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*J Immunol* 2005; 175:2366-2373; doi: 10.4049/jimmunol.175.4.2366

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Characterization of the Murine Immunological Signaling Network with Phosphospecific Flow Cytometry

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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-γ, 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 difficult 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-γ 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-γ, GM-CSF, IL-2, and IL-10) can lead to the phosphorylation of Stat1, Stat3, and Stat5 at the dimerization-inducing tyrosine residues. IFN-γ 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-γR 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-γ 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 γ-chain family, all of which share the common γ signaling receptor chain.
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-γR 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-α (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-κB 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 i.v. 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\textsuperscript{mid}, and CD11b\textsuperscript{low}) in response to four stimuli (IFN-γ, 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-FITC or -PE (H57-597), CD4-FITC or -PE (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; Molecular Probes). Recombinant murine cytokines included IFN-γ (10\textsuperscript{7} U/mg), GM-CSF (5 × 10\textsuperscript{7} U/mg), and IL-10 (5 × 10\textsuperscript{7} U/mg) from PeproTech and IL-2 (10\textsuperscript{8} U/mg) from BD Pharmingen. LPS (Escherichia coli 027: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.

\*Abbreviations used in this paper: Ax647, Alexa Fluor 647; MFI, median fluorescence intensity.

**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-μm pore size mesh, pelleted, and resuspended at 5 × 10\textsuperscript{6} cells/ml in room temperature to 37°C RPMI 1640 containing 10% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), and l-glutamine. Cells were maintained at 37°C in 5% CO\textsubscript{2} in conical tubes (to prevent monocyte/macrophase 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 μl. Typically, mice were killed after 15 min by a brief (30-s) CO\textsubscript{2} 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-μm 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\textsuperscript{7} 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, TCRβ-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 250,000–500,000 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\textsubscript{stimulated}/MFI\textsubscript{unstimulated}).

**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, TCRβ-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 TCRβ staining levels were then used to define B cells (B220\textsuperscript{+} TCRβ\textsuperscript{−}), T cells (TCRβ\textsuperscript{+} B220\textsuperscript{−}) and a double-negative population (B220\textsuperscript{+} TCRβ\textsuperscript{−}). These double-negative cells were gated according to CD11b and side scatter to define three additional populations, CD11b\textsuperscript{high}, CD11b\textsuperscript{mid}, and CD11b\textsuperscript{low}. For general profiling, CD11b\textsuperscript{mid} and CD11b\textsuperscript{low} cells were combined to form a CD11b\textsuperscript{+} population because the CD11b\textsuperscript{+} and CD11b\textsuperscript{high} populations did not always resolve sufficiently to discriminate them clearly after fixation and permeabilization. The CD11b\textsuperscript{high} population, which is also Gr-1 positive, includes monocytes and neutrophils, whereas the CD11b\textsuperscript{mid} and
being identified: CD11bhigh, CD11bmid, and CD11blow. Note that the
tion was then gated according to CD11b expression, with three populations
CD11bmid and CD11blow populations did not always resolve and were
double-negative population (B220−TCRβ−).

Protein phosphorylation was assessed by examining the fluores-
cence intensity of bound phosphospecific Abs in each of the afo-
mentioned 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, CD11blow cells had a large increase in Stat3 phosphoryla-
tion 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-γ, 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 fix-
ation and permeabilization, both in vitro and in vivo samples were
stained with the surface marker Ab mixture of CD11b-FITC,
TCRβ−PE, and B220-Cy5.5PerCP in addition to pStat1, pStat3,
pStat5, or p-p38 Abs conjugated to Alexa647 (Fig. 2). Phosphoryla-
tion of these four signaling proteins was analyzed in B cells, T
cells, CD11bhigh, and CD11blow cells in response to the four stim-
uli, generating a 64-point profile for comparison of in vitro and in
vivo stimulation. Shown in Fig. 2 are the histograms of phospho-
protein-specific Ab fluorescence in each of these cell types.

