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# Modulation of IL-4/IL-13 cytokine signaling in the context of allergic disease

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## Abstract

Aberrant activation of CD4 T helper 2 (Th2) cells and excessive production of Th2 cytokines like interleukin (IL)-4 and IL-13 have been implicated in the pathogenesis of allergic diseases. Generally, IL-4 and IL-13 utilize JAK-STAT signaling pathways for induction of inflammatory gene expression and the effector functions associated with disease pathology in many allergic diseases. However, it is increasingly clear that JAK/STAT pathways activated by IL-4/IL-13 can themselves be modulated in the presence of other intracellular signaling programs, thereby changing the overall tone and/or magnitude of IL-4/IL-13 signaling. Apart from direct activation of the canonical JAK-STAT pathways, IL-4 and IL-13 also induce pro-inflammatory gene expression and effector functions through activation of additional signaling cascades. These alternative signaling cascades contribute to several specific aspects of IL-4/IL-13-associated cellular and molecular responses. A more complete understanding of IL-4/IL-13 signaling pathways, including the precise conditions under which non-canonical signaling pathways are activated, and the impact of these pathways on cellular and host level responses, will better allow us to design agents that target specific pathological outcomes, or tailor therapies for the treatment of uncommon disease endotypes.

### Keywords

Allergic disease; cytokine signaling; IL-13; IL-4

## Introduction:

Allergic diseases such as allergic asthma, atopic dermatitis, and allergic rhinitis collectively affect over 10–15% of the global population and their prevalence has doubled in last

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decade[1]. Allergic diseases are characterized by aberrant activation of CD4 T helper 2 (Th2) cells in response to innocuous environmental proteins[2] - allergens - and subsequent production of Th2-derived cytokines such as interleukin (IL)-4, IL-5, and IL-13 at sites of allergic inflammation[3, 4]. Th2-derived cytokines are considered central to the pathology associated with allergic diseases, and as such excellent reviews on mechanisms driving Th2 development in the context of allergic diseases[5, 6], and the mechanisms through which Th2 cytokines influence disease pathology[7] are available. Accordingly, there has also been a great deal of interest in anti-Th2 cytokine biologicals for the treatment of these diseases[8, 9]. Moreover, as signaling pathways activated by Th2 cytokines typically use Janus kinase/Signal Transducer and Activator of Transcription (JAK/STAT) pathways for inducing expression of genes that contribute to their downstream effector functions, the use of JAK inhibitors is also being considered[10]. However, other understudied signaling intermediates have been reported to be activated in response to Th2 cytokines and recent work suggests that the intensity of Th2 cytokine signaling can be influenced by the presence of other inflammatory signals (i.e., Th17-associated cytokines). Given that the true power (and limitations) of targeting Th2-associated pathways *in their entirety* in the context of allergic diseases is still being deciphered, it is likely premature to speculate as to how and/or when specific pathways may represent unique and tractable targets for therapeutic intervention. Nonetheless, a more complete understanding of the multitude of pathways activated by disease relevant cytokines may lead to therapeutic interventions applicable to specific forms of allergic asthma (i.e., Th2 low or mixed Th2/Th17 responses[9, 11]), disease forms for which therapeutic options remain limited. Thus, the purpose of this article is to review evidence for activation of non-canonical IL-4/IL-13-induced signaling intermediates (such as MAPKs, PI3K/Akt, PKC and SRC), discuss negative and positive regulators of the IL-4/IL-13 signaling pathways, and describe interactions between IL-4/IL-13 signaling pathways and other pathways.

## Role of IL-4 and IL-13 in allergic disease:

IL-4 and IL-13 are central to the pathology of many allergic disorders and have a variety of cellular targets that contribute to these responses. IL-4 serves as a key cytokine promoting the development of allergic disease through its ability to prime the differentiation of naïve T helper cells into Th2 cells[12, 13] and its ability to promote immunoglobulin class switching to IgE in B cells[14–16]. In contrast, the role of IL-13 is primarily exerted in the effector phase via potent influences on structural cells such as epithelial cells, endothelial cells, fibroblasts, keratinocytes and smooth muscle cells[17–22]. On these cells, IL-13 induces a variety of effects, including induction of tissue remodeling[23], regulation of barrier function[24, 25], differentiation and proliferation of mucus-producing goblet cells[26], and induction of smooth muscle hypertrophy[19, 27]. Although several agents targeting IL-4, IL-13 or their receptors are being explored for the treatment of allergic disease[28–31], a complete understanding of the intracellular signaling cascades activated by these two allergic-disease relevant cytokines, and the biological processes controlled by various signaling pathways may inform potentially novel therapeutic options that can be tailored to control specific aspects of disease pathology.

## IL-4 and IL-13 receptor complexes

IL-4 and IL-13 utilize shared receptors for activation of intracellular signaling cascades (Figure 1). The IL-4/IL-13 receptor family consists of two distinct heterodimeric receptors: the type I IL-4R (consisting of the IL-4Rα and the common gamma chain (γc)) and the type II IL-4R (consisting of IL-4Rα and the IL-13Rα1). Although IL-4 can bind to, and signal through, both type I and type II IL-4Rs, IL-13 binding and signaling is restricted to the type II IL-4 receptor[23]. Importantly, the cellular distribution of IL-4Rα and IL-13Rα1 is variable - although most cell types express at least low levels of IL-4Rα[32], hematopoietic cells typically express higher levels of IL-4Rα, while non-hematopoietic cells express higher levels of IL-13Rα1[33]. This may explain the particularly pronounced effects of IL-13 on non-hematopoietic cells (e.g. epithelial cells or fibroblasts) compared to lymphocytes (for more information on this aspect of type I/II IL-4R biology please see[33, 34]). Importantly, IL-13 (but not IL-4) can also bind to a unique receptor called IL-13Rα2. IL-13Rα2 binds to IL-13 with a higher affinity than IL-13Rα1, and because IL-13Rα2 has a short cytoplasmic tail (as well as a cleavage-induced, or alternatively spliced soluble forms), many consider IL-13Rα2 a decoy receptor[35–37]. However, recent reports suggest that IL-13Rα2 can activate alternative signaling pathways[38, 39]. The canonical and alternative pathways activated downstream of these receptors are discussed below.

