Chapter 2

Constitutive STAT3 tyr705 and ser727 phosphorylation in AML cells caused by autocrine secretion of IL-6

Jan Jacob Schuringa\textsuperscript{1,2}, Albertus T.J. Wierenga\textsuperscript{2}, Wiebe Kruijer\textsuperscript{1}, and Edo Vellenga\textsuperscript{1}

\textsuperscript{1}University Medical Center Groningen, Department of Hematology, Hanzeplein 1, 9713 GZ, Groningen.
\textsuperscript{2}Biological Center, Department of Genetics, Kerklaan 30, 9751 NN, Haren.

Blood. 2000 Jun 15;95(12):3765-70
Summary

To explore the activation patterns of signal transducer and activator of transcription 3 (STAT3) in acute myeloid leukemia (AML), we examined whether phosphorylation of tyrosine705 and serine727 residues was abnormally regulated in cells from AML patients. In 5 out of 20 AML cases (25%), STAT3 was constitutively phosphorylated on tyrosine705 and serine727, which was not further upregulated by treatment with Interleukin-6 (IL-6). Furthermore, STAT3 was constitutively bound to the IRE response element in these cells as determined by EMSAs, and stimulation with IL-6 did not result in increased DNA binding. Interestingly, AML cells with constitutive STAT3 activation also secreted high levels of IL-6 protein. Treating these AML cells with anti-IL-6 resulted in restored IL-6-inducible STAT3 phosphorylations on both tyrosine705 and serine727 with low or undetectable basal phosphorylation levels in unstimulated cells. In contrast, treatment with anti-IL-1 did not result in altered STAT3 phosphorylation patterns. The constitutive IL-6 expression was associated with elevated levels of suppressor of cytokine signaling-1 (SOCS-1) and SOCS-3 mRNA expression, which were not downregulated by anti-IL-6. These data indicate that the constitutive STAT3 activation in the investigated AML blasts is caused by high IL-6 secretion levels, thus stimulating the JAK/STAT pathway in an autocrine and/or paracrine manner.
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Introduction

Acute Myeloid Leukemia (AML) is characterized by an accumulation of immature blasts in the bone marrow, finally resulting in a disturbed production of normal hematopoietic cells. Although little is known about the precise mechanisms of pathogenesis at the molecular level of this disease, AML is often associated with chromosomal translocations and inversions, affecting gene expression in ways that lead to defects in normal programs of cell proliferation, differentiation and survival. The most frequent targets of chromosomal translocations are transcription factors, resulting in the recombination of normally unrelated sequences from different chromosomes into hybrid genes that encode fusion products with altered function. However, the chromosomal translocations and inversions found in AML patients are highly divergent and the precise molecular defects in acute myeloid leukemia still have to be elucidated.

Interleukin-6 (IL-6) is a pleiotropic cytokine that can be constitutively expressed in AML cells. IL-6 initiates its action by binding to its receptor that is composed of two subunits: an 80-kDa IL-6 binding protein and a 130-kDa transmembrane signal transducing component (gp130). The gp130 receptor protein is also used by other members of the IL-6 cytokine family, including IL-11, Oncostatin M (OnM), Leukemia Inhibitory Factor (LIF), and Ciliary Neurotrophic Factor (CNTF). Activation of IL-6 signal transduction involves gp130 dimerization, ligand-dependent tyrosine phosphorylation of the gp130 associated protein-tyrosine kinases Jak1, Jak2, and Tyk2, as well as tyrosine phosphorylation of Signal Transducer and Activator of Transcription 3 (STAT3). Tyrosine phosphorylation of STAT3 occurs at a single residue tyrosine residue (tyr705) that is located in a conserved SH2 domain allowing STAT dimerization and transcription activation. In addition to tyrosine phosphorylation, STAT3 is serine phosphorylated at a single residue (ser727) in response to IL-6 as well as to other extracellular factors including interferon-γ (IFN-γ) and epidermal growth factor (EGF). Recently, it was demonstrated that maximal STAT3 transactivation requires phosphorylation on a unique serine residue 727, and that IL-6 induces ser727 phosphorylation of STAT3 via a signal transduction cascade involving Vav, Rac-1, MEKK and SEK.

