Published in final edited form as:

Curr Opin Immunol. 2011 October ; 23(5): 632–638. doi:10.1016/j.coi.2011.07.013.

Interpreting mixed signals: the cell's cytokine conundrum

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Abstract

Cytokines are essential for the activation, differentiation and control of the immune system. Many cytokines, alone or in combination with other cytokines, have multiple functions and can often act on many different cell types with distinct developmental or functional consequences. Despite the myriad of cytokines and cytokine receptors, there are relatively few signaling molecules that transduce these diverse cytokine signals. In this review, we will discuss the potential mechanisms used by cytokines to mediate distinct cellular outcomes from a small number of signaling molecules. Understanding this paradigm in cytokine signaling can aid in the development of potential therapeutic approaches involving cytokine targeting or use.

An inescapable problem in cytokine signaling

Cytokines provide communication between cells of the immune system to effect productive immunity. Through cytokine stimulation, cells can become activated, differentiate, alter their trafficking, and become suppressed. In essence, most of the physiologic outcomes observed in the immune system are mediated by cytokines.

The signaling cascade most associated with cytokine stimulation is the Janus kinase (Just Another Kinase; JAK) – Signal Transducers and Activator of Transcription (STAT) pathway. Most cytokine receptor chains associate with one or two of the four JAK family kinases (JAK1, JAK2, JAK3, TYK2) [1]. Cytokine binding to their respective cytokine receptor chains induces dimerization, and subsequent tyrosine phosphorylation and activation of the tethered JAK kinase(s) [2]. This subsequently recruits members of the STAT family of transcription factors (STAT1, 2, 3, 4, 5a, 5b, 6). Unphosphorylated STAT monomers, which are excluded from the nucleus and localize to the periphery of the cell, become phosphorylated by activated JAKs, inducing dimerization and translocation to the nucleus where they mediate transcription of their target genes [2].

The immune system utilizes at least fifty cytokines to deliver signals in the immune microenvironment. However, cells only possess four JAK proteins and seven STAT proteins with which to deliver these signals [3]. Downstream of the same STAT protein, extremely diverse physiologic events can occur. How can diverse inputs lead to diverse consequences if they are routed through a highly restricted group of signal transducers?

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Several broad mechanisms could be used to diversify downstream signaling via a limited number of signaling molecules and fine-tune the cytokine response:

Receptor modulation

Cells may regulate cytokine signaling at the level of the receptor, effectively modulating their sensitivity to a particular STAT activator.

Signaling modulation

Cells may utilize alternative mechanisms of STAT signaling, essentially qualitatively changing the STAT depending on the cytokine.

Epigenetic modulation

Cells may regulate signaling at the level of the target genes by regulating the accessibility of target genes through epigenetic modifications, as STAT proteins can only activate a particular genetic program when gene loci are available.

This review will examine these hypotheses set against the recent observations, specifically focusing on STAT3 signaling. STAT3 epitomizes the cell's cytokine dilemma: a multitude of cytokines, all causing diverse cellular events, do so through the activation of STAT3.

Shifting the focus to the receptor

The notion that cytokines bind receptors and receptors generally activate STATs is rather simplistic. Cytokine signaling is an orchestrated series of events that can be regulated via multiple mechanisms. In order for a cytokine to mediate its effect on a target cell, it must first possess a viable receptor. Some receptors are constitutively expressed, while others require additional signals in order to be upregulated. Furthermore, many cytokine receptors are formed from multiple chains that have distinct modes of regulation. Cells can temporally modulate the expression of certain receptors thereby altering the cytokines to which a particular cell is responsive. For example, the IL12R β 1 chain is present on resting T cells and lacks intrinsic signaling capacity [4]. T cell activation in the presence of IL-2 or IL-27 upregulates IL-12R β 2, forming a functional receptor for IL-12 and initiating Th1 differentiation [4,5]. T cell activation in which IL-6 predominates, however, upregulates the IL-23R α chain, forming a receptor for IL-23, which stabilizes Th17 differentiation [6]. Thus prior exposure to a different cytokine gives rise to a distinct cytokine receptor partner for IL12R β 1 and thus activation of a distinct STAT pathway. There may also be temporal differences which may alter downstream consequences. The availability of receptors for a cytokine provide another level of regulation through which cytokines can mediate distinct effects. Furthermore, competition between different cytokines for the same receptor chains, presumably due to affinity differences, may also serve to shape cytokine responses. Lastly, some cytokines, or their components, appear to act as negative regulators by competing for cytokine binding. For instance IL27p28 (IL-30), in the absence of Ebi3, can block IL-6 signaling by out competing its binding to gp130 [7].

