Regulation of hematopoietic cell development and function by Stat3

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1. ABSTRACT

Activation of the Jak/Stat pathway plays a central role in hematopoietic development. Here, we focus on unique functions for Stat3 in the development of the hematopoietic system. Recent studies highlight a critical role for Stat3 in the development of T helper cell and B cell subsets as well as dendritic cell development and maturation. Further, Stat3 appears to play an important function in limiting neutrophil numbers and in regulating hematopoietic stem cell self-renewal. In macrophages, Stat3 is essential in limiting TLR signaling and the inflammatory damage associated with classical macrophage activation. Stat3 also plays an essential role in leukemic development induced by viral oncogenes, chromosomal translocations and by the Friend erythroleukemia virus. We also discuss the mechanism by which Stat3 is activated by extracellular signals, and the current understanding of the role of post-translational modification in the regulation of Stat3 activity. Understanding the molecular events underlying Stat3 activation will be critical in generating targeted therapeutics aimed at modulating Stat3 function.

2. INTRODUCTION

The Jak/Stat signaling pathway has long served as a paradigm for understanding the molecular events that regulate gene expression in response to extracellular stimuli. There are four mammalian Jak kinases, Jak1, Jak2, Jak3 and Tyk2. These cytoplasmic tyrosine kinases transduce signals from type I and type II cytokine receptors in response to the four (α)-helix bundle family of cytokines. The architecture of these kinases is similar, with seven conserved domains, JH(Jak homology)1 –JH7. The JH1 domain is the kinase domain, while the JH2 domain is a pseudokinase domain that does not in itself have kinase activity, but appears to perform a regulatory function. The JH3-JH7 domains which make up the N-terminal region of the Jak kinases include an SH2 domain and a FERM domain. While Jak1, Jak2 and Tyk2 are ubiquitously expressed, Jak3 is expressed primarily in hematopoietic tissues and is mutated in some patients with severe combined immunodeficiency (SCID).

Cytokine binding to their cognate receptors leads to activation of the Jak kinases which, in turn,

phosphorylate tyrosines in the c-terminal tail of the cytokine receptors. These phosphorylated tyrosines then become docking sites for downstream SH2 domain containing signaling molecules including the Stat family of transcription factors. Phosphorylation of the Stat molecules results in homo or heterodimerization of Stat factors through reciprocal phosphotyrosine/SH2 domain interactions. The resulting dimers translocate to the nucleus where they bind to DNA and activate gene transcription. There are seven mammalian Stats, Stat1, Stat2, Stat3, Stat4, Stat5a, Stat5b and Stat6. Knockout studies have clearly identified unique functions for the individual Stats. Stat1 transduces signals for all three classes of interferons (IFNs) and is thus critical in the immune response to bacterial and viral infections(1, 2). Stat2 is the only Stat that does not appear to homodimerize and bind DNA directly, but rather interacts with Stat1 and IRF9 in response to type I interferons to direct antiviral target gene expression(3). Stat4 and Stat6 are activated by IL-12 and IL-4/IL-13, respectively, and play an essential role in the development of Th1 and Th2 cells(4-7). There are two isoforms of Stat5, Stat5a and Stat5b, and gene targeting studies have underscored specificity for Stat5a in prolactin responses, while Stat5b is essential in mediating growth hormone responses(8-10).

Negative feedback regulation of the Jak/Stat pathway is mediated, in part, by the induction of the suppressor of cytokine signaling (SOCS) family of target genes. There are eight members of the mammalian SOCS family, SOCS1-7 and CIS. They each contain an SH2 domain for phosphotyrosine binding and a SOCS box which mediates Elongin B/C binding and promotes target ubiquitination. SOCS proteins can inhibit the Jak/Stat signaling pathway in multiple ways. SOCS can bind directly to Jaks and act as a pseudosubstrate to inhibit Jak kinase activity, they can bind to the phosphorylated receptor and compete with Stats for receptor binding, and they can target Jak kinases or the receptor itself for proteasome-mediated degradation. Gene knockout studies have demonstrated unexpected specificity for SOCS-dependent Jak/Stat inhibition. SOCS1 is a critical negative regulator of IFNγ signaling *in vivo*(11), whereas SOCS3 plays a central role in the negative feedback regulation of the IL-6 family of receptors(12, 13), and SOCS2 specifically inhibits Stat5b activation in response to growth hormone(14).
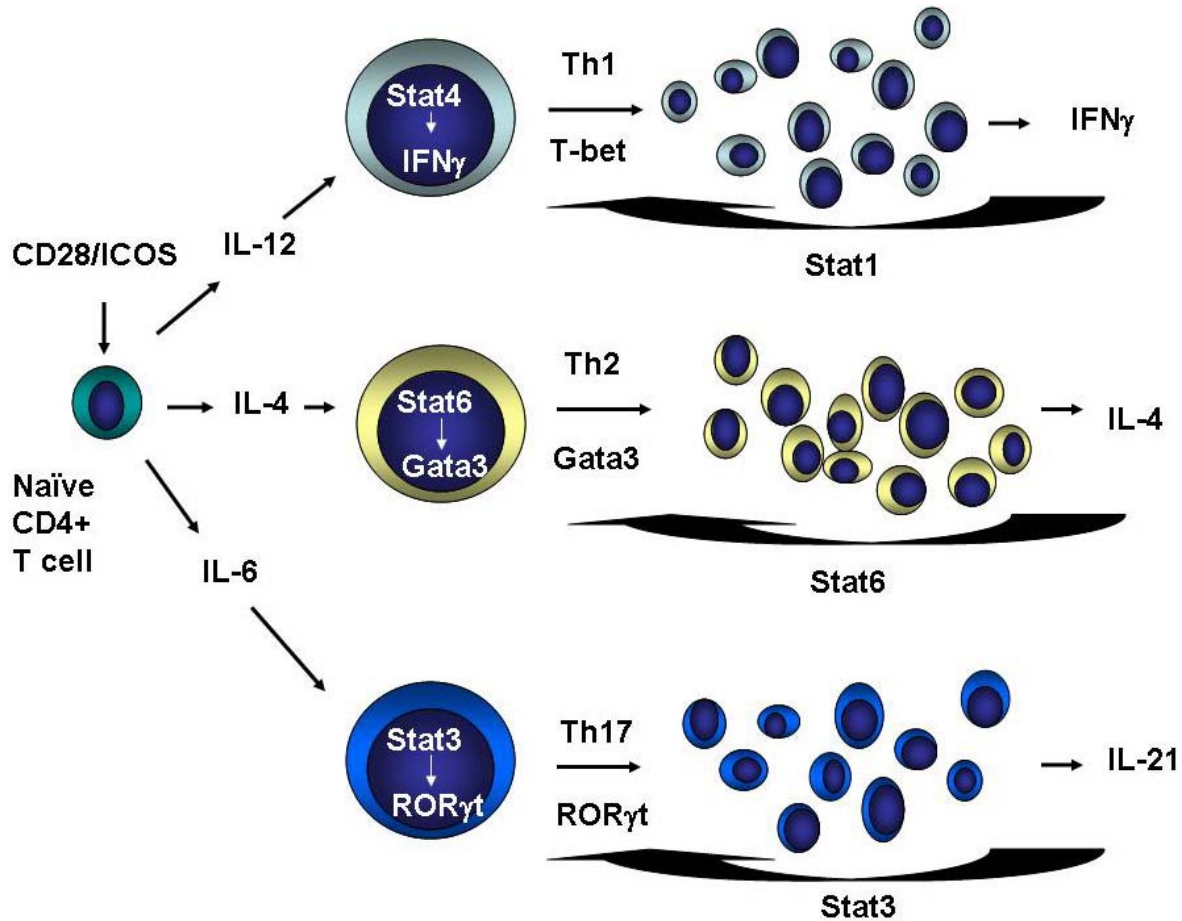
3. OVERVIEW OF STAT3 IN HEMATOPOIETIC DEVELOPMENT