## Type I IL-4R signaling pathway:

IL-4 binds with high affinity to IL-4Rα (KD = 0.1 nM)[40, 41] and formation of the IL-4/IL-4Rα complex facilitates the recruitment of γc to complete the formation of the type I IL-4R system (Figure 1). Assembly of this receptor complex allows phosphorylation and activation of JAK family members that are constitutively associated with IL-4Rα and γc: JAK1 and JAK3 respectively[42]. Once phosphorylated, JAK1 phosphorylates five tyrosine residues (Y497, Y575, Y603, Y631, and Y713) within the IL-4Rα cytoplasmic tail. These phospho-tyrosine residues subsequently act as docking sites for downstream phospho-tyrosine binding (PTB) signaling molecules[43, 44]. Specifically, pY497 residues form an insulin/interleukin-4 receptor (I4R) motif that interacts with PTB domains on insulin receptor substrate-2 (IRS-2)[45] and allows γc-dependent phosphorylation of IRS-2[46]. Phosphorylated IRS-2 binds to and activates the p85 domain of phosphatidylinositol-3-kinase (PI3K) leading to activation of Akt, PDK1/2 and p70S6 kinases[47]. In monocytes, IRS-2 activation is important for skewing towards an alternatively activated macrophage phenotype and expression of genes such as *Arg1*, *Chi3l* (Ym1), and *Chi3l1* (YKL-40)[46, 48]. IL-4 is the major inducer of alternatively activated macrophages which have been shown to enhance Th2 responses, elevate eosinophilic inflammation, promote tissue remodeling and fibrosis and exacerbate AHR[49–54]. Phosphorylation of Y575, Y603, and Y631 of IL-4Rα enables the recruitment of STAT6. Once recruited to the receptor, JAK1 and JAK3 phosphorylate the C-terminal tyrosine residue of STAT6 (Y631) leading to homodimerization of STAT6. Dimerized STAT6 translocates to the nucleus and activates the expression of many IL-4-responsive genes. In contrast to the activating role of the other tyrosine residues, tyrosine Y713 on the IL-4Rα is part of an immune-tyrosine-based inhibitory motif (ITIM) and helps in negative regulation of IL-4 signaling responses through recruitment of SHP1/2[55], important negative regulators of IL-4 signaling (see below).

## Type II IL-4R signaling pathway:

In contrast to the type I IL-4R, which binds only IL-4, the heterodimeric type II IL-4R binds both IL-4 and IL-13 (Figure 1). Although IL-4 has higher binding affinity towards IL-4Rα ($K_D$ = 0.1 nM)[41] than that of IL-13 to IL-13Rα1 ($K_D$ = 1.7 nM)[56], IL-13 is typically produced in higher quantities than IL-4. Binding of IL-4/IL-13 to type II IL-4R activates JAK1, which is constitutively associated with IL-4Rα, and tyrosine kinase 2 (TYK2), which is associated with IL-13Rα1. Activated JAK1 and TYK2 phosphorylate residues Y575, Y603, Y631, Y709 in the IL-4Rα leading to recruitment and activation of STAT6, as in the Type I IL-4R. Importantly, as γc is not present in type II IL-4R system, IRS-2 is not activated[46]. On the other hand, the cytoplasmic domain of IL-13Rα1 contains two tyrosine residues, Y402 and Y405, which have been proposed to act as a docking site for STAT3[55]. Unfortunately, the physiological consequences of potential STAT3 activation in this context are not well understood.

## IL-13Rα2:

IL-13Rα2 was initially described as a decoy receptor capable of limiting IL-13-induced pathology. This was based on several properties, including a higher binding affinity of IL-13 for IL-13Rα2 than IL-13Rα1 ($K_D < 10^{-15}$ M vs $K_D$ = 1.7 nM)[56, 57], and the observation that the cytoplasmic tail of IL-13Rα2 (17 amino acids[58]) is much shorter than that of IL-13Rα1 and IL-4Rα (60 & 785 amino acids respectively) and lacks the conserved JAK binding sites essential for signal transduction[56, 59]. The concept of IL-13Rα2 serving an inhibitory function is supported by studies demonstrating that 1) endogenous expression of IL-13Rα2 attenuated IL-13-induced gene expression in lung fibrosis and atopic dermatitis models[37, 38]; 2) endogenous expression of IL-13Rα2 decreased the sensitivity of the type II IL-4R to IL-13[37]; 3) antibodies blocking interactions between IL-13 and type II IL-4R in IL-13Rα2-sufficient mice did not induce IL-13 relevant gene expression[37]; 4) mice lacking IL-13Rα2 demonstrated increased IL-13-mediated cutaneous inflammation, $IgG_1$ levels, and trans-epidermal water loss in a mouse model of atopic dermatitis compared to wild-type mice[36]; and 5) IL-13Rα2 null mice had increased IL-13-mediated STAT6 activation compared to wild type mice[36]. Importantly, IL-13Rα2 expression is induced by IL-13 in multiple cell types (primary human keratinocytes, keratinocyte cell lines[60,36,61]), suggesting that expression of IL-13Rα2 may represent a negative feedback loop which can attenuate IL-13 signaling.

Although these studies suggest a negative role for IL-13Rα2 in regulation of IL-13 signaling, contradicting observations exist – we[39] and others[62] have demonstrated that IL-13Rα2 KO mice display *reduced* airway hyperresponsiveness (AHR), mucus production, and IL-13-induced gene expression in the lung compared to wild type mice in both HDM-challenge and IL-13-challenge models (Figure 1). Moreover, IL-13Rα2 overexpression in lung epithelial cells reconstituted airway inflammation comparable to HDM-challenged wild type mice, suggesting that pulmonary epithelial cell expression of IL-13Rα2 was the mediator of these effects[62]. Mechanistically, IL-13 interaction with IL-13Rα2 has been shown to activate the transcription factor activator protein-1 (AP-1), which then induced TGFβ production[63–65]. Other studies show that IL-13 binds to IL-13Rα2 and induces

extracellular signal-regulated kinases 1/2 (ERK1/2), and the downstream AP-1-related gene C-Jun in human nasal epithelial cells[65]. Another recent study examining potential IL-13Rα2 signaling demonstrated that IL-13 binding to IL-13Rα2 increased epidermal growth factor receptor vIII (EGFRvIII) tyrosine kinase activities, leading to increased RAS/RAF/MEK/ERK and STAT3 activation in a human glioblastoma cell line[66]. Although signaling via the IL-13Rα2 has been implicated in many tumor models[67–70], its role in allergic diseases remains understudied. Nonetheless, these studies suggest a complex role for IL-13Rα2 in regulating allergic diseases.

