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New technologies for autoimmune disease monitoring

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Abstract

Purpose of review—This article will review new technologies used to characterize the immune phenotype of cells and serum for potential use in studies of autoimmunity.

Recent findings—One area of recent development in studies of immune phenotyping is the contrast between cells of the immune system at rest and following activation. This simply involves comparing these cells at rest and following ligand-induced activation and measuring signaling system activation (phosphopeptide identification) or intracellular cytokine production or activation-induced gene expression. Preliminary data using these techniques have begun to identify signatures of disease (biomarkers) that are only seen when using these activation-induced assays. One of the most exciting new technologies, cytometry by time-of-flight mass spectrometry, couples a flow cytometer to a mass spectrometer, allowing many more parameters to be analyzed per cell, and without spillover between assay reagents, compared to conventional optical flow cytometry (heavy ions, mass, replaces fluorophore readout). Another new technology to analyze soluble proteins, bead-based immunoassays, can simultaneously measure up to 75 soluble analytes in a multiplexed array. Other technologies provide similar innovations in terms of analytical content, throughput, and miniaturization.

Summary—We believe that new cellular genomic and protein-based technologies can provide key insights into autoimmune disease pathogenesis, progression, and therapy, and that these assays need to be applied in a systematic way to samples from patients with autoimmune diseases.

Keywords

cytometry by time-of-flight mass spectrometry; intracellular cytokine assays; multiplexed immunoassays; phosphopeptide

Introduction

Much recent attention has been put on the discovery of disease-related biomarkers [1]. These are generally proteins or peptides whose expression level is correlated with a particular disease, or with its prognosis or therapeutic responsiveness. In the immune system, there are relatively few such disease-specific biomarkers identified, and only a handful that are used clinically to monitor disease (e.g., rheumatoid factor, anti-DNA antibodies, etc.). Yet, the immune system is integrally involved in the pathogenesis of infectious diseases, cancer, transplant rejection, and autoimmunity. In particular, the case for immune monitoring in autoimmune disease is direct, in that failures of immune regulation are causally linked to the disease pathogenesis. Thus, there are reasons to apply additional efforts to the search for biomarkers in autoimmunity.

One area of concern is the current reliance on single nucleotide polymorphism (SNP) analyses and high-throughput sequencing to identify disease-specific genetic signatures in autoimmune diseases. There are many reasons to question the use of these technologies: monozygotic twins have a concordance rate of less than 50% for most autoimmune diseases; no SNP has a relative risk that is disease-relevant in terms of biomarker development (the exception is the MHC and there the genes are not disease-specific, simply disease-associated); and recent studies have demonstrated, in an animal model of T1D, that the change in 'gene expression', driven by disease in a tissue-specific and age-dependent manner, identifies disease-associated gene expression, not polymorphisms or mutations, as relevant to disease pathogenesis [2*]. Thus, we must turn to alternative ways to identify disease-associated 'biomarkers' for autoimmune diseases.

As above, much of our knowledge of disease pathogenesis and therapy has come through testing in animal models, with translational studies attempting to bring such knowledge to the aid of humans. However, this methodology is often both slow and flawed, in that subtle differences between the model systems and human disease, and in the immune systems between experimental animals and humans, will consistently confound efforts at translational medicine [3]. The growth of so-called 'human immunology' attempts to circumvent this bottleneck by directly studying the immune system of humans and its perturbation in disease.

Unlike the animals used in studying models of human disease, humans are genetically heterogeneous, are exposed to vastly different environmental influences, and are not readily subjected to controlled experimentation. So much of human immunology is done as observational science, wherein a set of parameters is measured in a group of participants, and correlations between health and disease are drawn. To account for the vast genetic and environmental heterogeneity, not to mention age, gender, and ethnicity of the patients being studied, large cohorts must be studied. And because the human immune system is poorly understood, and clinical studies are expensive, one should arguably make as many measurements as possible per subject [4]. These aims can only be efficiently accomplished with the aid of technology that can measure many parameters at once (high content), and/or that can process many samples in a short time (high throughput). In addition, due to

limitations of sample volume collection from human subjects, assays that are miniaturized, or sample-sparing, are also important.

