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Development and application of ‘phosphoflow’ as a tool for immunomonitoring

Sheng Wu¹, Lei Jin¹, Luis Vence¹, and Laszlo G Radvanyi^{1,†}

¹Department of Melanoma Medical Oncology, The University of Texas, MD Anderson Cancer Center, Houston, TX 77030, USA

²Department of Breast Medical Oncology, The University of Texas, MD Anderson Cancer Center, Houston, TX 77030, USA

Abstract

Flow cytometry has revolutionized our ability to monitor immune responses by allowing us to simultaneously track a variety of cell surface and intracellular markers in discrete cell subsets in a highly sensitive and reproducible manner. This is especially critical in this new era of vaccinology trying to tackle the growing problems of chronic viral infections and cancer that not only evade host immune responses, but can negatively manipulate vaccine-induced immune responses. Thus, understanding how lymphocyte signaling is altered under normal and pathological conditions has become more critical. Over the last decade, a new flow cytometry technology called ‘phosphoflow’ (also sometimes called ‘phosflow’), is rapidly developing for tracking multiple intracellular signaling molecules in the immune system at a single-cell level. Antibodies and reagents for tracking both tyrosine-phosphorylated and serine/threonine-phosphorylated signaling intermediaries in key immune signaling pathways have been developed, and phosphoflow is now starting to be applied to a wide variety of both preclinical and clinical studies on lymphocyte responses, as well as the functioning of cancer cells and virally infected cells. Here, we review the development of phosphoflow technology, its modern applications in the field of immunomonitoring and its current limitations. We then provide a perspective on the future of phosphoflow and a vision of how it can be applied to emerging critical questions in human vaccinology and public health.

Keywords

flow cytometry; immunomonitoring; lymphocyte; phosflow; phosphoflow; phosphorylation; signaling

The sensitive and accurate measurement of cell-mediated immune responses has become increasingly important as a key part of vaccine development and immunotherapy research, especially for the prevention and treatment of chronic viral infection and cancer. Although past vaccine development efforts against most of our infectious diseases have been aimed at inducing neutralizing antibody (Ab) responses and memory B-cell responses, measured by serological assays, a new generation of vaccine development is tackling more difficult targets requiring strong cell-mediated immune responses through T-helper and cytotoxic T cells in order to mediate protection. In addition, a new generation of therapeutic vaccines against cancer and established chronic viral infections, especially HIV and hepatitis C virus (HCV), are areas of intense research [1,2]. Overall, many neutralizing Ab-dependent vaccines protecting us against routine pediatric and adolescent infectious disease have been developed. We are now

[†]Author for correspondence: Tel.: +1 713 563 9489 lradvanyi@mdanderson.org.

faced with the much more difficult challenge of developing a new generation of vaccines and immunomodulatory therapies generating stronger and longer-lasting cell-mediated immune response focusing on antigen-specific CD8⁺ T cells.

The development of vaccines for these new targets has also necessitated the parallel development of newer and more sensitive immunomonitoring assays able to measure the responses of antigen-specific effector T-cell responses, innate immunity and the effects of cytokine signaling. A number of assays, such as ELISpot and measurement of inflammatory cytokines produced by activated lymphocytes in blood and cell culture using ELISA, have now been routinely used for the last few decades [3,4]. However, these ‘bulk’ assays measure the overall activity of an entire peripheral blood lymphocyte or T-cell population, making it difficult to pinpoint the phenotype or real identity of the reactive cells involved. The application of flow cytometry to vaccine immunomonitoring has revolutionized the field, allowing for the highly sensitive and precise measurement of antigen-specific lymphocyte and innate lymphocyte responses over a wide range of frequencies. Two main flow cytometry-based assays have been tetramer staining using peptide-loaded, fluorescent-tagged class I or class II MHC/HLA molecules, and intracellular cytokine staining (ICS) for IFN- γ , TNF- α , IL-10 and other cytokines after *in vitro* restimulation of lymphocyte isolates with target antigens, cells or cytokines. Tetramer staining and ICS have also been combined in a single assay to track cytokine production specifically in the activated T-cell subpopulations. Another set of flow cytometry-based assays measure cytotoxic T-lymphocyte (CTL) or natural killer (NK) cell cytotoxic activity through tracking CD107 release out of effector cells using Ab-based trapping at the cell surface [5,6], or by measuring indicators of apoptosis initiation in target cells, such as caspase-3 cleavage [7].

Overall, these flow cytometry-based immunomonitoring tools have not only allowed for more sensitive measures of cell-mediated immunity in normal and diseased states, but have introduced the ability to track responses at a single-cell level in a high-throughput fashion. However, another dimension that needs to be addressed in measuring cellular responses and assessing the state of the immune system overall during vaccination and other forms of immunotherapy is the activation of intracellular signaling molecules triggered by antigen recognition through antigen receptors (T-cell receptor [TCR] or B-cell receptor [BCR]), the activation of cytokine receptors driving lymphocyte expansion and differentiation, and other accessory signals either costimulating (e.g., CD28), or inhibiting these positive signals (e.g., negative costimulatory signals through programmed death 1 [PD-1] and CTL antigen 4 [CTLA-4] on activated T cells). The activation of tyrosine and serine threonine kinases phosphorylating multiple intracellular targets, including adaptor proteins, scaffold proteins, and other downstream protein and lipid kinases, is a critical process in all of these signaling pathways [8,9]. These events happen quickly over seconds and minutes and can have sustained effects lasting for hours or days, depending on the nature of the pathway and the activity of phosphatases in the system [10,11]. An analysis of both activated and steady-state levels of phosphoproteins in lymphocytes can give us a snapshot of how cells are responding to their environment and has the potential to rapidly quantify the degree of activation of different signaling pathways, such as those triggered immediately after antigen contact. This approach can not only be applied to the cells of the ‘responding’ immune system, but also to damaged or diseased cell targets, such as virally infected cells and tumor cells where normal signaling pathways through protein kinases, in response to environmental cues, have been disrupted, resulting in these pathways either shutting down, being abnormally attenuated or being abnormally accentuated.

