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Research paper

High dimensional flow cytometry for comprehensive leukocyte immunophenotyping (CLIP) in translational research

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ABSTRACT

New paradigms in translational research are focused on deep understanding of all aspects of the human immune system in response to diseases or perturbations such as vaccination or therapy. To obtain this knowledge, coordinated, comprehensive assessments by genomics, proteomics, and cytomics are necessary. One component of this assessment is comprehensive leukocyte immunophenotyping (CLIP) that not only provides a deep and broad description of the entire immune system at any given moment, but also encompasses all leukocyte lineages, including activation states, functional markers, and signaling molecules. As envisioned, a CLIP panel could study nearly 400 antigens utilizing 17-parameter flow cytometry. The CLIP panel is structured in a manner that tubes are grouped by lineage and, within lineage each of the tubes, while having some redundant markers, characterize distinct populations. To date, a preliminary 10 tube CLIP panel has been developed with the following 17 parameter tubes: T_{reg}, T_{h17}, $T_{h1/2}$, $B_{general}$, $B_{naive/memory}$, $B_{intracellular}$, NK_1, NK_2, myeloid/monocyte, and dendritic cells (DC). Together these tubes have the potential to identify over 28,000 subsets of leukocytes. The feasibility of developing these tubes has been demonstrated, as well as their utility in describing complex alterations of the immune system in the context of disease and vaccination. The plethora of data accrued in the preliminary CLIP panel highlights the need for novel data analysis and reduction strategies, while at the same time illustrates the power of CLIP.

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1. Introduction

The human immune system is a highly complex, tightly regulated component of the body that is a key player in protection from infectious diseases and foreign antigens. It has a role in virtually every disease of man and dysregulation of this system can have severe consequences not only in terms of infection, but also in the forms of autoimmune disease or cancer. While this system is widely accepted as being highly complex, approaches for its study have historically been very simplistic. Quite often a specific cell type (e.g. dendritic cells, B cells, or T regulatory cells) is studied in great detail in the context of one disease or a limited number of diseases, or a very cursory examination is performed of multiple cell types in a similar setting. Studies of the immune system in mice, while at times more comprehensive, have limitations in their applicability to human immunology (Davis, 2008). Thus a new paradigm is beginning to emerge in which a comprehensive, more systems-based approach is used to study human immunology in both its basal state and upon perturbation by disease, therapy, or vaccination.

Comprehensive assessment of the immune system utilizes a multitude of technologies to examine as many facets as possible. Assays for these studies may include gene expression arrays, SNP genotyping, proteomics, ELISPOT assays, serologic studies, a vast assortment of functional assessments, serum cytokine analyses and flow cytometric analysis of a large number of cell surface markers, as well as intracellular

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antigens. In order to truly provide a comprehensive, detailed view of the immune system *in toto*, the flow cytometry assays must be far more in depth and comprehensive than any previously attempted, spanning all lineages but at the same time providing detailed information regarding all known leukocyte subsets. Previous approaches to immunophenotyping have selected either depth (detailed immunophenotyping of a single lineage or subset) or breadth (immunophenotyping of multiple lineages without significant depth), whereas the current approach seeks both depth and breadth. We term this comprehensive leukocyte immunophenotyping (CLIP). This comprehensive approach for immunophenotyping, in addition to all known leukocyte subsets, should also include activation markers, intracellular cytokines, phosphorylated signaling proteins, and in certain instances, tetramer analysis. In many aspects, this approach more closely resembles gene profiling studies than it does immunophenotyping of the past.

For such a panel to be developed in a meaningful manner necessitates the use of high dimensional immunophenotyping where as many of these markers as possible are studied concomitantly. To this end, our laboratory is developing an in-depth and comprehensive panel to immunophenotype as many components of the immune system as possible, with the end goal of better defining immune perturbations in both health and disease. This panel utilizes 15-color, 17-parameter flow cytometry to study known lineages and subsets within these lineages as well as permitting 'data mining' to characterize previously undiscovered subsets. The construction of the panel is centered on developing individual tubes within lineages that, when combined, will yield a comprehensive phenotype of all lineages.