This review will highlight a new generation of technologies that have the attributes of being high throughput, high content, and/or miniaturized. As such, these are ideal technologies to apply to studies of human immunology and to the search for biomarkers of autoimmune disease pathogenesis or therapeutic response. In addition, many of these assays are capable of measuring ‘induced states’ or phenotypes of cells upon stimulation of one or more signaling pathways *in vitro*. Such assays are particularly fruitful for discovering biomarkers of disease, as many disease-specific alterations are seen not in the resting phenotype of cells, but rather in their response to activating stimuli [5].

Phosphopeptide flow cytometry

Most signaling systems in immune cells employ a common post-translational modification, phosphorylation, to propagate the activation signal(s). In the last decade, many antibodies have been generated that recognize the phosphorylated form of these signaling proteins. Phospho-specific flow cytometry has thus become an approach for analyzing and interpreting signal transduction at the single-cell level. The use of phospho-specific flow cytometry typically involves four steps: isolation, preparation, and stimulation of cells of interest; fixation and permeabilization; staining for surface antigens and phospho-proteins; and multiparameter flow cytometry analysis. The techniques for practicing this technology have recently been published [6]. This technology enables the simultaneous and high-throughput examination of several phosphopeptides within multiple cell populations, such as distinct immune compartments in the spleen or blood. The approach can facilitate a broad range of novel studies aimed at deciphering intracellular signaling events within heterogeneous populations of primary cells.

The multiparameter nature and single-cell resolution of phospho-specific flow cytometry have already enabled advancements in diagnostic testing, statistical mapping of signaling networks, and high-throughput drug screening [7]. Phospho-specific flow cytometry has been used to analyze phospho-signaling networks within samples from acute myeloid leukemia (AML) patients, enabling the detection of intra-patient cancer cell heterogeneity and demonstrating a noteworthy correlation between phospho-signaling and disease outcome [5]. Another study has demonstrated the ability to use correlational data obtained by phospho-specific flow cytometry to build directional, statistical maps of phospho-signaling networks within primary cells [8]. Although not yet utilized in any studies of T1D, the technique has been utilized in studies of an animal model of systemic lupus erythematosus (SLE) [9]. SLE is a complex autoimmune disease of unknown cause that involves multiple interacting cell types driven by numerous cytokines and autoantigens. Although the initiating events leading to SLE pathology are not understood, there is a growing realization that dysregulated cytokine action on immune cells may play an important role in promoting the inflammatory autoimmune state. Investigators in Dr Nolan’s laboratory applied phospho-specific flow cytometry to characterize the extent to which regulation of cytokine signal transduction through the STAT family of transcription factors was disturbed during the progression of SLE. Using a panel of 10 cytokines thought to have

causal roles in the disease, they measured signaling responses at the singlecell level in five immune cell types from the MRLlpr mice [9]. Their results suggested that negative feedback regulation opposed inflammatory cytokines that have self-sustaining activities, and suggested to them that a cytokine-driven oscillator circuit may drive the periodic disease activity observed in many SLE patients.

Cytometry by time-of-flight mass spectrometry

When immunologists discuss or debate how the immune system operates, it is often in a language that has been guided, and built, by the flow cytometer. Cell types are defined by surface markers, driven by antigens or cytokines in the environment, or by internal clocks deciding cell fate and function. Name the immune system cell type, the cytokine it produces, the intracellular proteins, or ion levels one wants to understand, and it is likely a flow cytometric system based on fluorescence has been developed to shed light upon it. Flow cytometers come in a range of sizes and flavors from three-parameter machine workhorses often used for clinical settings or simple green fluorescent protein (GFP) experiments to the more exotic devices employing as many as seven lasers and claiming to be ‘capable’ of analyzing – and sorting upon – 22 parameters.