Stat3 is a functionally pleitropic member of the Stat family and transduces signals from the IL-10 and IL-6 families of cytokine receptors. There are two isoforms of Stat3, Stat3α and Stat3β, which is carboxy terminally truncated before the transactivation domain (TAD) (reviewed in (15)). Stat3 is the only Stat family member that is essential for development, however conditional knockouts have revealed a number of critical functions for Stat3 in hematopoietic development and immune regulation (reviewed in detail below). Further, while Stat1 generally inhibits proliferation and promotes apoptosis, Stat3

promotes cell growth and transformation. While the molecular mechanism behind these mutually antagonistic functions of Stat1 and Stat3 remain to be clarified, heterodimerization of Stat1 and Stat3, and competition for receptor binding sites have been implicated (reviewed in (16)).

T helper responses include at least three T cell subsets: Th1, Th2 and Th17. Recent studies have identified a critical role for Stat3 in the development of Th17 cells (Figure 1). The development of T regulatory and Th17 cells appears to be reciprocally regulated during differentiation and Stat3 plays a central role in mediating this lineage specification. IL-6 and TGFβ induce Th17 differentiation in a Stat3-dependent manner(17). Neither naturally occurring Th17 cells nor Th17-dependent autoimmunity occur when Stat3 is ablated in CD4 cells, and these cells are skewed toward a Th1 phenotype in the absence of Stat3(18). While expression of a hyperactive Stat3 promotes Th17 differentiation, Th17 differentiation is impaired in Stat3-deficient T cells, associated with a decrease in ROR-gamma expression and an increase in expression of Foxp3, which promotes T regulatory development(19, 20). Furthermore, IL-21 induced by IL-6 in activated T cells, induces Th17 differentiation, IL21 and IL-23 receptor expression, and suppression of Foxp3 in a Stat3-dependent manner (21). Finally, the inhibition of Th17 development by Foxp3 can be overcome by IL-6, due to Stat3 mediated inhibition of Foxp3 expression(20).

Cases of hyper-IgE syndrome, a compound primary immunodeficiency characterized by a highly elevated serum IgE, are both autosomal dominant and sporadic. Dominant negative mutations in the DNA binding domain of Stat3 have been found in patients with sporadic hyper-IgE syndrome, and mutations in both the DNA binding domain and SH2 domain of Stat3 have been isolated from both familial and sporadic cases(22-25). Increased levels of proinflammatory gene transcripts were observed in unstimulated peripheral blood neutrophils and mononuclear cells, and both unstimulated and LPS stimulated mononuclear cells from these patients cultured *in vitro* produced elevated levels of TNFα. However, peripheral blood cells from these patients were refractory to stimulation with IL-6 or IL-10. Interestingly, Th17 cells were low or absent in the peripheral blood of these patients, and purified T cells harboring the heterozygous Stat3 mutations failed to generate Th17 cells *in vitro* due to insufficient expression of ROR-gamma(24, 26, 27). These studies highlight the crucial role of Stat3 in the development of the Th17 lineage in the human population and may explain the devastating susceptibility of these patients to a narrow spectrum of infections including Staphylococcus aureus and Candida albicans.

In addition to Th17 cells, the development of T follicular helper (Tfh) cells, important in humoral immunity, requires Stat3, but is independent of the Th17 lineage(28). Stat3 also plays a critical role in the development of B cells at various stages. Deletion of Stat3 in the B cell lineage results in a cell automonous increase in pre-pro-B cells, but a decrease in the pre-B and pro-B
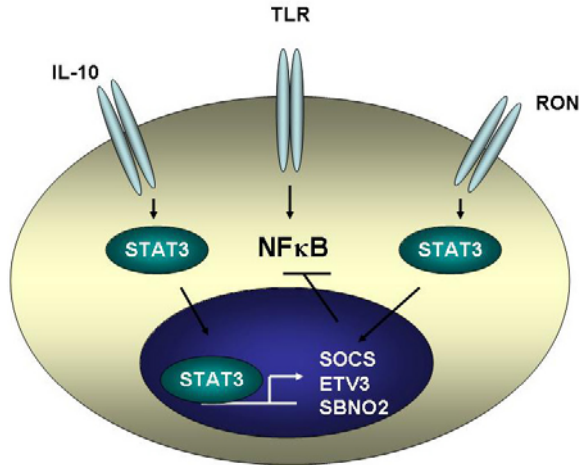
Figure 1. Stat3 promotes the differentiation of Th17 cells. The development of T helper cell subsets is dependent upon the cytokine environment. While IL-12 and IL-4 promote the development of Th1 and Th2 cells respectively, IL-6 plays a critical role in promoting Th17 cell development. Stat activation downstream of cytokine stimulation selectively and specifically mediates T helper cell differentiation. Stat4 is required for the development of Th1 cells, Stat6 for the development of Th2 cells and Stat3 for the development of Th17 cells. Cytokines produced by the developing T helper cells provide positive feedback through the activation of Stats to promote the expression of lineage-specific transcription factors. IFNγ produced by Th1 cells induces expression of T-bet in a Stat1-dependent manner. IL-4 produced by developing Th2 cells enhances Stat6 activation and the induction of Gata3 expression. IL-21 produced by developing Th17 cells feeds back to further induce Stat3 activation and the expression of RORγt.

compartments, suggesting that Stat3 regulates an early step in B cell development(29). In addition, pro-B cells from these animals exhibited enhanced apoptosis following cytokine withdrawal, suggesting that Stat3 may regulate both differentiation and survival of the B cell lineage. A critical role for Stat3 was also described in the T cell-dependent terminal differentiation of plasma cells(30). Consistent with this result, BCL-6, a gene involved in chromosomal translocations in B cell lymphomas, suppresses the differentiation of B cells to plasma cells, by inhibiting Stat3-dependent expression of B lymphocyte-induced maturation protein (Blimp-1), a major regulator of plasma cell development(31).

