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## Characterization of the Murine Immunological Signaling Network with Phosphospecific Flow Cytometry

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# Characterization of the Murine Immunological Signaling Network with Phosphospecific Flow Cytometry<sup>1</sup>

Peter O. Krutzik,<sup>2</sup> Matthew B. Hale,<sup>2</sup> and Garry P. Nolan<sup>3</sup>

The immune system is a multitiered network that at the first level uses changes to intracellular signaling proteins to commit cells to determined fates. At the second tier, cells interact with one another via specifically expressed surface receptors and their cognate signaling molecules. At the third level, the local environments of immune cells change the outcomes of intracellular signaling pathways and thereby the role of cells during immune challenge. The interplay among these three tiers allows the distinct cell types of the immune system to respond cohesively to eliminate foreign Ags. In this study, using phosphospecific flow cytometry, we analyze elements of these network tiers by generating profiles of single-cell phosphoprotein responses in B cells, T cells, and myeloid cells to a number of mechanistically and clinically relevant cytokines (IFN- $\gamma$ , GM-CSF, IL-2, and IL-10) as well as LPS at key regulatory interfaces (Jak-Stat and MAPK pathways). The stimuli typically induced phosphorylation of specific signaling pathways and exerted their effects on distinct subsets of immune cells. However, upon comparison of stimulation *in vitro* and *in vivo*, we noted that signaling pathway specificity and cell type specificity were influenced strongly by the external environment. When taken from the *in vivo* environment, certain cell subsets became hypo- or hyper-responsive, showed profound differences in sensitivity to cytokine levels, or displayed altered phosphorylation kinetics. Thus, simultaneous analysis of the three tiers of the immune system network illustrates the principles by which immune regulation is context dependent and how *in vitro* culture systems compare with the *in vivo* environment. *The Journal of Immunology*, 2005, 175: 2366–2373.

The mammalian immune system forms a multitiered signaling network composed of tightly regulated intracellular signaling, specialized interacting cell subsets, and highly organized, remotely communicating, immune compartments. Illustrative of one tier of the network, activation of surface receptors on a given immune cell modulates the activities of intracellular proteins. Such modulation adjusts signaling pathway structure, often via induced phosphorylation of cognate target proteins. At the second tier of regulation, extracellular ligands act specifically on one or more cell types, but the functional response (secretion, differentiation, and proliferation) is a consequence of integrating all local cellular outcomes to allow for a coordinate, rapid, and highly effective response to pathogen challenges. Modulating the properties of both intracellular signaling pathways and cell type specificity is the third level of the network, the local tissue environment of the immune cells.

The study of cell subsets by phenotypic surface markers has provided significant and potent understanding of immune processes. Despite this, a fuller appreciation of intracellular signaling would be required to supplement prior biochemical studies that could not access subset-specific differences at the single-cell level, because these assays are typically conducted by classical lysis techniques of large numbers of cells. Therefore, it has been diffi-

cult to analyze the first two tiers of the immune network simultaneously. Critically, because both levels of the network are designed to respond to foreign challenge, one can gain maximal understanding of the system by eliciting and measuring responses to external stimuli.

Phosphospecific flow cytometry is uniquely suited to interrogate the immunological signaling network at both the cell subset and intracellular signaling levels, because cell types can be discriminated by surface markers and simultaneously analyzed for phosphorylation states of signaling proteins (1–11). This allows signaling to be followed in multiple populations of immune cell lineages at the single-cell level, a critical element for building an accurate model of networked, multilevel signaling.

Among the many signaling pathways crucial to immune function, the Jak-Stat pathway plays a particularly important role in cytokine signaling (12–14). As an example of Jak-Stat signaling, binding of IFN- $\gamma$  to its receptor induces receptor dimerization, activation of Jak1 and Jak2, and subsequent phosphorylation of Stat1 on a conserved tyrosine residue. When phosphorylated, Stat1 proteins dimerize and translocate into the nucleus to activate transcription of cognate genes such as IFN regulatory factor-1.

A survey of the network signaling properties of the Jak-Stat pathway highlights how cytokines from multiple families (IFN- $\gamma$ , GM-CSF, IL-2, and IL-10) can lead to the phosphorylation of Stat1, Stat3, and Stat5 at the dimerization-inducing tyrosine residues. IFN- $\gamma$  is a member of the type II IFN family that is critical to innate and acquired host responses to viral infection and for Th1 cell development. The IFN- $\gamma$ R is found in nearly all immune cell types and signals through Jak1, Jak2, and Stat1. Because of its important role in Th bias and viral clearance, a more comprehensive understanding of the activation profiles in subsets of immune cells in response to IFN- $\gamma$  is desirable.

Another important cytokine, GM-CSF, is a member of the IL-3R family and induces myeloid differentiation and activation, acting through Stat5. IL-2 is a member of the common  $\gamma$ -chain family, all of which share the common  $\gamma$  signaling receptor chain.

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The IL-2R signals through Stat5 and is primarily restricted to NK, activated T and B cells, and regulatory T cells. Finally, the IL-10R is similar to the IFN- $\gamma$ R in sequence, but IL-10 modulates the immune responses of monocytes and macrophages to inhibit Th1 and is thought to enhance Th2 development. IL-10 signals via Jak1/Tyk2 and Stat3. Again, the complexities of how these factors act across multiple cell types to drive immune system function has been difficult to comprehend in multiple populations simultaneously.

The MAPK pathway also plays an important role in immune cell signaling by mediating TCR and BCR activation as well as responses to many inflammatory cytokines, such as IL-1 and TNF- $\alpha$  (15). Previous studies have shown the utility of phosphospecific flow cytometry in analyzing MAPK activation in T cell signaling (10, 11, 16). Other mediators of MAPK activity include TLR ligands, one of which is the bacterial cell wall component, LPS. TLRs are essential to innate immune responses and are able to recognize conserved motifs in bacterial and pathogenic organisms, such as lipopolysaccharides, lipoproteins, CpG DNA, and flagellin (17–19). Upon binding to LPS, TLR4 has been reported to induce both p38 and JNK as well as NF- $\kappa$ B activity. TLR4 appears to be restricted to APCs, including monocytes, macrophages, and dendritic cells (20). However, measurement of phosphorylation in response to LPS has typically been restricted to cell lines or has been assumed to be due to surface expression of TLR4.