But for anyone who has been in the business of immunology long enough, one knows the routine in designing an immunology experiment. What do we want to measure? What antibodies are at hand? What fluorophores do we have it conjugated to? How many parameters of measurement will capture the biology we desire to assay? What kind of flow cytometer is available and can it handle that many parameters? Is the time available on the machine? And then the process iterates, going back to the antibodies available, refining the fluorophores based on how available they are on the desired antibodies to match the wavelengths of the available laser systems on the available machines. Inevitably the fluorophores do not match, they bleed into each other’s spectra (requiring compensation), or one must wait an interminable period to get fresh antibody for appropriate conjugation. Even worse, for high-parameter experiments the frank fact is that it requires often years of training to efficiently and competently deal with six or more parameters and trust the data results are not mangled by some detector ‘blip’ or compensation artifact. The bottom line is that flow cytometry has reached a glass ceiling of around 12–15 parameters per cell, and neither quantum dots, nor Raman spectra dots, nor nano-wonder-not-invented-yet dots are going to solve the drive for the collection of higher parameter datasets.

Ask any research immunologist why they need so many parameters and the answer would be finger pointing to the latest issue of *Immunity* or *Journal of Immunology* – or to the lab budget. The field abounds in markers and cell subsets in dizzying variety and ascribed multiple functions. The problem often is we choose to look at a slice of biology with minimum parameters because we are limited – but not because we do not appreciate there is more information per cell we wish we could collect. We want to look at many cell types in the same experiment, or correlated against each other, but again we are limited by conventional cytometry.

Especially with regard to autoimmunity it can be readily appreciated that it is the immune system *state* that is at odds with itself. Picking any subset of cells to study (e.g., T cells, B cells, etc.) because it is one's bias to believe the entire network is driven by some dysregulation of that chosen cell does not obviate the fact that the rest of the immune system will be accommodating to the changes driven by that cell. Given this, it stands to reason that with more markers for more cell types to be measured, as well as markers of cell response states via phosphorylation (see section above), we can get a better composite view of immune states through which to search for relevant biomarkers of immunity or disease states.

Enter the cytometry by time-of-flight mass spectrometry (CyTOF), a conception of Scott Tanner at the University of Toronto [10*] and developed by DVS Sciences (Fig. 1). This machine substitutes for fluorophores the 'mass' of rare earth isotopes. Antibodies or binding agents are labeled with short-carbon-chain chelators via standard chemistry. Isotopic ionic 'salts' of the rare earths (obtainable from a variety of commercial vendors) are used as the labeling agent. Approximately 50 isotopes are available in a manner that can be readily used in the current CyTOF device.

Eventually, the CyTOF technology may have the capacity to measure up to 100 parameters per cell. Current state-of-the-art cytometers allow maximal detection of up to 15 parameters per cell. The CyTOF machine could significantly advance the research goals of many researchers studying autoimmune diseases and should enrich the field of clinical immunology as the technology is effectively implemented.

With the CyTOF in Garry Nolan's lab at Stanford, it is currently possible to demonstrate effective utilization of 25 separate markers (22 phospho-proteins, two-cell viability DNA isotope-labeled intercalators, and one-cell volume isotope reagent). With this device, in a single tube and with one antibody 'staining', it will ultimately be possible to readily detect and characterize all the major cell subsets in the blood (using antibodies that recognize at least 20 different surface molecules), and then be able to observe within each of those subsets another 80 intracellular phosphorylation events or other intracellular events that can be marked by specific antibodies. With this technology, autoimmune patient cell subsets will be mapped in a complete manner with the goal of informing the clinician of the diagnosis or diagnostic subset, prognosis or therapeutic indication, and response to treatment.

Notably, the CyTOF machine does not require compensation, allowing the application of statistical techniques that were here-to-fore impossible, given the constraints of fluorescence noise with traditional machines. The new mass spectrometer-based flow cytometer offers an unparalleled revolution in the analysis of immune system processes. The three-color to five-color 'traditional' flow cytometer initially defined the major cell subsets of the immune system we understand today (e.g., T cells, B cells, macrophages, etc.). The eight-color machine allowed us to characterize multiple immune system cells and led to important advances in immune cell biology. By merging this capability with intracellular staining, highend machines now allow 15-color analysis, opening possibilities for patient stratification of drug response outcomes. Within the year, CyTOF will enable measurement of up to 30 simultaneous parameters; within 2 years it will be possible to routinely monitor

as many as 50 parameters of a single cell (as many as 100 might be accomplished with an additional chelator chemistry that enables additional ions to bind to the antibodies). This will allow the dissection of biological mechanisms of many disease processes that are impacted by the immune system.

