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Flow cytometry as an integrative method for the evaluation of vaccine immunogenicity: A validation approach

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ABSTRACT

The applied bioanalytical assays used for the evaluation of human immune responses from samples collected during clinical trials must be well characterized, fully validated and properly documented to provide reliable results. Even though recommendations for the standardization of flow cytometry instrumentation and assay validation for its clinical application have been published by several organizations, definitive guidelines are not available yet. The aim of the present paper is to provide a validation approach for flow cytometry, examining parameters such as linearity, relative accuracy, repeatability, intermediate precision, range and detection limits and specificity, in order to demonstrate and document its applicability for clinical research purposes and its possible use as one of the methods for the evaluation of vaccine immunogenicity.

1. Introduction

The results of cell-mediated as well as humoral immune responses evaluated in samples collected during clinical trials are used to make critical decisions supporting the efficacy of a vaccine, medicinal drug substance or product. For this reason, the applied bioanalytical assays used must be well characterized, fully validated and properly documented to provide reliable results (i.e. consistent and reproducible data). As highlighted in the "Guideline on bioanalytical method validation" by Ref. [1], the validation of an analytical method is based on the achievement of the following criteria: accuracy, robustness, precision, specificity, linearity, sensitivity (limits of detection/quantification). Although recommendations for the standardization of flow cytometry instrumentation and assay validation for its clinical application have been published by several organizations, such as the AAPS Flow Cytometry Action Program Committee, International Council for Standardization in Haematology and the International Clinical Cytometry Society, so far definitive guidelines are not available yet [2,3]. Conversely to other laboratory assays which are represented by quantitative methods and can be fully validated, flow cytometry is defined as a semi-quantitative assay since the numerical result obtained from the test sample is proportional to the sample but not generated from a calibrator or reference material. The lack of qualified reference materials, the intrinsic cell variability, as well as their stability, the bioanalytical category of the data, the complexity of the technology and of standardizing data analysis, make flow cytometry validation difficult [4]. As a consequence of these lacks, the validation of the flow cytometry method requires a "validation strategy" which is based on the fundaments reported in Good Laboratory Practice (GLP) and Good Clinical Laboratory Practice (GCLP) guidelines, in order to demonstrate that the assay is Fit for purpose (FFP) within the Context of Use (COU) [5].

The aim of the present paper is to document the results obtained from the validation of the flow cytometry assay using fluorescent antibodies to characterize cell populations within PBMCs and their production of inflammatory markers (interleukins and cytokines) in human samples. In detail, we wanted to assess if the assay could be validated for the detection and quantitation of IL-2, IL-13, CD40L, IFN-γ and TNF-α cell markers in specific live, $CD3^+$ $CD4^+$ or $CD8^+$ positive human cell populations so that it could be used for the evaluation of such populations in human clinical samples collected from clinical trials performed for the evaluation of the efficacy of vaccines.

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2. Materials and methods

2.1. PBMCs thawing and stimulation

Commercially available human Peripheral blood mononuclear cells (PBMCs) were purchased from CTL Europe GmbH (CAT# CTL-CP1). PBMCs were rapidly thawed (about 2 min (min) at 37 °C) and transferred in a 15 ml sterile tube containing pre-warmed thawing solution (PBS w/o Ca^{2+} Mg^{2+} ((Gibco Life Sciences, CAT# 14190-250) containing 2.5 mM Ethylene Diamine Tetraacetic Acid (EDTA) (PanReacAppliChem, CAT# A3145) and 20 μg/mL Deoxyribonuclease I (DNAse I) ((Sigma, CAT# DN25-100 mg)). Samples were washed twice by centrifugation at 311*g* for 10 min at room temperature (RT) and then counted. PBMCs were resuspended in complete medium prepared as follow: RPMI-1640 with L-Glutamine (Gibco, CAT# 52400-025) additionated with 1% Foetal Bovine Serum (FBS) (Euroclone, CAT# ECS0180L), 1% Penicillin/Streptomycin 100X (Euroclone, CAT# LODE17602E), 1% Na pyruvate 100X (Gibco, CAT# 11360-039), 1% Non-essential amino acids 100X (Gibco, CAT# 11140-035), 1 μg/ml anti-CD28 (Purified NA/LE Mouse Anti-Human CD28, BD Biosciences, Clone CD28.2, CAT# 555725) and 1 μg/ml anti-CD49d (BD Pharmingen™ Purified NA/LE Mouse Anti-Human CD49d, BD Biosciences, CAT# 555501). Negative and positive controls were set up by incubating the cells with complete medium and complete medium supplemented with Staphylococcal enterotoxin B from *Staphylococcus aureus* (SEB) (Sigma Aldrich, CAT# S4881-5 mg) at the final concentration of 1 μg/ml, respectively. One million cells were cultured in a 96-well round bottom plate in complete medium for each of the conditions evaluated. Cells were incubated for 16 h (hs) at 37 °C (Celsius centigrade) in humidified atmosphere containing 5% CO_2. After 2 hs of incubation, Brefeldin A (BFA) (Sigma, CAT# B7651-5 mg) was added in each well at the final concentration of 5 μg/ml.