Upon consideration, the profiles could be characterized as dem-
onstrating 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-γ-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 CD11bhigh
cells responded strongly to IL-10 with increased Stat3 phosphory-
lation, whereas T cells had a minimal Stat3 phosphorylation at the
dose used and CD11bhigh cells showed no detectable response.
This indicated that IL-10 modulates B cells and CD11bhigh cells
much differently than T cells and CD11bhigh cells. In another ex-
ample, only the myeloid cell populations (CD11bhigh and
CD11blow) displayed Stat5 phosphorylation in response to GM-
CSF. These same CD11bhigh 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-γ and IL-10. To
our surprise, T cells consistently responded poorly to IFN-γ stimu-
lization 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 phosphory-
lation). B cells were also somewhat hyper-responsive in vitro, but
their response in vivo was more robust than that of T cells. IFN-γ
itself was active in vivo, because CD11bhigh and CD11blow 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, indi-
cating 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 CD11bhigh
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 CD11bhigh cells; in vivo, the response was
negligible, but in vitro, Stat5 phosphorylation was about half that
of GM-CSF. In striking contrast, the CD11bhigh 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 1. Surface gating used to define immune cell subsets for phos-
phoprotein analysis. Murine splenocytes were fixed in formaldehyde and
permeabilized in methanol, then stained with CD11b-FITC, TCRβ−PE,
B220-Cy5.5PerCP, and a phosphospecific Ab conjugated to Alexa647. Scatter
gating was used to eliminate cell debris and RBC, whereas B220 vs TCRβ
gating defined B cells (B220−TCRβ−), T cells (TCRβ+ B220+), and a
double-negative population (B220/TCRβ−). The double-negative population
was then gated according to CD11b expression, with three populations
being identified: CD11bhigh, CD11bmid, and CD11blow. Note that the
CD11bmid and CD11blow populations did not always resolve and were
therefore combined into a CD11bint 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 Alexa647. Phosphorylation was measured in each cell
population by analyzing the intensity of Alexa647 fluorescence, with in-
creased 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 CD11bint
population shows a large increase in Alexa647 fluorescence, and therefore Stat3
phosphorylation, upon IL-10 treatment.
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
CD11bint response to IFN-\(\gamma\)/H9253, 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\(^{\text{int}}\), whereas those that
did not respond tended to be lower in CD11b (CD11b\(^{\text{int}}\); data
not shown). Both CD11b\(^{\text{int}}\) and CD11b\(^{\text{low}}\) 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 exper-
iment with IL-10 (Fig. 3). IL-10 was chosen because we had ob-
served 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\(^{\text{int}}\) cells responding. The dose-response

![FIGURE 2. Global profile of immune cell signaling networks in vitro and in vivo.](http://www.jimmunol.org/)

![FIGURE 3. The immune network senses increasing levels of IL-10 with cell type-specific responses.](http://www.jimmunol.org/)
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^int cells appeared inconsistent during in vitro stimulations, while in vivo, the CD11b^int population returned to baseline levels of p38 phosphorylation more rapidly than the CD11b^high population. In vitro, the response of the CD11b^high 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^mid 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 within an otherwise uniform population, we chose to characterize CD4^+CD25^+ 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...
α-chain of the IL-2R and forms the high affinity version of the receptor along with the β- and γ-chains. The β- and γ-chains alone form an IL-2R with intermediate affinity, ~100-fold less than the high affinity form. CD4+CD25 T cells typically comprise ~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 TCRβ-FITC, CD25-PE, CD4-Cy5.5PerCP, and pStat5-Ax647 (Fig. 5). CD4+ TCRβ+ 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 (~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, CD25high cells also responded to lower doses of IL-2 (10 ng) than CD25low and CD25neg cells (Fig. 5B).

Notably, however, in vivo, the response was not homogeneous; only about half the CD25+ cells responded to the lower dose. At higher doses in vivo, all the CD25+ cells responded along with many CD25- cells. It is interesting that in vivo, the CD25+ cells only required 10-fold more cytokine than the CD25- population to induce Stat5 phosphorylation, whereas they required ~100-fold more in vitro. The total cell number of CD25+ 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 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 phosophspecific 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-γ, 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-γ, Stat5 phosphorylation by GM-CSF, and p38 phosphorylation by LPS. Cell type-specific responses were seen in myeloid cells (CD11bhigh and CD11bint) 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 CD11bint cells responded to IL-10, whereas T cells and CD11bhigh cells remained in their basal states. However, the magnitude of the response in CD11bint 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 CD11bhigh and CD11bint 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-γ, 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-γ responsiveness in T cells in vivo, or whether extrinsic factors in tissue culture medium up-regulate their responsiveness (manuscript in preparation). Because IFN-γ plays such a large role in the Th1 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 CD11bint cells to stimulation differed significantly in vitro and in vivo. In vivo, these cells responded uniformly to IFN-γ and GM-CSF with Stat1 and Stat3 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, CD11bint cells were affected in opposing directions by extrinsic factors. With IFN-γ 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 CD11bint cells (Fig. 3). Dose-response experiments with IFN-γ 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 distribution. Although IFN-γRs 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 CD11bhigh cells responded to a lower dose of IL-10 than B cells in vivo, but to a similar dose in vitro. The CD11bhigh 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, CD11bhigh cells responded rapidly and sustained signaling for 15 min, whereas CD11bmint cells showed a rapid and transient phosphorylation of p38 at 5 min. In vitro, the CD11bhigh population was essentially nonresponsive, whereas the CD11bmint 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. CD11bhigh cells become unresponsive in vivo, possibly by modulation of their TLR levels or by differentiation in response to culture conditions. CD11bmint 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 CD11bhigh 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 CD11bmint 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-2Rα) 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+ 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).

Acknowledgments
We thank Roland Wolkowicz, Jeff Fortin, and Tom Wehrman for critical reading of the manuscript and helpful discussions.

Disclosures
P. O. Krutzik has received consultancy payments from BD Pharmingen, and G. P. Nolan has patents pending on phosphospecific flow cytometry techniques.

References