While Stat3 promotes the development of B and T cell subsets, Stat3 activation by G-CSF appears to play a negative role in the regulation of granulopoiesis. Mice with a targeted deletion in Stat3 unexpectedly exhibit neutrophilia and are hyperresponsive to G-CSF(32). This phenotype is associated with the inability of G-CSF to induce the expression of SOCS3(33). SOCS3 is induced by Stat3 and functions as a feedback inhibitor of the IL-6 receptor by binding to the phosphorylated Y757 on the gp130 cytoplasmic domain(12, 13). Consistent with the lack of SOCS3 induction in Stat3 deficient animals, mice with a targeted deletion of SOCS3 also display neutrophilia and enhanced responses to G-CSF(34, 35). Furthermore, SOCS3 deficient progenitor cells stimulated with IL-6 or G-CSF are skewed toward macrophage development at the expense of neutrophil development(36). However, while G-CSF-induced Stat3 does not appear to be required for granulocyte development, Stat3 plays a crucial role in emergency granulopoiesis and mature neutrophil function(37). Taken together, these studies highlight both positive and negative roles for Stat3 in mediating signaling by G-CSF.

Figure 2. Stat3 inhibits classical macrophage activation. Stimulation of Toll-like receptors (TLRs) by pathogen associated molecular patterns (PAMPs) induces the activation of NFκB and the upregulation of pro-inflammatory gene expression. Several anti-inflammatory receptors, including IL-10 and the Ron receptor tyrosine kinase, induce activation of Stat3 which has anti-inflammatory properties. While the mechanism by which Stat3 inhibits pro-inflammatory gene expression is unclear, upregulation of genes including SOCS3, ETV3 and SBNO2 by Stat3 may contribute to the inhibition of TLR-induced NFκB activity.

Deletion of Stat3 causes a profound deficiency in the dendritic cell (DC) compartment and abrogates the effects of Flt3L on DC development. These mice exhibit increased numbers of common lymphoid and myeloid progenitors (CMP/CLP), but an absence of common DC progenitors, suggesting that activation of Stat3 by Flt3 regulates the commitment of CMP/CLP to the DC linage(38). Mice lacking the transcription factor Gfi1 also exhibit a decrease in myeloid and lymphoid DCs, associated with a decrease in Stat3 activity. Interestingly, *in vitro*, progenitor cells from these mice failed to differentiate into DCs, but rather differentiated into macrophages suggesting that Gfi1, and possibly Stat3, determine the lineage commitment of DCs vs. macrophages(39). However, while Stat3 is necessary for expansion of DC progenitors, it is not required for DC maturation(40). Conversely, IL-6 receptor knockout mice exhibit increased numbers of mature DCs suggesting that IL-6 blocks DC maturation, and this effect requires binding of Stat3 to the IL-6 receptor(41). Interestingly, DCs in the tumor microenvironment are usually immature. Elevated levels of cytokines such as IL-6, IL-10 and G-CSF produced by tumor cells induce activation of Stat3 in myeloid cells and inhibit DC activation resulting in the accumulation of immature myeloid cells(42, 43). Ablation of Stat3 signaling in DCs, either by using a phosphopeptide inhibitor of Stat3 or by deleting the Stat3 gene, abrogated tumor-induced inhibition of DC functional maturation, and decreased the number of immature DCs, indicating that Stat3 activity in the DCs is critical for this response(44, 45). Taken together, these studies suggest that targeting

Stat3 in cancer could promote DC maturation and tumor immunity.

Little is known regarding the role of Stat3 in the hematopoietic stem cell compartment. Transduction of primitive murine fetal liver cells with a dominant negative Stat3 markedly reduced the *in vivo* lympho-myeloid reconstituting ability of these cells, without affecting the short term repopulating ability of these cells, indicating a role for Stat3 in the regulation of the fetal stem cell compartment(46). This is supported by studies in which fetal cells from the AGM (aorta-gonad-mesonephros) region, the initial site of definitive hematopoiesis, from gp130 deficient mice were cultured in vitro. While the stem cell population in these cultures failed to expand, reintroduction of a wild-type, but not a mutant gp130 which cannot activate Stat3, rescued this defect, while expression of a dominant negative Stat3 in cells from wild-type mice suppressed the expansion of these cells(47). The deletion or constitutive activation of Stat3 in adult hematopoietic stem cells did not affect the in vitro proliferation or multilineage differentiation potential of these cells(48). However, expression of a constitutively activate form of Stat3 in adult mouse bone marrow stem cells increased their regenerative activity in lethally-irradiated recipients while a dominant negative form of Stat3 suppressed this activity. These studies indicate that Stat3 promotes hematopoietic stem cell self renewal in the early phase of hematopoietic stem cell regeneration, but not under homeostatic, conditions(49).

4. STAT3 IN MACROPHAGE ACTIVATION AND INNATE IMMUNITY

The pivotal role of Stat3 in the regulation of macrophages came from studies in which Stat3 was deleted in macrophages and neutrophils. These mice are highly susceptible to endotoxic shock, exhibit polarized Th1 immune responses and develop chronic enterocolitis with age(50). This phenotype is very similar to the phenotype of IL-10 knockout mice, which also develop chronic colitis(51). The suppressive effects of IL-10 on macrophage activation are completely abolished in the absence of Stat3 indicating that Stat3 plays a central role in mediating the anti-inflammatory effects of IL-10 (Figure 2). This observation is supported by expression studies which indicate that most, if not all, of the effects of IL-10 on LPS induced gene expression are Stat3 dependent(52, 53). Furthermore, expression of a constitutively active Stat3 can replicate the suppressive effects of IL-10 in human primary macrophages(54). Further studies indicated that the development of colitis in the mice lacking Stat3 in myeloid cells is dependent on the production of IL-12p40, and is mediated by TLR4-dependent signaling(55). A similar approach, in which Stat3 was deleted early in hematopoietic development, found that these mice develop Crohn's disease-like pathogenesis within 4-6 weeks of birth, with increased cell autonomous myeloid proliferation and enhanced NFκB activation(38). Taken together, these studies clearly identify the activation of Stat3 by IL-10 as a critical negative regulator of macrophage activation both *in vitro* and *in vivo*.