Recently, a family of cytokine-inducible inhibitors of signaling has been identified that downregulate the Jak/STAT signaling pathway. The proteins in this family, including cytokine-inducible SH-2 containing protein (CIS) and SOCS/Jak-binding protein (JAB)/STAT-induced STAT inhibitor proteins, are proteins containing SH2
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domains which interact with JAKs, thus preventing the activation of STATs\textsuperscript{17-19}. Specifically, SOCS-1 and -3 are implicated in the downregulation of the IL-6-induced activation of STAT3\textsuperscript{20-23}. Moreover, SOCS-1 and SOCS-3 can quickly be upregulated by IL-6\textsuperscript{18,19,21}.

However, the activation of STATs has not only been implicated in gp130 receptor downstream signaling, but might also be due to oncogene activation. Abnormal activation of STAT1, STAT3, STAT5 and STAT6 has been demonstrated in cells transformed by Src, Abl, and various other oncoproteins and tumor viruses\textsuperscript{24-32}. Also, in acute leukemia a spontaneous activation of STATs has been observed. Constitutive DNA binding of STAT1 and STAT5 was found in ALL, whereas constitutive DNA binding and tyrosine phosphorylation of STAT1, STAT3 and STAT5 was detected in several AML patients\textsuperscript{25,33-35}. Furthermore, constitutive STAT1 and 3 serine phosphorylation has been found in some AML and many CLL cases\textsuperscript{36}. Although it has not been demonstrated that constitutive STAT3 activation is contributive in the development of leukemias, the consistent finding of abnormal STAT3 activation in these cells suggests that STATs might fulfill a role in the ongoing process of transformation. Recently, it has been demonstrated that STAT3 plays a key role in G1 to S phase cell-cycle transition through upregulation of cyclins D2, D3 and A, and cdc25A, and the concomitant downregulation of p21 and p27\textsuperscript{37}. Thus, a constitutive STAT3 activation might lead to a growth advantage of the malignant counterpart.

Here, we report that a constitutive activation of STAT3 is observed in 25\% of the investigated AML patients which is due to an autocrine secretion of IL-6, thus leading to a continuous activation of the Jak/STAT pathway. The high expression levels of IL-6 protein are also associated with an increased expression of SOCS-1 and SOCS-3 mRNA. Blocking the action of secreted IL-6 by treatment with anti-IL-6 leads to a loss of constitutive STAT3 phosphorylation and normal IL-6-induced STAT3 activation patterns.
**Materials and methods**

**Patient population and isolation of AML cells**

Peripheral blood cells from 20 patients with AML were studied after informed consent. The AML cases were defined according to the classification of the FAB committee as M1 to M6 (Table 1)\(^3\). AML blasts were isolated by density-gradient centrifugation as described\(^3\). The cells were cryopreserved in aliquots of 20 to 50 \(\times\) \(10^6\) cells/ml in 10% dimethylsulfoxide (DMSO; Sigma, St Louis, MO) and 10% fetal bovine serum (FBS; Hyclone, Logan, UT), employing a method of controlled freezing and storage in liquid nitrogen. After thawing, T lymphocytes were depleted by 2-aminoethylisothiouronium bromide (AET)-treated sheep red blood cell (SRBC) rosetting. The cell population consisted of more than 98% AML blasts as determined by May-Grünwald-Giemsa staining. Fluorescence-activated cell sorting (FACS) analysis showed <1% CD3 (Becton Dickinson, Sunnyvale, CA) -positive cells.