2.2. Flow cytometric analysis (FACS)

After overnight incubation (16 hs), the plates containing PBMCs were centrifugated at RT at 699 g for 4 min (min). The supernatant was discarded and PBMCs were washed with 200 μl/well of PBS-2.5 mM EDTA and centrifuged at 699 g for 4 min. Cells were then stained with Live/Dead staining (ThermoFischer, CAT# L34976) (1:1000 in PBS) and incubated for 20 min at RT in the dark. Cells were then washed twice in PBS-2.5 mM EDTA and centrifuged at RT at 699 g for 4 min. Then the cells were permeabilized incubating them with 1X BD cytofix/cytoperm (CAT# 554714) in the dark for 20 min at 4 °C and after they were washed twice with 1x perm/wash buffer in PBS-2.5 mM EDTA + 1% BSA (CAT# 15260037) and centrifuged at 699 g for 4 min, at 4 °C (+2/+8 °C). To identify T cell subsets, the supernatant was removed and single-cell suspension was stained with the appropriate combination of the following directly conjugated monoclonal antibodies (MoAb) whose have been previously titrated to select their correct dilution: CD3 BV786 Clone SK7 (CAT# 664527), CD4 BB700 Clone SK3 (CAT# 566392), CD8 BV510 Clone RPA-T8 (CAT# 563256), CD40L APC Clone TRAP1 (CAT# 555702), IFN-γ A-700 Clone B27 (CAT# 557995), TNF-α PE CY7 Clone MAb11 (CAT# 557647), IL-13 BV421 Clone JES10-5A2 (CAT# 563580) and IL-2 PE Clone MQ1-17H12 (CAT# 559334) (BD Biosciences) diluted in perm/wash buffer. Perm/wash buffer was previously prepared diluting it in PBS-2.5 mM EDTA + 1% BSA + 2% normal rabbit serum (CAT# 10510). The cells were incubated with the mixture containing fluorescent antibodies for 20 min at RT in the dark. Cells were then washed twice with perm/wash buffer 1x, centrifuged at 699 g for 4min at RT, resuspended in PBS-2.5 mM EDTA and samples were acquired at the FACS machine (BD LSR II 4 LASER, interfaced to PC FACS Diva software 8.0.1 (BD Biosciences). 500,000 events per sample were analyzed. Dead cells were excluded from the analysis. Initial gating selected only live cells using an amine reactive dye (Live/Dead staining). The lymphocytes were gated using a drawn gate using SSC-A (granularity) and FSC-A (size). The single cells were selected using SSC-A and SSC-W gate. Subsequent gating allowed to select $CD3^+$ cells and within this $CD3^+$ lymphocyte gate, CD4 and CD8 T cells were identified (Fig. 1). The number of $CD4^+$ and $CD8^+$ cells expressing each marker (IL-2, IL-13, CD40L, IFN-γ and TNF-α) was evaluated.

2.3. Flow cytometry validation parameters

The assay parameters examined included: Range and Detection Limits, Repeatability/Intra-assay Precision, Intermediate Precision, Specificity, Linearity, Relative Accuracy, as summarized in Table 1. Robustness was not evaluated due to the lack of multiple lots of fluorescent antibodies and PBMCs.

The assay validation criteria evaluated were selected from European Medicines Agency (EMA) "Guideline on bioanalytical method validation" [1], Food and Drug Administration (FDA) "Guidance for Industry; Q2B Validation on Analytical Procedures Methodology" [6,7], and International Conference on Harmonization (ICH) guidelines "Validation of analytical procedures: text and methodology; ICH Harmonised Tripartite Guideline, Q2(R1)" [8].

2.4. Statistical analysis

The percentage of CD4 and CD8 cells positive for each marker was calculated versus (*Vs*) the live cell population. For each parameter evaluated R2, GM or CV were calculated as described in Table 1.

3. Results

The validation protocol design allowed different components contributing to the overall variability of the assay to be distinguished guaranteeing the flow cytometry assay for its dilutional linearity, relative accuracy, repeatability, specificity, intermediate precision, range and detection limits.

3.1. Range and detection limits

From the evaluation of range and detection limits it has been observed that the acceptance criteria were met for all the cytokines. Regarding $CD4^+$ IL-13+ and $CD8^+$ IL-13+, special considerations have been made (see "3.1.1") since even for cells stimulated with SEB, the number of positive cells for this marker was too low to be detected and comparable to the unstimulated condition.

3.1.1. Special considerations for $CD4^+$ and $CD8^+$ IL-13 subpopulations

The measurements on the unstimulated cells (Blank) returned a mean = 0.009 and standard deviation (SD) = 0.003. A bootstrap method with 100,000 resamples from normal distribution was used for estimating the threshold in the blank distribution which identifies the limit of detection (LOD). Assuming that LOD = mean(Blank) + 3.3xSD (Blank), the threshold was calculated as the quantile of the normal distribution with probability $p = 0.9995$ (Fig. 2A).

The extreme values observed in the right tail of the distribution of the blank samples starting at the threshold value (red dotted line) equal to 0.021 constituted the distribution of the expected LOD values (Fig. 2B). The 99% confidence interval of the LOD distribution was in the range [0.021: 0.024]. From the measurements on SEB-stimulated cells we calculated a mean = 0.023 and a SD = 0.003 (Fig. 2B). When analyzing the IL-13 sample for the $CD8^+$ cells population we calculated a mean and a SD for the unstimulated cells equal to 0.005 and 0.003, respectively. The bootstrap distribution of blank samples is reported in Fig. 2C. The lower limit of LOD (i.e., the threshold) was 0.014 and the 99% confidence interval of the LOD distribution was in the range [0.014: 0.017] (Fig. 2D). The returned a mean equal to 0.005 and a SD equal to 0.004 with an expected value below the threshold of the LOD distribution (Fig. 2C and D). As for all fluorescent antibodies used in these validation