STAT modifications: it's not just tyrosine phosphorylation

While receptor- and JAK-mediated tyrosine phosphorylation is an essential part of STAT activation, alternative inputs can alter the level of activation of a STAT protein (Figure 1). This could effectively allow the same STAT to be qualitatively different depending on the cytokine receptor being triggered. For instance, this could distinguish IL-2-induced STAT5 from IL-7-induced STAT5. All of the STAT proteins except STAT6 carry a conserved serine phosphorylation site, distal to the tyrosine site, which is required for optimal

transcriptional activity [8]. Mutation of these serine residues results in decreased binding of STATs to target genes. For instance, serine phosphorylation of STAT4 is important for IFN- γ production but dispensable for cell proliferation, suggesting serine phosphorylation may help modulate target gene transcription [9].

Serine phosphorylation of STATs generally occurs independently of JAK activation and is caused by a secondary signaling cascade emanating from distinct signaling pathways, thus integrating signals from multiple inputs. Furthermore, different serine/threonine kinases appear to phosphorylate different STATs, adding another potential layer of control. Serine phosphorylation of STAT1 and STAT4 occurs via p38, a member of the serine/threonine-specific mitogen-activated protein kinase (MAPK) family [10,11]. In contrast, the Akt-mammalian target of rapamycin (mTOR) pathway appears to play a pivotal role in STAT3 serine phosphorylation [12]. Tyrosine and serine phosphorylation represent a possible integration point for multiple signaling pathways; one cytokine may activate both tyrosine and serine phosphorylation, while another may only induce tyrosine phosphorylation, leading to distinct transcriptional outcomes.

Although STAT6 lacks the canonical STAT family serine phosphorylation motif, it does possess multiple, alternate serine phosphorylation sites that are thought to negatively regulate its transcription activity [13]. Recently, c-Jun N-terminal kinase (JNK), another member of the MAPK family, was shown to phosphorylate a single inhibitory serine site in STAT6 following IL-1 β but not IL-4 stimulation, which was suggested to control the balance between IL-1 β and IL-4 stimulations [14].

Phosphorylation is not the only mechanism that can regulate STAT activity. Several studies have implicated acetylation in the regulation of STAT responses. Using mutant STAT proteins deficient in a lysine acetylation site, STATs were shown to be deficient in their ability in dimerizing and binding to target genes. However, the role of STAT acylation remains controversial. While some have suggested that acetylation acts as a switch for the dephosphorylation of STAT1 [15], others found no role for acetylation in the regulation of STAT phosphorylation [16]. Future studies are needed to elucidate the role of acetylation in STAT signaling.

Sumoylation is another mode of STAT modification which has been shown to inhibit its activity. SUMO is conjugated to proteins by members of the Protein Inhibitor of Activated Stat (PIAS) E3 ligase family in manner analogous to ubiquitylation [17]. Sumoylation of STAT1 by PIAS ligases limit some aspects of its transcriptional activity, but spare other STAT-mediated transcriptional programs [18]. Furthermore, recent studies have implicated the serine phosphorylation of STAT proteins in their sumoylation [11].

Unfortunately, STAT-mediated signaling events have not yet been rigorously interrogated in diverse settings. Tyrosine phosphorylation is an essential step for cytokine-mediated transcription, but STATs may be further fine-tuned by numerous alternate, post-translational modifications that have yet to be fully assessed. Furthermore, some studies have suggested that STATs may have non-transcriptional activity, such as regulating the cell cycle progression [19]. There may also be additional signaling pathways induced at the receptor that could further modulate the downstream consequences of cytokine signaling. It will be important for future studies to consider these alternative STAT modifications in mediating diverse cytokine-driven programs.