Subsequent studies have further characterized the role of Stat3 in the regulation of innate and acquired immune responses. Disruption of Stat3 in macrophages and neutrophils results in enhanced susceptibility to septic peritonitis associated with an increase in production of inflammatory cytokines by resident macrophages, but impaired bactericidal activity(56). Leukocyte infiltration and enhanced expression of TNFα, MIP-2, KC and MCP-1 in this model of acute peritonitis were enhanced, and this response could be recapitulated by adoptive transfer of resident macrophages lacking Stat3, indicating that Stat3 plays an important role in the regulation of resident macrophages(57). In addition to innate immune responses, Stat3 signaling in antigen presenting cells plays a critical role in the induction of antigen-specific T cell tolerance. While targeted disruption of Stat3 in antigen presenting cells promoted the response of CD4+ T cells to tolerogenic stimuli, increased Stat3 activity in antigen presenting cells lead to impaired antigen-specific T cell responses(58). The importance of macrophage Stat3 in the regulation of T cell responses is highlighted by the observation that mice lacking Stat3 in macrophages fail to develop colitis when crossed to RAG knockout animals(55, 59). In contrast, T cell specific Stat3 knockout mice show impaired T cell proliferation through preventing apoptosis(60), and inactivation of the IL-6/Stat3 signaling cascade in CD4+ T cells results in the suppression of acquired immune (T cell) mediated colitis, suggesting that the function of Stat3 in regulating the progression of IBD is cell-type specific(61-63).

IFNγ efficiently primes macrophages for inflammatory activation by TLRs and other cytokines through the activation of Stat1. Activation of Stat1 by IFNγ promotes the expression of pro-inflammatory genes induced by TLR stimulation and opposes the activities of Stat3 induced by IL-10(64). Other cytokines, including IFNα/β and IL-6 can activate both Stat1 and Stat3, and the balance between these transcription factors can determine the balance between pro- and anti-inflammatory functions(65). While IL-10 induces its own expression via a Stat3-dependent mechanism(66), IFNγ suppresses TLR-mediated induction of IL-10 expression and downstream Stat3 activation, thereby interrupting Stat3-mediated feedback inhibition(67). In addition, IFNγ switches the balance of IL-10 Stat activation from Stat3 to Stat1(68), while the increase in Stat1 expression during priming with IFNγ inhibits the induction of Stat3-dependent pathways by subsequent IFNγ stimulation(69). These studies highlight the critical role of Stat3 in attenuating the potentially tissue-damaging effects of Stat1. Toxoplasma gondii mediates the IL-10-independent activation of Stat3 and inhibits macrophage IL-12 production in a Stat3-dependent manner(70), thus underlining the potential for invading pathogens to alter this balance to promote their survival.
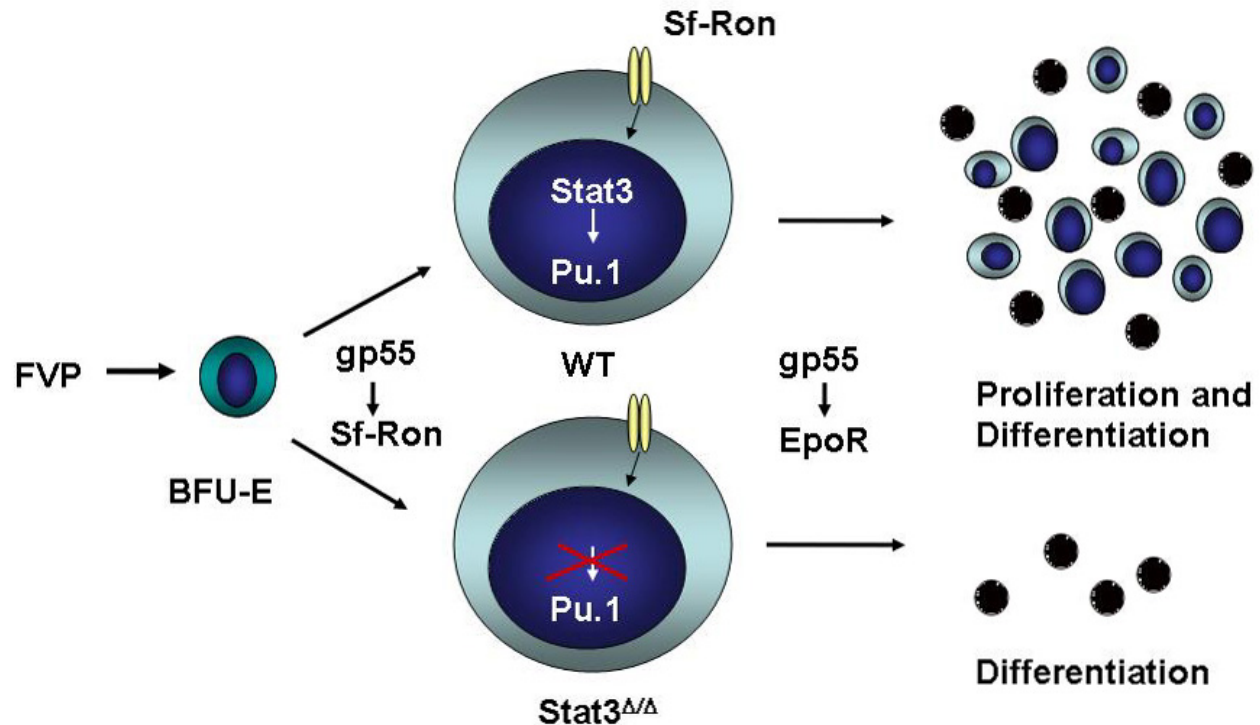
Receptor tyrosine kinases also play a critical role in regulating macrophage activation and innate immune responses. Activation of the Ron receptor tyrosine kinase by its ligand macrophage stimulating protein (MSP) inhibits TLR-induced NFκB activity(71), and IL-12p40 expression in an IL-10-independent manner(72), and mice with a targeted deletion in Ron exhibit enhanced susceptibility to septic shock due, in part, to increased IL-12-mediated IFNγ production by NK cells(73). Signaling through the Ron receptor inhibits IFNγ-induced Stat1 activation, while promoting the phosphorylation of Stat3(73), thus tipping the balance of these pro- and anti-inflammator mediators (Figure 2). Likewise, the TSC-mTOR signaling pathway is a negative regulator of innate inflammatory responses. While inhibition of mTOR promotes pro-inflammatory cytokine production and inhibits release of IL-10 mediated by Stat3, activation of mTOR diminishes NFκB activity and enhances Stat3 activation, thus reversing the proinflammatory cytokine shift(74). Mice deficient for the Mer receptor tyrosine kinase are also more susceptible to septic shock due to enhanced macrophage activation(75) and a triple mutation in the related Tyro3, Axl and Mer (TAM) receptors exhibit a severe lymphoproliferative disorder and systemic autoimmunity(76). However, while both Ron and the TAM receptors induce expression of SOCS1 and SOCS3 in macrophages, the TAM receptors paradoxically induce the activation of Stat1, but not Stat3, in these cells(77).