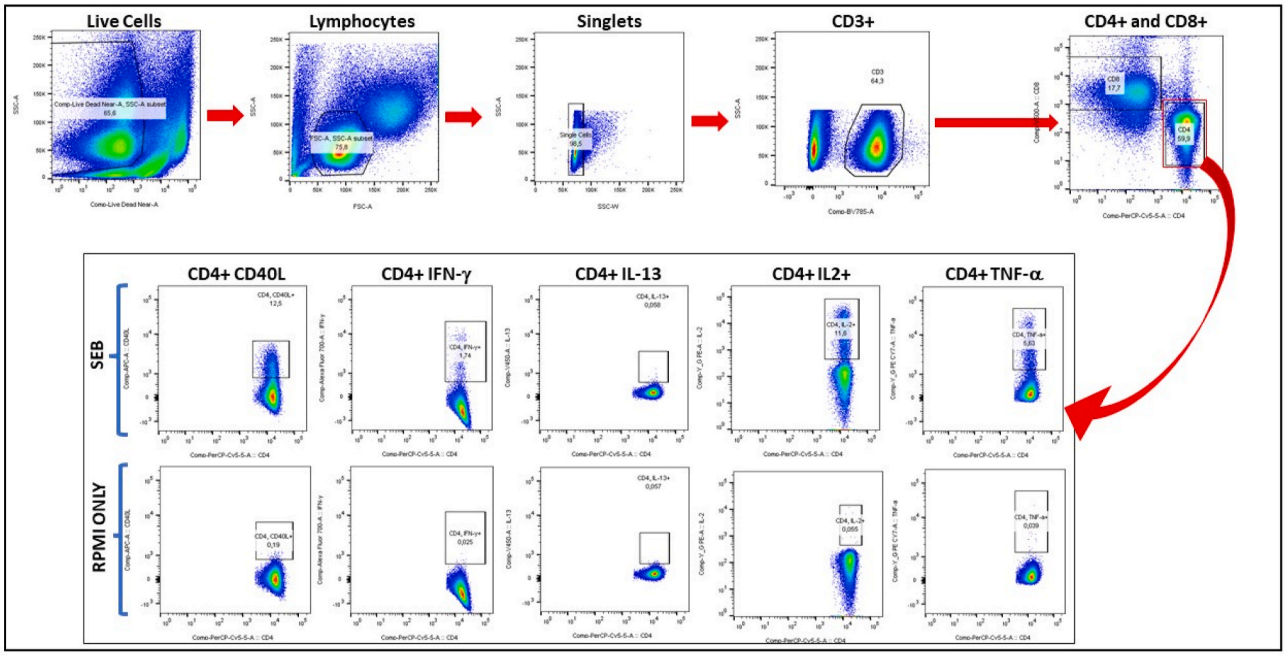

Fig. 1. Representative gating strategy for flow cytometry-based evaluation of cytokine production in SEB-stimulated $CD4^+$ cells *vs* medium.

experiments, also for IL-13 the choice of the optimal concentration to be used in the staining mix was previously chosen in a set-up experiment by calculating the Mean of Fluorescence Intensity (MFI) at different dilutions (from 1:20 to 1:320) (Table 2). The calculation of MFI allowed to choose 1:40 as the best concentration for IL-13 to be used in validation experiments.

3.2. Precision- repeatability

For the SEB-stimulated $CD4^+$ subpopulation, the assay met the acceptance criteria for repeatability for all the cytokines evaluated. For the unstimulated $CD4^+$ subpopulation, the assays met the criteria for INF-γ+, IL-2+ and TNF-a+ cells. However, the assay for CD40L+ and IL-13+ cells failed to meet the acceptance criteria for repeatability with CVs% of 33.54% and 23.82%, respectively. These findings suggest that for these two cytokines a relative variability higher than 20% was present in unstimulated $CD4^+$ cells.

For the SEB-stimulated $CD8^+$ subpopulation, the assay was repeatable for all the cytokines evaluated with the exception of IL-13. For the unstimulated $CD8^+$ subpopulation, the assay met the acceptance criteria for repeatability for all the cytokines evaluated (Table 3).

3.3. Precision- intermediate precision

Whereas for the SEB-stimulated $CD4^+$ subpopulation, the assay met the acceptance criteria for intermediate precision for all the cytokines evaluated, for the unstimulated $CD4^+$ subpopulation, none met the acceptance criteria for intermediate precision.

Concerning the SEB-stimulated $CD8^+$ subpopulation, the assay met the acceptance criteria for intermediate precision for all the cytokines evaluated with the exception of IL-13, whereas for the unstimulated $CD8^+$, the assay for IL-2+ and TNF-α+ met the acceptance criteria for intermediate precision (Table 4).

These data were generated in two independent experiments in each of which 6 biological replicates were performed for each condition (n = 12).

3.4. Specificity

Regarding specificity, when the denominator in the signal to noise (s/n) ratio obtained was 0, the ratio could not be calculated and the s/n was reported as n/a. For the $CD4^+$ subpopulation, the test resulted to be specific for all the cytokines evaluated with the exception of cells stained for IL-13 and cross-evaluated for CD40L and IFN-γ. The IL-13 signal/noise ratio for CD40L and the IL-13 signal/noise ratio for IFN-γ were 1.389 and 0.568, respectively (See "3.1.1").

Concerning the $CD8^+$ subpopulation the test was specific for all the cytokines evaluated (Table 5).

3.5. Dilutional linearity

For both the $CD4^+$ and $CD8^+$ subpopulations, the assay met the acceptance criteria for dilutional linearity for all the cytokines evaluated except IL-13 (Figs. 3 and 4). For IL-13 it was not possible to narrow the range of dilutions for the calculation of linearity as the number of positive cells for this cytokine obtained in the undiluted SEB-stimulated samples was small (See "3.1.1").

These data were generated in two independent experiments in each of which 2 biological replicates were performed for each condition. Total replicates n = 4.