The recent development of flow cytometry-based approaches to measure changes in the phosphorylation status of key intracellular signaling molecules such as STATs, members of the MAPK and stress-activated protein kinase families, other cell survival kinases and adaptor

molecules has greatly added to our ability to track the activity of lymphocytes and other relevant cell types in response to different conditions. This new approach, called ‘phosphoflow cytometry’ (phosphoflow), has great potential as a powerful tool to open up new avenues of investigation in human immunology through the rapid and sensitive tracking of signaling pathways in individual antigen-specific lymphocytes during vaccination and screening the responses of multiple lymphocyte subsets simultaneously, such as different T-cell effector and memory subsets, in response to different activating signals in a high-throughput fashion [12]. This will pave the way towards newer and better population-based studies to discern why different people respond differently to vaccines and immunomodulators, and how aging and chronic diseases, such as diabetes and arthritis, can affect vaccine responses. Traditional approaches using bulk assays on cell lysates, such as immunoblotting and ELISA, to measure phosphorylation states that do not allow for the discrimination between different cell subpopulations are now being rapidly substituted by phosphoflow assays as more applicable Abs, fluorescent tags, and improved fixation and staining reagents become available. Although newer methods such as reverse protein arrays [13,14] have been a major improvement to more sensitively determine the levels of multiple phosphoproteins in lysates of lymphocytes and other leukocytes in a high-throughput fashion on chips, a major drawback at present is their inability to discriminate between individual cells and discrete cell subsets in a sample.

In this article, we will examine the current status of phosphoflow technology and its current application to monitoring immune responses and the signaling status of cells. Shortcomings in our current methods and reagents will be pointed out as well as possible avenues for improvement. Finally, we will give an overview of where we see the field heading in the future, and what current and new basic and clinical research areas may be positively impacted by developments in phosphoflow technology.

Development of phosphoflow staining methods

Staining of phosphorylated molecules for flow cytometry has been explored by different groups since 1999. Different methods were explored for molecules, such as STAT1 [15,16], STAT4 [17], AKT/PKB [18,19], ERK, MEK [20], p38, cJun [21] and so on. These studies established a number of key criteria needed for successful detection of intracellular phosphoproteins. First, the cells need to be rapidly fixed in order to keep the original phosphorylation status of target proteins. Second, the specific Abs must be able to get access to the target epitopes by effective cell permeabilization. Third, Abs used to stain cell surface proteins for subset discrimination and phosphorylated moieties in intracellular proteins should specifically recognize and stably bind to epitopes that have been denatured by fixation [22]. In order to develop a general protocol for phosphoprotein staining, Nolan and colleagues were the first academic group to systematically compare different methods, and develop the first effective and reproducible approach for intracellular phosphoprotein detection using flow cytometry [22]. They initially tested the MAPK cascade in Jurkat T cells [23] and the JAK–STAT pathway in the monomyelocytic cell line U937 [24,25] as the two model systems to develop these staining flow cytometry analysis methods. Different stimulation conditions of multiple signaling pathways were evaluated by phosphoflow analysis. The results correlated well with immunoblotting [22]. They also compared different organic solvents and detergents to fix and permeabilize cells simultaneously, such as methanol, ethanol and acetone. Alternatively, a two-step method, fixation with formaldehyde prior to a permeabilization step with methanol, ethanol, acetone, Triton-X 100 or saponin was also tested. The fold change in median fluorescence intensity between stimulated and unstimulated cells revealed that fixation with formaldehyde and permeabilization with methanol was the best combination, which was confirmed in multiple signaling pathways [22]. Further experiments suggested that the concentration of formaldehyde, and the time and temperature of incubation, resulted in minor changes of phosphoprotein staining [22]. The stability of different phosphoepitopes was also

evaluated in these initial studies and found to remain 70–90% stable overnight in staining buffer (phosphate-buffered saline with 1% bovine serum albumin) after fixation and permeabilization [22]. In fact, Ab binding to these phosphoepitopes was found to protect them from decomposition [26]. Small molecular fluorophores, such as fluorescein isothiocyanate, Alexa Fluor 488® and Alexa Fluor 647® (Molecular Probes Inc., OR, USA) were also found to be optimal for phosphoflow, because they facilitate easier entry of Abs into the cells and their minimal influence on the binding of Abs to target proteins [11]. The choice of permeabilization agents (methanol vs detergent-based) for phosphoflow staining also depends on the target of choice. In this respect, milder saponin-based permeabilization methods have been found to be suitable for detecting phosphorylated MAPKs (ERK and p38) residing mostly in the cytoplasm, while an optimal readout of nuclear translocated phosphoproteins, such as the STATs, favor the use of methanol permeabilization systems [27,28].

Simultaneous measurement of multiple active kinase states was also explored by Nolan's group [12]. Phosphorylations of p38, p44/42 and JNK/stress-activated protein kinase in the MAPK family, AKT/PKB in cell survival signaling, and tyrosine kinase 2 were detected in subpopulations of peripheral blood mononuclear cells (PBMCs) using phosphoflow. T cells stimulated by chemical agents, phorbol myristate acetate and ionomycin, and anti-CD3 or anti-CD8 Abs, showed that all the three pathways could be monitored at the same time by phosphoflow [12]. Different phosphorylation states were found between CD3⁺-gated cells and CD3⁺-sorted cells, which suggested that prior purification of each subpopulation might affect the endogenous signal pathways [12]. These phosphoflow studies also found that the timing and extent of phosphorylation of key signaling molecules in naive T cells and memory T cells after activation were different, which also proved that phosphorylation of different signaling pathways in heterogeneous subpopulations could be analyzed at the same time [12].

Following these initial studies and demonstration of the feasibility of phosphoflow, multiple groups started to investigate this technology. A total of 36 different murine lymphocyte cell surface marker monoclonal Abs (mAbs) were tested for their ability to stain cell surface proteins after fixation [29]. These studies also found that the type of fluorophore linked to the Ab is another factor that could be affected by dehydration and denaturation conditions. Small molecular fluorophores, such as fluorescein isothiocyanate, Alex Flour 488 and Alex Flour 647, can resist the effects of methanol, while fluorophores (e.g., phycoerythrin, PerCP and PerCP-Cy5.5) can lose their fluorescence in some cases. Therefore, the choice of fluorophores should be taken into account when sequential staining is used [29]. The side scatter property of cells is another key parameter that was found to be affected during phosphoflow. For example, scatter properties of B and T cells from murine bone marrow remain unaffected, while those of CD11b⁺ cells from the spleen and peritoneal cavity are modified in different ways [29]. The mechanism of this difference is still unclear and other markers should be combined to identify those subpopulations.