## IL-4 and IL-13 mediated activation of non JAK/STAT-mediated pathways:

Apart from direct activation of above-described JAK-STAT pathways, IL-4 and IL-13 also induce pro-inflammatory gene expression and effector functions through activation of additional signaling cascades, including AKT/phosphoinositide 3-kinase (PI3K)/mammalian target of rapamycin (mTOR), mitogen activated protein kinases (MAPK), or SRC, through STAT6-dependent and - independent signaling pathways (Figure 1). These alternative signaling cascades contribute to important aspects of IL-4/IL-13-associated cellular and molecular responses.

### PI3K activation:

IL-4 and IL-13 have been shown to induce activation of a PI3K/AKT/mTOR cascade, which has profound effects on cell biology. PI3K is a lipid kinase that is typically activated by receptor tyrosine kinases (RTKs) binding to the P85 subunit of PI3K[71]. Activated PI3K then phosphorylates the plasma membrane lipid phosphatidylinositol (4,5)-bisphosphate (PIP2) to generate phosphatidylinositol (3,4,5)-trisphosphate (PIP3), which is responsible for further activation and initiation of signaling cascades such as protein serine/threonine kinases, AKT, and phosphoinositide-dependent kinase (PDK)[71]. Activated AKT initiates downstream gene expression that contributes to cell growth, proliferation, and anti-apoptotic activity through engagement of mTOR signaling[72]. Studies have shown that IL-4-induced IRS-2 activates PI3K and the downstream protein serine/threonine AKT[46] and that this contributes to IL-4-induced changes such as alternatively activated macrophage differentiation and enhanced vascular remodeling and muscularization[54, 73–75]. Interestingly, although IL-13 signals exclusively through the type 2 IL-4R complex, and thus does not activate IRS-2 (as it utilizes IL13Rα1/TYK2 rather than γc/JAK3, which are required for phosphorylation of Y647 and docking of IRS-2 to the IL-4Rα), IL-13 has also been shown to activate PI3K (via the p110 subunit), which then phosphorylates AKT in murine smooth muscle tracheal cells[27, 76, 77]. IL-13-driven activation of a PI3K/AKT/Protein Kinase C (PKC) cascade contributes to induction of Tenascin-C expression in a mouse bleomycin-induced fibrosis model[77]. Interestingly, Tenascin-C is a glycoprotein found to be upregulated in bronchial tissue of asthmatic patients[78], suggesting that this pathway may contribute to IL-13-induced lung remodeling in the context of asthma. The mechanisms through which IL-13 supports activation of PI3K, remain unclear.

### MAPK activation:

Both IL-4 and IL-13 also activate members of the MAPK family - a family of serine threonine kinases that induce expression of inflammatory-, growth factor- and osmotic stress-related genes[79]. MAPKs are classified into 3 subfamilies: 1) extracellular signal related protein kinase 1/2 (ERK1/2), 2) Jun N-terminal kinases (JNKs) and 3) p38 MAPK[80–82]. Activation of these signaling intermediates contributes to the increased activity of transcriptional regulators including nuclear factor of activated T-cells (NFAT), cyclic AMP-response element binding protein (CREB), and AP-1, and have been described to mediate specific aspects of IL-4/IL-13 responses. IL-4-mediated induction of p38 MAPK stabilized IL-6 mRNA in human keratinocyte cell line[83], suggesting that IL-4 can regulate innate inflammatory responses in structural cells. IL-4-mediated activation of ERK has been reported in a human T cell line, and inhibition of ERK was found to inhibit IL-4-driven STAT6 activation and subsequent Th2 differentiation[84], suggesting an important role for IL-4-induced MAPK activation in Th2 skewing. Using IL-13-transgenic mice, it was demonstrated that IL-13 induces phosphorylation of ERK1/2 (but not JNK and p38) through a STAT6-independent pathway[85]. Interestingly, chemokine production via canonical STAT6 signaling versus ERK1/2 activation varied by chemokine, with some chemokines demonstrating complete dependence upon STAT6 (CCL11, CCL2, CCL6), complete dependence on ERK1/2 (CXCL1) or equivalent induction by STAT6 and ERK1/2 (CCL3, CCL4, CCL5)[85]. In a human keratinocyte cell line, inhibition of ERK1/2 completely abrogated IL-4- and IL-13-mediated induction of IL-13Rα2[79], suggesting ERK1/2 is also important for IL-4/IL-13-mediated IL-13Rα2 expression. Thus, specific inhibition of IL-13 or IL-4 driven ERK may have selective effects on IL-4 and IL-13-induced cellular recruitment and disease pathology.

### SRC Activation:

A final signaling intermediate implicated in IL-4/IL-13 signaling is the proto-oncogene tyrosine-protein kinase SRC. SRC signaling has been implicated in cell proliferation, growth, and survival[86] as well as downstream activation of various STAT proteins[86–89]. Consistent with reports that SRC can promote activation of some STAT proteins[86], overexpression of v-SRC in mouse fibroblasts induced constitutive STAT6 phosphorylation and activation, while fibroblasts lacking SRC displayed reduced IL-4-driven activation of STAT6, suggesting that SRC might be involved upstream of STAT6[90]. Interestingly, although IL-4 and IL-13 both utilize STAT6 as an important signaling intermediate, the activation and involvement of SRC in IL-13-induced signaling pathways has not been explored. Nonetheless, these studies implicate SRC as an important upstream activator of STAT6 in response to IL-4. Given the interest in development of therapeutics based on targeting SRC[91], it would be important to further delineate the relationship between IL-4/IL-13 signaling and SRC activity.