This experiment is representative of two independent experiments wherein 2 biological replicates were performed in each. Total replicates n = 4.

3.6. Relative accuracy

For the $CD4^+$ subpopulation, the assay met the acceptance criteria for relative accuracy for all the cytokines evaluated at all the dilutions tested with the exception of IL-13 for all dilutions tested (1:2 to 1:128) and the highest dilution tested (1:128) for IL-2 and TNF-α.

Regarding the $CD8^+$ subpopulation, only a few dilutions tested for some of the cytokines met the acceptance criteria for relative accuracy. The acceptance criteria were not met for CD40L from 1:32 to 1:128 (the highest dilutions tested), for INF-γ from 1:8 to 1:32 dilutions, for IL-13 from 1:2 to 1:128 dilutions (all dilutions tested), for IL-2 for 1:2 and from 1:8 to 1:32 dilutions and for TNF-α from 1:8 to 1:32 dilutions.

Table 1
Summary of Validation Parameters examined and Acceptance Criteria.

PARAMETER	DESCRIPTION	ACCEPTANCE CRITERIA
Dilutional Linearity	Linearity was evaluated by spiking SEB-stimulated stained cells into unstimulated stained cells. In particular, SEB-stimulated stained cells were evaluated neat and mixed in a 50% (1:2), 25% (1:4), 12.5% (1:8), 6.25% (1:16), 3.13% (1:32), 1.56% (1:64) and 0.78% (1:128) ratio in unstimulated stained cells in order to obtain 2 replicates from one operator. Dilutional Linearity samples were tested by two operators in two different days. The number of cells expressing each marker (IL-2, IL-13, CD40L, IFN-ɣ and TNF-α) was evaluated. The coefficient of determination (R2) of the regression between the Log of Geometric Mean (GM) of the percentages of the 4 values obtained from the positive cells for each marker evaluated respect to the Log of sample dilution was calculated.	R2 of the regression line had to be ≥ 0.95
Relative Accuracy	For the evaluation of Relative Accuracy, the data of Dilutional Linearity were used and GM between replicates was calculated.	GM of the obtained percentages for each marker had to be within 80–120% respect to the calculated value from the neat sample results. The formula used was 100*(GM Observed/GM Calculated)
Precision –Repeatability	The intra-run variability was determined by one operator within one run. In detail, 6 repetitions (6 wells containing 1×10^6 cells from one characterized subject) were seeded, stimulated with SEB and stained with the full fluorescent antibody panel. In addition, 6 repetitions (6 wells containing 1×10^6 cells from one characterized subject, unstimulated) were seeded and stained with the full fluorescent antibody panel.	The CV% was calculated between the percentage of cells positive for each marker among the 6 repetitions and it had to be >20% [7]
Precision – Intermediate Precision	Intermediate Precision was determined as the variability across two operators across different runs (Day 1 and Day 2). In detail, 6 repetitions (6 wells containing 1-1x10^6 cells from one characterized subject) were seeded, stimulated with SEB, in addition 6 repetitions of the same cells, without stimulants (negative), were included in each run. All wells received a full antibody panel staining.	The CV% calculated between the percentage of cells positive for each marker among the 6 SEB repetitions and the 6 negative repetitions, among the two operators had to be $\leq$ 20% [7]
Range and Detection Limits	The detection and quantitation limits were defined by the technical characteristics of the assay. The Flow Cytometer instrument could detect a single cell, set as Lower Limit of Quantitation (LLOQ); the layout of the experiment was thought to include 1×10^6 cells for each well, and this value could be assumed as Upper Limit of Quantitation (ULOQ). These assumptions generated a range of detection and quantitation of 1 to 1×10^6 cells. A derived percentage value in terms of cells expressing marker *Vs* the number of live cells could be calculated having a theorical range of 0.001–100.000%.	1-10^6 cells,0.001–100.000% positive cells *Vs* live cell population
Specificity	The specificity was evaluated on single cytokine-stained cells: once the correct dilution for each antibody has been established in titration experiments, the specificity was evaluated on the results of that specific dilution. In detail, one series of single-color staining (for each marker) was performed on SEB stimulated PBMCs, and the percentage of cells positive for each marker was calculated *Vs* the live cell population. To evaluate the specificity, the same calculation was performed on markers not represented by the antibody used for the staining, this approach evidenced non-specific fluorescence signals (e.g. Cells stained for CD40L were evaluated for IL-2, IL-13, IFN-ɣ and TNF-α).	The ratio between the percentage of specific marker cells and the percentage of non-specific marker cells had to be $\geq$ 10; this value was referred to the signal to noise ratio (s/n ratio $\geq$10) specified into the "FDA Validation on Analytical Procedures Methodology" document.

4. Discussion

All the results obtained in this validation study met the acceptability criteria evaluated with the exception of IL-13 in both $CD4^+$ and $CD8^+$, for which specific considerations have been made, and relative accuracy evaluated in CD8 subpopulation.