Signaling pathway- and cell type-specific attributes of stimuli are largely cell-intrinsic properties, but can be modulated by the external environment of the cells. By delivering cytokines *in vivo* or adding them to *ex vivo* cultures of splenic cells, we explored the contributions of extrinsic factors such as stromal cells, tissue organization, and serum/medium components in regulating pathway and cell type specificity of stimuli. We therefore profiled multiple tiers of the immune cell signaling network by measuring the phosphorylation of four signaling proteins (Stat1, Stat3, Stat5, and p38) in four cell types (B cells, T cells, CD11b<sup>high</sup>, and CD11b<sup>int</sup>) in response to four stimuli (IFN- $\gamma$ , GM-CSF, IL-10, and LPS) across time and concentration gradients. Cell-specific expression of cytokine receptors correlated with responsiveness to their cognate cytokines, suggesting that phosphospecific flow cytometry can complement analysis of cytokine receptor expression by measuring actual functional activity in addition to surface expression. Taken together, the results underscore a tightly regulated network with notable and unexpected cases of cell type- and signaling pathway-specific responses that are modulated by the external environment. These results provide a comprehensive and physiologically relevant framework for comparison with signaling networks that have become altered in pathological disease states (21).

## Materials and Methods

### *Abs and reagents*

All murine and phosphospecific Abs were supplied by BD Pharmingen. The following surface Abs were used: CD11b-FITC (M1/70), B220-Cy5.5PerCP or -PerCP (RA3-6B2), TCR $\beta$ -FITC or -PE (H57-597), CD4-Cy5.5PerCP (RM4-5), and CD25-PE (PC61). The following phosphospecific Abs were used: pStat1 (Y701, clone 14 or 4a), pStat3 (Y705, clone 4), pStat5 (Y694, clone 47), and p-p38 (T180/Y182, clone 30 or 36). All phosphospecific Abs were supplied conjugated to Alexa Fluor 647 (Ax647;<sup>4</sup> Molecular Probes).

Recombinant murine cytokines included IFN- $\gamma$  (10<sup>7</sup> U/mg), GM-CSF (5  $\times$  10<sup>6</sup> U/mg), and IL-10 (5  $\times$  10<sup>5</sup> U/mg) from PeproTech and IL-2 (10<sup>8</sup> U/mg) from BD Pharmingen. LPS (*Escherichia coli* 0127:B8) was purchased from Sigma-Aldrich. Formaldehyde ampules (16% in water) were obtained from Electron Microscopy Sciences and used up to 1 wk after opening.

### *In vitro stimulation*

For all experiments, male BALB/c mice from the Stanford University animal core facility in-house colony were used at 6–9 wk of age. All animals were treated in accordance with university and Administrative Panels on Laboratory Animal Care guidelines. Spleens were excised after killing mice by brief carbon dioxide asphyxiation and cervical dislocation. The spleens were homogenized in PBS and 5 mM EDTA, filtered through 70- $\mu$ m pore size mesh, pelleted, and resuspended at 5  $\times$  10<sup>6</sup> cells/ml in room temperature to 37°C RPMI 1640 containing 10% FBS, penicillin (100 U/ml), streptomycin (100  $\mu$ g/ml), and L-glutamine. Cells were maintained at 37°C in 5% CO<sub>2</sub> in conical tubes (to prevent monocyte/macrophage adherence) for 1–2 h before stimulation. Stimuli were typically added for 15 min at the indicated concentrations. Formaldehyde was added directly to the culture medium to a final concentration of 1.6%, and cells were fixed for 15 min at room temperature. Methanol was then added directly to a final concentration of 80%, and samples were stored at 4°C for 30 min to 1 h before being transferred to –80°C for storage. Samples were typically analyzed within 24 h, but could be stored for 1–2 wk (2).

### *In vivo stimulation*

Stimuli were delivered to mice by tail vein injection in a volume of 200  $\mu$ l. Typically, mice were killed after 15 min by a brief (30-s) CO<sub>2</sub> administration and cervical dislocation. Spleens were removed and homogenized directly in 10 ml of PBS containing 1.6% formaldehyde. After 15 min, the cell suspension was passed through 70- $\mu$ m pore size mesh, and methanol was added directly to a final concentration of 80%. Methanol was added directly to accommodate *in vivo* experiments where samples were offset by 5–10 min and thus required a method that avoided pelleting cells after fixation. Samples were cooled as in the *in vitro* experiments.

### *Surface and intracellular staining*

Cell suspensions in methanol were washed twice with staining medium (PBS containing 0.5% BSA and 0.02% sodium azide), then resuspended in staining medium at 10<sup>7</sup> cells/ml for intracellular phosphoprotein staining as previously described (2, 22). Samples were then divided to allow for analysis of multiple phosphoproteins (because one phosphospecific Ab was added per stain). The same surface mixture (typically CD11b-FITC, TCR $\beta$ -PE, and B220-Cy5.5PerCP) and different phosphospecific Abs were added simultaneously for 20–30 min, then washed with 15–20 vol of staining medium. Cells were resuspended in staining medium and kept at 4°C before analysis on a FACSCalibur instrument (BD Biosciences) equipped with a 633 helium-neon laser to allow for Ax647 excitation and detection. Data were analyzed in FlowJo (TreeStar). Typically 250,000–300,000 events were gathered per experiment to give >1,000 cells for all populations analyzed. The median fluorescence intensity (MFI) was used to determine fold change upon stimulation (fold change = MFI<sub>stimulated</sub>/MFI<sub>unstimulated</sub>).