Over the last 10 years, a number of academic groups together with scientists from industry have developed optimized fixation and permeabilization reagents and techniques suitable to detect a number of different phosphoproteins, many of which are now commercially available [12,27–29]. This work has also identified more suitable Abs capable of staining cell surface markers after fixation, and a growing number of established Ab clones compatible with phosphoflow fixation and permeabilization methods have now been identified. Many of these are unpublished, but can be found in technical documents, available on websites of different vendors. Thus, phosphoflow has emerged as a critical new assay technology in the field of flow cytometry that can be seriously evaluated as a vaccine immunomonitoring tool able to be performed by most immunology labs worldwide. A set of defined reagents able to track the tyrosine or serine/threonine phosphorylation of a host of different lymphocyte signaling molecules, together with cell surface Abs compatible with the different fixation/

permeabilization methods needed, are now available for both human and murine experimental systems. These Abs can be purchased from a number of vendors who also have websites introducing the technology for the novice with detailed protocols, phosphoflow mAbs and reagents, and lists of phosphoflow-compatible cell surface staining Abs. However, it is important to point out that many Ab clones against lymphocyte cell surface and intracellular molecules (e.g., transcription factors and cytokines) still remain untested and may be compatible with the phosphoflow systems presently used. For example, we have recently found an anti-CD56 Ab (clone N901; Beckman Coulter, CA, USA) that shows excellent staining properties in these assays that has not yet been reported. Thus, each investigator needs to empirically test different Abs as needed, depending on the specific needs of their system.

Preclinical applications of phosphoflow

Over the last decade, phosphoflow technology has evolved, allowing for the simultaneous tracking of the phosphorylation status of multiple molecules in different leukocyte subsets [30–32]. Phosphoflow has also begun to be applied in a variety of sub-disciplines. Table 1 summarizes some of the major signaling molecules and cell types that have been studied. Much activity has focused on the MAPK pathway (p34, p38, ERK1/2 and JNK) and STAT (especially STAT1 and STAT5) signaling pathways, with some more recent emphasis on proximal antigen-receptor signaling pathway intermediaries in T and B cells (lymphocyte-specific protein tyrosine kinase [LCK], FYN, spleen tyrosine kinase [SYK], ζ -chain-associated protein kinase [ZAP]70 and linker for activation of T cells [LAT]) and the AKT pathway.

Phosphoflow in activated lymphocytes & other leukocyte subsets

Phosphoflow has provided a new tool to quantitatively measure in a high-throughput fashion how different signaling pathways are activated by specific lymphocyte activators and how these pathways interact in real-time [10,33]. Phosphoflow has been used to study downstream signaling in T cells following TCR activation by monitoring changes in key phosphotyrosine residues. For example, a ‘phosphosignature’ of TCR activation using CD3 ligation together with costimulation via CD28 or lymphocyte function-associated antigen (LFA)-1 accessory molecules has been obtained in CD4⁺ T cells [34]. This study aimed at further understanding the role of LFA-1 in T-cell activation. LFA-1 plays an important role in the interaction between T cells and antigen-presenting cells as part of the immune synapse. However, its exact mechanism of action in terms of downstream signaling in lymphocytes is still unclear. CD4⁺-naive T cells were stimulated with CD3 plus CD28, or CD3 and CD28 plus LFA-1. The profile of phosphorylated kinases was monitored simultaneously by phosphoflow at different time points [34]. LFA-1 coligation was found to increase the phosphorylation of PI3K pathway intermediaries, the ERK1/2 pathway, as well as both sarcoma proto-oncogene tyrosine protein kinase and JNK [34].

Phosphoflow can also be applied to map the kinetics of signaling events downstream of BCR triggering [10,33]. Dynamic responses of two different subtypes of B cells to BCR stimulation, IgM and IgG isotype, have been tested by phosphoflow [10]. B cells were stimulated with anti-IgM or anti-IgG and the time course of phosphorylated ERK1/2 and SYK was measured. Compared with CD20⁺ cells in PBMCs, IgM⁺ B cells showed rapid, transient phosphorylation of ERK1/2 and SYK in the first 4–16 min then declined rapidly afterwards. By contrast, phospho-ERK1/2 and phospho-SYK appeared more rapidly in IgG⁺ B cells on a per-cell basis and their peak levels maintained for over 2 h [10]. In this study, dose–response and time course experiments of BCR signaling were also done to investigate the influence of H₂O₂ on phosphatases [10]. Results suggested that addition of H₂O₂ after BCR stimulation amplified and enhanced BCR signaling. Most importantly, because phosphoflow could analyze the phosphorylation status at a single-cell level, ERK1/2 and SYK were found to be simultaneously phosphorylated in the same population of B cells [10].

The immune system is a highly interactive network with many feedback loops, in which one immune response can be regulated by multiple factors [35]. Intracellular signaling proteins, intercellular interactions and local environments can coordinate to determine the fates and responses of immune cells [35]. The advantages of phosphoflow make it feasible to simultaneously analyze different populations without the need to isolate cell subsets and interrupt these layers of regulation to perform the analysis. For example, phosphoflow has been used to discern the phosphorylation profiles of B cells, T cells and myeloid cells in response to a number of cytokines (IFN- γ , GM-CSF, IL-2 and IL-10) and lipopolysaccharide [35]. Results showed that specific pathways were activated in distinct populations according to different stimuli [35]. Interestingly, comparison of signaling profiles from stimulation *in vivo* and *in vitro* found that the external environment strongly influenced the sensitivity of certain cell subtypes to cytokines and the kinetics of phosphorylation [35]. This work emphasized the importance of the local environment in immune responses and proved that the phosphoflow method can reflect the *in vivo* status of phosphorylation in multiple cell subsets. In addition to lymphocyte activation, phosphoflow has been used to study what it is that regulates a state of 'anergy' or hyporesponsiveness of T cells to TCR stimulation. One recent study looked at the Notch pathway activated by Jagged-1 fusion protein in purified human CD4⁺ and CD8⁺ cells [36]. Inhibition of T-cell activation by Notch signaling correlated with upregulation of gene related to anergy in lymphocytes but not blockade of TCR signaling, as determined by measuring phosphoprotein signatures by phosphoflow for CD3 ζ , LAT, LCK, ZAP70 and SLP-76 [36].

