Determinants of the extent and duration of STAT3 signaling

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Keywords: STAT3 regulation, tyrosine phosphorylation, constitutive activity, pathway deregulation, cancer

Submitted: 06/15/12
Revised: 07/12/12
Accepted: 07/12/12

http://dx.doi.org/10.4161/jkst.21469
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Multiple molecular mechanisms have been identified that are responsible for the deregulation of the quantitative aspects of JAK-STAT signaling. These mechanisms enhance the extent and the duration of, e.g., STAT3 activation and have profound consequences on the phenotypes of the affected cells. The fine tuning of STAT3 signaling is required to maintain its physiological functions and its deregulation is associated with diverse pathological states. Deregulation can be exerted by the gain of function of components mediating the activation of STAT3 or the loss of function of molecules involved in the deactivation steps of STAT3. Gain of function mutations can involve tyrosine kinases that phosphorylate STAT3, mutations in cytokine and growth factor receptors causing their ligand independent activation, mutations in STAT3 that enhance and prolong its tyrosine phosphorylation and the autocrine or paracrine production and secretion of cytokines, most notably IL-6. Diminished deactivation of phosphorylated STAT3 can be due to the reduced expression of tyrosine phosphatases, inactivating mutations in these enzymes, silencing or functional inactivation of SOCS molecules, post-transcriptional inhibition of Pias3 expression or deletion mutations in the lymphocyte adaptor protein, LNK. STAT3 variants that exhibit autonomous transactivation potential have been detected in 40% of patients with T-cell large granular lymphocytic leukemia in clonally expanded CD8+ T cells. These patients also were preferentially affected by neutropenia and rheumatoid disorders and the results suggest that activating STAT3 mutations in T lymphocytes could be a cause of autoimmune diseases.

Extent and Duration of STAT3 Activation

STAT3 is a transcription factor that is activated by extracellular ligands, e.g., the cytokines IL-6, IL-10, IL-21, IL-27, G-CSF and leptin, but also by the growth factors EGF and HGF, through specific binding to transmembrane receptors and the induction of receptor associated and cytoplasmic tyrosine kinases. STAT3 activation can be observed in multiple organs and cell types, e.g., in immune cells, mammary epithelial cells, adipocytes, neural cells, cardiomyocytes, hepatocytes, stem cells and tumor cells, and is correlated with such diverse cellular phenotypes as differentiation, proliferation, apoptosis regulation, angiogenesis, malignant transformation, metastasis formation and drug responsiveness. How can a single transcription factor influence so many different functions? The cellular contexts, defined by the activity of interacting signaling pathways and the epigenetic state most likely play determining roles, but the discrete levels and the duration of STAT3 signaling also cooperate and contribute to the manifestation of distinct cellular outcomes. In skeletal muscle cells for example, the induction of STAT3 by exogeneously supplied IL-6 is rapid and transient. It reaches a maximum after about 1 h of cytokine stimulation and dampening mechanisms cause a return of activated STAT3 to basal levels within about 2 h. In tumor cells, a different activation pattern can be observed and...
strong activation of STAT3 is being maintained over long periods of time.6 Persisting and high levels of STAT3 signaling seem to be associated with cellular proliferation and transformation.2,7 Since the strength of STAT3 signaling and the duration of its activation appear as central determinants of the phenotypic cellular functions of this transcription factor, these parameters are tightly controlled. Several mechanisms have been described that regulate these parameters and either affect the activation step of STAT3 or the subsequent deactivation events.

Mechanisms and Components Affecting STAT3 Activation and Deactivation

The diversity of the molecular mechanisms contributing to the finetuning of STAT3 signaling probably reflects the importance of the quantitative aspects of this signaling pathway for the cellular physiology, but also provides vulnerability and possibilities for disturbance. Infringement with the finetuning of STAT3 activation is frequently associated with pathological states. Enhanced STAT3 signaling output can be traced to two basic steps in the regulation of extent and duration of STAT3 activation: (1) Mechanisms that enhance the activation step. They can be based on genetic alterations in molecular components that result in intracellular “gains of function” or effects on intercellular communication events that result in the exposure of cells to high levels of kinase activating signals. Both mechanisms cause higher rates of tyrosine phosphorylation of STAT3. (2) Mechanisms and mutations that impede the negative regulation of STAT3, i.e., alterations that result in a “loss of function” of molecular components that are involved in the downregulation of the cells. The mutation at position Y640 occurs in the dimerization domain of STAT3 and promotes its activation status.14 A similar observation has been made in T-cell large granular lymphocytic leukemia cells.15 This lymphoproliferative disorder is characterized by a high percentage of clonal CD3+CD8+ cytotoxic T lymphocytes (CTLs) and is associated with autoimmune disorders and immune-mediated cytopenias. Forty percent of the patients with large granular lymphocytic leukemia express constitutively activated STAT3 variants with mutations in the dimerization domain of STAT3 and aberrant activation.16 The inappropriate activation of STAT3, however, can also be explained by the deregulation of linked signaling events that are not directly attributable to distinct mutations in components of the JAK STAT pathway, but by deregulated expression levels of accessory protein.