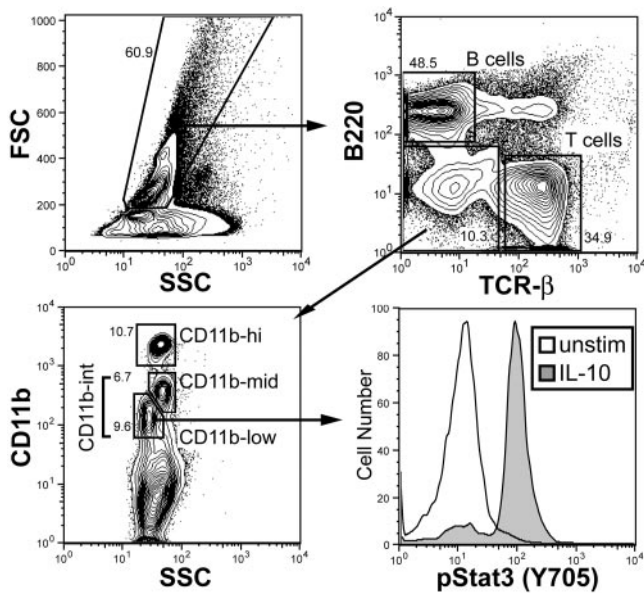
## Results

### *Surface characterization*

To analyze signaling events across multiple cell types, we first defined cell subsets according to surface marker expression. After fixation with formaldehyde and permeabilization with methanol, splenocytes were simultaneously stained with CD11b-FITC, TCR $\beta$ -PE, B220-Cy5.5PerCP, and one of several phosphospecific Abs conjugated to Ax647 (Fig. 1). These surface Abs gave accurate and expected subpopulations after methanol permeabilization of cells (we have observed varying efficiency of staining for certain murine surface markers after treatment of cells with methanol (22)). Forward and side scatter properties were used to eliminate RBC and debris. B220 and TCR $\beta$  staining levels were then used to define B cells (B220<sup>+</sup>TCR $\beta$ <sup>–</sup>), T cells (TCR $\beta$ <sup>+</sup>B220<sup>–</sup>) and a double-negative population (B220<sup>–</sup>TCR $\beta$ <sup>–</sup>). These double-negative cells were gated according to CD11b and side scatter to define three additional populations, CD11b<sup>high</sup>, CD11b<sup>mid</sup>, and CD11b<sup>low</sup>. For general profiling, CD11b<sup>mid</sup> and CD11b<sup>low</sup> cells were combined to form a CD11b<sup>int</sup> population because the CD11b<sup>mid</sup> and CD11b<sup>low</sup> populations did not always resolve sufficiently to discriminate them clearly after fixation and permeabilization. The CD11b<sup>high</sup> population, which is also Gr-1 positive, includes monocytes and neutrophils, whereas the CD11b<sup>mid</sup> and

<sup>4</sup> Abbreviations used in this paper: Ax647, Alexa Fluor 647; MFI, median fluorescence intensity.





**FIGURE 1.** Surface gating used to define immune cell subsets for phosphoprotein analysis. Murine splenocytes were fixed with formaldehyde and permeabilized in methanol, then stained with CD11b-FITC, TCR $\beta$ -PE, B220-Cy5.5PerCP, and a phosphospecific Ab conjugated to Ax647. Scatter gating was used to eliminate cell debris and RBC, whereas B220 vs TCR $\beta$  gating defined B cells (B220<sup>+</sup>TCR $\beta$ <sup>-</sup>), T cells (TCR $\beta$ <sup>+</sup>B220<sup>-</sup>), and a double-negative population (B220<sup>-</sup>TCR $\beta$ <sup>-</sup>). The double-negative population was then gated according to CD11b expression, with three populations being identified: CD11b<sup>high</sup>, CD11b<sup>mid</sup>, and CD11b<sup>low</sup>. Note that the CD11b<sup>mid</sup> and CD11b<sup>low</sup> populations did not always resolve and were therefore combined into a CD11b<sup>int</sup> population for phosphoprotein analysis in some cases. Phosphorylation events induced by cytokines and LPS were measured by staining cells with Abs against pStat1, pStat3, pStat5, and p-p38 conjugated to Ax647. Phosphorylation was measured in each cell population by analyzing the intensity of Ax647 fluorescence, with increased fluorescence correlating with an increase in specific protein phosphorylation. Shown is a sample in which splenocytes were treated with IL-10, and pStat3 levels were measured. Analysis of the CD11b<sup>low</sup> population shows a large increase in Ax647 fluorescence, and therefore Stat3 phosphorylation, upon IL-10 treatment.

CD11b<sup>low</sup> populations contain monocytes, macrophages, neutrophils, and CD11c<sup>+</sup> dendritic cells (23).

Protein phosphorylation was assessed by examining the fluorescence intensity of bound phosphospecific Abs in each of the aforementioned cell subsets. An increase in fluorescence correlates with an increase in protein phosphorylation (2). As an example, in Fig. 1 splenocytes were stimulated *in vitro* with IL-10, then stained with the above surface markers and a phosphospecific Ab against Stat3. Cell types were gated, and phosphorylation was measured by comparing unstimulated cells to those treated with IL-10. As shown, CD11b<sup>low</sup> cells had a large increase in Stat3 phosphorylation in response to IL-10. Similar analyses were used to generate a more comprehensive profiling (see below) of immune system responses to stimulation both *in vitro* and *in vivo*.

#### Global comparison of signaling *in vitro* and *in vivo*

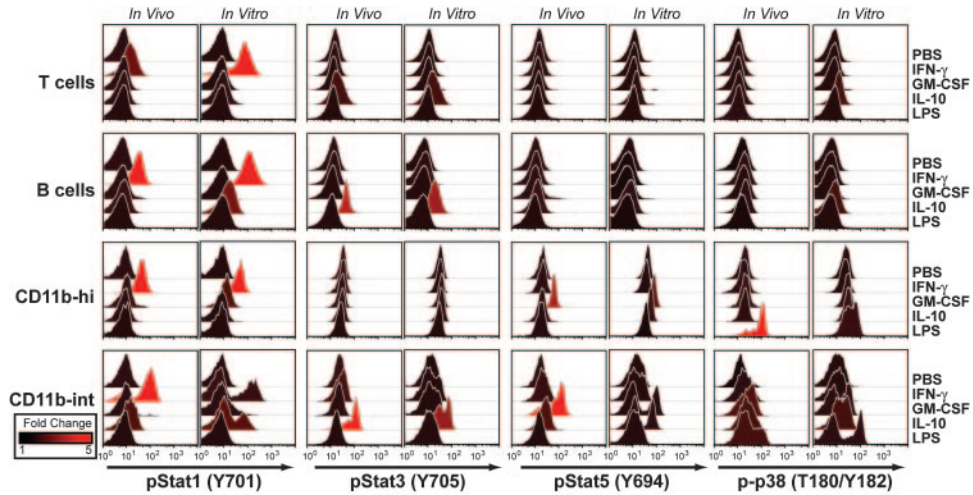
To probe the immune cell signaling network, we applied a panel of stimuli that would generate a profile of signaling responses in the cell types defined above. For *in vitro* experiments, we added IFN- $\gamma$ , GM-CSF, IL-10, or LPS to cultures of splenocytes for 15 min before fixation and permeabilization. For *in vivo* analysis, the stimuli were administered *i.v.* 15 min before preparation of the splenic cells (see *Materials and Methods*). The spleen was homog-

enized in formaldehyde, then cells were permeabilized. After fixation and permeabilization, both *in vitro* and *in vivo* samples were stained with the surface marker Ab mixture of CD11b-FITC, TCR $\beta$ -PE, and B220-Cy5.5PerCP in addition to pStat1, pStat3, pStat5, or p-p38 Abs conjugated to Ax647 (Fig. 2). Phosphorylation of these four signaling proteins was analyzed in B cells, T cells, CD11b<sup>high</sup>, and CD11b<sup>int</sup> cells in response to the four stimuli, generating a 64-point profile for comparison of *in vitro* and *in vivo* stimulation. Shown in Fig. 2 are the histograms of phosphoprotein-specific Ab fluorescence in each of these cell types.