Phosphoflow has been used to analyze other leukocytes besides lymphocytes, such as granulocytes [27,37]. For example, a combination of immunophenotyping and intracellular signaling staining with phosphoflow has been used to study the effects of anti-IgE and allergens on human peripheral blood basophils [27]. Basophils stimulated with anti-IgE and allergen showed phosphorylation of p38 5 min prior to upregulation of CD63, which is a surface activation marker for basophils [27]. By contrast, specific inhibition of p38 phosphorylation greatly reduced upregulation of CD63 [27].

Phosphoflow to evaluate Pathological conditions in cells

The delicate balance in signaling pathways is needed for proper cell function. External pathogens and internal mutations can irreversibly break the balance in different growth-regulatory signaling pathways and result in disordered signaling associated with different immune system-related diseases and cancer. In addition to being extensively applied in basic research to investigate the mechanisms of signaling pathways, phosphoflow has also been used in translational research to screen the effects of aberrant events or mutant molecules on signaling pathways in pathological conditions [37–46].

Significant efforts have been made to compare the signaling profiles between normal and cancer cells. In general, alterations of signal pathways in cancer cells usually result in enhanced survival and proliferation, impaired negative regulatory pathways (e.g., cell cycle blockade and apoptosis), and aberrant responses to external stimuli [38]. For example, a length mutation of FMS-like tyrosine kinase 3 with a duplicated Y591 phosphorylation site was found using phosphoflow in a population of acute myeloid leukemia patients, which correlated with overexpression of the antiapoptotic mediator bcl-2 and suppression of p53 that usually mediates cell cycle arrest and apoptosis in response to DNA damage [39]. Enhanced signaling through MAPK and STATs found using phosphoflow correlated with greater expression of bcl-2 [39] and poor clinical outcome [40]. Not only basal or constitutive signaling pathways, but also aberrant responses under stimulated conditions have been found in cancer cells by phosphoflow [40]. Point mutations in RAS genes have been shown to be associated with many cancers. In primary c-kit⁺ lin^{-low} hematopoietic stem/progenitor cells, the influence of the oncogenic

Kras^{G12D} allele on the phosphorylation profile was evaluated in a transgenic mouse model [41]. Hyperphosphorylation of ERK, STAT5 and p70S6 kinase were observed using phosphoflow in both basal level and in response to GM-CSF [41]. Similarly, hypersensitivity to low doses of GM-CSF, as measured by detection of Y694 phosphorylation in STAT5 by phosphoflow, was found in juvenile myelomonocytic leukemia, chronic myelomonocytic leukemia and acute myeloid leukemia [42]. Interestingly, this hypersensitivity, as monitored quantitatively by phosphoflow, could be an indicator of a disease status and may be a useful clinical marker, as this hypersensitive population disappeared in patients achieving clinical responses and remaining in remission [42]. The effects of cytokines on solid tumor cells has also been monitored using phosphoflow, especially STAT3 and STAT5 that are critical in mediating the expression of growth promoting and survival genes in cancer cells, as well as the production of immunosuppressive factors such as IL-10 and VEGF [46].

Detection of abnormal signaling signatures has been carried out by phosphoflow in other disease states besides cancer, such as the tracking of STAT5 activation in chronic viral infection [43,45] and MAPK activation in T cells during atopic reactions (rBet v 1) [37]. An imbalanced signaling profile was identified in monocytes of HIV-1-positive donors [43]. Reduced phosphorylation of STAT5 in monocytes in response to GM-CSF was found in HIV-infected patients [43]. By contrast, MAPK pathway intermediaries activated by lipopolysaccharide, was enhanced in the same population [43]. In HCV-infected patients, phosphorylation of STAT5 was found upregulated in CD4⁺CD25⁺Foxp3⁺ Tregs following blockade of PD-1 signaling, helping demonstrate that PD-1 can negatively regulate Tregs and that blocking PD-1 signaling may enhance Treg function [45].

Phosphoflow as a screening tool to identify drugs & genes effecting key intracellular signaling pathways

Conventional drug screening systems are usually based on purified enzymes or specific cell lines, which do not account for the cross-talk between signaling networks and interactions between different cells that normally occur. With the development of phosphoflow, properties of drugs on activation or inhibition of different intracellular signaling pathways by tracking differential phosphorylation states of specific proteins can be analyzed more rapidly, accurately and precisely in a high-throughput fashion. Phosphoflow can also be applied to rapidly and sensitively screen gene libraries to identify genes able to turn intracellular signaling on or off in leukocytes and other cell types in normal or diseased states [47]. To complement these applications of phosphoflow, a fluorescent cell barcoding (FCB) technique has been developed to increase the high-throughput capacity of flow cytometry [48]. The FCB technique facilitates the simultaneous analysis of samples by prelabeling the different samples with different intensities of various fluorophores before Ab staining [48]. When analyzing by flow cytometry, each sample can be differentiated according to the prelabeled fluorophore and then analyzed for the expression of the specific markers being measured in the different samples. This method can reduce sample-to-sample variation in both conventional flow cytometry analysis and phosphoflow during large-scale screening of immune responses, and can reduce reagent consumption and sample acquisition time [48]. Using this approach, phosphoflow has been used to screen a library of small-molecule natural compounds to identify cell type-specific and pathway-specific inhibitors [49]. For example, several compounds showed selective inhibition of JAK-STAT signaling pathway in B and T cells [49]. The specific inhibition was further confirmed by *in vivo* experiments in mice, both in splenocytes and in blood cells [49], which proved that phosphoflow was a reliable method to screen drugs.

Phosphoflow has been used to screen the effects of different drugs on T-cell activation in mouse models of graft-versus-host disease (GvHD) [50]. Phosphoflow staining revealed that phosphorylation of ERK1/2 and STAT3 played an important role in alloactivation of T cells

in GvHD [50]. SL327, a small-molecule inhibitor of ERK1/2 phosphorylation, was found to decrease T-cell alloactivation [50]. In addition, cucurbitacin I and cucurbitacin E, inhibitors of STAT3 phosphorylation, were also found to attenuate GvHD reactions mediated by T cells [50]. Phosphoflow has also been applied in drug screening for treatment or prevention of HIV infection. Here, the induction of CCR5 phosphorylation mediated by HIV infection of CD4⁺ T cells has been used to screen small-molecule and Ab inhibitors against CCR5 activation by phosphoflow [51]. Dose–response curves and time courses of phosphorylation in the cytoplasmic domain of CCR5 were monitored [51].