Genomic topography: examining the epigenetic landscape of STAT targets

Considerable plasticity is exhibited by the epigenetic landscape of the genome. Consequently, the developmental and functional program driven by a particular cytokine-

STAT combination may simply be limited by the loci they can access at a given time. Thus, the effects observed may be secondary to the epigenetic landscape at the time of cytokine signaling, which is likely to vary between different cell types and/or developmental stages. This could, in part, explain how many cytokines can have distinct effects on different cell types.

Recent studies have shed light on how these events might shape the functional consequences of cytokine signaling by using chromatin immunoprecipitation (ChIP) combined with massive parallel sequencing (ChIP-Seq). Differential histone modifications are a primary mechanism of epigenetic gene regulation, with histone H3 trimethyl Lys4 (H3K4me3) functioning as an activating histone mark while histone H3 trimethyl Lys27 (H3K27me3) acts as a repressive histone modification. Global mapping of H3K4me3 and H3K27me3 by ChIP-Seq has been used to assess the epigenetic landscape following cytokine-driven T cell differentiation [20]. These studies have highlighted specific loci responsible for T helper cell differentiation, implicating a role for target gene accessibility in cytokine signaling. ChIP-Seq has also been used to obtain a genomic map of STAT binding under different T cell differentiating conditions [21–23]. A common theme emerging from these experiments was that while some of the expected STAT targets were highly enriched (for instance, STAT4 at the *Ifng* locus), there were many STAT targets that were previously unknown, increasing speculation regarding their contribution to T helper differentiation. Furthermore, STAT binding to the so-called “master regulators” of T helper differentiation (*Tbx21* [T-bet; drives Th1 development], *Gata3* [Th2], *Rorc* [ROR γ t; Th17]) was not as clear as previously thought. Additional studies which combine multiple approaches could reveal how key features of the epigenetic landscape correlate with STAT binding following cytokine stimulation.

The mixed signals of STAT3

STAT3 can be activated by many cytokines. This is in part because STAT3 can be activated by frequently utilized receptor chains. For instance in T cells, gp130 (IL-6 signal transducer; *Il6st*), which is used by IL-6, IL-11, IL-27, Leukemia Inhibitory Factor (LIF), Oncostatin M (OSM), Ciliary Neurotrophic Factor (CNTF), Cardiotrophin 1 (CT-1) and Cardiotrophin-like Cytokine (CLC), is an essential cytokine receptor that activates STAT3 predominantly but also activate STAT1 [24]. STAT3 is also activated by non-gp130 receptors such as the G-CSFR [24]. Some cytokines that utilize the common γ chain (interleukin 2 receptor, gamma chain; *Il2rg*) can also activate STAT3, including IL-21 and IL-2 [24,25,26]. The IL-23R, which is composed of the IL-23R α chain and IL-12R β 1, also activates STAT3 and STAT4 [6]. Finally, IL-10, an immunoregulatory cytokine that potently suppresses T cells, also activates STAT3 via its IL10R1/IL10R2 receptor chains. Thus, many cytokines that modulate T cell function do so through STAT3 (Figure 2).

As IL-6, IL-21, and IL-23 contribute to T helper 17 (Th17) differentiation, it is not surprising that STAT3 is essential for Th17 cell development, including in humans with Job's Syndrome, which involves mutations in *STAT3* [27]. Indeed, T cell-specific deletion of STAT3 prevents Th17 development [21,28]. However, the story has recently become more complex. While STAT3-deficient cells skew away from Th17 cells and towards a T helper 1 (Th1) phenotype [28], STAT3 has also been shown to be important for T helper 2 (Th2) differentiation. STAT3-deficient T cells fail to become Th2 cells because STAT3 and STAT6 cooperate to promote Th2 differentiation and cytokine production [29]. What is not clear is how STAT3 is being activated in conditions that skew towards a Th2 response. Interestingly, a recent study has suggested that IL-2 is required for Th2 development [23], which could induce STAT3, although further elucidation is clearly required.

STAT3, as a critical downstream target of IL-21 stimulation, is crucial for the generation of T follicular helper (Tfh) cells [30]. In STAT3-deficient T cells the ability to generate Tfh cells, and consequently germinal centers, is profoundly inhibited. ChIP-Seq analysis revealed considerable overlap between the binding sites for IL-21-induced STAT3 and interferon response factor 4 (IRF4) in Tfh cells, implicating a cooperative role for these transcription factors [31]. Thus, STAT3 may modulate its function by synergizing with other transcription factors.