(1) G-CSF receptor activation controls survival, proliferation and differentiation of myeloid progenitor cells via JAKs. JAKs in turn control the levels of cytokine receptor expression and increased JAK expression can confer growth factor independent STAT3 activation and hematopoietic cell transformation.16

(2) Elevated expression of sphingosine-1-phosphate receptor-1 (S1PR1), a G protein-coupled receptor for the lysophospholipid sphingosine-1-phosphate (S1P), has been found in tumors with activated STAT3.17 STAT3 induces the transcription of the S1pr1 gene and enhanced S1pr1 expression activates STAT3 and IL-6 expression. These reciprocal regulatory events are thought to maintain the persistent activation STAT3 in cancer cells.

(3) Autocrine and paracrine mechanisms, especially the secretion of IL-6 by tumor cells and cells of the tumor microenvironment, contribute to STAT3 activation.18 Ras activation, e.g., induces the secretion of IL-6 and can act in a paracrine fashion to promote angiogenesis and tumor growth.19 Similarly, EGF activation in lung carcinoma cells causes IL-6 expression and secretion, and thus the paracrine and autocrine stimulation of STAT3 in cells of the tumor microenvironment.20,21 Finally, STAT3 activation induces IL-6 gene transcription and thus establishes a positive feedback loop in tumor cells in vitro and tumor tissues in vivo.22

Mechanisms resulting in the enhancement of STAT3 signaling are not restricted to the activation steps, but can also be founded in the molecular events governing the downregulation of activated STAT3 and the cessation of signaling. These events are based on diverse components and their loss of function can result in the maintenance of the activated state and the persistence of STAT3 signaling.

(1) The direct reversion of STAT3 activation can be accomplished by protein tyrosine phosphatases, PTP. At least three of them have been identified that can catalyze STAT3 dephosphorylation, TC-PTP, SHP1 and SHP2.23 The loss of PTP function has been observed in tumor cells with inappropriately activated STAT3. The receptor protein tyrosine phosphatase
delta (PTPRD), for example, is frequently inactivated in glioblastoma multiforme (GBM), head and neck squamous cell carcinomas and lung cancer. The inactivation of the PTP can be the result of intragenic deletions or of epigenetic silencing by promoter methylation.\textsuperscript{24}

(2) Direct negative feedback is being used to regulate STAT3 signaling. The suppressor of cytokine signaling (SOCS) 1 and 3 genes are STAT3 targets and their products bind to JAK or cytokine receptors, thereby suppressing further signaling. SOCS-1 and SOCS-3 are strong inhibitors of JAKs, with kinase inhibitory regions at their N-terminus.\textsuperscript{25} Hypermethylation of the SOCS-3 promoter and transcriptional silencing was frequently detected in lung and breast cancer and mesotheliomas. Restoration of SOCS-3 expression in lung cancer cells resulted in the downregulation of activated STAT3, induction of apoptosis and growth suppression.\textsuperscript{26}

(3) Bacterial proteins can affect STAT3 activation. Pasteurella multocida toxin (PMT) is a highly mitogenic protein that affects cellular signaling through its modulation of heterotrimeric G proteins. It also activates STAT signaling in a persistent fashion. Enhanced STAT3 activity seems to be promoted by the induction of the serine/threonine kinase Pim-1, which in turn phosphorylates SOCS-1. This modification disrupts the interaction with the elongin BC complex, which normally allows the SOCS proteins to shuttle activating components of STAT signaling to the proteasome.\textsuperscript{27} The negative regulatory function of SOCS-1 is subverted by Pim-1 dependent phosphorylation and results in the sustained activity of STAT3.\textsuperscript{28}