Mechanisms of drug action have also been investigated by phosphoflow. For example, the role of statins in cancer has been evaluated. Shachaf *et al.* found that atorvastatin prevented and reversed MYC-induced lymphomagenesis; however, in the presence of constitutively activated K-Ras^{G12D}, this effect was inhibited [52]. Phosphoflow data showed that atorvastatin could inactivate Ras-induced ERK1/2 phosphorylation, but could not do so when constitutively activated K-Ras^{G12D} was present [52]. These findings suggested that heterogeneous cancer cells could respond differently to one drug, and multiple drugs should be combined to obtain the optimum anticancer effects. Overall these examples point to the tremendous potential of applying phosphoflow technology in drug screening and pharmacodynamic studies in different human diseases.

Phosphoflow as a tool to monitor immune responses in humans

The development of new immunotherapies and vaccines are currently being developed against cancer and chronic viral infections (e.g., HCV and HIV). These approaches aim to stimulate strong cell-mediated immunity using active and passive immune modulation and represent a major new paradigm in vaccine design and cancer treatment in the 21st Century. Increasingly more and more types of immunotherapies, including antigen-specific vaccines with new adjuvant formulation activating Toll-like receptors (TLRs), cytokine therapies (e.g., IL-2, GM-CSF, IFN- α and IL-15), and mAb therapies activating or inhibiting specific immunomodulatory pathways in T cells are being evaluated for their therapeutic efficacy against cancer and viral infection. This has generated a need to not only evaluate the effects of different immunotherapy regimens on lymphocytes in treated individuals, but also to develop biomarkers that track cell-mediated immune responses that correlate to clinical responses that may also predict for whether an individual will respond or not to a given therapy. These newer immunotherapies are emerging to be quite ‘selective’, with only small subsets of people exhibiting clinical responses during therapy (e.g., IL-2 therapy for melanoma, renal cancer, and HIV; and anti-CTLA-4 for multiple solid tumor types) [53–57]. Phosphoflow technology may be tremendously helpful here by being able to measure the effects of vaccines and immunomodulators on different relevant signaling pathways in T cells and other leukocytes from patients *in vitro* and as a screening tool to determine who may be more sensitive to a certain immunotherapy or have immune cell complications during treatment. In addition, by being able to accurately measure the activation of different intracellular signaling molecules in multiple T cells and NK-cell subsets in response to cytokines and other immunomodulators, such as TLR agonists, phosphoflow can also be merged with genomics approaches to identify unique patient subpopulations that have more acute responses based on mutations or polymorphisms in key signaling molecules or cell surface receptors. In cancer, another class of drugs has entered on center stage as well; these are specific tyrosine kinase inhibitors (TKIs) that inhibit cancer growth [58,59], but also can have unforeseen side effects on the immune system by inhibiting the competence of T and B cells to respond to stimulation (so-called ‘immunocompetence’) [60,61]. Thus, in each case, sensitive assays that can rapidly and reproducibly measure the effects of immunomodulators and TKIs are needed.

The design of new vaccines and immunomodulators (e.g., TLR agonists) activating CD8⁺ CTLs against chronic virus infections is proving to be more difficult than expected and ways of tracking antigen-specific T cells and predicting who will respond adequately to these vaccines are needed. Although phosphoflow has not yet been systematically used to track antigen-specific T-cell responses during vaccination, we predict that as the technology grows it will have a significant impact in this area of immunomonitoring. Phosphoflow can be used to detect phosphorylated signaling molecules downstream of TCR–HLA/MHC–peptide interactions after vaccination and is now being combined with tetramer staining to track changes in antigen-specific T-cell populations [Nolan GP, Pers. Comm.]. It will be essential to determine whether clinically relevant routes of vaccination or vaccine boosters cause rapid systemic effects (such as cytokine secretion) that can be detected in the blood using phosphoflow. This can also be applied to determining immediate effects (in ‘real-time’) of immunomodulators, such as IL-2, in cancer patient lymphocytes *in vivo*. Although all these applications are still in their infancy, a number of studies have begun to examine patient lymphocyte responses using phosphoflow as correlative markers during patient treatment.

Recently, a critical new area has opened up that is applying phosphoflow in assessing similarities or differences in lymphocyte signaling pathways between diseased and aged versus healthy human populations. At present, the published work in this area has been carried out on cancer patients, although initial work looking at how aging in the human population affects phosphoflow profiles in lymphocytes in response to proinflammatory cytokines (e.g., IL-1, IL-6, TNF- α and interferons [IFNs]) has also begun [Davis M, Pers. Comm.]. We and others have begun to systematically analyze metastatic melanoma patients receiving cytokine therapies, such as IL-2 and IFN- α therapy for the sensitivity of their lymphocytes to activate STAT5 and STAT1 tyrosine phosphorylation [62–64]. These responses are also being compared with responses to IL-2 from normal donor PBMCs. A recent study using phosphoflow found that STAT1 tyrosine phosphorylation was defective in lymphocytes from a majority of melanoma patients versus normal donors after treatment with type I IFNs [64]. Patients who did respond needed much higher type I IFN concentrations to trigger STAT1 activation [64]. Interestingly, no defect in STAT1 activation in response to IFN- γ was found in lymphocytes or the CD4⁺ and CD8⁺ T-cell subsets [64]. Our group has been interested in the responses of lymphocytes to high-dose IL-2 therapy and has used phosphoflow to look at STAT5 and STAT1 activation in response to IL-2. We have found no obvious difference between melanoma patients and normal donors in the ability of high-dose IL-2 *in vitro* to trigger STAT5 phosphorylation in the major lymphocyte subsets, including CD4⁺, CD8⁺ and CD8⁺CD56⁺ T cells as well as the CD56^{high} and CD56^{low} NK-cell subsets [Wu S, Radvanyi LG, Jin L *et al.*, Unpublished Data]. By contrast, STAT1 phosphorylation was reduced [Wu S, Radvanyi LG, Jin L *et al.*, Unpublished Data]. Using this phosphoflow technique, we have also been able to measure the activation of STAT5 in ‘real-time’ in the whole blood of melanoma patients during the very first bolus infusion of IL-2 and have found a rapid and consistent activation all major non-B-lymphocyte subsets. Figure 1 is an example of a representative experiment from a metastatic melanoma patient receiving high-dose IL-2 therapy at MD Anderson Cancer Center (TX, USA) showing a rapid and extensive tyrosine phosphorylation of STAT5 during the first bolus IL-2 infusion detected immediately *ex vivo* compared with IL-2 activation of the same patients' cryopreserved PBMCs. Extensive activation of STAT5 is seen in the T-cell and NK subsets shown. Although some studies have found that melanoma patients can exhibit a defect in the ability of their T cells to activate STAT5 in response to IL-2 [63], after screening PBMCs from multiple metastatic melanoma patients receiving high-dose IL-2 therapy, we have not found any appreciable defects in the activation of STAT5 at high doses of IL-2 mimicking conditions *in vivo* during IL-2 infusion. We have also applied phosphoflow to study how the activation of STAT5 and STAT1 change over longer periods of times in response to IL-2. As shown in Figure 2, phosphoflow revealed that STAT5 gets tyrosine phosphorylated immediately at early time points and remains

