To Flow or Not to Flow: Immune Cell Profiling in Translational Research

PRECISION for medicine

Introduction

With advances in our understanding of the complex, multifaceted role of the immune system in the development and progression of cancer and other immune-related diseases and disorders, immune cell profiling is becoming an increasingly indispensable part of the drug development process. Insights gained from profiling of the immune system can be used to optimize therapeutic design and treatment strategies—as well as to monitor disease progression and treatment response—in oncology, hematology, autoimmune conditions, and infectious diseases.

Since its commercialization in the early 1970s, flow cytometry has become the gold standard for immunophenotyping.[1] In addition to being a workhorse in the research laboratory, flow cytometry has evolved into a tool widely used in clinical trials for immune monitoring.[2] While flow cytometry is the oldest, most studied method of quantifying and typing immune cell populations, it is not the only one, and it is not without its challenges. During the past decade, epigenetic immune monitoring has emerged as a complementary approach. This technique is based on the highly cell type–specific methylation imprints at individual gene loci, each of which allows the specific distinction of a given immune cell type from other cell populations. More specifically, cell counting with this technology is based on the fact that all human cells have a known and stable number of each gene and quantitative analysis of methylation strictly associates with cell numbers.

For drug developers, identifying the right approach to immune profiling at each stage of clinical development—including the later stages, when it is often considered impractical to include these approaches—may be critical for advancing promising candidates, identifying predictors of response, or monitoring therapeutic efficacy. In this review, we compare and contrast flow cytometric and epigenetic immune monitoring, highlighting the opportunities and challenges of each of these immune profiling methods. We also provide guidance on when and how these technologies might be used to help move clinical drug development programs forward.

The growing need for immune monitoring

With the advent of cell-, gene-, and antibody-based therapies for immune-related diseases and disorders, the role of immune monitoring in both research and clinical care has increased exponentially. Individual parameters, or even comprehensive views, of immune status are central to diagnosing patients, screening eligibility for certain treatments, and predicting treatment response and susceptibility to infection.

These parameters are also essential for monitoring both adverse events (eg, rejections in transplantation) and efficacy (eg, activation of specific cell types in cancer treatment and monitoring of B cell levels with rituximab treatment). Of note, having an antibody-independent test for confirming the effects of therapeutic (depleting) antibodies would be appealing for confirming results.

> **"...identifying the right approach to immune profiling at each stage of clinical development—including the later stages, when it is often considered impractical to include these approaches—may be critical for advancing promising candidates, identifying predictors of response, or monitoring therapeutic efficacy"**

The value of flow cytometry

At present, flow cytometry is often the go-to technology to measure immunological parameters for translational research. Flow cytometry is highly suitable for analyzing a limited set of samples since a wide range of antibodies and fluorophore combinations are commercially available, which can be adapted and combined with the right expertise. In addition, flow cytometry can be used to address many different questions, including depletion and repopulation of specific immune cell populations, cell type–specific activation status, drug/receptor binding or specific modification of proteins (eg, phosphorylation), and antigen experience via tetramer/dextramer reagents. While flow cytometry is the gold standard for immunophenotyping, it faces significant logistical challenges, is hard to standardize among different laboratories, and can be cost-prohibitive for certain clinical trial programs.

Opportunities with flow cytometry

The key advantage of flow cytometry over competitive technologies is its advanced stage of development. With antibody production and labeling established at an industrial level, flexibility with respect to the antibody target is virtually limitless. At the same time, flow counters have undergone various generations of improvement and optimization, achieving clean cell-to-cell resolution in a multidimensional, high-complexity fashion. Highly skilled research-based labs now routinely measure upwards of 18 cell surface markers simultaneously, enabling the identification of many defined immune cell types, while commercial labs are able to measure 8 to 10 colors on clinically validated flow cytometry instruments. Analysis of multiple markers in combination on the same cell types allows the acquisition of information regarding cell activation states, antigen experience, etc.