STAT3 also plays a major role in the development and function of different regulatory T cell (T_{reg}) populations. In addition to its well known effects in suppressing macrophage activation and cytokine production, IL-10, a STAT3 activator, is required by T_{regs} to mediate their maximal suppressive capacity *in vivo*, particularly in limiting inflammation driven by Th17 cells [32,33]. Furthermore, using a T_{reg} -specific deletion of IL-10R, it was shown that IL-10 induced STAT3 is critical for the suppressive capability of T_{regs} and that T_{reg} -derived IL-10 is essential for the suppression of IL-10R⁺ Th17 cells [32,34]. Likewise, STAT3-deficient T_{regs} fail to suppress Th17 responses and ameliorate colitis [35]. These studies also suggested that the cytokine environment, sensed by T_{regs} , induces a program of suppression that is optimized for that inflammatory environment, and may also contribute to appropriate homing.

Inhibitory cytokines can also induce the development of regulatory T cells from conventional T cells [36,37]. IL-10-induced T_{regs} (iT_{regs}), which do not express Foxp3, appear to play a key role in oral tolerance and their development is STAT3-dependent [38]. Conversely, STAT3 appears to be important in limiting TGF β - iT_{reg} development as signaling via IL-6R and gp130 can prevent Foxp3 upregulation and inhibit T_{reg} conversion [39]. The IL-27R, which signals through gp130 to STAT3, can also inhibit the development of T_{regs} , but this effect is not due to limiting Foxp3 expression but rather through the inhibition of IL-2 production [40]. Thus, STAT3 can inhibit T_{reg} development in distinct ways, modulated by the receptor that is utilized. Taken together, these data paint a complex picture in which STAT3 can facilitate nT_{reg} -mediated repression of Th17-driven inflammation yet block TGF- β -driven iT_{reg} development.

These studies clearly implicate STAT3 as a critical regulator of both helper and regulatory T cell development and function. What remains unclear is how these individual cell types utilize STAT3 to effect different functional programs. While STAT3 is central to the events described above, the receptors utilized to activate STAT3 are often different. These receptors must therefore impart additional information to the T cell, either through alternative modification of STATs or through other signaling pathways, as detailed above.

Cytokine signaling can be fine-tuned by other regulators that we have not yet discussed. The suppressors of cytokine signaling (SOCS) proteins play an important role modulating cytokine signals. SOCS3 is a STAT3 target gene that negatively regulates numerous STAT3-dependent signaling pathways [3]. Whether a particular cytokine is inhibited by SOCS3 is controlled at the level of the receptor. For example, Gp130 and the G-CSFR possess a SOCS3 binding sites. Thus, IL-6 stimulation activates STAT3 and induce SOCS3 expression, which binds to Gp130 thereby inhibiting its own signaling [41]. Likewise IL-27, which also utilizes gp130, can activate STAT3 and be inhibited by SOCS3 [42]. By contrast, the IL-10R, which lacks a SOCS3 binding site, induces SOCS3 expression in a STAT3-dependent way, but is not inhibited by SOCS3 [3]. So while STAT proteins are biochemical effectors of cytokine signaling, differential utilization of regulatory proteins like SOCS can help to shape downstream consequences.

Knockout locally, assay globally: a holistic approach to studying cytokine signaling

Utilizing knockout systems to study STAT proteins has revealed essential roles for these molecules in T cell function. However, STAT proteins contribute to multiple diverse roles in signal transduction in T cells and thus their deletion can give rise to complicated phenotypes. Utilizing more ‘surgical’ genetic deletions or knockins would likely prove more informative. Genetic models of receptor deletion can yield much cleaner and more interpretable results than analysis of STAT knockout mice, especially if performed in a cell type-specific manner. Furthermore, if a receptor activates multiple STATs (which many do), a receptor-deficient cell provides a mechanism to prevent all signaling events via specific receptor, while leaving other signaling events unaffected. Ideally, even more restricted mutant models should be generated. For instance, specific motifs or residues could be mutated in receptors (eg. a JAK- or SOCS-binding site) or STATs (tyrosine and serine phosphorylation sites, sumoylation motifs, etc.) in a conditional manner. Inclusion of cell-type specific CreER lines would generate mice that would allow for cell-type specific, temporal deletion of a single facet of cytokine signaling. Substantial insights into cytokine signal have already been made from non-condition versions of mutated receptors. For example, point mutations in Y757 of Gp130 were decisive in establishing this residue as the SOCS3 binding site [43,44].