phosphorylated for up to 24 h (1440 min) in T cells and NK cells. By contrast, STAT1 phosphorylation is delayed, mostly occurring in the T-cell subset with little activation of STAT1 in NK cells. This suggests that STAT1 activation is indirect and not directly activated through IL-2. Indeed, we have found that IL-2-induced IFN- γ production in response to IL-2 within 12 h drives this later activation of STAT1 [Wu S, Radvanyi LG, Jin L *et al.*, Unpublished Data]. These results point to the potential of phosphoflow as a screening tool to gauge the sensitivity of patients to cytokines and shows how the technique can reveal subtle indirect cytokine regulatory networks activating different signaling molecules through time. These studies have also revealed that pools of phosphorylated transcription factors, such as STAT5 in lymphocytes, can persist for prolonged periods of time *in vivo* well after the cytokine is washed out of the system and this prolonged STAT5 activation may correlate to clinical responses [62].

Expert commentary: current limitations of phosphoflow

Although phosphoflow has begun to emerge as a new and powerful tool to analyze the activity of intracellular signaling pathways by rapid, sensitive and high-throughput detection of intracellular protein phosphorylation, a number of technical limitations still exist that need to be addressed to improve the utility of this approach as an immunomonitoring and screening tool.

First, in many cases, the fluorescent intensities of the phosphorylated proteins of interest are still quite weak in most phosphoflow applications. This may limit the sensitivity of detecting and measuring more subtle changes in signaling pathways and signaling intensity in cells, especially when contemplating the tracking of antigen-specific T- and B-cell responses *ex vivo* during vaccination or immunotherapy where the frequencies and/or magnitude of the antigen-specific response may be very low, such as in responses against weak (self) antigens and subdominant epitopes. Better fixation and permeabilization methods/reagents coupled with brighter fluorochrome conjugates or staining methods will be needed in this respect. An added issue is that in some situations only small sample sizes are available for monitoring, especially from tissue sources, such as monitoring T-cell responses in tumor-infiltrating lymphocytes in cancer patients *ex vivo*. Newer flow cytometry technology compatible with smaller sample sizes with slower flow rates and increased sensitivity will be needed in order to allow adequate time to adjust the acquisition parameters before these limited samples run out.

Second, another limitation of phosphoflow is measuring responses in signaling pathways in lymphocyte subsets. This requires either cell-surface staining or intracellular staining for specific phenotypic markers to discern different lymphocytes and T cells (naïve, memory and Treg) together with phosphoflow using Abs able to stain cells that have undergone prior fixation under conditions needed to optimally preserve phosphorylated moieties. Although a number of mAb clones reactive against cell surface leukocyte CD and non-CD markers capable of staining cells following fixation are available for phosphoflow, more work is needed to identify more mAb clones or generate new clones that are compatible with these phosphoflow applications. The current list of cell surface markers compatible with phosphoflow applications is still short and newer Ab clones are needed in a greater variety and in more discrete leukocyte and lymphocyte subsets found in low frequencies, such as Tregs, NK cells, B cell and antigen-specific T-cell subsets delineated by specific cell surface activation and effector-memory markers. In the case of Tregs and Th17 cells, where key nuclear transcription factors (Foxp3 and ROR γ t, respectively) are needed to reliably detect these cells, new Abs that can be combined in these phosphoflow assays will be required. In the mouse system, Abs adequately delineating Tregs in phosphoflow applications have been identified and methods tracking STAT phosphorylation has been developed [65], but for human Treg cells, this is still a

problematic area requiring more optimized reagents. Although there are some recent reports of simultaneous staining of human Foxp3 together with phosphorylated proteins [45], in our experience these protocols result in greatly reduced signal strength of the phosphorylated target protein and are not easily reproducible. Development of new Abs and protocols in this regard would greatly boost our understanding of human Treg biology in pathological states involving self-antigen reactivity, such as cancer and autoimmune disease.

Third, there is still a problem with staining other intracellular markers simultaneously, such as cytokines together with phosphorylated proteins. For example, one key current limitation is linking phosphoflow profiling with monitoring cytokine production through intracellular staining. Here, the differential timing of phosphorylation events in response to activation signals versus transcriptional activation of cytokine genes and cytokine protein production is a key issue.

A fourth area in need of development is assay optimization and then eventually standardization. This is especially important in immunomonitoring and screening the effects of drugs and immunomodulators using phosphoflow. Currently, there is a momentum at both the national and international levels to optimize and standardize flow cytometry assays used for measuring cell-mediated immune responses utilizing tetramer and ICS as well as other assays technologies (e.g., ELISpot) [66]. As phosphoflow enters more and more into the realm of immunomonitoring, optimized and reproducible staining methods will need to be developed depending on the parameters measured and the unique needs of individual research centers. Eventually, however, there will have to be national or international effort to establish a set of standardized reagents and protocols to ensure accuracy and consistency across research and clinical trial sites. For some signaling molecules, such as the phosphorylated STATs and MAPKs, where optimized Abs and protocols already exist, it will be critical to make sure that different centers all follow these same optimized protocols if data are to be meaningfully compared across sites. This may also avert the appearance of contradictory results in the scientific literature. For example, currently, contradictory results have appeared on the ability of melanoma patients to activate STAT5 and STAT1 in response to IL-2 and IFNs [62–64]. This could be due to the different reagents (Abs, and fixation and permeabilization) and staining protocols used.