Collectively these studies suggest that IL-4 and IL-13 have the potential to activate several signaling pathways beyond their well described influence on JAK/STAT6 signaling. Confirming the importance of these pathways, identifying situations where they are preferentially induced, and delineating the specific molecular processes that are activated in response to their signaling will be important to fully understand the molecular mechanisms

driving disease in situations where excessive IL-4/IL-13 production are expected to play a role.

## Regulators of IL-4 and IL-13 signaling:

As described above, IL-4 and IL-13 signaling utilizes multiple signaling pathways. These pathways can themselves be modulated in the presence of other intracellular signaling programs, thereby changing the overall tone and magnitude of IL-4/IL-13 signaling. Both positive and negative regulators of IL-4/IL-13-induced responses have been described. Below we discuss these positive and negative regulators of IL-4/IL-13 signaling, the mechanisms they utilize to alter these signaling pathways, and the expected influence of these pathways on the allergic response.

## Negative regulators of IL-4 and IL-13 signaling:

The key negative regulators of JAK-STAT signaling pathways are Protein Tyrosine Phosphatases (PTPs), Src homology 2-containing protein tyrosine phosphatase (SHP-1), and Suppressor of Cytokine Signaling (SOCS) proteins (Figure 2).

### Protein Tyrosine phosphatases (PTPs):

Some of the most widely recognized negative regulators of IL-4/IL-13 signaling are the Src homology 2-containing phosphatases, including tyrosine-protein phosphatase non-receptor type 6 (PTPN6/SHP-1)[92–94], PTPN11 (SHP-2)[95, 96], and SH2-containing inositol phosphatase (SHIP)[97–99]. The 5$^{th}$ tyrosine residue (Y709) of the IL-4Rα cytoplasmic domain serves as a docking site for regulatory phosphatases including SHP-1, SHP-2 and SHIP, and as such, this region of the IL-4Rα has been termed the Immunoreceptor Tyrosine-based Inhibitory Motif (ITIM)[100]. Once recruited to the IL-4 receptor complexes, these phosphatases have the capacity to attenuate or terminate these signaling pathways by removing phosphate groups on activated signaling intermediates owing to their intrinsic phosphatase capacity. In support of their role as a negative regulator of IL-4 and IL-13, transgenic expression of SHP-1 in mouse fibroblasts (NIH3T3) significantly reduced IL-4-induced STAT6 phosphorylation and STAT6 responsive gene expression compared to control cell line[94]. Additionally, bone marrow derived macrophages (BMDM) from mice with a spontaneous mutation rendering SHP-1 inactive (“motheaten”, or me$^{v}$ mice) demonstrated enhanced IL-4-mediated phosphorylation of STAT6 compared to cells from wild type mice[94]. Similarly macrophages from SHIP deficient mice demonstrated more substantial IL-4-driven skewing towards an alternatively activated phenotype with enhanced Arg1 activity[101]. Finally, ITIM or SHP-1 deletion in mice led to a dramatic decrease in dephosphorylation of IL-4/IL-13-induced STAT6[92, 102–104]. *In vivo* studies also suggest these PTPs negatively regulate allergic inflammation. Me$^{v}$ mutant mice had increased eosinophils, elevated IgE levels, and higher AHR compared to wild type mice in OVA-induced allergic asthma models[102, 103, 105–107]. Likewise, SHP-2 deletion in myeloid cells decreased OVA-induced eosinophil recruitment and AHR compared to wild type mice[108]. Finally, mice in which the ITIM on the IL-4Rα chain is mutated to be resistant to phosphorylation (IL-4Rα Y709F mice) and thus unable to activate SHP-1/SHP-2 during IL-4/IL-13 signaling demonstrate increased Th2-driven inflammation, such as

increased STAT6 phosphorylation, increased activation of alternative macrophages, elevated eosinophil recruitment, and exacerbated AHR compared to WT mice in an OVA-induced allergic asthma model[102, 109, 110].

PTP1B, another PTP, had also been reported to modulate cytokine signaling by dephosphorylating JAK2 and TYK2[111]. Overexpression of PTP1B led to dephosphorylation of STAT6 and decreased STAT6 transcriptional activity, while PTP1B deficiency led to prolonged STAT6 phosphorylation[112]. Co-precipitation studies demonstrated a direct interaction between PTP1B and both JAK1 and STAT6, suggesting multiple different pathways through which PTP1B might influence IL-4 signaling. Importantly, IL-4 also induces expression of PTP1B through a STAT6-independent, PI3K-dependent signaling pathway[112]. As IL-4 and IL-13 both utilize JAK1 and STAT6, PTP1B is likely to inhibit IL-13-induced JAK and STAT6 phosphorylation as well, although such regulation has not been formally tested.

### SOCS-mediated inhibition of IL-4/IL-13 signaling:

SOCS proteins are negative regulators of JAK-STAT signaling[113]. The SOCS family of proteins contains 8 unique members (SOCS1 – 7 and Cytokine Inducible Sh2 protein (CIS)). This family is defined by the presence of a C-terminal SH2 domain, and a SOCS box which contains both kinase inhibitory and ubiquitin ligase functions[114]. This domain also allows association with elongin BC, an adaptor protein which helps recruit E3 ubiquitin ligase scaffold (cullin5)[115]. Patterns, and cell type-specific expression of SOCS proteins varies amongst cell types, but expression is typically of an inducible nature, and often can be induced by STAT transcription factors, suggesting that these represent feedback inhibitors of inflammatory cytokine signaling that prevent prolonged STAT signaling (recently reviewed in[116]). SOCS proteins regulate JAK-STAT activation through direct inhibition of JAK activity via a unique short motif within the SOCS box called the kinase inhibitory region (KIR)[117, 118]. Following interaction with activated kinases (via SH2-mediated phosphotyrosine binding), SOCS proteins promote ubiquitination of associated targets and subsequent proteasomal degradation[73,119].