While it is clear that Stat3 plays a critical role in macrophage activation, the mechanism by which Stat3 mediates these effects remains unclear. Several studies suggest that sustained activation of Stat3 may be critical for its anti-inflammatory effects. Both IL-6 and IL-10 induce Stat3, however IL-6 has pro-inflammatory effects while IL-10 is anti-inflammatory. The difference could be due to the induction of SOCS3 which inhibits IL-6-, but not IL-10-, induced Stat3 resulting in transient activation of Stat3 by IL-6 but sustained activation by IL-10. Interestingly, deletion of the SOCS3 binding site in gp130 results in anti-inflammatory signaling by IL-6(78, 79). SOCS3 has also been implicated in directly mediating the inhibitory effects of IL-10 on LPS-induced macrophage activation(80). In addition, administration of SOCS3 by intracellular protein therapy or gene delivery protected mice from the lethal effects of LPS(81, 82). IL-10 also activates the expression an ETS family transcriptional repressor, ETV3, and a helicase family corepressor, SBNO2, in a Stat3-dependent manner (Figure 2), and expression of ETV3 and SBNO2 repressed NFκB-activated transcriptional reporters(83). Furthermore, a constitutive active Stat3 interacted with alphaCP-1, a novel RNA binding protein with specificity for C-rich pyrimidine tracts and coexpression of alphaCP-1 augmented the suppressive effect of Stat3C on an NFκB reporter whereas knockdown of alphaCP-1 reduced this effect(84). Further studies will be needed to clarify the molecular mechanisms involved in the suppression of macrophage activation by Stat3.

5. ROLE OF STAT3 IN HEMATOPOIETIC TRANSFORMATION

Following the initial discovery that Stat3 is required for cellular transformation by v-src(85, 86), and that a constitutive active Stat3 molecule itself can lead to cellular transformation(87), evidence for a critical role for Stat3 in transformation has steadily accumulated. v-Src induces an erythroleukemia in mice, and while v-src

Figure 3. Stat3 is essential for the progression of Friend erythroleukemia. Infection of erythroid progenitor cells (BFU-E) by Friend erythroleukemia virus induces a multi-stage erythroleukemia in mice. The viral glycoprotein, gp55, interacts with the erythropoietin receptor (EpoR) and a truncated form of the Ron receptor tyrosine kinase (Sf-Ron) to promote disease. While activation of Sf-Ron promotes the proliferation of infected erythroblasts, EpoR activation promotes the terminal differentiation of these cells. Phosphorylation of Stat3 by Sf-Stk following Friend virus infection induces the expression of the transcription factor Pu.1, which counteracts the differentiation signals induced by EpoR activation to promote the expansion of infected erythroblasts prior to terminal differentiation. In the absence of Stat3, Pu.1 expression is low and the cells fail to expand in response to viral infection, rendering these mice resistant to the resulting erythroleukemia.

induces Stat3 activation and cellular transformation in wild-type, but not Stat3-/- MEFs(88), c-Src overexpression in Csk-/- MEFs does not induce considerable Stat3 activation(89). Similarly, the critical role of Stat3 in hematopoietic transformation is underscored by the observation that other oncogenic retroviruses that induce hematologic malignancies also induce aberrant activation of Stat3. The Mer receptor tyrosine kinase, cloned from a B-lymphoblastoid library and found ectopically expressed in pediatric ALL patients(90), is the mammalian orthologue of the chicken retroviral oncogene v-Eyk(91). Transgenic overexpression of Mer in hematopoietic cells induces a lymphoblastic leukemia/lymphoma(92). Interestingly, a single amino acid substitution in the v-Eyk intracellular domain, resulting in the generation of a Stat3 binding motif, promotes activation of Stat3 and enhances cellular transformation(93). The Ron receptor tyrosine kinase is aberrantly expressed in patients with mediastinal large B cell lymphoma and classic Hodgkin lymphoma(94, 95). Our studies demonstrate that Stat3 is tyrosine phosphorylated downstream of a truncated form of the Ron receptor tyrosine kinase, Sf-Ron, activated by the viral oncoprotein, gp55, encoded by the Friend erythroleukemia virus(96). Using Stat3$^{fl/fl}$ mice, we demonstrated that Stat3 plays a critical role in promoting the polyclonal expansion

of infected cells in the early stages of Friend disease, but is dispensable at later stages of leukemic transformation. In this model, the primary role of Stat3 is to inhibit erythroid differentiation, in part through upregulation of Pu.1 expression, thus promoting the expansion of infected erythroblasts (Figure 3).

Constitutive activation of receptor tyrosine kinases mediated by mutations in the kinase and juxtamembrane domains is a hallmark of many maliganancies, and many of these mutations also lead to alterations in Stat activation. The Asp(816) mutant of the Kit receptor, found in patients with mastocytomas, induces the constitutive activation of Stat1 and Stat3, and activation of Stat3, but not Stat1, is required for the ability of the mutant Kit receptor to promote tumorigenicity and cytokine-independent growth of leukemic cells(97, 98). Interestingly, Stat proteins are also preferentially phosphorylated by juxtamembrane domain mutants of Kit found preferentially in patients with gastrointestinal stromal tumors (GIST) (99). Alternatively, the Flt3 receptor, harboring an internal tandem duplication (Flt3-ITD) in the juxtamembrane domain found in patients with AML, but not the wild-type receptor or an activated receptor harboring a mutation in the kinase domain of Flt3 (Flt3-

TKD), induces the activation of Stat5(100), which appears to be a direct target of Flt3-ITD(101). In a bone marrow transplantation model, Flt3-ITD induces a myeloproliferative syndrome, however Flt3-TKD induces a lymphoid disorder with longer latency(102). These distinct phenotypes could be due to differential activation of Stats by the Flt3 mutants.

Many chromosomal translocations that promote leukemic development have also been shown to activate Stat3. The Alk kinase is constitutively activated in anaplastic large cell lymphomas (ALCLs) through a chromosomal translocation which joins the nucleoplasmin (NPM) gene to the Alk kinase(103). ALK activates Stat3 and protects hematopoeitic cells from cell death(104), and studies using mice in which Stat3 is deleted in B and T cell populations demonstrated that Stat3 is required for the development of B cell lymphoma in NPM-ALK transgenic mice and for the growth and survival of human and mouse NPM-ALK-transformed B and T cells(105). Furthermore, administration of antisense oligonucleotides targeted against Stat3 impaired the growth of NPM-ALK tumors *in vivo*, demonstrating the potential for Stat3 as a pharmacological target in the treatment of lymphoma. NPM-ALK also promotes the expression of IL-22, which binds to a heterodimeric receptor composed of IL-22R1 and IL-10R2, and contributes to the activation of Stat3 and tumorigenicity in ALk+ ALCL in an autocrine fashion(106). Interestingly, Stat3 expression also contributed to impaired immune surveillance by conferring properties of regulatory T cells to the T cell lymphoma(107). Constitutive tyrosine and serine phosphorylation of Stat3 was also detected in chronic myelogenous leukemia (CML) patients harboring the BCR-ABL translocation(108), and Bcr-Abl was able to induce both tyrosine and serine phosphorylation of Stat3 in cell lines and in primary CD34+ CML cells(109). Activation of Stat3 by other products of chromosomal translocations including TEL-JAK2, TEL-ABL, and TEL-protein tyrosine phosphatase receptor-type R (PTPRR) have also been reported(110, 111).

