Elaboration of Polychromatic Flow Cytometry Quality Assurance Program in the Context of Immune-Monitoring Platform

Kim Ellefsen, Gregory Gonzalez, Maud Zuchuat, Giuseppe Pantaleo and Alexandre Harari

Vaccine and Immunotherapy Center, Service of Immunology and Allergy, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland

ABSTRACT

Objectifs: Cellular immune responses induced by vaccination/immunotherapies are complex and require a polychromatic flow cytometry analysis to accurately characterize the phenotype and functionality of T-cell responses. Here we review the procedures of validation, standardization and quality control of all aspects of the operation of a clinical flow cytometry analysis.

Methods: In order to generate reproducible and reliable detection of polychromatic flow cytometry assays, we have first implemented a quality assurance program to optimize, calibrate and monitor the multiparameters flow cytometer (4 lasers, 20 parameters). Second, in collaboration with Dr Roederer at VRC/NIH and other experts in this field we have validated SOPs for stimulating, staining and acquiring complex evaluations of T-cell responses. Finally, guidelines for gating strategy and quality control for the samples analysis were optimized, validated and implemented.

Results: The standardization of all aspects of the flow cytometry allowed us to achieve high levels of reproducibility, specificity and sensitivity with regard to T-cell assays. For instance, mean intra-assay variability for intracellular cytokine assays (ICS) was assessed using 20 replicates per assay and 4.1 % and 2.6 % of variability have been measured for CD4 and CD8 T-cell responses, respectively. Mean Inter-assay variability for ICS (assessed by performing 5 experiences on 3 subjects and 3 different stimulations) ranged between 9.9 % and 15.9 % for CD4 and CD8 T-cell responses, respectively.

Conclusion: The elaboration of a quality assurance program for the polychromatic flow cytometry assays is a critical issue in clinical trial which is fundamental to achieve reliable immune assessments.

INTRODUCTION

The Vaccine and Immunotherapy Center (VIC) and the Laboratory of AIDS Immunopathogenesis are renowned for their pioneer activity in the characterization of cellular immunity, in particular in antiviral and vaccine-induced immunity, tolerance in allergy and transplantation, mucosal immunity and T-cell activation. As a fundamental research platform, our laboratory has specifically developed several assays to measure, characterize, quantify and monitor virus-specific or vaccine-induced T-cell responses. The VIC is a laboratory and clinical research platform specialized in vaccinology, clinical research, immune-monitoring and basic immunology. **Our laboratory has implemented a strict methodology to develop, standardized and validated reliable diagnostic tools according to Good Laboratory Practices (GLP) compliance (ISO/IEC 17025).**

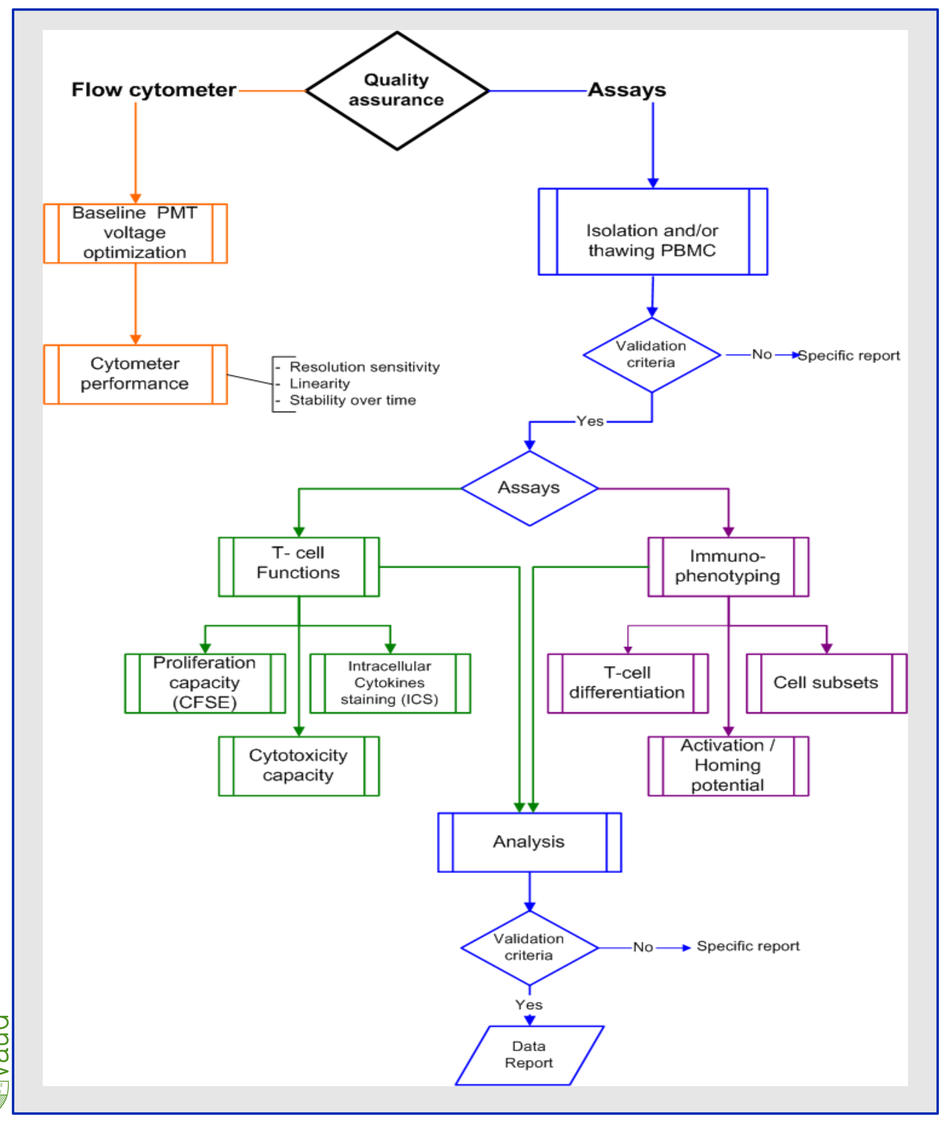
The flow cytometry T-cell assays have become instrumental in the pre-clinical and clinical phases of vaccines or therapeutic evaluation. The major advantages of flow-based assays include rapid assessment of over a dozen distinct markers on lymphocytes but also on different cell subsets. In this context, measurement of so many markers increasingly aims to assess T-cell responses but they also increase the likelihood of errors. In this regard, the establishment of **good quality program assurance** for both the instrument (flow cytometer) and the assays is crucial since the data from polychromatic system may provide insight relevant for the clinic.