Among SOCS proteins, SOCS1 has been demonstrated to have direct inhibitory activity relevant to IL-4/IL-13 signaling. SOCS1 directly limits JAK phosphorylation and facilitates the ubiquitination of IRS-2 following activation of the type I IL-4R. In monocytes, IRS-2 tyrosine phosphorylation is important for alternative macrophage activation through induction of IL-4-dependent gene expression. As SOCS1 suppresses IRS-2 tyrosine phosphorylation by ubiquitinating IRS-2 and targeting IRS-2 for proteasomal degradation[120], this directly limits alternative macrophage activation. In B cells, SOCS1 negatively regulates IL-4- and IL-13-induced STAT6 activity by direct inhibition of JAK1[121], suggesting that SOCS1 can also regulate IgE production. Not surprisingly, the role of SOCS1 as a negative regulator of IL-4 and IL-13 signaling is supported by many studies[114, 120–124]. In an OVA-induced allergic asthma model, mice expressing SOCS1 that is unable to enter the nucleus (which spares them from the early lethal phenotype observed in complete SOCS1-deficient animals[125]) had enhanced Th2 inflammation, such as elevated eosinophils, airway epithelial cell remodeling, and increased Th2 cytokines in response to

OVA-albumin exposure compared to wildtype mice[126]. Similarly, human nasal epithelial cells taken from individuals with severe eosinophilic asthma had reduced SOCS1 expression levels compared to healthy controls[122].

Like SOCS1, SOCS3 directly binds to and inhibits the catalytic domain of JAK1, JAK2 and TYK2 through its KIR motif. Apart from inhibiting JAK kinase activity, SOCS3 also inhibits JAK1, IRS-2 through E3 ubiquitination and proteasomal degradation[127]. E3 ubiquitin ligase activity of SOCS3 was particularly important for inhibition of IL-4-induced ERK phosphorylation and the C-Jun[128]. Not surprisingly, overexpression of SOCS3 in a rat mast cell line reduced calcium-induced ERK1/2 phosphorylation and C-Jun transcription factor activity resulting in reduced IL-4-induced gene expression. Conversely, knockdown of SOCS3 triggered higher ERK1/2 phosphorylation and C-Jun transcription factor expression, suggesting SOCS3 negatively regulates IL-4-induced outcomes by inhibiting ERK signaling[128].

Collectively, these studies provide a rationale for use of PTP or SOCS-activating therapeutics for treatment of allergic disease. Interestingly, transcellular liposomal delivery of SOCS3 in lung epithelial cells inhibited the allergen-induced IL-4/IL-13-mediated phosphorylation of STAT6 and STAT3[129], revealing the therapeutic potential of such an approach.

## Positive modulators of IL-4 and IL-13 signaling:

In addition to the negative regulators of IL-4/IL-13 signaling, factors which positively influence the intracellular signaling pathways activated by these cytokines have been described, including post-translational modification of STAT6 (via PARP-ylation), cyclic AMP (cAMP), sphingosine receptor signaling and IL-17A signaling (Figure 2).

### PARP-mediated modulation of IL-4/IL-13 induced STAT6 activation

Poly (ADP-ribose) polymerase (PARP) is a nuclear enzyme that mediates a unique post-translational modification by attaching ADP-ribose polymer chains to target proteins. Classically, PARP proteins have been implicated in DNA repair as many PARP targets include enzymes important in these processes (e.g. DNA ligase III). Recent studies on asthma suggest PARP activity levels are enhanced in asthma patients' lungs, possibly as a result of reactive oxygen species (ROS)-mediated DNA damage in pulmonary epithelial cells[130]. However, beyond its role in DNA repair, PARP proteins have been reported to contribute to asthma pathology by influencing cellular capacity to respond to, and produce, Th2 cytokines[131, 132]. Interestingly, PARP1 deficiency results in markedly reduced levels of STAT6 in spleen cells, despite comparable levels of STAT6 mRNA expression, suggesting that PARP1 is an important promoter of STAT6 stability[133]. Although the mechanism behind PARP1-mediated regulation of STAT6 integrity remain unclear, it is possible that PARP-ylation of STAT6 inhibits proteolytic cleavage of STAT6 induced after activation. In support of this possibility, cellular exposure to a calpain inhibitor (but not a proteosome inhibitor) reversed the rapid degradation of STAT6 seen in cytokine stimulated PARP1$^{-/-}$ cells[133].

Like PARP1, PARP14 was implicated in the progression of asthma pathogenesis. PARP14 has been described as a transcriptional factor switch for STAT6-associated gene expression: when IL-4 is absent, PARP14 is present at the promoters of IL-4 responsive genes and recruits HDAC2/3 to limit the expression of these genes[134]. However, upon IL-4 signaling, PARP14 PARP-ylates itself as well as HDAC2/3, resulting in the loss of HDAC2/3 at these sites and simultaneous recruitment of transcriptional activators with HAT activity. These transcriptional activators facilitate more efficient STAT6 binding to promoters of IL-4 responsive genes and increase IL-4-dependent gene epression[134]. PARP14 was found to directly PARP-ylate STAT6 itself, suggesting an additional means whereby PARP14 might regulate STAT6 activity[134]. Not surprisingly then, overexpression of PARP14 promotes IL-4-induced Th2 differentiation and gene expression, while PARP14 deficient mice had evidence of decreased OVA-induced airway inflammation due to decreased Th2 differentiation[135, 136]. These findings suggest that blocking PARP activity could act as potential therapeutic for Th2-mediated allergic disease.

### cAMP in Th2 immunopathology:

3',5'-cyclic-adenosine monophosphate (cAMP) is a secondary messenger that plays diverse signaling roles in cells[137]. Intracellular cAMP levels are regulated through the balance of adenylyl cyclase (AC) activity, which creates cAMP, and phosphodiesterase 4 (PDE4), which converts cAMP to 5'-AMP[138]. Importantly, regulation of intracellular cAMP levels has roles in asthma: activation of $G_{\alpha s}$-linked G protein coupled receptors (GPCRs – e.g. $\beta_2$-adrenergic receptor[139]) lead to smooth muscle relaxation[140], while $G_{\alpha i}$-linked GPCRs (e.g. Muscarinic Acetylcholine receptor 2) lead to activation of PDE4 and inhibition of smooth muscle relaxation[141, 142]. cAMP has been shown to regulate gene expression downstream of IL-4 - particularly the expression of Arginase I (*Arg1*), a key protein associated with alternatively activated macrophages. Specifically, cAMP[143,144] and IL-4 synergistically induce arginase I expression in RAW264.7 macrophages and primary BMDM. The effects of cAMP on arginase I expression were found to be dependent on increased nuclear localization of C/EBPβ (independent of C-EBPβ expression levels), which in turn facilitated increased STAT6 binding to the *Arg1* promoter[143, 144]. Collectively, these findings suggest cAMP could function as a both positive and negative regulator of allergic inflammation. However, cAMP acts as a positive regulator of Th2 signaling.