Activation of Stat3 in leukemic transformation can also occur indirectly via the inhibition of negative regulators of Stat signaling. Several translocations associated with acute promyelocytic leukemia (APL), including Stat5b-RARalpha, PML-RARalpha and promyelocytic leukemia zinc finger (PLZF)-RARalpha have been shown to enhance IL-6 induced Stat3-dependent reporter activity(112). PML normally forms a complex with Stat3, inhibiting its DNA binding activity. While PML/RARalpha does not associate with Stat3, it dissociates PML from Stat3 and restores Stat3 activity, resulting in enhanced gp130-dependent growth(113). In addition, several studies have identified methylation of the Stat inhibitors Socs1 and Shp1 as a mechanism by which Stat activation could be enhanced in AML(114-116). Interestingly, Stat3 induces DNA methyltransferase 1 (DNMT1) expression in malignant T cells(117), and Stat3, DNMT1 and histone deacetylase 1 form complexes that bind to the Shp1 promoter, resulting in epigenetic silencing of Shp1(118). Patients with severe congenital neutropenia
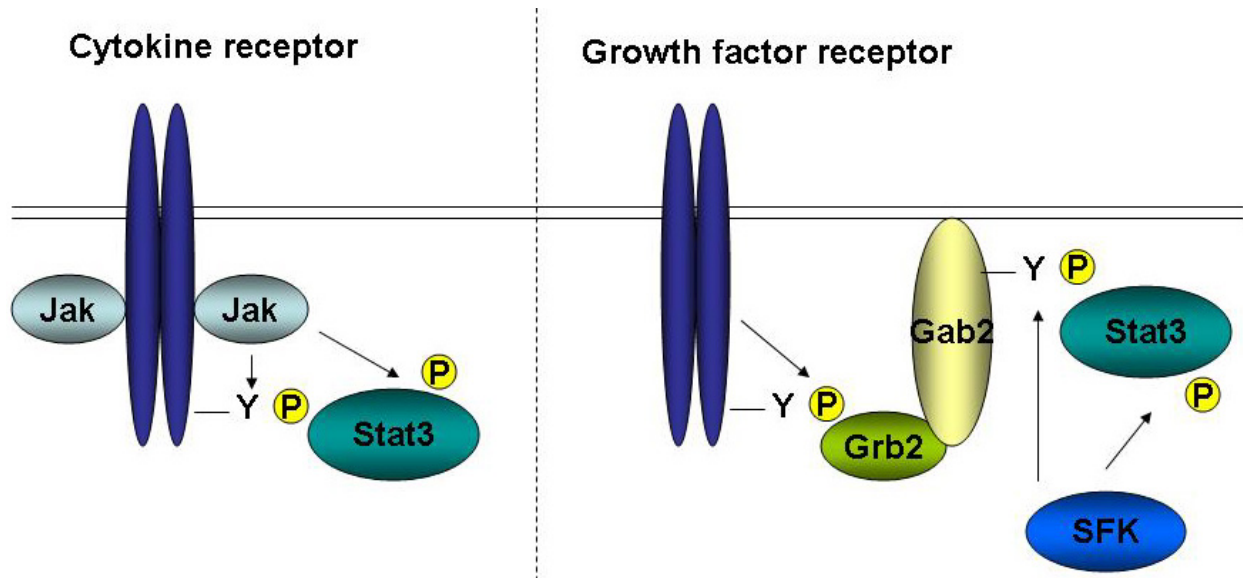
due to c-terminal truncations in the G-CSF receptor are predisposed to AML, and this region of the G-CSF receptor has been shown to negatively regulate Stat activation in a Shp1-dependent manner(119). Conversely, SOCS3, a negative feedback regulator of Stat3 induced by Sat3, is highly expressed in ALK+ anaplastic large cell lymphoma cell lines(120). Studies using Socs3-deficient MEFs suggest that, in the absence of Socs3, Stat3 function changes from anti-apoptotic to pro-apoptotic, indicating that co-expression of Socs3 and Stat3 may be critical for the survival of transformed cells in which Stat3 is constitutively activated(121).

Finally, Stat3 transcription itself can be a target of oncogenic transformation. Recent studies have demonstrated that the Stat3 gene is a target of the HMGA1 (high-mobility group A1) oncogene. HMG1a induces expression of a Stat3 promoter in transfection experiments and binds to a conserved region of the Stat3 promoter in leukemic cells as demonstrated by ChIP. HMG1a transgenic mice develop an aggressive lymphoid malignancy, and blocking Stat3 function induced apoptosis in the transgenic leukemia cells(122).

6. ACTIVATION OF STAT3 BY CYTOKINE AND GROWTH FACTOR RECEPTORS

Recent studies using peptide immunoblot affinity assays demonstrated that only phosphotyrosine peptides containing +3 Gln (not Leu, Met, Glu or Arg) bind to the SH2 domain of Stat3. Mutation of three amino acids within the SH2 domain of Stat3 resulted in loss of YxxQ binding. The side chains of Lys591 and Arg609 interact with pTyr. Glu638 amide hydrogen bonds with oxygen within the +3 Gln side chain(123). Several cytokine receptors lead to recruitment and activation of Stat3 via the presence of the canonical YxxQ motif in the cytoplasmic tail of the receptor. Gp130, a subunit of the IL-6, IL-12, IL-27 and LIF family of cytokine receptors harbors the signature YxxQ motif which is required for the activation of Stat3 downstream of this family of cytokines(124). In addition, the G-CSF receptor contains a YxxQ motif as well as an alternate binding motif, YxxC, in the c-terminal tail of the receptor(125). Chimeric analysis revealed that two YxxQ motifs in the c-terminal tail of the LIF receptor are also critical for Stat3 activation by this receptor(126). The recruitment of Stat3 to these receptors results in the phosphorylation of Stat3 by associated Jak kinases. Jak kinases have also been shown to directly recruit Stat5 through the JH2 domain of Jak and the carboxy terminus of Stat5, however this direct interaction has not been demonstrated for Stat3(127).