It is also clear that studying STAT activation with a single readout, such as tyrosine phosphorylation, may not be sufficient to examine cytokine signaling. Advances in the field of flow cytometry have allowed single-cell analysis of phospho-STAT signaling [45]. These techniques can be harnessed to study the entire repertoire of STATs and STAT modifications in response to cytokine stimulation. Recent advances in single-cell analysis using mass cytometry, a technique using isotope-labeled antibodies read out using a high throughput mass spectrometer (CyTOF), may provide methods to examine many more signaling events at once [46]. In theory, multiple modifications of all the receptor chains, JAK/STAT proteins and regulators could be assayed at the same time in a single cell, while still allowing for multiplexed analysis of multiple signaling pathways under different experimental conditions over time. This could reveal previously unappreciated signaling networks that we are simply unable to interrogate with current techniques. However, it is evident that this will require the generation of many more site-specific antibodies than currently available.

Recent studies have utilized ChIP-Seq to globally examine the downstream consequences of cytokine signaling. While this provides large amounts of data detailing where a particular STAT binds, this approach only tells us what is binding to where, not whether this has any physiological consequence. Thus, future mutant models may also employ STAT binding site deletion mutants in specific loci. Furthermore, combinatorial analysis of multiple ChIP-Seq datasets (eg. same STAT proteins activated by different cytokines), repressive and activating chromatin marks, and potential binding partners could reveal how these complex cytokine regulatory networks synergize to control T cell differentiation and productive immunity.

Conclusions

The immune response is regulated by an incredibly complex cytokine environment. While these cytokines are many and varied, the signaling molecules utilized by these cytokines, the JAK and STAT proteins, are relatively few. How do cells interpret these signals to modulate the immune response? A key aspect of this dilemma lies at the receptor and the multiple modifications and regulatory proteins that collectively shape a cytokine response. While some receptor chains are shared, there is generally more diversity in receptor chain

utilization than in the number of available JAK/STATs. Furthermore, receptor expression can be dynamically modulated by the cell, based on cell type, stimulation, and cytokine activity. Utilizing targeted, conditional deletions of receptors or specific receptor/STAT signaling motifs could facilitate more in-depth dissection of cytokine signaling. In addition, the signaling events themselves need to be more thoroughly investigated, particularly the various STAT modifications and how they shape their functions. As many therapeutic approaches either use or target cytokines, a complete understanding of the intricacies of cytokine signaling and function will allow researchers and physicians to better predict the impact of therapeutic intervention in human disease.

Acknowledgments

Supported by NIH grants R01 AI52199 and AI091977, an NCI Comprehensive Cancer Center Support CORE grant (CA21765), and the American Lebanese Syrian Associated Charities (ALSAC) to D.A.A. Vignali.

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HIGHLIGHTS

- A myriad of cytokines drive diverse responses but utilize few signaling molecules
- Signaling via the same STAT can have diverse downstream consequences
- Receptor, signaling and/or epigenetic modulation shapes the cytokine response
- STAT3 illustrates how a single molecule can orchestrate diverse cellular events
- Future studies should use focused, targeted mutations assayed using global approaches

Figure 1.

STAT modifications. STATs can be modified by many mechanisms; JAK-dependent tyrosine phosphorylation primes dimerization and allows for nuclear entry. Serine phosphorylation occurs via secondary signaling pathways such as Akt-mTOR or MAPK and permits alternative transcriptional programs. SUMOylation can inhibit certain transcriptional programs while sparing others. Acetylation is a controversial modification that may affect dimerization or nuclear import.

Figure 2. STAT3 signaling in T cells. Many cytokines utilize STAT3 to transduce their signals in cells. Despite the fact that all these cytokines utilize STAT3, the outcome of these cytokine signals is distinct between them. This variation can occur at the level of the receptor, at the level of negative regulation (such as SOCS3), or further downstream at the level of gene accessibility. Regarding the 'Other STATs activated' note that not all of these pathways have been evaluated genetically to confirm their roles in cytokine function.