....flow counters have undergone various generations of improvement and optimization, achieving clean cell-to-cell resolution in a multidimensional, high-complexity fashion

Challenges of flow cytometry

The most practical limitation of flow cytometry stems from sample preparation and the technical requirement for viable cells. This potentially biases results toward longer-living, more stable cell subpopulations, rather than directly reflecting the immune status in the blood of patients. Moreover, non-viable cells have greater autofluorescence and increased nonspecific antibody binding, which can lead to false positives and reduce the dynamic range, making identification of weak positive samples and rare populations difficult. Cells that have lost their integrity may also release DNA, which can lead to cell clumping. Hence, preservation of cells is the most important objective in the process leading to flow cytometric analysis. However, this preservation is only possible for limited windows or upon complex stabilization processes, such as cryopreservation or use of partial fixation that does not allow cells to be stimulated prior to assay. Given that the samples may need to be shipped to the site of analysis and patients may not be in places where real-time flow cytometry can be performed, the logistical demands for clinical trials—particularly multicenter studies—can become extremely complex. In the case of global clinical trials, sample handling issues or the exorbitant cost of these logistics may prevent flow cytometry from being a viable solution.

Other challenges that drug developers should be aware of include:

- Flow cytometry results are only as good as the antibodies that are available and how they are handled
- Data from the same sample may differ significantly depending on both the operator and the machine
- Gating flow data is subjective, and the magnitude of the problem varies depending on intensity differences between cells (eg, bright or dim versus on or off) and the skill of the analysts
- Stimulation of the cells may be required to see some markers, which requires viable, intact cells that can respond to treatment or drug
- When surface antigens are not abundantly expressed on target cells and show residual expression on control cells, there may be difficulty in rendering a clear separation
- Antibody reagents typically have a shelf life of 6 months, so it is often necessary to bridge studies between new and old batches or reagents
- Applicability of flow cytometry is doubtful when the antibodies used target the same antigens as the therapeutic antbodies

The value of epigenetic immune cell quantification

Since all cells in an individual have the same DNA blueprint, the functional distinction between different cell types is determined at the gene expression level. Epigenetic modification of DNA by methylation is one of the mechanisms by which gene expression is controlled. These (de)-methylation phenomena can be exploited for the quantitative assessment of immune cell populations using quantitative polymerase chain reaction (qPCR)-based assays. When used in loci with cell type-specific epigenetic patterns, this technique can be used to detect and quantify a wide range of different immune cells.[3-5] Use of epigenetic patterns for detection and quantification is a relatively new concept for immune monitoring and only a limited set of highly specific markers are currently available. However, those markers available as a DNA-based technology can improve and streamline global logistics with simplified sample preparation and reduced overall costs.

“epigenetic immune cell quantification can be performed in an automated, operator-independent manner and is less susceptible to the variability of reagents used and therefore, represents an efficient, economically viable and convenient global solution for immune monitoring, particularly for large multicenter studies”

Opportunities with epigenetic immune cell quantification

Since DNA is highly stable, epigenetic immune-cell quantification methods can be applied to fresh, frozen, or paper-spotted dried blood and other body fluids or tissues, eliminating the need for special care during sample storage and transport. This creates opportunities for immune monitoring in studies where flow cytometry presents a logistical challenge. In addition, unlike flow cytometry, which uses arbitrarily defined thresholds for positiveness, epigenetic immune cell quantification measures signal for each analyzed cell and, depending on the locus, indicate 1 of 2 states for each cell. As a result, the method is digital and provides reproducible data.

Further, epigenetic immune cell quantification can be performed in an automated, operator-independent manner and is less susceptible to the variability of reagents used since the components required for qPCR are synthetically produced and standardized. As such, this technique represents an efficient, economically viable, and more convenient global solution for immune monitoring, particularly for large multicenter studies. Global Phase 2 and Phase 3 immuno-oncology and autoimmune studies benefit from this technology due to simplified sample handling and logistics for sites across the globe that have access to eligible clinical trial participants but limited sample handling expertise. Given that there is no need for real-time assays or complex cryopresentation protocols, epigenetic immune cell quantification can provide valuable results at a fraction of the cost and complexity.