(4) A small family of proteins, most descriptively named Pias (protein inhibitors of activated STAT), directly interacts with their targets, and Pias3 has shown specificity for STAT3 recognition. This interaction results in the inhibition of STAT3 mediated gene activation most likely by blocking the DNA binding activity.\textsuperscript{29} The expression of Pias3 correlates with STAT3 activation, and Pias3 controls the extent and the duration of STAT3 activity in normal cells. In cancer cells, the expression of the Pias3 protein is post-transcriptionally suppressed and promotes the oncogenic effects of activated STAT3.\textsuperscript{6}

(5) The lymphocyte adaptor protein (LNK) is a negative regulator of thrombopoietin and erythropoietin mediated JAK2 activation. Deletion mutations in this gene were observed in patients with myeloproliferative neoplasms (MPNs). These LNK mutants caused augmented and sustained thrombopoietin dependent signaling and STAT3 activation due to the loss of LNK negative feedback regulation.\textsuperscript{30}

### A Hyperactive STAT3 Variant in T-Cell Large Granular Lymphocytic Leukemia Cells

Advanced DNA sequencing technology allows the comparison of the genomes of normal cells and tumor cells and bioinformatic analysis the determination and interpretation of mutations consistently associated with cellular transformation.\textsuperscript{31,32} The investigation of DNA derived from 77 patients with T-cell large granular lymphocytic leukemia, a lymphoproliferative disorder characterized by the presence of a large fraction of clonal CD3\textsuperscript{+}CD8\textsuperscript{+} cytotoxic T lymphocytes (CTLs), revealed that in 40% of the cases mutations in the STAT3 gene could be detected.\textsuperscript{15} The mutations were all clustered in exon 21 encoding the SH2 domain of STAT3 and resulted in a more hydrophobic dimerization domain. The most frequent mutation found was Y640F in 17% of the cases, followed by D661V in 9%, D661Y in 9% and N647I 4%. The mutant STAT3 molecules were preferentially phosphorylated on tyrosine 705 and present in the nucleus of the leukemic cells. The Y640F and D661V variants were further analyzed and exhibited enhanced transactivation potential for known STAT3 target genes. These observations assign a “gain of function” phenotype to these molecules. The mutation at position 661 is very reminiscent of the STAT3 variant, which was originally obtained in mutagenesis experiments and defined STAT3 as an oncogene.\textsuperscript{33}

Why would the expansion of CD8\textsuperscript{+} T cells be affected by inappropriately strong STAT3 signaling? Non-redundant functions in the immune system and development of lymphocytes have been assigned to STAT signaling. They influence cell fate decisions of differentiating naive T cells and regulate the intensity and duration of inflammatory responses. T helper cell differentiation, Th1, Th2, Th17 and Treg, requires the functions of STAT1, STAT3, STAT4, STAT5 and STAT6\textsuperscript{34,35} and STAT3 determines the differentiation of naive T cells into the regulatory (Treg) or inflammatory (Th17) T cell lineages. Th17 cells produce IL-17, act in the host defense against bacteria and fungi and contribute to autoimmune diseases. STAT3 also regulates cell growth, apoptosis and the transcription of inflammatory genes and contributes to the development of chronic inflammatory diseases and malignant and neurodegenerative diseases.\textsuperscript{36} It promotes pro-oncogenic inflammation and suppresses anti-oncogenic Th1 immune responses.\textsuperscript{2} Targeted deletion of the STAT3 gene in the CD4\textsuperscript{+} T cell compartment of mice impaired the experimental induction of autoimmune conditions, for example, uveoretinitis or encephalomyelitis, most likely because of a reduction in the expression of activated \( \alpha/\beta \) integrins on CD4\textsuperscript{+} T cells.\textsuperscript{37}

Insights into the role of STAT3 signaling in CD8\textsuperscript{+} T cells have recently been gained from studies in mice and humans\textsuperscript{38} in which STAT3 signaling was negatively impaired through genetic manipulation of mice\textsuperscript{39} or a STAT3 gene mutation in human patients.\textsuperscript{40} CD8\textsuperscript{+} T cells can be distinguished into short lived effector CD8\textsuperscript{+} T cells important for immediate pathogen control and memory CD8\textsuperscript{+} T cells, which can self-renew, persist and provide for long-term immunity. STAT3 activation, through the cytokines IL-10 and IL-21, seems to be directly involved in the cell fate decision of activated T cells, their differentiation into functional CD8\textsuperscript{+} memory T cells and the maintenance of this cell pool. These conclusions were derived from experiments in a mouse model in which the STAT3 gene had been conditionally deleted in activated CD8\textsuperscript{+} T cells.\textsuperscript{39} They were corroborated by observations in a cohort of patients with autosomal dominant hyper IgE syndrome (AD-HIES) in which a dominant negative mutation in the STAT3 gene has been detected.\textsuperscript{40} These
patients show a reduced number of central memory CD4+ and CD8+ T cells when compared with healthy controls and a decreased ability to control bacterial and viral infections.