RESULTS

We have implemented a **quality assurance program to optimize, calibrate and monitor flow cytometers**. Basically, following installation or maintenance services, the more efficient detectors (e.g., PMT) for fluorescence detection, and the more efficient the transmission and reflection properties and reflection properties of the optical filters and mirrors are adjusted with specific beads. The minima PMT are fixed and these PMT require daily monitoring to ensure overall signal quality. This day to day operation allows the evaluation of a wide array of critical instrument parameters: e.g. resolution sensitivity, linearity and stability over time for each laser.

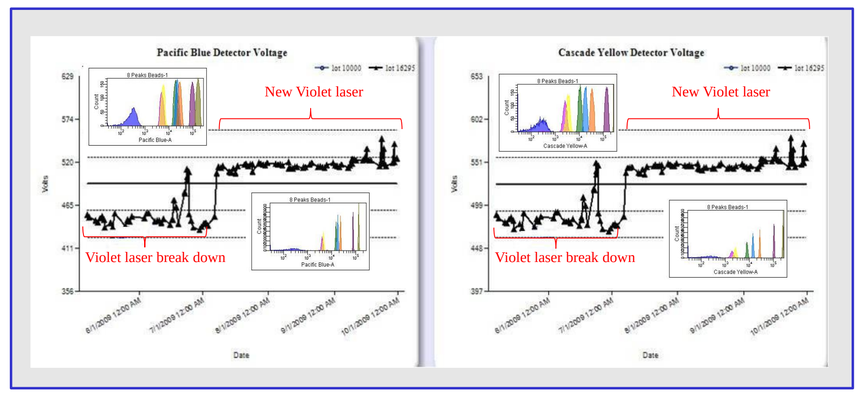
We also implemented **quality assurance program for the flow cytometry assays** (see flow chart). Appropriate handling of blood samples is a critical step. The collection, processing, cryopreservation, storage and manipulation of peripheral blood mononuclear cells (PBMCs) are all key steps for assessment of vaccine/immunotherapies or disease-induced immune responses.

The first step of this program is to monitor the quality of PBMC following isolation and/or thawing to ensure reliable results in functional and phenotypic T-cell assays. For example, for frozen cells, It has been shown that the relevance of functional assays is strictly dependent on the viability of the cryo-preserved PBMC, such that samples with <75% viability are not suitable for cytokine production following stimulation. If the validation criteria are acceptable, the assays will be performed according to the standard operating procedures (**SOPs**). **The second step**, the gating strategy will be applied for each samples in the same way and samples with a viability ≤75% will be excluded PBMC (viability dye (LIVE/DEAD stain kit). In the case of T-cell responses assessment, the samples will be stimulated with SEB and the responses will be compared between each time point for each single subject. Samples with inconsistent SEB T-cell responses are excluded from the analysis.