### Role of Sphingosine 1-phosphate in Th2 cytokine signaling:

Sphingosine 1-phosphate (S1P) is a bioactive lipid known to activate 5 unique GPCRs, $S1P_1$ through $S1P_5$[145]. S1P binds to receptors on multiple cell types involved in allergic diseases to mediate changes such as stimulation of airway smooth muscle cell proliferation[146] and FcεRI-mediated mast cell activation[147] which disrupts the integrity of airway epithelium[148]. Recently, S1P receptor 2 ($S1P_2$) was found to be implicated in exacerbating allergic diseases through interactions with IL-4 and IL-13 downstream signaling intermediates. In a bleomycin-induced, IL-13-mediated model of lung fibrosis, $S1P_2$ deficient mice demonstrated decreased fibrosis following administration of bleomycin[149]. Further examination showed reduced activation of STAT6 in the lungs compared to controls despite equivalent levels of IL-13 protein production and IL-13 receptor expression[149], suggesting that responsiveness to IL-13 was limited in the absence

of $S1P_2$ signaling. Supporting this possibility, knocking out $S1P_2$ from PMA-differentiated THP-1 cells in vitro, or inhibiting the Rho kinase pathway (a pathway strongly activated by S1P2 signaling[150]), reduced the IL-4/IL-13-mediated phosphorylation of STAT6, JAK1, and JAK3[151]. Decreased IL-4/IL-13-induced STAT6 activation observed in the context of limited $S1P_2$ signaling was associated with increased SOCS3 expression[128] – and although not formally tested, the authors suggest that the $S1P_2$ signaling limits SOCS3 expression, thereby enhancing IL-4/IL-13 signaling. Together, this evidence suggests that $S1P_2$-Rho pathway is necessary for optimal activation of STAT6.

### Effects Th17 and Th1 cytokines on IL-13 signaling cascades:

Recent studies on the severe asthma endotype identified elevated levels of mixed Th2/Th17 cells in bronchioalveolar lavage fluid (BALF) and lungs of more severe asthmatic patients[152–154] and that IL-17A can augment IL-13-induced pathology in mouse models[39]. Our group has explored mechanisms behind these observations by examining the effects of IL-17A on IL-13-induced signaling cascades. We demonstrated that, even though IL-17A utilizes a completely JAK/STAT-independent signaling pathway, the presence of IL-17A enhanced IL-13-mediated STAT6 phosphorylation and expression of downstream genes in both human and mouse cells of various types (hematopoietic, non-hematopoietic). The effects of IL-17A on IL-13 signaling are very rapid (occurring in <5 minutes of stimulation), do not require additional protein expression, and require concurrent IL-13 and IL-17A signaling in the same cells. Interestingly, in contrast to the synergistic effects of IL-17A on IL-13 induced pathways, IL-13 downregulated IL-17A-related gene expression, suggesting reciprocal coregulation of IL-13 and IL-17A[39]. Although another IL-17A family member, IL-17E (IL-25) has clear pro-Th2 supporting activities, these appear to be through direct activity on immune initiating cells (ILC2s, Th2 cells) rather than by modulating signaling in response to other Th2 cytokines[155]. These findings suggest that IL-17A (but not IL-17E) modulates IL-13-mediated intracellular signaling pathways through direct effects on signaling intermediates activated in response to IL-13.

Although Th1-derived cytokines are known to counter the development of Th2 cells[156, 157], and thus can negatively regulate production of both IL-4 and IL-13, Th1 cytokines like IL-12 and IFNγ can directly antagonize IL-4R signaling. Pre-treatment of both epithelial cells[158] and monocytes[159, 160] with IFNγ prior to IL-4 stimulation reduced IL-4-induced STAT6 activation. In monocytes, IFNγ pre-treatment triggered inhibition of STAT6 activation was a result of increased SOCS1 expression[159], while in epithelial cells it was associated with increased expression of both SOCS1 and SOCS3[158]. Collectively, these observations are consistent with SOCS proteins acting as inducible, negative regulators of IL-4/IL-13 cytokine signaling. However, our group has shown a direct inhibitory effect of IL-12 on IL-13-mediated STAT6 phosphorylation and IL-13-induced gene expression in murine BMDCs and primary murine tracheal epithelial cells[161]. Importantly, the IL-12-mediated inhibition of IL-13-induced STAT6 activation occurred when IL-12 and IL-13 were added simultaneously to the culture (i.e., not in pre-treated cells) and within 15 minutes - too rapidly to be due to de novo IFNγ synthesis – suggesting that IL-12 signaling was directly antagonizing IL-13-induced STAT6 activation. The mechanisms responsible for this remain unclear.

Further identification of disease endotypes where enhancing pathways are activated to potentially amplify IL-4/IL-13 signaling may present novel therapeutic options for the treatment of disease. In support of this idea, therapeutic inhibition of PARP1 with Olaparib was found to ameliorate HDM/OVA-induced airway inflammation[162]. In addition, our group demonstrated that combination therapy of anti-IL-13 and anti-IL-17A (at levels too low to individually impact IL-13- or IL-17A-induced disease processes) ameliorated HDM-induced allergic asthma in a model of Th2/Th17-driven allergic asthma[163].