While most tyrosine kinase receptors also activate Stat3, few contain a canonical Stat3 binding motif. We have identified a novel YxxQ motif in Gab2, and demonstrated a requirement for this site in the activation of Stat3 by the Ron receptor tyrosine kinase(96). Gab2 is a large adaptor protein (related to the insulin substrate family of adaptors) that is recruited to receptor complexes through a proline-rich motif that binds to the N-terminal SH3 domain of Grb2, and a PH domain that promotes

Figure 4. Activation of Stat3 by cytokines and growth factors. Cytokine receptors, including gp130, contain canonical Stat3 binding motifs. Upon cytokine stimulation, these motifs become tyrosine phosphorylated by Jak kinases, resulting in the recruitment and activation of Stat3. Most growth factor receptors are reported to activate Stat3, however, in most cases, these receptors do not harbor Stat3 binding sites. One possible mechanism for the activation of Stat3 by growth factor receptors is through the recruitment of a Grb2/Gab2 complex. Gab2 contains a canonical Stat3 binding site which could result in the indirect recruitment of Stat3 to the receptor signaling complex. The activation of Stat3 by growth factor receptors requires Src family kinases (SFKs), suggesting that SFKs may promote the phosphorylation of the Stat3 binding site on Gab2, Stat3 itself, or both.

recruitment to the plasma membrane in a PI3kinase-dependent manner. There are three mammalian Gab proteins, Gab1, Gab2 and Gab3, which all contain five conserved tyrosines in the c-terminal region, two of which bind to Shp2 and three of which are docking sites for p85. However, recruitment of Gab2, but not Gab1, to the Ron receptor is sufficient to induce cytokine-independent growth of primary erythroblasts infected with Friend virus(128), and this transforming event is dependent upon the Stat3 binding motif in Gab2, which is not found in Gab1(96). A c-fms interacting protein, STAP2, which is phosphorylated following EGF stimulation, also contains a YxxQ motif(129) suggesting that other adaptors could also mediate Stat3 activation downstream of growth factor receptors.
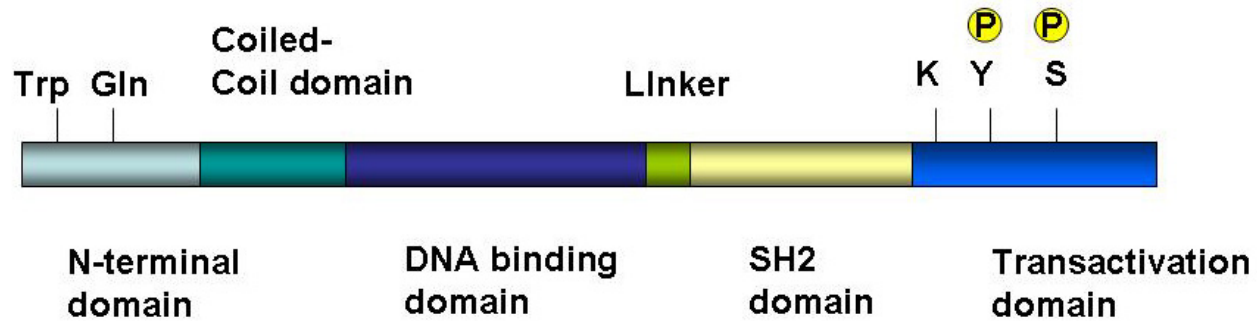
While the mechanism by which most tyrosine kinases lead to activation of Stat3 remains unclear, the Src family of non-receptor tyrosine kinases have been implicated in the activation of Stat3 downstream of the EGF, ErB2 and PDGF receptors(130-132). Interestingly, Src family kinases have also been implicated in the phosphorylation of Gab2. Hck has been shown to phosphorylate Gab2 in multiple myeloma cells(133), whereas Lyn and Syk are required for the phosphorylation of Gab2 downstream of FcεR1(134) and GCSF-induced Gab2 phosphorylation is dependent on the Lyn kinase(135). These studies raise the possibility that recruitment and activation of Stat3 by a variety of cell surface receptors could be mediated by a Grb2/Gab2 complex and dependent on the phosphorylation of Gab2 by Src family kinases (Figure 4). A Grb2/Gab2 signaling complex has also been implicated in the development of chronic myelogenous leukemia (CML) induced by expression of the Bcr/Abl fusion protein(136), and the Lyn kinase phosphorylates Gab2 in BCR/ABL+ CML cells that are resistant to imanitib, an inhibitor of the Abl kinase(137). Furthermore, constitutively active forms of Shp2, found in juvenile myelomonocytic leukemia (JMML), induce transformation of murine bone marrow cells and cause a fatal JMML-like disorder in a Gab2-dependent manner(138). It will be interesting to determine whether activation of Stat3 mediates transformation by Gab2 in these experimental models.

7. REGULATION OF STAT3 BY POST-TRANSLATIONAL MODIFICATION.

While it is long recognized that tyrosine phosphorylation of Stat3 on Y705 (Figure 5) promotes the dimerization and nuclear localization of Stat3 monomers resulting in DNA binding and the subsequent induction of gene expression, it is becoming evident that unphosphorylated Stat3 can also induce transcriptional responses. The induction of Stat3 phosphorylation induces an early wave of gene expression, including the expression of Stat3 itself. The accumulation of unphosphorylated Stat3 in these cells can induce a second wave of gene expression via mechanisms that are both NFκB-dependent and NFκB–independent(139). In the induction of NFκB-dependent gene expression, unphosphorylated Stat3 binds to unphosphorylated NFκB in competition with IκB and the resulting Stat3/NFκB dimmer localizes to the nucleus and induces expression of a subset of NFκB-dependent

Figure 5. Domain organization of Stat3. Stat3 contains an N-terminal domain, followed by a coiled-coil domain, a DNA binding domain, a linker region, an SH2 domain and a transactivation domain (TAD). Post-translation modification of the TAD on Tyr705 promotes the dimerization and nuclear localization of Stat3, whereas phosphorylation of Ser727 in the TAD promotes transcriptional activation. Acetylation of Lys685 in the TAD domain as also been reported to play a critical role in Stat3 dimerization. The N-terminal domain mediates Stat3 tetramerization and recruitment of co-activators or co-repressors. Trp37 and Gln66 in the N-terminal domain are reported to play a critical role in tetramer formation.

genes(140). However, while Stat1 can dimerize in an anti-parallel conformation via extended interfaces of the globular N domain and bind DNA in a tyrosine phosphorylation-independent manner(141), this interface is absent in the Stat3 structure, and studies indicate that the core fragment of Stat3, unlike Stat1, is primarily monomeric(142). In addition, a constitutive active form of Stat3 in which cysteine residues are engineered into the carboxy terminus of each Stat3 molecule to promote dimerization, still requires tyrosine phosphorylation of the preformed dimers to transactivate Stat3 target genes(143). These results suggest that tyrosine-independent homodimerization of Stat3 is not likely to mediate the transcriptional responses induced by the unphosphorylated form of Stat3.

