TNF-α+ (vs. 27%), 23% IFN-γ+ (vs. 7%); together with CD8+ cells 3% IL-2+ (vs. 25%), 25% TNF-α+ (vs. 7%) and 43% IFN-γ+ (vs. 8%) (35). Such significant differences are very common between ICS protocols and, to a certain extent, between other flow cytometry experiments. Regarding the sex and age of our subjects, we selected 8 women and 8 men aged 21-26 years. Statistical analysis revealed no significant differences in CD4:CD8 ratio or cytokine production between men and women (data not shown). However, since cytokines play such an important role in immunity, changes in the cytokine profile are expected in accordance with the natural evolution of the immune system from the immune naivety of the newborns to the mature immunity of the adults and consequent immune senescence of the elderly. Thus, multiple studies have investigated the dynamics of the human cytokine profile in relation to age, showing that cytokine production suffers continuous modifications from birth and throughout life. A recent review was published by Marie-Luise Decker et al. which extensively covers this topic both for soluble and intracellular cytokines (50) in children (0-17 years) and in adults (18+ years). This paper showed that, throughout literature, intracellular IFN-γ and TNF-α production by activated CD4+ and CD8+ cells positively correlates with age, regardless of cell stimulant (PMA/ION, LPS, TLR-agonist), sample type (CBMC, PBMC, whole cord/peripheral blood) and duration of stimulation (4-24h). For intracellular IL-2, the literature is controversial, but evidence pleads for a negative age correlation for this cytokine. These conclusions are consistent with multiple studies where the same age-dependent variations were also observed for soluble cytokine levels. However, there are too many variables to be taken into consideration in order to meta-analyze the data from literature, such as: age and other particularities of the subjects, sample type, cell stimulant, duration of stimulation etc. Therefore, as Marie-Luise Decker et al. concluded, the heterogeneous results and the lack of consistency and comparability frequently seen between these studies is a major issue (50). This knowledge emphasizes the great need for optimization and standardization in this niche of research and the importance of adequate reference intervals. Moreover, each subject/patient should

be regarded as a unique individual, not only in relation to age, but to heredity, sex, environment, etc. Only then could such complex techniques be confidently implemented in routine clinical practice for the assessment of patient IIP.

Intracellular staining – a tool for precision medicine

Precision medicine is an emerging health care approach that takes into consideration genetic, environmental, and lifestyle individual variability when preventing and treating disease. Since the immune system is involved directly or indirectly in virtually every disease, assessing IIPs is an important prerequisite for precision medicine. Despite continuous progress in this area, IIP assessment remains a challenging task. This is especially due to the interindividual variability of the immune system, but also to the technical complexity and lengthiness of such experimental procedures. Several IFAs have been developed, but only a few are implemented in routine clinical practice. Therefore, there is an increasing demand for optimization and standardization of IFAs so that more practicable alternatives for clinical use can be established.

The assessment of IIP should always be followed by an interpretation with respect to the clinical context of the subject/patient. In our study, all subjects were apparently healthy, as we only aimed to collect enough data for the establishment of a preliminary T-cell cytokine production reference range specific to this activation protocol. However, considering ICS-IFA as a tool for precision medicine, the interpretation of the results requires a certain degree of knowledge and understanding of the intricate cytokine dynamics observed in different diseases. Thus, we conducted a literature search in order to identify disease-specific alterations in the cytokine profile of CD4+ and CD8+ T-cells, as reported by other ICS experiments. Finally, we summarized our literature findings in Table 4 where, in accordance with the present study, cytokine alterations are only presented for IL-2, TNF-α and IFN-γ.

Alterations in cytokine production and/or activity occur in many pathological processes, but arguably more persistently and to a greater extent in the development and evolution of autoimmune diseases. Consequently, cytokine-production IFAs are a critical tool for assessing IIPs, especially in the setting of precision medicine. ICS is an advanced IFA most notably used for evaluating T-cell immune response by assessing intracellular cytokine synthesis. The main advantage of ICS is that cytokine production can be tracked at the single cell level, but this versatile technique also enables the simultaneous assessment of cell phenotype and various differentiation and functional parameters (e.g. transcription factors, phosphoproteins etc.). Moreover, recent technological advances in flow cytometry have increased the number of markers that can be analyzed simultaneously in experiments using state-of-the-art devices. Also, a practical advantage is that ICS protocols can be conveniently paused at certain stages of the process, without affecting the overall experiment and its results. These characteristics make ICS a valuable tool for precision medicine, particularly useful for the in-depth analysis of the immune response and assessment of IIPs.