Finally, with the rapid accumulation of signaling data by phosphoflow together with the development of newer phosphoflow markers and the increased variety of fluorochrome conjugates for multiparameter flow cytometry, it will be an increasing challenge to interpret and organize the increasing amount and complexity of experimental data on multiple signaling pathways in a single experiment. As a result, newer bioinformatics tools analyzing signaling pathways and networks in cells specifically tailored to phosphoflow data will be needed [67].

Five-year view: the future of phosphoflow & critical areas in need of development

Over the last decade, phosphoflow has emerged as a powerful methodology to measure intracellular signaling networks and is showing great promise as a new tool in our immunomonitoring toolbox for tracking vaccine responses and the effects of immunotherapies in cancer patients, and patients with chronic viral infection and autoimmunity. Improvements in reagents and assay methodologies will make phosphoflow more widely used not only in preclinical research, but also in monitoring immune responses and immune status in response to vaccines and immunomodulators. One of the key areas in need of further research is to evaluate the potential of phosphoflow as a sensitive tool to monitor antigen-specific T-cell responses in vaccines and other immunotherapy development efforts for HIV and cancer. It will be important to also discern how intracellular signals downstream of the TCR and cytokine

receptors are affected by immunomodulators and immunosuppressive factors inhibiting T-cell responses in individuals with cancer, chronic viral infections and autoimmune disease. A number of Abs are currently available to detect the phosphorylation of LCK, LAT, ZAP70, TCR ζ and other intermediaries downstream of antigen-receptor triggering that are in need of further evaluation for their utility in tracking antigen-specific T-cell responses over a wide frequency range (1/10,000–1/10) in the blood after vaccination or treatment with cytokine therapies. Brighter fluorochromes or dual fluorochrome conjugates and improved flow cytometry analyzer sensitivity may be needed to accurately and reproducibly stain for the relatively small changes in phosphorylation status of different signaling molecules in low-frequency populations. A method utilizing multiple intracellular staining mAb conjugated to same fluorochrome may yield the signal strength needed for this application since TCR activation usually activates many signaling molecules downstream of the TCR and in the CD3 complex more or less simultaneously. This may be combined with tetramer staining and markers of T-cell differentiation (memory versus naive) for a powerful combination assay system. Another potential approach to increase the sensitivity and specificity of phosphoflow to monitor signaling changes in low-frequency lymphocyte subsets is using tyramide-based catalyzed reporter deposition signal amplification currently employed with *in situ* hybridization [68,69]. This technique uses a first-step horseradish peroxidase-conjugated Ab binding to the phosphorylated protein of interest followed by addition of tyramide, a horseradish peroxide substrate that is catalytically deposited on to the horseradish peroxide on the Ab. The tyramide can be directly fluorochrome-conjugated (e.g., Cy3.29–tyramide), or biotin-labeled. In the case of biotin–tyramide, addition of fluorochrome-labeled streptavidin is used as a final visualization step [68,69]. Catalyzed reporter deposition signal amplification has facilitated a 500–1000-fold increase in sensitivity of *in situ* hybridization signals and its application to flow cytometry and phosphoflow is a promising new approach to improve signal strength especially in time course experiments where signals wane over time [69].

Another new technology to watch out for in the next 5 years that can be applied to phosphoflow is a novel combination of flow cytometry with elemental mass spectrometry (CyTOF™ [DVSSciences, ON, Canada]) being developed and soon to be marketed by scientists at the University of Toronto, ON, Canada [70]. This new instrument uses a novel set of Abs labeled with multiple different metal isotopes that can be detected by time-of-flight mass spectrometry. Stained cells are run through a fluidics system (flow cytometer portion) into the mass spectrometer portion where each individual signal is detected. The system is much more sensitive and eliminates the traditional problems of autofluorescence and compensation plaguing traditional flow cytometry. In addition, many metal isotopes are currently available theoretically allowing for the staining of 50 or more parameters simultaneously in a single sample [70]. Currently, CyTOF is being developed as a tool to detect intracellular phosphorylated signaling molecules in lymphocytes [Nolan GP, Pers. Comm.] and it will be exciting to watch how this new area unfolds in the next few years.

Finally, as a new and powerful method to monitor lymphocyte responses to cytokines, small molecules, drugs and other environmental factors, phosphoflow can be applied to broaden the application of human immunology to public health. Over the last few decades, almost all of our emphasis has been on mouse models of the immune system and much less knowledge has been gained on the human immune system due to limited funding and resources. However, we have begun to reach the limit of what mouse models can do and suitable mouse models for the many of current human health problems do not exist. In addition, critical differences between the human and murine immune systems have come more to light recently [71]. All of these have now placed a renewed emphasis on human immunology and understanding how the immune system changes in individuals during aging and chronic disease such as obesity and diabetes. In this respect, together with other assays, phosphoflow can be applied in screening efforts to measure how key signaling molecules such as STATs are activated in response to

different cytokines and other immunomodulators as a measure of ‘immune health’ or ‘immunocompetence’ over a large population [71]. For example, comparing the signaling signatures of immune cells between young and elderly people may disclose the correlation between the immune system and aging and identify factors associated with a more stable functioning immune system (especially innate immunity) and better health in old age. These screening efforts may identify ‘at-risk’ populations. In pathological conditions, we also envision phosphoflow profile being applied as a biomarker to measure the sensitivity or potential efficacy of different drugs and immunotherapies in cancer in our drive towards more ‘personalized medicine’.