Phosphorylation of Stat3 on serine 727 (Figure 5) also plays a critical role in the regulation of Stat3 activity. Mice harboring knock-in alleles at S727A were grossly normal, and MEFs from these animals displayed a 50% reduction in transcriptional responses, suggesting that this residue is not essential for Stat3 function. However, when these mice were crossed to Stat3 null animals to generate heterozygotes (S727A/-), these mice showed growth retardation and a high incidence of perinatal lethality(144). While the remaining mice failed to show any developmental abnormalities, these mice were more sensitive to LPS-induced septic shock(145). Stat3 exists in two isoforms, the full-length Stat3α and a truncated Stat3β in which the transactivation domain and S727 are missing. Consistent with the knock-in studies, experiments in mice in which these isoforms were ablated individually by gene targeting indicate that Stat3α is the primary mediator of IL-10 function in macrophages(146). Interestingly, phosphomimetic mutation of S727 in the presence of a Y705 to phenylalanine mutation resulted in enhanced anchorage-independent growth of human prostate cancer cells in soft agar and increased tumorigenicity in NOD/SCID mice, associated with increased nuclear localization of Stat3(147). Furthermore, recent studies indicate that serine, but not tyrosine, phosphorylation of Stat3 is required for RANKL-induced osteoclastogenesis,

whereas tyrosine phosphorylation by IL-6 lead to the development of macrophages instead of osteoclasts(148). In addition, constitutive phosphorylation of serine, but not tyrosine, on Stat3 has been demonstrated in B lymphocytes from patients with chronic lymphocytic leukemia (CLL)(149). These studies suggest that tyrosine and serine phosphorylation of Stat3 may play both overlapping and distinct roles in the regulation of Stat3 function.

Stat3 can promote its own serine phosphorylation by acting as a scaffold for the kinases TAK1 and NLK following binding to gp130 which, in turn, promote S727 phosphorylation of Stat3(150). However, the mechanism by which serine phosphorylation of Stat3 regulates gene expression is not entirely clear. Sequences in the c-terminal domain of Stat1 and Stat3 are responsible for regulating stimulus specific phosphorylation of S727 and differentially affecting target gene expression(151). The region from amino acid 752-761 on Stat3 is required for S727 phosphorylation and recruitment of SRC-1(152). S727 itself is found within a LPMSP motif that is conserved among the transcription activation domains of several Stats, and S727 as well as other sites within this motif are required for the recruitment of the transcription co-activator CBP/p300 to promoters of Stat3 target genes(153). Recent studies indicate that the peptidyl-prolyl cis/trans isomerase 1 (Pin1) interacts with Stat3 upon cytokine stimulation. Overexpression of Pin1 promotes target gene expression and recruitment of p300 and this activity of Pin1 is dependent on S727 in Stat3(154). Alternatively, S727 mediates association of Stat3 with GRIM-19 which inhibits transcription driven by activated Stat3, but not Stat1(155). Thus serine phosphorylation of Stat3 has the potential to regulate interactions with both positive and negative regulators of transcription.

The potential for other post-translational modifications to regulate Stat3 transcriptional activity have recently been explored. Stat3 was shown to be acetylated at K685 (Figure 5) following cytokine stimulation, and a lysine to arginine substitution at this position demonstrated that this site was critical for Stat3 to form stable dimers

required for DNA binding and transcriptional regulation(156). The acetylation of K685 was confirmed by generation of a acetyl-specific antibody against Stat3(157). Additionally, acetylation of Stat3 was reported to promote the processing of NFκB p100 to p52 and this processing required the acetyltransferase activity of CBP/p300(158). However, the role of acetylation in the regulation of Stat activity remains controversial(159). In addition, recent studies have suggested a role for Arg31 methylation (Figure 5) in promoting Stat1activity by inhibiting the association of Stat1 with PIAS1(160). The increased association of PIAS1 with Stat1 in the absence of arginine methylation was also reported to prolong the half-life of tyrosine phosphorylated Stat1 by decreasing its association with the protein tyrosine phosphatase, TC-PTP(161). However, subsequent studies failed to detect arginine methylation of either Stat1 or Stat3, or a role for protein methyltransferases in the regulation of Stat transcriptional activation(162, 163). More studies will need to be performed to determine the role of acetylation and/or methylation in the regulation of Stat3 function.

The N-domain of Stat3 has also been implicated in regulating transcriptional activation of Stat3 target genes. The N-terminal domain of Stat3 has been reported to regulate the association of Stat3 with the p300 bromodomain thus stabilizing enhanceosome assembly. Despite equivalent levels of Stat3 binding, cells expressing an N-terminally truncated Stat3 mutant (Stat3-deltaN) were unable to recruit p300 and RNA polymerase II to the native Socs3 promoter as efficiently as those expressing full-length Stat3(164). Alternatively, association of the N-domain of Stat3 with histone deacetylase 1 (HDAC1) leads to transcriptional repression associated with a decrease in the nuclear localization of Stat3(165). Both the N- and C-domains of Stat3 are required for complex formation with Cdk9, which binds to the proximal gamma-FBG promoter and increases loading of RNA polymerase II on the TATA box and coding regions and Cdk9-dependent phosphorylation of CTD RNA pol II(166). Residues in the N-terminal domain of Stat3 are also critical for stable tetramer formation of purified phosphorylated Stat3 and for optimal activation of the alpha2-macroglobulin gene(167). However, tetramer formation through adjacent Stat binding motifs does not appear to be required for induction of SOCS3 gene expression by Stat3(168). Our data indicate that the N-terminal domain of Stat3 plays a key role in the induction of Pu.1 expression, and cytokine-independent colony formation, in primary bone marrow-derived cells infected with Friend erythroleukemia virus. These studies suggest that regulation of gene expression by the N-domain of Stat3 is likely to be highly context dependent.

8. SUMMARY

Stat3 plays a central role in hematopoietic cell development, immune cell function and leukemic transformation. Thus, Stat3 is an attractive candidate as a therapeutic target for a wide range of diseases. The potential for targeting Stat3 activity in cancer, both in cancer cells themselves and in the tumor microenvironment to regulate inflammation and tumor immunity, has received a great deal of attention in recent years. However the development of effective therapeutics against Stat3 has remained a challenge. A better understanding of the mechanism by which Stat3 regulates these responses, will allow for the development of more effective therapeutics and/or lead to the discovery of additional therapeutic targets in cancer and other immune-related diseases.

9. ACKNOWLEDGMENT

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Abbreviations: signal transducer and activator of transcription (Stat), janus kinase (Jak), suppressor of cytokine signaling (SOCS), interferon (IFN), toll-like receptor (TLR), jak homology (JH), severe combined immunodeficiency (SCID), transactivation domain (TAD), src homology 2 domain (SH2), DNA binding domain (DBD), dendritic cell (DC), granulocyte colony stimulating factor (G-CSF), common myeloid progenitor (CMP), common lymphoid progenitor (CLP), inflammatory bowel disease (IBD), lippopolysaccharide (LPS), Grb2-associated binding protein 2 (Gab2)

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