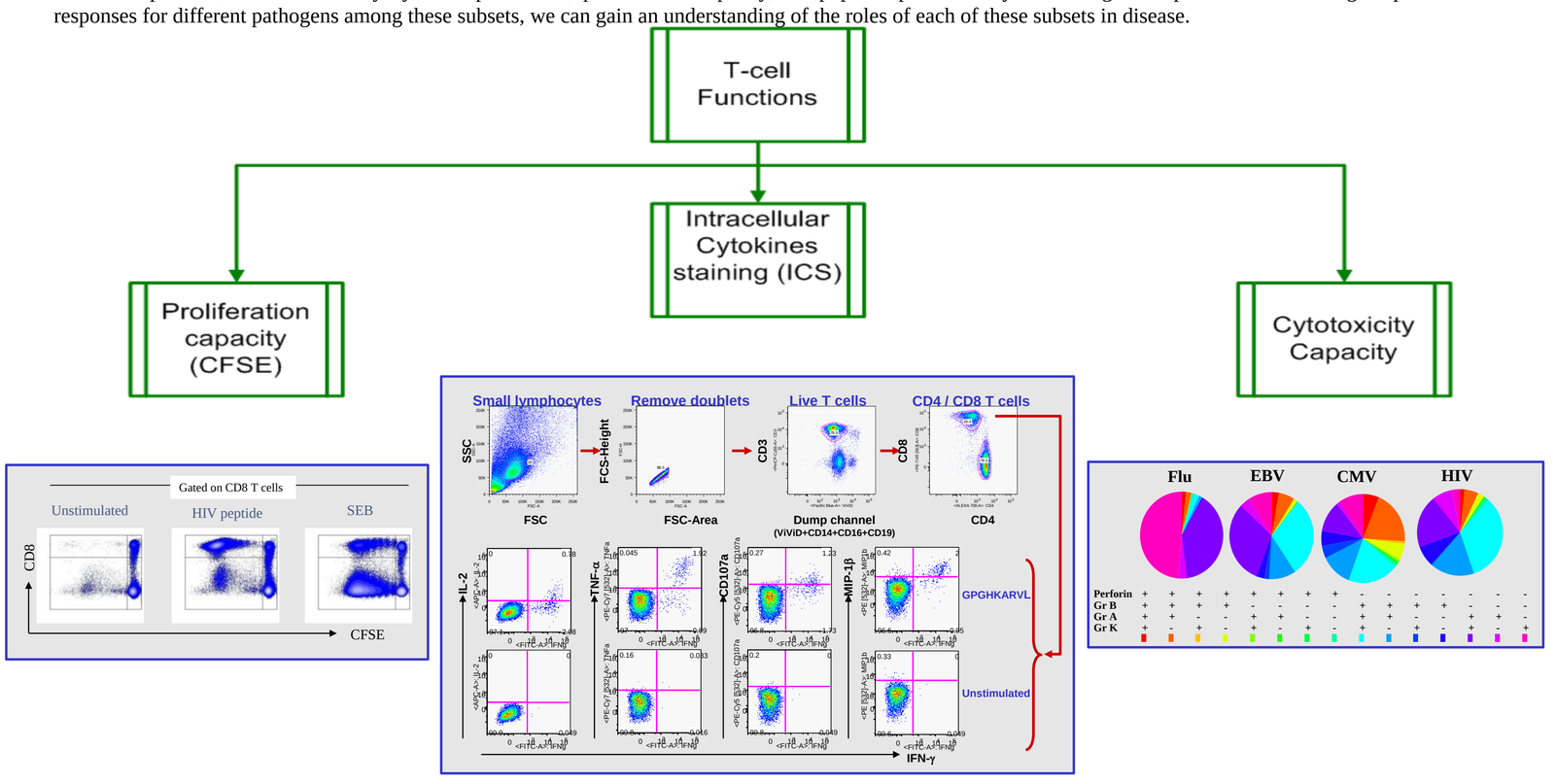


For each flow cytometry assay we performed a qualitative validation to assess precision, reproducibility and limit of robustness.
In this example, quantitative validation of the ICS includes three cytokines, only IFN-γ and TNF-α responses were evaluated in terms of the validation parameters (inter- and intra-assays). Overall, these validation experiments demonstrate that ICS assay is robust and can be used to assess T-cell responses.