Although the systems employed to arrive at the conclusion that STAT3 is a central signaling factor in the establishment and maintenance of memory CD8+ T cells both rely on diminished STAT3 signaling, they complement the observations reported by Koskela et al.15 Enhanced and prolonged STAT3 signaling, emanating from the mutated STAT3 variant, seems to mimic the functions of IL-10 and IL-21 and result in the expansion of a stable CD8+ memory T cell pool. This may eventually lead to T cell large granular lymphocytic leukemia, a clonal disorder of large granular lymphocytes. The observation that patients with activating STAT3 mutations frequently also suffered from neutropenia and rheumatoid disorders indicate that the expansion of the CD8+ T cell population can trigger adverse autoimmune reactions.

**Conclusions**

The summary of the molecular mechanisms responsible for the enhanced activation and the diminished deactivation of STAT3, shown in Tables 1 and 2, indicates that the fine tuning of the extent and duration of STAT3 activation can easily be derailed and that this deregulation is associated with diverse pathological states. The variants that have been detected in the STAT3 gene in T-cell large granular lymphocytic leukemia15 and in inflammatory hepatocellular adenomas14 gene have been designated as “constitutively active” and the ones found in the autosomal dominant hyper IgE syndrome (AD-HIES) as “dominant negative.”40 This description is probably too apodictic. The “constitutively active” variant is probably still partially regulated by cytokine signaling and affected by the deactivation mechanisms; similarly, the “dominant negative” variant is probably not entirely inactive and still has some residual activity. They are deregulated in extent and duration of signaling. Such observations have also been made with hyperactive and muted variants of STAT541,42 and emphasize the quantitative aspects of STAT regulation. The presence of an inappropriately strong STAT3 signal is sufficient to trigger the clonal expansion of CD8+ T cells, the emergence of large granular lymphocytic leukemia and the occurrence of autoimmune disorders.

### Table 1. Molecular alterations resulting in the enhanced activation of STAT3 signaling

| Components and mechanisms | Cells and phenotypes | References |
|--------------------------|----------------------|------------|
| v-src, activated oncogenic version of the tyrosine kinase c-src | Fibroblast transformation | 8 and 9 |
| JAK2 (V617F), activating mutation in the tyrosine kinase domain | Myeloproliferative neoplasms | 10, 11 and 12 |
| gp 130, activating deletion of the ligand binding site in the IL-6 coreceptor | Inflammatory hepatocellular adenoma | 13 |
| STAT3 (Y640F and D661V), activating mutations in the dimerization domain | Hepatocellular adenoma, T cell large granular lymphocytic leukemia | 14 and 15 |
| G-CSF receptor activation through increased JAK2 expression | Hematopoietic cell transformation | 16 |
| Sphingosine 1 phosphate receptor overexpression, auxiliary in IL-6 dependent STAT3 activation | B16 mouse melanoma cells, human breast cancer | 17 |
| EGF receptor, mutation in the kinase domain, induces IL-6 secretion | Human lung adenocarcinomas | 20 |
| Ras, oncogenic mutation induces IL-6 secretion | Pancreatic cancer | 19 |
| IL-6, autoregulation, autocrine induction of IL-6 secretion | Lung adenocarcinoma cells | 22 |

### Table 2. Molecular alterations resulting in the diminished deactivation of STAT3

| Components and mechanisms | Cells and phenotypes | References |
|--------------------------|----------------------|------------|
| PTP, protein tyrosine phosphatases, transcriptional silencing, deletion mutations | Glioblastoma multiforme, head and neck squamous cell carcinoma, lung cancer | 2 |
| SOCS, suppressors of cytokine signaling, promoter methylation and transcriptional silencing | Lung and breast cancer, mesotheliomas | 26 |
| SOCS-1, PMT-induced expression of Pim-1, inhibition of SOCS-mediated E3 ubiquitin ligase activity | Rat-1 fibroblasts, enhanced cell proliferation | 28 and 27 |
| PIAS, protein inhibitors of activated STAT, post-transcriptional silencing | Glioblastoma and breast cancer | 6 |
| LNK, lymphocyte adaptor protein, deletion mutations | Myeloproliferative diseases | 30 |
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