Upon consideration, the profiles could be characterized as demonstrating cell type- or signaling pathway-specific outcomes upon stimulation. Signaling pathway-specific effects were defined as those in which the stimulus induces phosphorylation of a single analyzed phosphoprotein across multiple cell types. For instance, IFN- $\gamma$  induced phosphorylation of Stat1 in all cell types tested, and GM-CSF induced Stat5 phosphorylation in myeloid cell subsets. However, in most situations we observed that only certain subsets of cells were responsive to a given cytokine. We defined such cell type-specific effects as those in which only particular cell subsets responded to a given stimulus. For example, B cells and CD11b<sup>int</sup> cells responded strongly to IL-10 with increased Stat3 phosphorylation, whereas T cells had a minimal Stat3 phosphorylation at the dose used and CD11b<sup>high</sup> cells showed no detectable response. This indicated that IL-10 modulates B cells and CD11b<sup>int</sup> cells much differently than T cells and CD11b<sup>high</sup> cells. In another example, only the myeloid cell populations (CD11b<sup>high</sup> and CD11b<sup>int</sup>) displayed Stat5 phosphorylation in response to GM-CSF. These same CD11b<sup>+</sup> populations were the only subsets that responded to LPS with increased p38 phosphorylation. Thus, GM-CSF and LPS specifically and differentially drove activation of the myeloid arm of the immune network.

It was important to compare corresponding *in vitro* and *in vivo* profiles, because it has long been considered that there might be important differences between the two. Critically, we noted several cases of hyporesponsiveness and hyper-responsiveness after cells were placed *in vitro* for stimulation. Hyper-responsive signaling states *in vitro* were observed for two stimuli, IFN- $\gamma$  and IL-10. To our surprise, T cells consistently responded poorly to IFN- $\gamma$  stimulation *in vivo* (~2-fold increase in Stat1 phosphorylation), whereas they responded the most strongly of all cell types tested when placed *in vitro* (8- to 12-fold increase in Stat1 phosphorylation). B cells were also somewhat hyper-responsive *in vitro*, but their response *in vivo* was more robust than that of T cells. IFN- $\gamma$  itself was active *in vivo*, because CD11b<sup>high</sup> and CD11b<sup>int</sup> cells showed comparable pStat1 inductions both *in vivo* and *in vitro*. The result is not limited to splenic T cell populations, because T cells from peripheral blood are also hyporesponsive *in vivo*, indicating that the hyporesponsiveness is not entirely explained by access issues of T cells in splenic microenvironments (data not shown).

In the case of IL-10 signaling, hyper-responsive signaling *in vitro* was characterized by the induction of Stat5 phosphorylation in myeloid cells in addition to the Stat3 phosphorylation seen *in vivo*. *In vivo*, IL-10-induced Stat5 phosphorylation in CD11b<sup>int</sup> cells was 4-fold less than that induced by GM-CSF, but *in vitro*, the two stimuli were comparable. This augmented response to IL-10 was also seen in CD11b<sup>high</sup> cells; *in vivo*, the response was negligible, but *in vitro*, Stat5 phosphorylation was about half that of GM-CSF. In striking contrast, the CD11b<sup>high</sup> population had a diminished p38 response to LPS stimulation *in vitro*. These cells responded strongly *in vivo*, but their response *in vitro* was subdued and inconsistent (this prompted us to perform kinetic experiments with LPS; see Fig. 4). Thus, it is clear that signaling pathways



**FIGURE 2.** Global profile of immune cell signaling networks in vitro and in vivo. For in vivo experiments, mice were injected i.v. (via the tail vein) with IFN- $\gamma$  (800 ng), GM-CSF (200 ng), IL-10 (1  $\mu$ g), or LPS (5  $\mu$ g) in a volume of 200  $\mu$ l. Mice were killed 15 min after injection, except for LPS treatment, where mice were killed after 5 min. Spleens were removed, homogenized, and fixed immediately. For in vitro experiments, splenocytes ( $5 \times 10^6$ /ml) were treated with IFN- $\gamma$  (50 ng/ml), GM-CSF (10 ng/ml), IL-10 (100 ng/ml), or LPS (1  $\mu$ g/ml) for 15 min before fixation and permeabilization. Cells were simultaneously stained with CD11b-FITC, TCR $\beta$ -PE, B220-Cy5.5PerCP, and pStat1 (Y701), pStat3 (Y705), pStat5 (Y694), or p-p38 (T180/Y182) phosphospecific Abs conjugated to Ax647. Displayed are histograms of phospho-Ab fluorescence for each of the cell types defined in Fig. 1: B cells, T cells, CD11b<sup>high</sup>, and CD11b<sup>int</sup> (combining CD11b<sup>mid</sup> and CD11b<sup>low</sup> cells). Histograms are colored according to the change in median fluorescence intensity relative to control, unstimulated cells (black indicates a fold change of 1 (e.g., no increase in phosphorylation), and red indicates a fold change of 5). The experiments shown are representative of at least three experiments in vivo and in vitro for each stimulus.

respond differently in vitro and in vivo, underscoring a need for determining the physiological relevance of what is determined in vitro. In this study we provide a method for understanding the relationship between in vitro and in vivo experiments and show that they complement one another in understanding signaling regulation.