Regarding $CD4^+$ and $CD8^+$ IL-13+ subpopulations, the application of the bootstrap method revealed that since on average the values of SEB cells were inside the 99% confidence interval of the LOD, we could not either reliably detect the percentage of $CD4^+$ cells or met the precision criterion. From the analysis of the undiluted SEB-stimulated $CD8^+$ IL-13+ cells, it has been observed that the mean value calculated was below the 99% confidence interval of the LOD estimated over the undiluted unstimulated $CD8^+$ IL-13+ cells. Since the expected value for IL-13 in both $CD4^+$ and $CD8^+$ subpopulations was below the threshold of

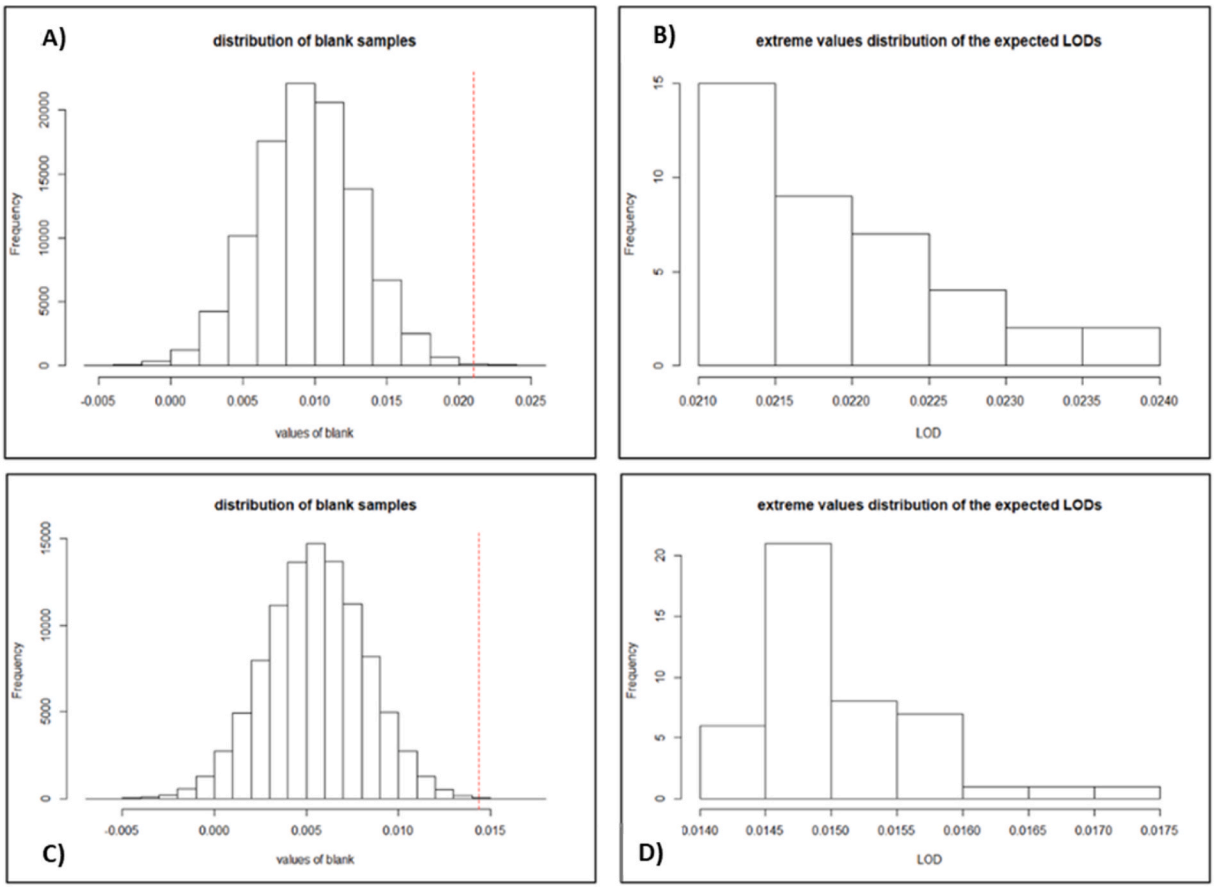

Fig. 2. Special Considerations for $CD4^+$ and $CD8^+$ IL-13+ subpopulations: 2 A) $CD4^+$ IL13: distribution of measurements over the unstimulated cells; 2 B) $CD4^+$ IL13: distribution of the extreme values of the LOD assuming normal distribution of blank samples; 2C) $CD8^+$ IL13: distribution of measurements over the unstimulated cells; 2D) $CD8^+$ IL13: distribution of the extreme values of the LOD assuming normal distribution of blank samples.

Table 2
Calculation of MFI for $CD4^+$ IL-13+ and $CD8^+$ IL-13+ cells.

CD4 cells					CD8 cells				
Cytokine	**Dilution**	**Mean+**	**Mean-**	**Ratio**	**Cytokine**	**Dilution**	**Mean+**	**Mean-**	**Ratio**
IL-13	1:20	757	770	0.983	IL-13	1:20	794	737	1.077
IL-13	1:40	644	611	1.054	IL-13	1:40	710	611	1.162
IL-13	1:80	577	600	0.962	IL-13	1:80	669	597	1.121
IL-13	1:160	540	543	0.994	IL-13	1:160	537	565	0.950
IL-13	1:320	487	510	0.955	IL-13	1:320	509	502	1.014

Table 3
Repeatability for $CD4^+$ and $CD8^+$ cells.

Repeatability $CD4^+$		Repeatability $CD8^+$	
Samples	*CV%*	*Samples*	*CV%*
CD40L SEB	0.079	CD40L SEB	2.36%
CD40L Unstim.	0.335	CD40L Unstim.	195.42%
IFN-γ SEB	0.053	IFN-γ SEB	7.39%
IFN-γ Unstim.	0.131	IFN-γ Unstim.	87.87%
IL-13 SEB	0.168	IL-13 SEB	33.25%
IL-13 Unstim.	0.002	IL-13 Unstim.	39.55%
IL-2 SEB	0.057	IL-2 SEB	12.65%
IL-2 Unstim.	0.187	IL-2 Unstim.	35.96%
TNF-α SEB	0.021	TNF-α SEB	4.66%
TNF-α Unstim.	0.172	TNF-α Unstim.	28.60%

These data were generated in one experiment wherein 6 biological replicates (n = 6) were performed for each condition.

