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Semi-automated and standardized cytometric procedures for multi-panel and multi-parametric whole blood immunophenotyping

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Abstract Immunophenotyping by multi-parametric flow cytometry is the cornerstone technology for enumeration and characterization of immune cell populations in health and disease. Standardized procedures are essential to allow for inter-individual comparisons in the context of population based or clinical studies. Herein we report the approach taken by the *Milieu Intérieur* Consortium,

Abbreviations: DC, dendritic cell; CM, central memory; CV, coefficient of variation; EM, effector memory; EMRA, effector memory expressing CD45RA; FcR, Fc receptor; FMO, fluorescence minus one; FSC, forward scatter; FVD, Fixable Viability Dye; MFI, mean fluorescence intensity; NK, natural killer cell; PBMC, peripheral blood mononuclear cell; PMN, polymorphonuclear cell; PMT, photomultiplier tube; RBC, red blood cell; RT, room temperature; SOPs, standard operating procedures; SSC, side scatter; T_N, naïve T cell

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Automation;
Standardization

highlighting the standardized and automated procedures used for immunophenotyping of human whole blood samples. We optimized eight-color antibody panels and procedures for staining and lysis of whole blood samples, and implemented pre-analytic steps with a semi-automated workflow using a robotic system. We report on four panels that were designed to enumerate and phenotype major immune cell populations (PMN, T, B, NK cells, monocytes and DC). This work establishes a foundation for defining reference values in healthy donors. Our approach provides robust protocols for affordable, semi-automated eight-color cytometric immunophenotyping that can be used in population-based studies and clinical trial settings.

1. Introduction

Multiparametric flow cytometry is widely used for phenotyping immune cell populations in human blood samples. The abundance of reagents and growing technical innovations in the field of cytometry (e.g., mass cytometry, imaging cytometry and spectral analyzers) has further enhanced the enthusiasm for applying these approaches to the management of patients and the phenotyping of healthy individuals. Flow cytometric techniques have been used for more than two decades in clinical laboratories for the enumeration of $CD4^+$ and $CD8^+$ T cells, in the diagnosis of AIDS [1,2], and also in the characterization of lymphoma and leukemic immune cell expansions [3,4]. However, the implementation of standardized procedures within academic research laboratories has recently become a concern for the community, as the absence of such standardization has precluded comparison between studies and experimental settings. Indeed, greater attention is now placed on the requirement for optimized approaches and harmonization of methods [5,6]. Several international initiatives have supported increased standardization of flow cytometry protocols and applications across multiple laboratories that share common scientific or clinical interests. These include the EuroFlow Consortium, which focuses on laboratory procedures for the phenotyping of malignant leukocytes [7,8]; the Human Immunology Project Consortium (HIPC) and European Network for Translational Immunology Research and Education (ENTIRE), which are working together to develop panels for the phenotyping healthy donors [5,6,9]; the ONE study consortium, which is addressing cellular phenotyping in the setting of transplantation [10]; and the Association for Cancer Immunotherapy (CIMT), which have established proficiency panels for different cell populations [11].

The reproducibility of cytometric data depends on five principle criteria: sample type, sample handling, choice of reagents, instrument selection and qualification, and data analysis. In three coordinated reports, we detail the steps that have been taken by the *Milieu Intérieur* Consortium to control for the pre-analytic aspects of cellular phenotyping (reported here), to optimize the analysis of multi-dimensional data [Chen et al. co-submission], which applied together have allowed the characterization of immune phenotype variation in a population of healthy donors [Urrutia et al., in preparation]. Our approach to immune cell phenotyping supports our Consortium's long-term efforts in utilizing cytometric data as a quantitative intermediate phenotype for association studies. Only with accurate and reproducible methodologies can we begin to establish, integrate and share large data-warehouses of phenotypic and genetic data.

Several prior and ongoing efforts have contributed to the challenge of harmonizing methods in academic research laboratories. Particular attention has been given to sample type, with comparative assessments of fresh or frozen purified peripheral blood mononuclear cells (PBMCs) and whole blood [12–14]. Additional parameters that have been considered include panel design [6,8,10], the use of liquid, lyophilized or freeze-dried reagents [15] and the calibration and settings for the optical bench of multi-laser cytometers that permit longitudinal, multi-user or inter-laboratory standardization [7]. In academic studies, however, there is less attention given to the variability introduced by sample handling. In many instances, sample collection is not proximal to core facilities and despite the use of standard operating procedures (SOPs), studies have not evaluated the impact of manual sample handling on the measured cellular phenotypes such as size, granularity and activation state.

In this report, we detail the steps that were taken to establish a robust protocol for immunophenotyping from 100 μl of fresh whole blood, using four eight-color cytometry panels. We present the design of cytometry panels used for phenotyping and quantifying major cell populations present in human blood — T cells, B cells, NK cells, monocytes, dendritic cells, neutrophils, basophils and eosinophils. These data establish the foundation for the analysis of six hundred healthy donors, analyzed over a six-month time interval.

2. Materials and methods

2.1. Human subject materials, reagents and instrumentation used

For optimization studies and panel development, whole blood samples were collected from healthy volunteers enrolled at the Institut Pasteur Platform for Clinical Investigation and Access to Research Bioresources (ICAReB) within the Diagmicoll cohort. The biobank activity of ICAReB platform is NF S96-900 certified. The Diagmicoll protocol was approved by the French Ethical Committee (CPP) Ile-de-France I, and the related biospecimen collection was declared to the Research Ministry under the code N° DC 2008-68. Samples collected as part of the *Milieu Intérieur* population based study were procured by investigators working at BioTrial, Rennes [Thomas