Multiplexed bead immunoassays

The use of flow cytometry to create multiplexed immunoassays is now well established as a research methodology [11], but its use in immune monitoring of human disease is still in its infancy. The methodology is conceptually relatively simple. A set of uniform particles ('beads'), generally polystyrene, are dyed to discreet intensities of one or more labeling dyes, and/or created in a set of discreet sizes that can be distinguished by their light scattering properties. In one of the most commonly used platforms, up to 100 distinguishable bead 'addresses' are created by combining 10 levels of fluorescence of each of two labeling dyes. Each type of bead within this matrix can then be used to create its own immunoassay for detection of a unique soluble analyte. Commonly, a sandwich immunoassay format is used, such that a specific capture antibody is immobilized on the bead, and after incubation with the sample of interest, a labeled detector antibody specific for a different epitope of the same analyte is added. Usually, indirect detection is used; for example, the detector antibody is biotin-labeled and is followed by incubation with streptavidin-PE. Using this format, many different beads can be mixed and resolved via their individual addresses, and a cocktail of detector antibodies for all specificities being probed can be created. This makes for a relatively simple multiplexed assay that can be used for virtually any combination of soluble proteins for which specific antibody pairs exist.

The range of multiplexed bead immunoassays is wide, and there are both commercial kits with predefined specificities and the possibility of creating custom assays of the user's choosing. The most common targets are cytokines and chemokines, usually analyzed in serum, plasma, or cell culture supernatants. Other bodily fluids, including saliva, urine, tears [12], wound exudates, cerebrospinal fluid, tissue homogenates, and so on, can also be used.

When analyzing healthy human serum or plasma, the levels of most cytokines are extremely low, generally at or near the detection limit of the assay [13]. Sensitivity can thus become a major issue because of the desire to detect readings within the normal range, or at least to detect subtle elevations that may characterize a disease state. It is inherently simpler to optimize sensitivity for a single analyte assay than to simultaneously optimize the sensitivity of a large number of analytes in a multiplexed assay. Nevertheless, investigators have found that multiplexing up to 50 cytokine and chemokine assays does not noticeably decrease the sensitivity of the vast majority of analytes, compared to a smaller multiplex.

Inhibitory factors in the matrix are an equal or even greater source of lost sensitivity in some assays. For example, detection of a number of cytokines is decreased several fold in the presence of normal human serum (unpublished data from Holden Maecker). This can be seen by dilution of assay standards in serum versus dilution in buffer recommended by the manufacturer, with sometimes dramatic loss of signal in the former versus the latter case. Ways to mitigate this problem need to be further investigated.

In addition to analyzing ‘resting’ levels of cytokines in serum, plasma, etc., investigators have begun to explore another possible monitoring methodology, namely, stimulated cytokine assays. Here whole blood or PBMC is incubated with a cocktail of stimuli, which could include one or more cytokines, mitogens, and so on. After overnight stimulation, the supernatant is analyzed for a wide variety of induced cytokines by a multiplex bead assay. Because the cells are perturbed from their resting state, induced phenotypes corresponding to higher or lower levels of cytokine induction can be read. These may be more sensitive biomarkers of diseases such as autoimmunity, where resting levels of cytokines in blood are still mostly undetectable, but where dysregulation of cell signaling pathways may be visualized as changes in the induced state [9^{*}]. The information thus gleaned is not as precise as that from, say, intracellular cytokine staining, where the production of cytokines is analyzed on a per-cell basis for each cell subset being interrogated. However, the multiplex bead assay offers relative simplicity, and the ability to read out a much larger array of analytes than conventional cell-based flow cytometry.

In addition to cytokines, multiplex bead assays are increasingly being used to detect phosphorylated signaling intermediates, in much the same way as phospho-flow, but using cell lysates. Again, the resolution of cell subset specificity is lost, but the number of analytes that can be simultaneously measured is very high. Of course, normalization of total protein levels in lysates, or some other way of normalizing signals in lysate-based assays, is critical to achieving precise quantitation.