## Conclusions:

IL-4 and IL-13 can both signal through the type II IL-4R, which leads to the activation of STAT6 and expression of STAT6-dependent genes. Despite this similarity, these two cytokines have been ascribed different functions in the context of allergic disease. These differences may arise due to unique activation of additional signaling pathways by IL-4 signaling through the type I IL-4R (IRS-2) and differential expression of the type I and type II IL-4R on hematopoietic, verses non-hematopoietic cells. Moreover, additional inputs provided by either positive or negative regulators of IL-4/IL-13 signaling pathways can selectively fine tune responses to these cytokines, thereby subtly altering the biological outcomes of local signaling in response to these molecules. However, it is also clear that additional signaling pathways may be activated by these cytokines (MAPK, PI3K, SRC), and that activation of these unique pathways may in turn influence IRS-2/STAT6 activation or promote altered biological outcomes in response to these paradigmatic Th2 cytokines. Although a great deal of attention has been paid to the effects of IL-4/IL-13-driven activation of IRS-2 and STAT6, the role of these additional signaling pathways is poorly understood. It is our belief that a more complete understanding of IL-4/IL-13 signaling pathways, including the precise conditions under which non-canonical signaling pathways are activated, and the impact of these pathways on cellular and host level responses, will better allow us to design agents that target specific pathological outcomes, or tailor therapies for the treatment of uncommon disease endotypes.

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## Abbreviations used:

| | |
|---|---|
| **Th2** | CD4 T helper 2 |
| **IL** | Interleukin |
| **JAK** | Janus kinase |
| **STAT** | Signal Transducer and Activator of Transcription |
| **$\gamma c$** | common gamma chain |
| **$K_D$** | Dissociation constant |
| **PTB** | phospho-tyrosine binding |

| | |
|---|---|
| **IRS-2** | insulin receptor substrate-2 |
| **PI3K** | phosphatidylinositol-3-kinase |
| **MAPK** | mitogen activated protein kinases |
| **Akt** | serine threonine protein kinase |
| **PKC** | protein kinase C |
| **SRC** | proto-oncogene tyrosine-protein kinase |
| **I4R** | insulin/interleukin-4 receptor |
| **TYK2** | tyrosine kinase 2 |
| **mTOR** | mammalian target of rapamycin |
| **RTK** | receptor tyrosine kinase |
| **PIP2** | phosphatidylinositol (4,5)-bisphosphate |
| **PIP3** | phosphatidylinositol (3,4,5)-trisphosphate |
| **PDK** | phosphoinositide-dependent kinase |
| **EGFRvIII** | epidermal growth factor receptor vIII |
| **ERK** | extracellular signal-regulated kinases |
| **JNK** | Jun N-terminal Kinase |
| **NFAT** | nuclear factor of activated T-cells |
| **CREB** | cyclic AMP-response element binding protein |
| **AP-1** | activator protein-1 |
| **mRNA** | messenger ribonucleic acid |
| **PTPs** | Protein Tyrosine phosphatases |
| **SOCS** | Suppressor of Cytokine Signaling |
| **SHP-1** | Src homology 2-containing protein tyrosine phosphatase |
| **SHIP** | SH2-containing inositol phosphatase |
| **ITIM** | immune-tyrosine-based inhibitory motif |
| **BMDM** | bone marrow derived macrophages |
| **KIR** | kinase inhibitory region |
| **AHR** | airway hyper responsiveness |
| **IgG** | immunoglobulin G |

| | |
|---|---|
| **IgE** | immunoglobulin E |
| **CIS** | cytokine Inducible Sh2 protein |
| **ADP-ribose** | adenosine diphosphate ribose |
| **PARP** | poly (ADP-ribose) polymerase |
| **DNA** | deoxy ribonucleic acid |
| **cAMP** | cyclic AMP |
| **S1P** | Sphingosine 1-phosphate |
| **ROS** | reactive oxygen species |
| **HDAC** | histone deacetylase |
| **HAT** | histone acetyltransferase |
| **OVA** | ovalbumin |
| **HDM** | house dust mite |
| **BALF** | bronchoalveolar lavage fluid |

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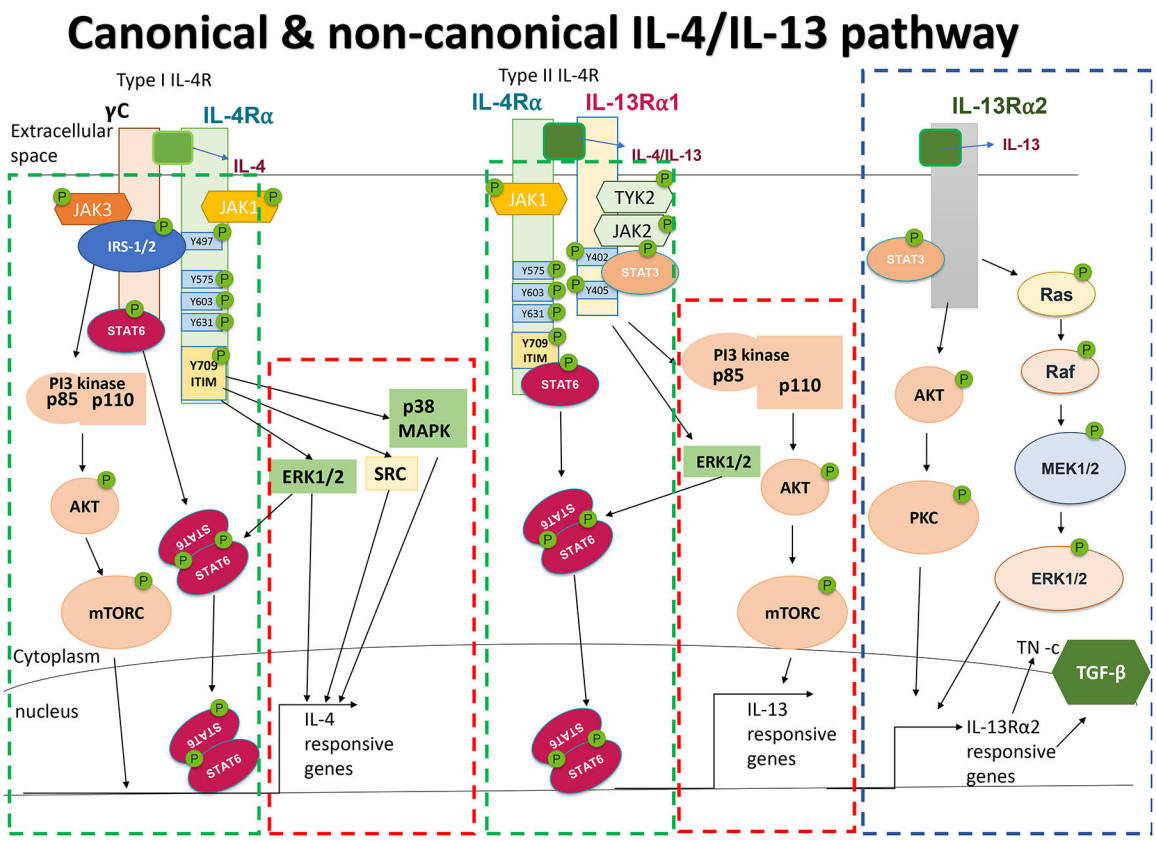