Limitations of epigenetic immune cell quantification

As a relatively new technique, epigenetic immune cell quantification does not yet have a broad array of well-defined, cell type-specific biomarkers. Whereas congruence of epigenetic measurements to flow cytometry has been shown for various markers, new epigenetic markers for various other cell types need to be identified and such congruence or divergence with flow panels have to be determined. For markers where no congruence between flow cytometry and epigenetics is observed, neither technique can be considered right or wrong since both clearly show relevant biological phenomena. Unlike flow cytometry, which is supported by years of observation in studies, the limited experience with epigenetic immune cell quantification makes it more difficult to integrate epigenetic data into currently available data sets. These limitations suggest that, in highly exploratory studies that aim to identify new, indication-specific cell types, epigenetic qPCR currently can only provide limited insight.

Leveraging epigenetic immune cell quantification for clinical trials

In this era of precision medicine, combining epigenetic immune cell quantification with other immune monitoring solutions can be a powerful way to unlock value in clinical trials. However, leveraging this rapidly advancing technology requires specialized expertise. The process involved in developing a validated epigenetic immune cell assay for clinical trial immune monitoring involves:

1. Identifying epigenetic marker regions of high specificity
2. Performing biological validation of the marker
3. Developing a qPCR-based assay for the marker
4. Validating the assay for accuracy, specificity, stability, and robustness

There are already a number of established assays for immune identification and quantification, with many more in development. In some cases, drug developers can even work with experts in epigenetic immune monitoring technology to design and develop customized epigenetic assays for a particular cell type.

Selecting the appropriate immune monitoring technology for your clinical trial

When considering the relative value of these 2 immune monitoring approaches in clinical trials, the general view is that flow cytometry remains the tool of choice in early exploratory studies with wide and unspecific cell targets. However, in larger studies with clearly identified target cell populations and in clinical practice, the ease of sample logistics, laboratory-independent robustness, and high reproducibility of epigenetic immune monitoring make it the preferred method.

To provide a framework for selecting an appropriate immune monitoring tool, flow cytometry is best-suited for answering questions such as:

- My drug should modulate the immune system, but we are not sure which cellular subsets are affected. Therefore, we want to cast a wide net and look at multiple 18-color flow panels, with a view to reducing the panels when we can focus on a specific signature
- Receptor occupancy, that is, the need to see that a particular cell surface marker and how many of them have been engaged
- Change in activation state of an intracellular pathway, such as phosphorylation of a specific protein

On the other hand, epigenetic immune cell quantification is designed to address needs such as:

- Precise quantification of depletion/reconstitution of specific cell types, particularly when depleting antibodies have been used
- Immunophenotyping for large numbers of samples
- Immune cell profiling within solid tissues where extraction of viable cells can be particularly challenging
- Immune cell profiling in multicentric studies particularly when involving clinical sites in remote locations where isolation and shipment of viable cells is a significant obstacle

Conclusion

The role of flow cytometry in translational research and clinical medicine is well established, and this technology is expected to remain a key platform for decades to come, whereas epigenetic immune monitoring is a relatively novel approach with highly complementary advantages. Understanding the nuances of these technologies—and the situations in which each is best applied—is critical in the development of therapeutics for immune-related diseases, as well as the selection and monitoring of patients who are most likely to benefit from these innovations.

References

1. Herzenberg LA, Parks D, Sahaf B, Perez O, Roederer M, Herzenberg LA. The history and future of the fluorescence activated cell sorter and flow cytometry: a view from Stanford. *Clin Chem.* 2002;48(10);1819-1827.
2. Jaye DL, Bray RA, Gebel HM, Harris WA, Waller EK. Translational applications of flow cytometry in clinical practice. *J Immunol.* 2012;188(10):4715-4719.
3. Baron U, Floess S, Wieczorek G, et al. DNA demethylation in the human FOXP3 locus discriminates regulatory T cells from activated FOXP3(+) conventional T cells. *Eur J Immunol.* 2007;37(9):2378-2389.
4. Wieczorek G, Asemissen A, Model F, et al. Quantitative DNA methylation analysis of FOXP3 as a new method for counting regulatory T cells in peripheral blood and solid tissue. *Cancer Res.* 2009;69(2):599-608.
5. Sehouli J, Loddenkemper C, Cornu T, et al. Epigenetic quantification of tumor-infiltrating T-lymphocytes. *Epigenetics.* 2011;6(2):236-246.
6. Baron U, Werner J, Schidknecht K, et al. Epigenetic immune cell counting in human blood samples for immunodiagnostics. *Sci Transl Med.* 2018;10(452).