Table 4
Intermediate precision for $CD4^+$ and $CD8^+$ cells.

Intermediate Precision $CD4^+$		Intermediate Precision $CD8^+$	
Samples	*CV%*	*Samples*	*CV%*
CD40L SEB	0.090	CD40L SEB	0.91%
CD40L Unstim.	0.779	CD40L Unstim.	74.91%
IFN-γ SEB	0.018	IFN-γ SEB	0.92%
IFN-γ Unstim.	0.376	IFN-γ Unstim.	70.04%
IL-13 SEB	0.103	IL-13 SEB	89.66%
IL-13 Unstim.	0.383	IL-13 Unstim.	29.92%
IL-2 SEB	0.086	IL-2 SEB	0.84%
IL-2 Unstim.	0.221	IL-2 Unstim.	13.26%
TNF-α SEB	0.009	TNF-α SEB	0.52%
TNF-α Unstim.	0.402	TNF-α Unstim.	6.80%

the LOD distribution we could not reliably distinguish the results of SEB-stimulated cells from the results of the unstimulated cells. The failure to detect IL-13 expressing cells was not due to an incorrect choice of the

Table 5
Specificity for $CD4^+$ and $CD8^+$ cells.

Specificity $CD4^+$	
SEB STIMULATED $CD4^+$ STAINED FOR CD40L+	Signal/noise ratio
Signal/noise ratio for IFN-γ	n/a
Signal/noise ratio for IL-13	n/a
Signal/noise ratio for IL-2	n/a
Signal/noise ratio for TNF-α	9840.18
SEB STIMULATED $CD4^+$ STAINED FOR IFN-γ+	**Signal/noise ratio**
Signal/noise ratio for CD40L	1663.46
Signal/noise ratio for IL-13	n/a
Signal/noise ratio for IL-2	n/a
Signal/noise ratio for TNF-α	3326.92
SEB STIMULATED $CD4^+$ STAINED FOR IL13	**Signal/noise ratio**
Signal/noise ratio for CD40L	1.39
Signal/noise ratio for IFN-γ	0.57
Signal/noise ratio for IL-2	29.90
Signal/noise ratio for TNF-α	59.81
SEB STIMULATED $CD4^+$ STAINED FOR IL2	**Signal/noise ratio**
Signal/noise ratio for CD40L	91.34
Signal/noise ratio for IFN-γ	55.64
Signal/noise ratio for IL-13	5666.67
Signal/noise ratio for TNF-α	1304.90
SEB STIMULATED $CD4^+$ STAINED FOR TNF-α	**Signal/noise ratio**
Signal/noise ratio for CD40L	599.08
Signal/noise ratio for IFN-γ	209.20
Signal/noise ratio for IL-13	4798.17
Signal/noise ratio for IL-2	n/a
Specificity $CD8^+$	
SEB STIMULATED $CD8^+$ STAINED FOR CD40L+	Signal/noise ratio
Signal/noise ratio for IFN-γ	n/a
Signal/noise ratio for IL-13	40.46
Signal/noise ratio for IL-2	n/a
Signal/noise ratio for TNF-α	n/a
SEB STIMULATED $CD8^+$ STAINED FOR IFN-γ+	**Signal/noise ratio**
Signal/noise ratio for CD40L	61.54
Signal/noise ratio for IL-13	123.08
Signal/noise ratio for IL-2	n/a
Signal/noise ratio for TNF-α	20.00
SEB STIMULATED $CD8^+$ STAINED FOR IL-13	**Signal/noise ratio**
Signal/noise ratio for CD40L	n/a
Signal/noise ratio for IFN-γ	n/a
Signal/noise ratio for IL-2	n/a
Signal/noise ratio for TNF-α	23.92
SEB STIMULATED $CD8^+$ STAINED FOR IL-2	**Signal/noise ratio**
Signal/noise ratio for CD40L	122.22
Signal/noise ratio for IFN-γ	305.13
Signal/noise ratio for IL-13	152.78
Signal/noise ratio for TNF-α	26.54
SEB STIMULATED $CD8^+$ STAINED FOR TNF−α	**Signal/noise ratio**
Signal/noise ratio for CD40L	134.15
Signal/noise ratio for IFN-γ	n/a
Signal/noise ratio for IL-13	n/a
Signal/noise ratio for IL-2	n/a

These data were generated in one experiment, 1 biological replicate for each condition (n = 1).

dilution for IL-13 antibody used for the present validation protocol, and for testing of the clinical trial samples, as the optimal dilution of antibody to detect IL-13 on $CD4^+$ and $CD8^+$ cells was selected based on the MFI. After the calculation of MFI, a 1:40 dilution of the IL-13 antibody was selected as this dilution had the highest MFI value obtained from CD4+IL-13+ SEB-stimulated cells/CD4+IL-13+ unstimulated cells and CD8+IL-13+ SEB-stimulated cells/CD8+IL-13+ unstimulated cells as reported in Table 2.