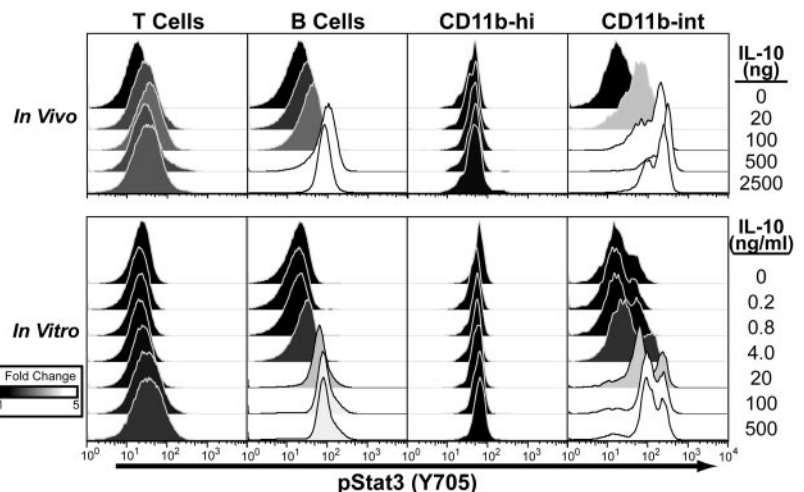
Interestingly, the responses to stimuli delivered in vivo were characteristically unimodal, that is, all cells in a given population responded equally well to a given stimulus (indicated by a single uniform peak). However, in vitro, the responses to some stimuli were strongly bimodal, suggesting that ex vivo, important, environmentally determined signaling elements are missing for the subsets of cells that appear to be nonresponsive or that negative regulators are present in the culture medium. For example, the CD11b<sup>int</sup> response to IFN- $\gamma$ , GM-CSF, and LPS was essentially split between cells that responded strongly and those that showed little or no response (Fig. 2). More detailed analysis and additional experiments have shown that the cells responding in vitro were

slightly higher in CD11b expression (CD11b<sup>mid</sup>), whereas those that did not respond tended to be lower in CD11b (CD11b<sup>low</sup>; data not shown). Both CD11b<sup>mid</sup> and CD11b<sup>low</sup> cells remained viable in vitro, because both populations responded uniformly to IL-10. The uniformity of responses in vivo is surprising, considering the complexity of tissue distribution of cell types and accessibility to injected cytokines.

*Dose responses in vitro and in vivo*

In vivo, cell responses to cytokines are determined not just by the presence or the absence of cytokines, but by graded responses to their concentrations. We determined whether the effective dose of cytokines added to in vitro cultures was similar to the dose needed to elicit responses in vivo. We performed a dose-response experiment with IL-10 (Fig. 3). IL-10 was chosen because we had observed it induced a robust and consistent phosphorylation of Stat3 and had been observed in the profile to be cell type specific, with only B cells and CD11b<sup>int</sup> cells responding. The dose-response

**FIGURE 3.** The immune network senses increasing levels of IL-10 with cell type-specific responses. In vivo, mice were injected i.v. with increasing doses of IL-10 from 20 to 2500 ng for 15 min before being killed. In vitro, cultures of splenocytes ( $5 \times 10^6$  cells/ml) were treated with 0.2–500 ng/ml IL-10 for 15 min before fixation and permeabilization. Cells were stained with CD11b-FITC, TCR $\beta$ -PE, B220-Cy5.5PerCP, and pStat3(Y705)-Ax647 phosphospecific Ab. Histograms are shaded according to the fold change in median fluorescence relative to PBS controls (black indicates no change; white indicates a 5-fold increase in fluorescence). The experiments shown are representative of two experiments in vivo and three in vitro.



experiment would therefore confirm cell type specificity and simultaneously compare the sensitivities of cells *in vitro* and *in vivo*. The level of Stat3 phosphorylation titrated with the dose of IL-10 both *in vitro* and *in vivo*. *In vivo*, B cells responded maximally at a dose of 500 ng, whereas the CD11b<sup>int</sup> population appeared near maximal at a 5-fold lower dose of 100 ng. T cells showed a small shift that saturated at 20–100 ng. *In vitro*, both B cells and CD11b<sup>int</sup> cells showed maximal stimulation between 20 and 100 ng/ml. The small T cell response *in vitro* required a higher dose of 100–500 ng/ml.

The results indicate that B cells and CD11b<sup>int</sup> cells respond to a similar dose of IL-10 *in vitro*, but that the CD11b<sup>int</sup> population is more sensitive than B cells *in vivo*. To compare the effective doses *in vitro* and *in vivo*, one can approximate the blood volume of a male 8-wk-old BALB/c mouse as 2 ml, such that the 100–500 ng used *in vivo* correlates to 50–250 ng/ml. This dose is remarkably similar to *in vitro* experiments considering the much larger number of cells that are stimulated *in vivo* as well as cytokine access to splenic compartments.

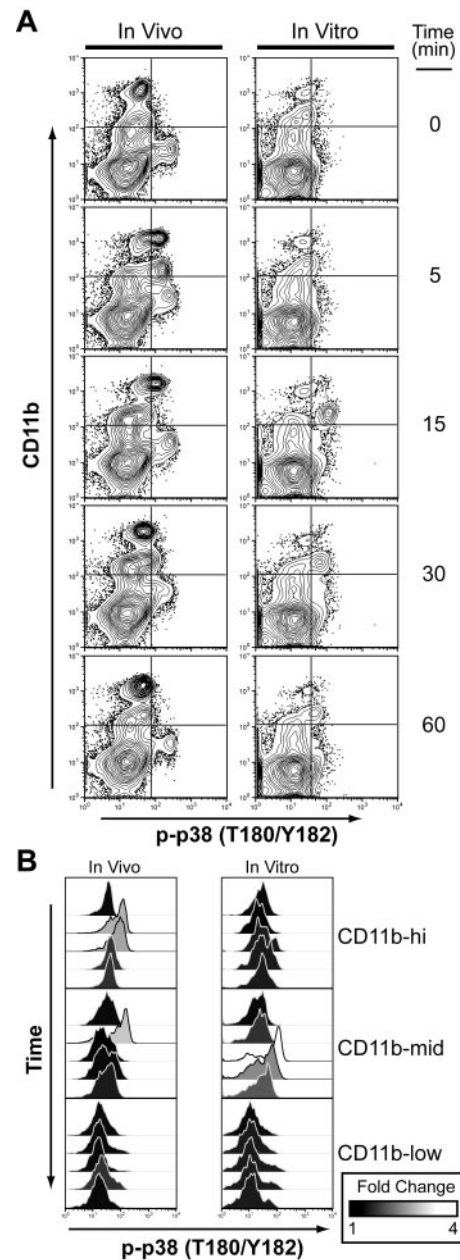
#### Kinetic response profile

The dose-response experiment examined how the immune signaling network senses the external environment by correlating levels of Stat3 phosphorylation to the concentration of IL-10. Similarly, the immune network must control its responses in a kinetic fashion, for instance, to maintain resistance to pathogens as long as the pathogen is present. Therefore, to complement the dose-response experiment with IL-10, we followed the kinetics of p38 phosphorylation in response to LPS (Fig. 4). Initial experiments indicated that this phosphorylation event may be very rapid and transient *in vivo*, but slower and more prolonged *in vitro*. Also, the response of CD11b<sup>high</sup> cells appeared inconsistent during *in vitro* stimulations, showing some responsiveness, but not as great as that seen *in vivo* (typically 2- to 3-fold less).