Strengths and limitations

Like many IFAs, ICS is a complex, lengthy, and costly procedure: a reason why most papers on this topic only report small sample sizes. Our study was conducted on a relatively large group of subjects and each sample was activated and analyzed in triplicate, rendering the results more robust. The main limitation of this study was dictated by the ICS kit factory design, since all anti-cytokine antibodies were conjugated with the same fluorochrome (PE). Consequently, staining/analysis was performed in distinct wells/tubes for each cytokine of interest (see Figure

Table 4. A short list of disease-specific alterations in the IL-2/TNF-α/IFN-γ cytokine profile of Th and Tc lymphocytes, as reported by other ICS experiments. Diseases were grouped in four main categories (top to bottom): infections, autoinflammatory and autoimmune diseases, malignancies, and transplantations. Upwards(↑)/downwards(↓) arrows indicate an increase/decrease in the proportion (%) of the specified cytokine-producing cell type (CD4+/CD8+). Abbreviations: TB – tuberculosis, HIV – human immunodeficiency virus, CMV – cytomegalovirus, FLU – influenza virus, VcV – vaccinia virus (smallpox vaccine virus), SLE – systemic lupus erythematosus, MS – multiple sclerosis, PP/RR/SPMS – primary progressive/relapsing-remitting/secondary progressive multiple sclerosis, cITP – chronic immune thrombocytopenia, UCTD – undifferentiated connective tissue disease, SSc – systemic sclerosis, l/dSSc – limited cutaneous/diffuse cutaneous systemic sclerosis, MCTD – mixed connective tissue disease, IBD – inflammatory Bowel Disease, ALL – acute lymphoblastic leukemia, B-CLL – B cell chronic lymphocytic leukemia, GI – gastrointestinal, AdCC – adenocarcinoma, NST – nonmyeloablative allogenic stem cell transplantation, aGVHD – acute graft-versus-host disease, SOT – solid organ transplantation.

		IL-2	TNF-α	IFN-γ	
Infections	TB	↓CD8+	↑CD8+ ↑CD4+	↑CD4+	[21]
		↑CD4+TNF+IL2+ ↑CD4+TNF+IFN+ ↑CD4+TNF+IL2+IFN+			
	HIV	↑CD8+IL2+IFN- and ↑CD4+IL2-IFN+ in HIV-controllers; CD4+IL2+IFN- for viremia control			[19]
	CMV FLU VcV	Each virus has an individual profile with different proportions of double- and triple-secretory CD4+ cells Triple-secretory CD4+ cells are the most functionally active cells			[20]
	General	Absent in CD8+ M3 (memory)	Single secretion in CD8+ N/M1, double-secretion in M2 and M3		[13]
Inflam./autoimm.	SLE	↑CD4+		↑CD4+ in SLE with arthritis	[16]
	MS	↓CD4+IL2+ in PPMS v. RR/SPMS		↑CD4+ ↑CD8+	[11]
	cITP			↑CD4+	[15]
	Behcet				[14]
	UCTD				[10]
	SSc	↑CD8+ in limited cutaneous (lSSc)		↑CD8+ in lSSc and dSSc	
	MCTD			↑CD8+	
	Crohn's			↓CD4+	[17]
	IBD			↑CD3+	[18]
Malignant	ALL	↓CD4+ ↓CD8+		↓CD4+ ↓CD8+	[22]
	B-CLL	↓CD4+ ↓CD8+	↑CD8+ in advanced disease	↑CD8+ in advanced disease	[23]
		↓CD8+ in progressive disease (PD)	↑CD8+ and ↑CD4+ in PD		[25]
	solid GI	↓CD4+		↓CD4+	[26]
	AdCC		↑CD8+ in immunized subj.	↑CD8+ in immunized subjects	[24]
Transpl.	NST	↓CD4+IFN+ and ↓CD8+IFN+ as indicators of relapse ↑CD4+IFN+ and ↑CD8+IFN+ as indicators of aGVHD			[12]
	SOT	Therapeutic immunodefficiency is dependant on drug dose and duration of treatment			[27]
		CMV-CD8+ as predictors of CMV viremia, disease severity and relapse			[28]
		↓CMV-CD8+CD69+IFN+ as predictor of CMV infection in SOT patients (kidney and liver)			[29]

1B), denying the assessment of intracellular co-localization of multiple cytokines. However, this issue can be easily avoided in future research by using customized antibodies instead. Another limitation was the lack of other cell surface markers besides CD4 and CD8. A more in-depth phenotyping would have enabled the analysis of the various subpopulations of Th cells (Table 4).

Abbreviations

CBC complete blood count
CTRL control unstimulated cells
FBS fetal bovine serum
FSC forward scatter
hsCRP high-sensitivity C-reactive protein
ICS intracellular cytokine staining
IFA immune functional assay
IFN-γ interferon gamma
IIP individual immune profile
IL-2 interleukin 2
ION ionomycin
Iso isotype control
LAC leukocyte activation cocktail
LPS lipopolysaccharide
PBL peripheral blood lymphocytes
PBMC peripheral blood mononuclear cells
PHA phytohemagglutinin
PI propidium iodide
PMA phorbol myristate acetate
SSC side scatter
TNF-α tumor necrosis factor-alpha
US unstained

Authors' contributions

MIB participated in the design of the study, carried out PBMC isolation and ICS assays, performed data acquisition and statistical analysis and drafted the manuscript. MDR and SGM participated in the design of the study, carried out PBMC isolation and ICS assays, performed data acquisition and revised the manuscript. DM conceived the study, participated in PBMC stimulation assays, coordinated the acquisition/interpretation of data, assisted the statistical analysis and significantly contributed to draft the manuscript. All authors agreed to be accountable for all aspects of the work and have read and approved of the final manuscript.

Conflicts of interest

The authors declare no conflict of interest.

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