The main reason the $CD8^+$ subpopulation failed to meet the relative accuracy acceptance criteria was the extremely low values of $CD8^+$ cells positive for the cytokines evaluated, even in the undiluted SEB-stimulated sample. This observation was in line with published literature. In fact, even though it is reported in literature that IL-13 is secreted not only by T helper cells, but also by $CD8^+$ T cells following activation, it was possible that the low IL-13 production observed during these experiments could be related to its main role in the pathogenesis of IgE-mediated allergic diseases [9] and not specifically in response to antigens. Hence, the poor relative accuracy was not an inherent property of

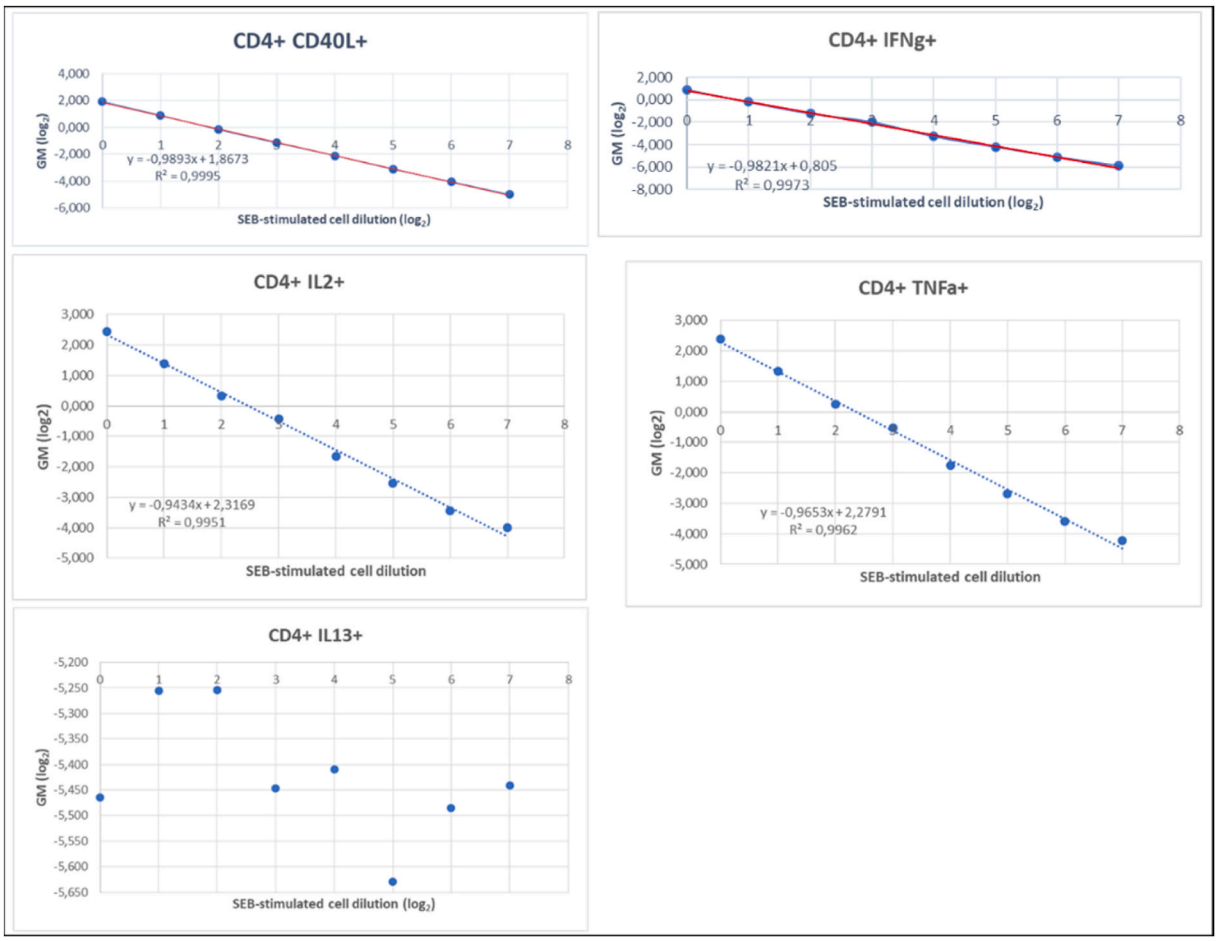

Fig. 3. Linearity for $CD4^+$ subpopulation.

the flow cytometry assay rather it was a consequence of trying to measure a rare event. Concerning the other cytokines, the acceptance criteria was not met for CD40L from 1:32 to 1:128 (the highest dilutions tested), for INF-γ from 1:8 to 1:32 dilutions, for IL-2 for 1:2 and from 1:8 to 1:32 dilutions and for TNF-α from 1:8 to 1:32 dilutions.

5. Conclusions

The evaluation of cell mediated immunity through multicolor flow cytometry can provide further information regarding the development of immunity induced by a vaccine as well as for the follow up of patients in clinical trials. In most cases, the evaluation of vaccine immunogenicity is performed by using antibody titers in serum samples. However, in view of the complexity of immune response and since new vaccines including internal viral antigen, produce a low level of antibody response, this unique evaluation cannot be sufficient to determine the effectiveness of a vaccine. For this reason, cell-mediated immune response upon a vaccination is increasingly being investigated, although currently it represents an exploratory endpoint since no correlates of protection regarding either the phenotype or the magnitude of the immune cell response following vaccination have yet been established [10–13]. Cell-mediated immune response can be investigated by flow cytometry assay, a method that allows a multiparametric analysis at a single-cell level of both major immune cell subsets and rare cell populations. The evaluation of cellular immune responses in subjects immunized with different vaccines, including influenza and SARS-CoV-2, is a commonly used and an indispensable technique [14–18]. At the same time several studies have been performed with the aim to harmonize experimental steps of such technique as well as gating approach [19] in order to reduce the variability across laboratories. In order to obtain solid evidence regarding the suitability of flow cytometry as tool for the evaluation of clinical samples and immune monitoring, the method [20], and thus also reagents used [21], has to be validated. The objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose. To this end, the validation protocol must describe criteria evaluated with a detailed protocol for sample preparation and the cell sample to be analyzed. Usually, typical validation characteristics which are considered for bioanalytical methods are: accuracy, precision (repeatability and intermediate precision), specificity, detection limit, quantitation limit, linearity and range [22]. Both the validation of bioanalytical methods as well as the analysis of study samples for clinical trials in humans should be performed following the principles of Good Clinical Practice (GCP) [1]. As previously described, a standard validation procedure cannot be applied to flow cytometry due to the lack of standard materials, the nature of the cells, and the complexity of a standardization of data analysis. This means that a validation strategy must be used by the laboratory to ensure the reliability of the data that will be obtained from the following analysis of clinical samples. The present validation protocol has been developed in order to demonstrate the suitability of the assay for its following use to process clinical samples derived from clinical trials; unfortunately, the unavailability of different human PBMC donor samples did not allow the evaluation of robustness criteria, representing a limit of the present study.