We therefore performed a time course from 5 to 60 min of LPS stimulation. We plotted our data in two dimensions, showing CD11b expression vs phospho-p38 (Fig. 4A). This was done to visualize which subpopulations of CD11b<sup>+</sup> cells were responding. As shown in Fig. 1, CD11b<sup>+</sup> cells can be separated into three populations, CD11b<sup>high</sup>, CD11b<sup>mid</sup>, and CD11b<sup>low</sup>. Histograms of phospho-p38 activation of these three subpopulations are detailed (Fig. 4B). *In vivo*, only the CD11b<sup>high</sup> and CD11b<sup>mid</sup> populations responded to LPS. The response was extremely rapid, peaking at 5 min and disappearing by 15–30 min. Interestingly, *in vivo* the CD11b<sup>mid</sup> population returned to baseline levels of p38 phosphorylation more rapidly than the CD11b<sup>high</sup> population. *In vitro*, the response of the CD11b<sup>high</sup> cells was heterogeneous and not as robust. These effects did not appear to depend on the dose of LPS used (1 μg/ml), because using 10-fold less LPS showed a similar response kinetic (data not shown). The CD11b<sup>mid</sup> population, in contrast, showed a very strong and reproducible response to LPS from 15 to 30 min *in vitro*, with levels still above those in unstimulated samples at 60 min. These experiments show that kinetic regulation of signaling differs substantially *in vitro* and *in vivo* and once again indicates the presence of other factors that modulate the responsiveness of immune cells to stimulation *in vivo* that are critically missing during *in vitro* analysis.

#### Correlation of cytokine receptor expression with responsiveness

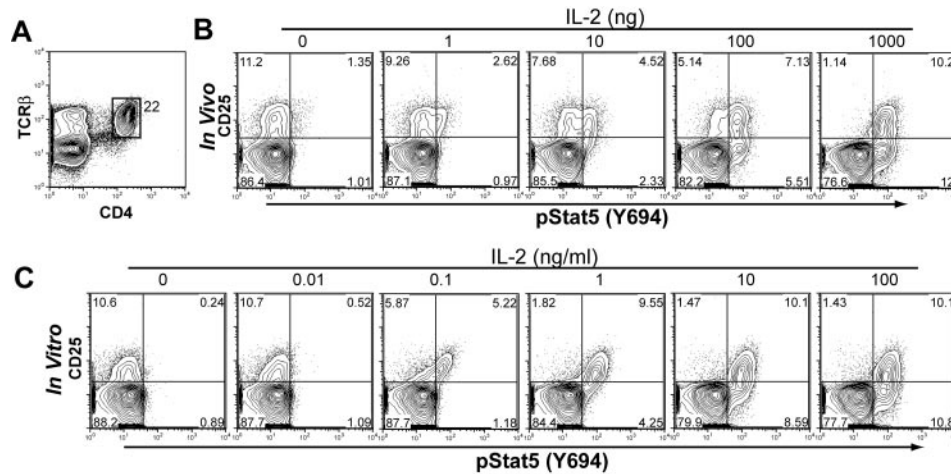
A final mechanism by which the immune network regulates signaling is via cell type-specific expression of cytokine receptors. In this manner, the immune system can restrict the involvement of particular cell subsets to specific immune challenges. To examine whether we could discern subpopulations of responsive cells



**FIGURE 4.** Kinetics of LPS signaling are modulated by extrinsic environmental factors both *in vitro* and *in vivo*. *In vivo*, mice were injected i.v. with 5 μg of LPS for 5, 15, 30, or 60 min before being killed. *In vitro*, splenocytes ( $5 \times 10^6$  cells/ml) were treated with 1 μg/ml LPS for the same time periods before fixation. Permeabilized cells were stained with surface Abs and phospho-p38 (T180/Y182) Ab. **A**, Two-dimensional analysis showing CD11b expression vs p-p38 levels. Note the presence of the two CD11b-int populations and their different response patterns to LPS both *in vitro* and *in vivo*. **B**, Histogram analysis showing p-p38 fluorescence for CD11b<sup>high</sup>, CD11b<sup>mid</sup>, and CD11b<sup>low</sup> populations. Time is shown increasing from *top* to *bottom* of each set of overlaid histograms. Histograms are shaded according to the fold change in MFI relative to unstimulated cells (black indicates no change; white indicates a 4-fold increase in fluorescence). B cells and T cells did not show a measurable increase in p38 phosphorylation in response to LPS. Data shown are representative of three experiments *in vivo* and four *in vitro*.

within an otherwise uniform population, we chose to characterize CD4<sup>+</sup>CD25<sup>+</sup> T cell responsiveness to IL-2. These cells have recently gained attention due to a correlation of high CD25 expression with regulatory/suppressor function (24–26). CD25 is the





**FIGURE 5.** Surface expression of CD25 (IL-2R  $\alpha$ -chain) correlates with responsiveness to IL-2. In vivo, mice were treated i.v. with 1–1000 ng of IL-2 for 15 min before death. In vitro splenocyte cultures ( $5 \times 10^6$  cells/ml) were stimulated with 0.01–100 ng/ml IL-2 for 15 min before fixation. Cells were fixed and permeabilized, then stained with TCR $\beta$ -FITC, CD25-PE, CD4-Cy5.5PerCP, and pStat5(Y694)-Ax647. *A*, Lymphocytes were gated according to TCR- $\beta$  and CD4 expression. CD4+TCR $\beta$ + T cells were then analyzed for CD25 expression and Stat5 phosphorylation in response to IL-2. *B*, In vivo dose response to IL-2. *C*, In vitro dose response to IL-2. Data are representative of two experiments in vivo and three in vitro.