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Key issues

- Phosphoflow is a new tool in flow cytometry to monitor immune responses that can simultaneously detect surface markers and the activation of intracellular signals in different subpopulations of cells in a sensitive and high-throughput fashion.
- Phosphoflow is beginning to be applied to a wide variety of areas in immunology research and immunomonitoring in both physiological and pathological conditions for mapping interacting signaling networks within discrete cell populations and as a new tool to screen signaling pathway inhibitors, such as tyrosine kinase inhibitor drugs, in cancer cells and lymphocytes.
- Some key shortcomings in current phosphoflow technology still limit its more widespread application in more areas due to the lack of suitable antibodies able to specifically bind to cellular proteins after the harsher fixation and permeabilization conditions used in phosphoflow. More phosphoflow-compatible antibodies able to stain cell surface and intracellular proteins for subset analysis are especially needed.
- A thorough evaluation of the potential of phosphoflow to monitor antigen-specific T-cell responses occurring at low frequencies in the blood during vaccination and immunotherapy is an unmet need. More sensitive assay systems will be useful in this area.
- As phosphoflow becomes more widely used, especially in clinical studies and vaccine research in monitoring immune responses, there will be a need to develop new data-analysis tools and optimize staining methodologies and analysis methods across research sites to ensure consistency and reproducibility.
- Wider application of phosphoflow will require the merging of newer, more sophisticated, analysis technology with antibodies or probes able to more reliably quantify low-abundance and low-frequency phosphosignals in discrete

lymphocyte populations. A whole new technology merging flow cytometry with mass spectrometry (e.g., CyTOF™) currently under development may revolutionize the field in this respect.

- With an emerging emphasis on human immunology research, phosphoflow promises to be a powerful screening tool on a population-wide scale to assess ‘immunocompetence’ in the human immune system to identify ‘at-risk’ individuals for certain immune-related diseases or predict therapeutic outcome.

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Figure 1. Rapid and extensive tyrosine phosphorylation of STAT5 (Y694) in different lymphocyte subsets in peripheral blood mononuclear cells of melanoma patients during high-dose IL-2 treatment

High-dose IL-2 therapy is a powerful immunotherapy against metastatic melanoma. However, since only a fraction of treated patients (15–16%) exhibit clinical responses (tumor regression) during high-dose IL-2 therapy, it has become increasingly important to understand the mechanism of action of IL-2 and develop predictive assays that can select responding versus nonresponding patients. Monitoring the responses of lymphocyte subsets to IL-2 in patients by phosphoflow technology may be a useful tool in this regard. The question of ‘immunocompetence’ of melanoma patients has also arisen that whether these patients can adequately activate signaling pathways in response to IL-2 in T cells and NK cells. Here we show the typical response of the major non-B-cell lymphocyte subsets (CD4⁺, CD8⁺, CD8⁺CD56⁺, CD56^{high} and CD56^{low} NK cells) to IL-2 treatment in the peripheral blood of metastatic melanoma patients. The top panel shows an examination of STAT5 activation in whole blood in ‘real-time’ 10 min of the first IL-2 bolus infusion into a patient (IL-2 is given as a bolus infusion at 720,000 IU/kg over a 15-min period). Peak IL-2 serum concentrations achieve 6000 IU/ml or more during this time. The bottom panel shows the responses of these same lymphocyte subsets in cryopreserved peripheral blood mononuclear cells from the same patient to 6000 U/ml of IL-2 in a phosphoflow assay *in vitro*. The results show a consistent high degree of STAT5 activation. NK: Natural killer.

Figure 2. Time course of phosphorylated STAT1(Y701) and STAT5(Y694) in different lymphocyte subsets in peripheral blood mononuclear cells of normal donors after stimulation with high-dose IL-2

Cryopreserved normal donor peripheral blood mononuclear cells were used as an example to show time course of changes in (A) intracellular STAT1 and (B) STAT5 phosphorylation in response to high-dose IL-2 (6000 U/ml) in CD3⁺CD56⁺ natural killer cells, and CD4⁺ and CD8⁺ T cells over a 24-h period.

Table 1
Some recent studies using phosphoflow to track the activation of specific signaling molecules in different cell types

Cell types	Target molecules	Ref.
Jurkat cells, human CD4 ⁺ naive T cells	PLC- γ 1, LCK, AKT, ELK-1, SRC, p38, RAF-1, ERK1/2, PIP3, PIP2	[34]
U937	STAT1	[47]
Human IgM B cells, IgG B cells	SYK, ERK1/2, p38	[10]
U937, K562, HUT78	AKT, CrkL	[33]
Murine B cells, T cells, myeloid cells	STAT1, STAT3, STAT5, p38	[35]
Human CD4 ⁺ and CD8 ⁺ T cells	LAT, LCK, ZAP70	[36]
Human basophils	p38	[27,37]
Acute myeloid leukemia	p53	[39]
Acute myeloid leukemia	STAT1, STAT3, STAT5, STAT6, p38, ERK1/2	[40]
Murine hematopoietic stem/progenitor cells	STAT5, ERK, S6	[41]
Juvenile myelomonocytic leukemia	STAT5	[42]
Human monocytes, microglial cells	STAT3, STAT5	[46]
Human monocytes	STAT1, STAT3, STAT5, ERK1/2	[43]
Murine splenocytes and blood cells	ERK1/2, p38, MAPKAPK2, NF κ B, IKK α / β , STAT1, STAT3, STAT4, STAT5, STAT6	[44]
Human Treg cells	STAT5	[45]
U937, Jurkat cells, murine splenocytes	STAT1, STAT3, STAT5, STAT6, ERK1/2, p38	[48,49]
Murine splenocytes	STAT3, ERK 1/2	[50]
MYC-induced lymphoma in mouse	ERK1/2, VAV, p38, I κ B α , STAT5, NF κ B, AKT, RAF, IKK α , STAT1, STAT3, AKT, JNK, ELK, MEK2, STAT3, PLC γ , BAD	[52]
Whole blood of normal human, normal cynomolgus macaque and normal rhesus macaque	CCR5	[51]

AKT: Protein kinase B; BAD: Bcl-2-associated death promoter protein; CCR: C-C chemokine receptor; CrkL: Crk-like; ELK: Ets-like; ERK: Extracellular signal-regulated kinase; I κ B α : Nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor, α ; IKK: I κ B kinase; JNK: Jun N-terminal kinase; LAT: Linker for activation of T cells; LCK: Lymphocyte-specific protein tyrosine kinase; MAPKAPK2: MAP kinase-activated protein kinase 2; MEK: Microtubule-associated protein kinase kinase; MYC: c-Myc oncogene; PIP2: Phosphatidylinositol 4,5-bisphosphate; PIP3: Phosphatidylinositol 3,4,5-trisphosphate; PLC: Phospholipase C; RAF: RAF proto-oncogene serine/threonine kinase; SRC: Sarcoma proto-oncogene tyrosine protein kinase; STAT: Signal transducer and activator of transcription; SYK: Spleen tyrosine kinase; VAV: Vav proto-oncogene protein; ZAP: ζ -chain-associated protein kinase.