There are many other types of analytes amenable to detection by multiplexed bead assays. Immunoglobulins are of course an obvious target, whether simultaneously probing for antibodies of different antigen specificity, or determining levels of various isotypes. Complement and acute phase proteins can be targeted, as can a number of other targets of immunological interest. Although other immunoassay platforms may provide greater simplicity and, in some cases, greater sensitivity, bead-based assays are unparalleled for their ability to create large multiplexed arrays. As such, they are tools of choice for exploratory human immunology studies, where soluble mediators such as cytokines are prime targets, and there is a desire to track as many of these proteins as possible in a multiplexed fashion.

Intracellular cytokine analysis

The ability to block secretory pathways in living cells has allowed cytokine production to be readily detected via intracellular staining with specific antigens. This was first done with mitogen stimulation [14], in which the responding cell population is large and the cytokine signal robust. However, it is now clear that specific antigen stimulation, either with proteins or with peptides, can also induce a detectable response, albeit in a potentially very small population of cells (e.g., 0.1% of CD4⁺ or CD8⁺ T cells) [15,16]. Optimized staining protocols, optimal gating strategies, and collection of sufficient events are critical to achieving interpretable results in such settings [17].

Autoantigen-specific T cells provide a particular challenge to this technology because the frequency of responsive cells in blood may be much lower than that for many viral or

bacterial antigens. However, with sensitive assays (tetramer or CFSE proliferation assays), they can be detected in the blood of healthy donors [18,19]. Because of the potential role of regulatory T cells (Tregs) in suppressing these responses [18], it may be helpful to deplete Tregs from blood or PBMC before stimulation for functional assays such as intracellular cytokine staining. Recent studies in both human T1D patients' cells and cells from NOD mice suggest that there may be a defect in the CD4 T effector rather than the CD4 T regulator [20,21] accompanying T1D. These studies must be verified, but if true, this opens a new avenue for the analysis of defects in signaling response of T effectors to regulation, rather than searching for a defect in the Tregs *per se*.

It can also potentially be useful to track mitogen responses as well as antigen-specific T-cell responses because dysregulation of signaling networks in autoimmunity can extend beyond the self-reactive T cells [9^{*}]. CD3⁺CD28 stimulation, or an activating combination of CD2 antibodies, is potentially appropriate, physiologically relevant stimuli to use in this regard.

Conclusion

We believe that new cellular genomic and proteinbased technologies can provide key insights into autoimmune disease pathogenesis, progression, and therapy, and that these assays need to be applied in a systematic way to samples from patients with autoimmune diseases.

Here we have highlighted several new immunological technologies that have the combined attributes of:

1. high-content analysis, that is, measurement of many parameters simultaneously, and
2. ability to read out an induced state (i.e., a response to stimulation *in vitro*).

We postulate that these will be particularly important concepts for human immunology research in general, and for autoimmune disease in particular, as we begin to define new human biomarkers for diagnosis, prognosis, and monitoring the response to therapeutic intervention.

The technologies presented here can provide complementary information. Multiplexed bead assays are particularly relevant for measuring soluble or secreted proteins, whereas the other techniques are cellular in nature. Intracellular cytokine analysis and phosphopeptide flow cytometry provide different readouts in the pathways of immune stimulation. And CyTOF has the potential to provide both of these readouts in a more highly multidimensional manner, without issues of optical spillover. It may truly be the cellular analysis platform of the future.

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References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest

Additional references related to this topic can also be found in the Current World Literature section in this issue (p. 389).

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Figure 1. Cytometry by time-of-flight mass spectrometry principle

(a) Principle of cytometry by time-of-flight mass spectrometry: antibody labeling with heavy metal isotopes, binding of labeled antibodies to cells, and conversion of labeled cells to plasma for quantitation of heavy isotopes by time-of-flight mass spectrometry. (b) Limitation of the visible spectrum is shown by the overlap in emission spectra of eight common fluorescent dyes. (c) By contrast, elemental isotopes of different mass are exquisitely separated by time-of-flight mass spectrometry, allowing many more labeled antibodies to be combined, without interference between labels.