$\alpha$ -chain of the IL-2R and forms the high affinity version of the receptor along with the  $\beta$ - and  $\gamma$ -chains. The  $\beta$ - and  $\gamma$ -chains alone form an IL-2R with intermediate affinity,  $\sim$ 100-fold less than the high affinity form. CD4<sup>+</sup>CD25<sup>+</sup> T cells typically comprise  $\sim$ 1% of the total splenocytes, but appear to be critical in regulating autoimmunity and transplant rejection/tolerance (26).

We analyzed cells with a combination of TCR $\beta$ -FITC, CD25-PE, CD4-Cy5.5PerCP, and pStat5-Ax647 (Fig. 5). CD4<sup>+</sup>TCR $\beta$ <sup>+</sup> cells were analyzed for their CD25 expression and Stat5 phosphorylation simultaneously (Fig. 5A). In vitro testing showed a strong correlation of CD25 levels to IL-2 responsiveness (Fig. 5C). At low doses (0.1 ng/ml), only the most CD25-positive cells showed a large increase in Stat5 phosphorylation ( $\sim$ 6-fold). However, at higher doses (10 ng/ml), intermediate and lower CD25 populations also showed increases in phospho-Stat5. Note that cells that appeared to be CD25 negative responded to high concentrations of IL-2. In vivo, CD25<sup>high</sup> cells also responded to lower doses of IL-2 (10 ng) than CD25<sup>low</sup> and CD25<sup>neg</sup> cells (Fig. 5B).

Notably, however, in vivo, the response was not homogeneous; only about half the CD25<sup>+</sup> cells responded to the lower dose. At higher doses in vivo, all the CD25<sup>+</sup> cells responded along with many CD25<sup>-</sup> cells. It is interesting that in vivo, the CD25<sup>-</sup> cells only required 10-fold more cytokine than the CD25<sup>+</sup> population to induce Stat5 phosphorylation, whereas they required  $\sim$ 100-fold more in vitro. The total cell number of CD25<sup>+</sup> cells did not change significantly between in vivo and in vitro analyses (data not shown). Thus, it is clear that cell surface expression of a cytokine receptor was not sufficient in making a cell highly responsive to low systemic cytokine levels in vivo, but may be sufficient in vitro.

## Discussion

By using signaling pathway- and cell type-specific responses in a combinatorial manner, the immune system signaling network is able to generate a highly diverse set of functional responses despite a limited number of cytokines, cell types, and signaling proteins. In addition to receptors that are coupled to intricate intracellular pathways, specialized cell types express their own set of receptors, and the activation of the same receptor on two different cell types can result in disparate functional responses. This allows sophisticated network behavior despite a limited repertoire of conserved signaling proteins (primarily of the Jak-Stat pathway). Modulating both

these levels of the network is the organization of the immune system into distinct compartments, further refining the immune response and ensuring that it is appropriate for the tissue in which the cells resides.

The analysis of immune cell signaling at the network level can be enhanced by approaches that allow cell activities to be discerned individually within heterogeneous populations. In this study we applied phosphospecific flow cytometry both in vitro and in vivo to interrogate the three tiers of the immune network: intracellular signaling (signaling pathway specificity), cell type specialization, and the effects of the external environment. The resulting signaling map serves as a guide for future investigations of the immune network and addresses a key concern underlying many experiments that support our understanding of modern immunobiology: that is, how well do in vitro cultures replicate the in vivo environment?

A profile of signaling responses to IFN- $\gamma$ , GM-CSF, IL-10, and LPS demonstrated clear cases of the first two tiers of the network: signaling pathway and cell type specificity. Signaling pathway specificity was highlighted by the induction of Stat1 phosphorylation by IFN- $\gamma$ , Stat5 phosphorylation by GM-CSF, and p38 phosphorylation by LPS. Cell type-specific responses were seen in myeloid cells (CD11b<sup>high</sup> and CD11b<sup>int</sup>) in their response to LPS. In this case, it was expected that the presence of TLRs on myeloid cells and their absence on B and T cells would dictate responsiveness (17). In another example, B cells and CD11b<sup>int</sup> cells responded to IL-10, whereas T cells and CD11b<sup>high</sup> cells remained in their basal states. However, the magnitude of the response in CD11b<sup>int</sup> cells was greater than that in B cells, indicating differences in protein level or the presence of negative regulators within the cells. Because myeloid cells did not respond uniformly to IL-10, it is possible that the CD11b<sup>high</sup> and CD11b<sup>int</sup> populations play different roles during immune challenges that involve IL-10 secretion. It is important to note that this is the first time such differences have been observed in primary cells. Indeed, one would expect that with more stimulation conditions, more similarities and differences will be found and will better define the role of each particular cell type in the greater immune network.

This broad profiling also showed the influence of a third tier of the immune network: the influence of the external environment/tissue compartment on internal signaling events. In vitro, extrinsic

factors include tissue culture medium additives (such as amino acids, sugar, salts, antibiotics, and glutamine) and serum composition. In vivo, extrinsic factors are probably more complex and include the location of cells within the spleen, accessibility to cytokines, proteins present in the blood, cell-cell interactions, and local concentration of signaling molecules. Systemic delivery of cytokines for in vivo experiments may not replicate perfectly the signaling responses to endogenous sources of these stimuli, because cell-cell interactions and targeted secretion can result in very high local concentrations of some cytokines. However, systemic delivery in parallel with in vitro stimulation sheds light on how signaling responses to these stimuli are regulated by extrinsic factors.

In vivo, T cells were largely unresponsive to IFN- $\gamma$ , showing a modest induction of Stat1 phosphorylation, whereas they were among the most responsive cells in vitro. The length of culture of cells ex vivo affected T cell responsiveness, with maximal responses seen after 2 h in culture (data not shown). We are investigating whether intracellular, soluble, or cell-associated factors act to negatively regulate IFN- $\gamma$  responsiveness in T cells in vivo, or whether extrinsic factors in tissue culture medium up-regulate their responsiveness (manuscript in preparation). Because IFN- $\gamma$  plays such a large role in the Th bias of T cells, this discrepancy between in vitro and in vivo stimulation is of great importance. It is interesting to note that a previous study has also shown differences between T cell activation events in vitro and in vivo using the phosphospecific flow cytometry approach (11). It was shown that although c-Jun and p38 phosphorylations were dependent on CD28 cross-linking in vitro, they did not require the CD28 molecule in vivo.