**Figure 1: Canonical and non-canonical IL-4 and IL-13 signaling pathways:**

Canonical pathways (marked in dashed green box) activated in response to type I and type II IL-4R signaling involve JAK/STAT-mediated phosphorylation of STAT6. Activation of the type 1 IL-4R also leads to activation of IRS-2 and downstream activation of PI3K, Akt and mTOR. Non-canonical activation (marked in red dashed box) of SRC, ERK1/2 and p38 MAPK in response to IL-4, or ERK1/2 and PI3K in response to IL-13, have also been described to contribute important biological outcomes. As activation of PI3K/AKT/mTOR is directly attributable to γc/JAK3-driven phosphorylation of Y647 in IL-4Rα in the type 1 IL-4R complex, but IL-13Rα1/TYK2-driven phosphorylation of Y647 has not been described for the type II IL-4R, PI3K/AKT activation is considered canonical downstream of the type I IL-4R, but non-canonical downstream of the type II IL-4R complex. IL-13 can also signal through IL-13Rα2 (marked in dashed blue box) to activate/phosphorylate Akt/PKC and Ras/ERK1/2 pathway to induce TGFβ, Tenascin-c and other IL-13Rα2-associated genes.

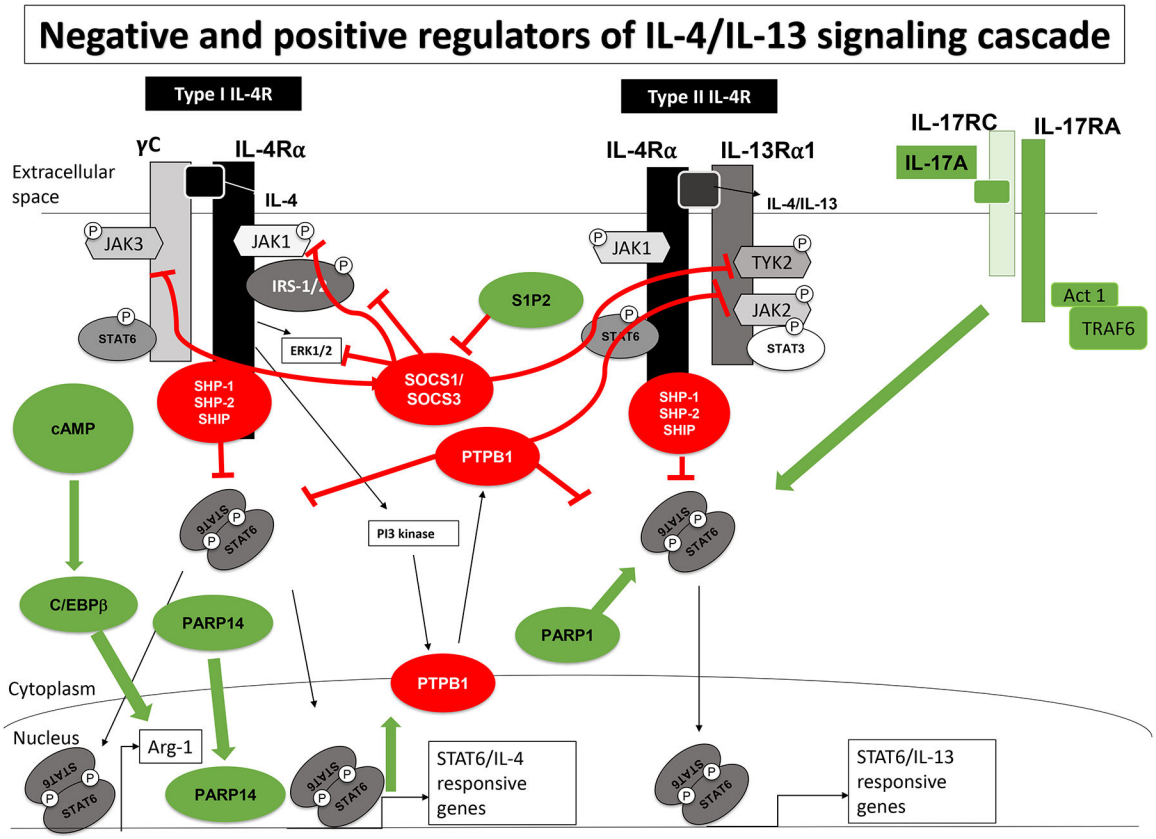


**Figure 2: Negative and positive regulators of IL-4 and IL-13 signaling:**

IL-13 and IL-4 signaling intermediates and receptors are marked in black and grey. Negative regulators (marked in red circles and red lines) of IL-4/13 signaling (SOCS proteins, protein tyrosine phosphatases) function by inhibiting JAK kinase activity, targeting activated signaling intermediates for proteolysis, and removing phosphate groups from activated signaling intermediates. Positive regulators (marked in green circles and green arrows) of IL-4/13 signaling pathway (PARP proteins, cAMP, S1P2 and IL-17A) facilitate efficient STAT6 DNA binding, protect STAT6 from proteolytic degradation, enhance STAT6 phosphorylation, enhance IL-4-induced gene expression through recruitment of C/EBPβ, and suppress SOCS3-mediated inhibition.