Optimally, this panel possesses recurring markers within the tubes of a given lineage in order to permit a deep level of inter-tube concatenation of immunophenotypes. Currently in its infancy, this panel consists of ten 15-color, 17-parameter tubes, but could well reach sixty or more in the next several years. Herein we present details concerning the development of this comprehensive panel and the progress to date in this effort.

2. Materials and methods

2.1. Specimens

This panel is created to study the immune subsets in peripheral blood. While many, if not all of the markers studied will be relevant in other specimens such as bone marrow, lymph nodes, synovial or ascites fluid, and so forth, the utility of each marker, and tube, ultimately must be validated for each type of specimen. Peripheral blood is collected in sodium heparin and processed within 2 h of collection. For these studies, the plasma is removed by centrifugation and the resulting cell pellet is lysed using ACK Lysing buffer (Quality Biological Inc., Gaithersburg, MD). Cells are subsequently washed in isotonic PBS and counted. For development of this panel, peripheral blood from healthy donors was used (IRB approved protocol 07-H-0113). In some experiments, fresh peripheral blood mononuclear cells (PBMCs) were compared to cryopreserved PBMCs from the same draw of the same donor. Further, this approach is preferentially designed for use on fresh blood, rather than cryopreserved. While this panel can be utilized on cryopreserved mononuclear cells (as will be shown later), there are many differences between the intensities of various markers in fresh versus frozen material, and extreme caution should be taken in comparing samples handled differently (Macey et al., 1998; Costantini et al., 2003; Weinberg et al., 2009). Furthermore, certain cell types such as myeloid cells and activated NK subsets may not be present in density gradient, prepared, cryopreserved specimens, and thus the entire immunophenome cannot be studied.

2.2. Sample staining

Antibodies used in these stainings were purchased from numerous manufacturers including Becton Dickinson (San Jose, CA), Beckman Coulter (Miami, FL), Life Sciences (Carlsbad, CA), Miltenyi Biotec (Bergisch Gladbach, Germany), R&D (Minneapolis, MN), BioLegend (San Diego, CA) and Ebioscience (San Diego, CA). Design of each tube included procedures recommended by Mahnke and Roederer (2007). All antibodies were titered prior to use. Fig. 1 shows an overview of the process of a tube development. Table 1 shows a matrix of the antibodies and fluorochromes used for the initial 10 tubes. Cells were lysed using ACK Lysing buffer, except where comparative studies were conducted with density gradient-prepared cells. After lysis of the RBCs, cells were washed twice with isotonic PBS and counted. In general, viability staining was performed for 30 min in the presence of LIVE/DEAD Aqua fixable viability dye (Life Sciences) followed by a wash in FACS staining buffer (PBS 2% Normal Mouse serum (Gemini Bioproducts, West Sacramento, CA)). Surface marker staining followed and was performed for 30 min in FACS staining buffer. Some tubes had intracellular staining. In these cases intracellular staining followed surface marker staining, and cells were permeabilized with either Ebioperm buffer (Ebioscience) or Cytofix/Cytoperm buffer (BD), and the buffer usage was dictated by the antigen being stained. Between one and two million cells were used for staining within each tube, and in the case of dendritic cell staining, up to 10 million cells per tube were used if sufficient blood was available.

2.3. Cytometry and data analysis

Samples were acquired on a Becton Dickinson LSR II equipped with four lasers (407 nm, 488 nm, 532 nm, and 633 nm wavelengths) with 18 PMT detectors, optimized as described by Perfetto et al. (2006). Between 500,000 and 5 million events were collected per FCS file for each tube, depending on the number of cells available, in order to have sufficient events for statistical analysis of rare subsets defined by multiple markers. Data were acquired using DIVA 6.1.2 software (BD, San Jose, CA) and the analysis was performed using Flowjo (Treestar Inc., San Carlos, CA) and Gemstone (Verity Software House, Topsham, ME) software programs (Bagwell, 2010b).

2.4. Theory/calculation

Construction of a CLIP panel using high dimensional flow cytometry presents a myriad of technical challenges

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