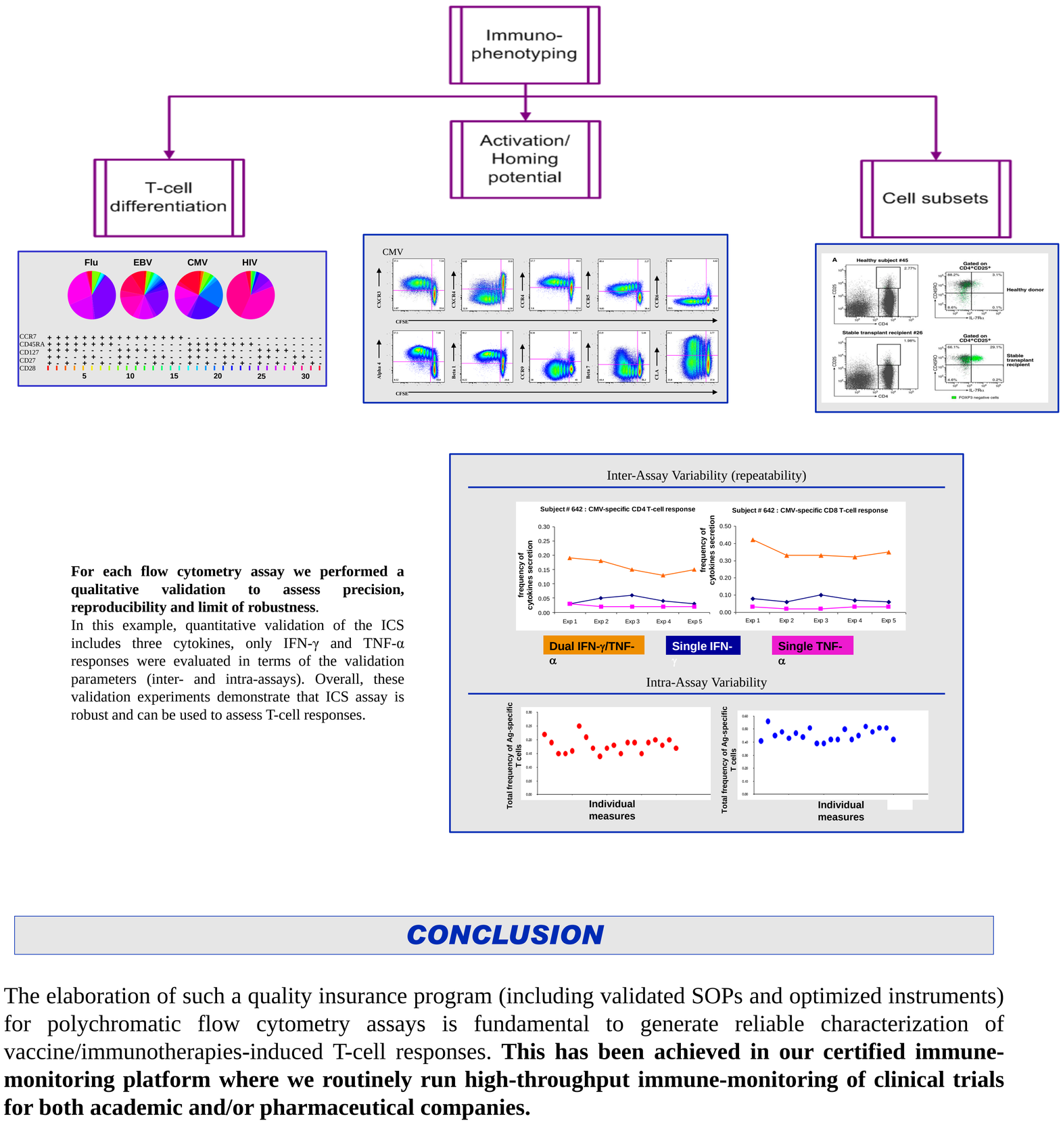
Shown are examples of time plots of accuracy, precision and sensitivity of violet laser. The monitoring voltage as a function of time illustrates the accuracy of violet laser has not acceptable variance due to the laser brake down. In this case on the first 2 days a loss of instrument sensitivity and precision (CV-8x rainbow beads) occurred until we change this laser.



Using **19-color flow cytometry**, we can identify hundreds of phenotypically distinct subsets of lymphocytes in peripheral blood. Most of these have unique functional profiles, as determined by cytokine production, proliferative capacity, or apoptotic potential. By examining the representation of antigen-specific responses for different pathogens among these subsets, we can gain an understanding of the roles of each of these subsets in disease.



We characterize the T cells that are induced by vaccination or natural infection both in terms of phenotype - differentiation markers - as well as function. In these experiments, we will make 4-6 distinct functional measurements on each cell (for example, production of multiple cytokines, cytotoxic activity, helper activity, proliferation), and use the remaining colors to identify the phenotypic identity of the cells. This gives us a detailed picture of the functional response as well as the differentiation stage of the antigen-specific T-cells, measurements that may ultimately provide us with clinical correlates.



CONCLUSION

The elaboration of such a quality insurance program (including validated SOPs and optimized instruments) for polychromatic flow cytometry assays is fundamental to generate reliable characterization of vaccine/immunotherapies-induced T-cell responses. **This has been achieved in our certified immune-monitoring platform where we routinely run high-throughput immune-monitoring of clinical trials for both academic and/or pharmaceutical companies.**