The response of CD11b<sup>int</sup> cells to stimulation differed significantly in vitro and in vivo. In vivo, these cells responded uniformly to IFN- $\gamma$  and GM-CSF with Stat1 and Stat5 phosphorylation, respectively. However, in vitro, the responses were bimodal, with approximately half the cells responding, and the other half remaining in the basal state. In addition, in vitro about half the population responded to IL-10 by phosphorylating Stat5 to levels similar to those after GM-CSF stimulation. Thus, CD11b<sup>int</sup> cells were affected in opposing directions by extrinsic factors. With IFN- $\gamma$  and GM-CSF stimulation, they are relatively hyporesponsive in vitro. However, when stimulated with IL-10, they induce Stat5 phosphorylation in vitro, but not in vivo. These differences highlight the ability of the immune network to regulate signaling in a nonintuitive manner depending on the extracellular milieu. Questions arise as to how these small differences might affect the signaling network when performing long term experiments in culture, and what factors are gained or lost to generate these hyper- and hyporesponsive states. Because in vitro culture conditions can be controlled relatively well, it will be interesting to determine whether the addition of soluble factors or endothelial cell types can more closely simulate the in vivo environment and help delineate the factors critical to its appropriate regulation. The availability of a known in vivo response makes such analysis possible.

For any signaling network to be effective, it must be able to monitor and respond to different levels of stimuli. In a dose-response experiment with IL-10, we found striking similarity between doses required in vitro and in vivo for maximal induction of Stat3 phosphorylation in B cells and CD11b<sup>int</sup> cells (Fig. 3). Dose-response experiments with IFN- $\gamma$  did not show such a strong correlation, because much higher concentrations (~100-fold) were required to elicit responses in vivo than in vitro (our manuscript in preparation). Indeed, we expected in vivo experiments to require more cytokine because of reduced cellular access to cytokines due to adsorption by other cell types and the effects of tissue distribu-

tion. Although IFN- $\gamma$ Rs are present on nearly all cells (including endothelial cells of blood vessels), the expression of the IL-10R is more restricted. Thus, more IL-10 may reach the spleen because it is not adsorbed on its passage through the cardiovascular system.

The effects of extrinsic environmental cues were also observed when CD11b<sup>int</sup> cells responded to a lower dose of IL-10 than B cells in vivo, but to a similar dose in vitro. The CD11b<sup>int</sup> population may have better access to the cytokine in vivo or may lose responsiveness as it is placed in vitro by down-regulating the IL-10R or other key intracellular components of the Stat3 signaling cascade. With these experiments, we have shown that the immune network is able to monitor cytokine levels and that certain cell types may be more sensitive than others, thereby generating immune responses that may involve different cell types depending on the severity of the immune challenge.

Another level of regulation in the immune system is the kinetics of responses to immune modulators. The rapidity of response to a stimulus as well as the duration of that response play critical roles in determining the magnitude of downstream signaling and transcription that occur. Kinetic analysis of LPS signaling revealed interesting differences between in vitro and in vivo conditions (Fig. 4). In vivo, CD11b<sup>high</sup> cells responded rapidly and sustained signaling for 15 min, whereas CD11b<sup>mid</sup> cells showed a rapid and transient phosphorylation of p38 at 5 min. In vitro, the CD11b<sup>high</sup> population was essentially nonresponsive, whereas the CD11b<sup>mid</sup> population showed a slower and more prolonged response from 15 to 30 min. These differences in kinetics are another example of regulation by extrinsic factors. CD11b<sup>high</sup> cells become unresponsive in vitro, possibly by modulation of their TLR levels or by differentiation in response to culture conditions. CD11b<sup>mid</sup> cells show a more prolonged response in vitro and therefore may have modulated the negative feedback loops that terminate signaling rapidly in vivo. Thus, it appears that although CD11b<sup>high</sup> cells respond strongly to LPS in vivo in the spleen, they may not mediate culture responses when placed in vitro. Rather, it may be the CD11b<sup>mid</sup> population that performs this role.

Because cell surface expression of cytokine receptors is a critical intrinsic difference between cell types that regulates their responses to cytokines, we examined the correlation between CD25 (IL-2R $\alpha$ ) expression and IL-2 sensitivity (Fig. 5). In vitro, high levels of the receptor correlated almost linearly with IL-2 induction of Stat5 phosphorylation. In vivo, interestingly, the response of CD25<sup>+</sup> cells was bimodal, with approximately half the cells requiring 100-fold more cytokine to induce a response. It is possible that some of these cells do not have access to IL-2 because of their location within the spleen, or that another cofactor or coreceptor inhibits their response to low doses. In vitro, these cofactors might be lost, or critical cell-cell interactions may be eliminated. We have observed this bimodal response in T cells in response to other stimuli and are investigating its significance (manuscript in preparation). The utility of in vivo experiments is quite evident in this case, because the heterogeneity in response may help delineate regulatory T cells from other cells expressing CD25. Perhaps only regulatory T cells have all the necessary intracellular components to be responsive to low doses of IL-2, and the expression of CD25 alone is not sufficient for responsiveness in vivo. Thus, as a system by which additional hypotheses of immune function are generated, the profiling and data presented show unprecedented opportunity for utility.

Immune cell signaling behaves as a tightly regulated, multitiered network. Individual cell types act as nodes within the signaling network and integrate signals from cytokines, environmental cues, cell-cell interactions, and other soluble factors to give appropriate functional responses on cell and system levels. Immune cells are



highly specialized, and the manner in which a cell processes incoming information and responds to it differs greatly by cell type. In this study we examined regulation of the immune network and intracellular, cell type, and organism levels. We present new evidence that crucial signaling responses of great immunological significance differ in vitro and in vivo, illustrating the caveats of making conclusions from in vitro experiments. By examining the Jak-Stat and MAPK pathways in the various cell types of the murine immune system both in vitro and in vivo, this study helps to define the multitiered nature of the immune network in its basal, preimmune state. Although not exhaustive, the profile gained provides a better understanding of the immune system and provides a means to detect alteration of signaling in pathological disease states, such as cancer and autoimmunity. Such analyses will accelerate development of therapeutic interventions for these diseases and provide a basis for further mapping of the landscape of signaling geography across the complexity of immune system processes (21).

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## Disclosures

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