In conclusion, it has been demonstrated that the ICS assay was able to detect and quantify IL-2, CD40L, IFN-γ and TNF-α cell markers in specific live, $CD3^+$ $CD4^+$ (or $CD8^+$) positive cell populations and it could be used for the assessment of the immune response in clinical samples from

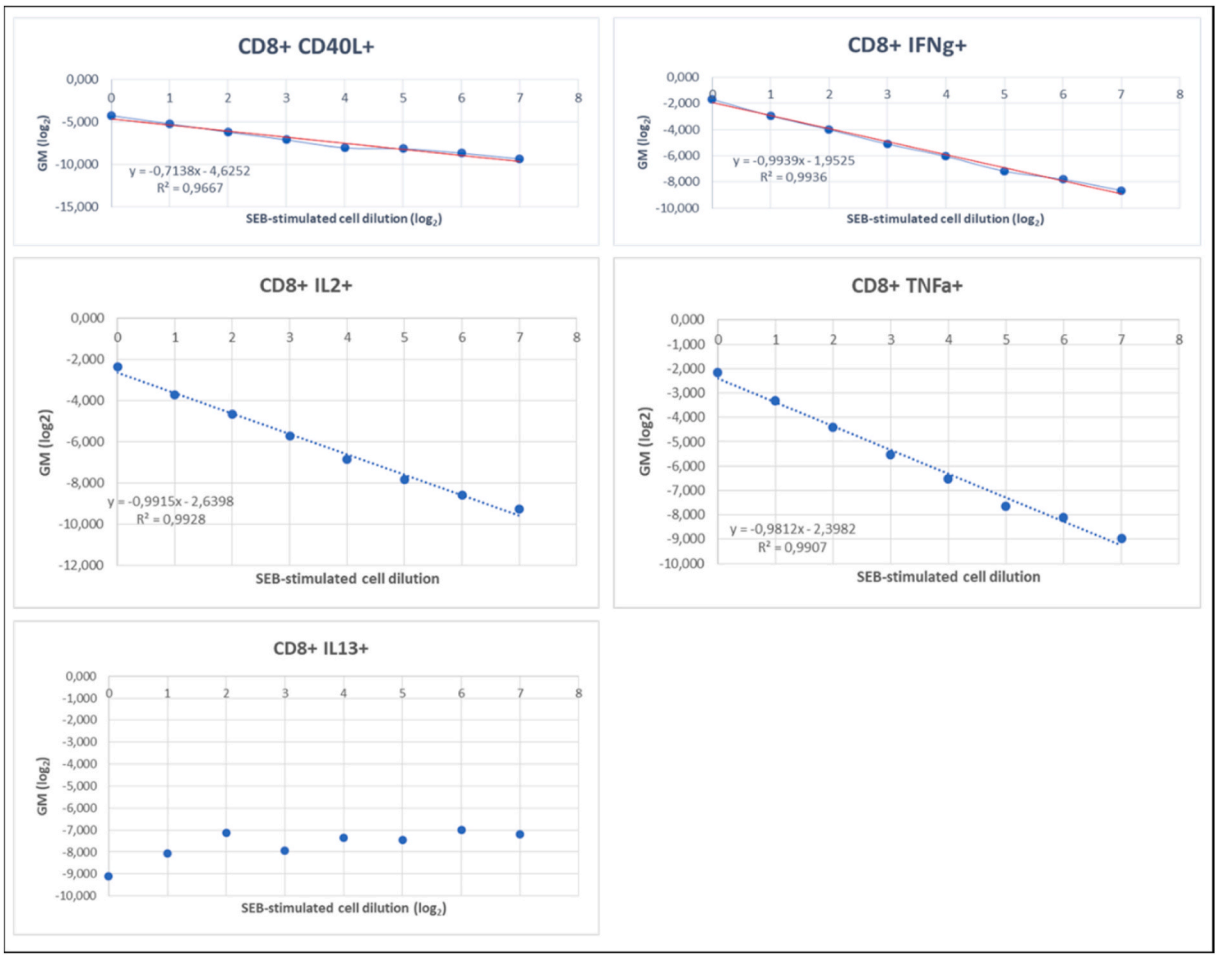

Fig. 4. Linearity for $CD8^+$ subpopulation.

epidemiology studies and vaccine clinical trials after stimulation with stimulating agents. Concerning IL-13, it has been established that for the evaluation of clinical samples it would have been measured as positive (when above the LOD) or negative (when at or below the LOD).

Authors' contributions

EG, CB and LG performed the set-up and validation experiments; EG, AT and PP evaluated the results; PP performed the statistical analyses; EG and AT designed the experiments; EG prepared the draft of the manuscript; AT and EM supervised the study. All authors have approved the final version of the manuscript.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Authors EG, PP, CB, LG and EM are employees of VisMederi srl; AT is employee of CSL Seqirus.

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