<table>
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Cell culture, reagents and antibodies

HepG2 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin and 10% FCS. AML blasts were cultured at 37°C at a density of $2 \times 10^6$/mL in RPMI 1640 media (Flow, Rockville, MD) supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin and 10% FCS. For RT-PCR analysis, AML no 11 was cultured for 24 hrs in RPMI 1640, the supernatant was collected, and this was used to culture AML 7 as indicated in the text. Cells were stimulated with 10 ng/ml human recombinant IL-6 (a generous gift from Dr. S.C. Clark, Genetics Institute, Cambridge, USA) or 10 ng/ml IFN-γ (Endogen, Woburn, MA, USA). Antibodies against STAT3 (C-20 and K-15, Santa Cruz) were used in dilutions of 1:4000, antibodies against STAT3( tyr705) and STAT3(ser727) were obtained from New England Biolabs and used in a 1:1000 dilution. Anti-IL-6 was a gift from L. van Aarden (CLB, Amsterdam, The Netherlands) and was used in a dilution of 1:1000, which was shown to inhibit the biological activity of >100 ng/ml IL-6 (data not shown). Anti-IL-1α was a gift from S. Gillis (Immunex Corp., USA) and anti-IL-1β was purchased from R&D systems (Minneapolis, USA). Both antibodies were used in a dilution of 1:250.

Western Blotting

Cells were plated in 12-wells culture plates, stimulated, washed and collected in PBS. Cells were lysed in Leammli sample buffer and boiled for 5 min prior to separation on 7.5% SDS-polyacrylamide gels. The proteins were transferred to a PVDF membrane (Millipore, Etten-Leur, The Netherlands) in Tris-glycine buffer at 100 mA for 1.5 h using an electroblotter (Pharmacia, Woerden, The Netherlands). Membranes were blocked with PBS buffer containing 5% non-fat milk prior to incubation with antibodies. Binding of each antibody was detected by HRP labelled pig-anti-rabbit secondary antibodies using Enhanced Chemiluminescence (ECL) according to the manufacturer’s recommendations (Amersham Corp.). Western blots were quantified using Image-Pro (Media Cybernetics, Silver Spring, USA).

EMSA

Nuclear extracts were prepared from $10^7$ cells as described previously according to the rapid Dignam method. A double-stranded synthetic oligo comprising the IL-6RE of the ICAM-1 promoter (upper strand: 5’-
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CGCGTAGCTTAGGTTTCCGGGAAAGCACG-3’) was $^{32}$P-labelled by filling in the 5’-protruding ends with $\alpha^{32}$P-dATP and Klenow enzyme. 5 µg nuclear extract was incubated with 20,000 cpm labeled probe for 20 min at 26°C and gel retardation analysis was performed on native 4% polyacrylamide gels in 0.5 x TBE. In supershift experiments, 1 µl of anti-STAT3 antibodies (C-20 supshift reagent, Santa Cruz) or anti-STAT1 antibodies (Transduction Labs, Lexington, KY, USA) were added.

RNA extraction and RT-PCR

For RT-PCR, total RNA was isolated from $10^7$ cells using Trizol according to the manufacturer’s recommendations (Life technologies). 3 µg of RNA per sample was reverse transcribed with M-MuLV Reverse transcriptase (Boehringer Mannheim). For PCR, 2 µl of cDNA was amplified using beta-2-globulin primers (forward: 5’-CCAGCAGAGATGGAAAGTC-3’; reverse: 5’-GATGCTGCTTACATGTCTCG), SOCS-1 primers (forward: 5’-CACGCACCTTCCGCACATTCC-3’; reverse: 5’-TCCAGCAGCTCGAAGAGGCA-3’) or SOCS-3 primers (forward: 5’-TCACCCACAGCAAGTTTCCCGC-3’; reverse: 5’-GTTGACCGTCTTCCGACAGAGATGC-3’) in a total volume of 50 µl using 2 units of Taq polymerase (Boehringer Mannheim). After 25 cycles, 15 µl aliquots were run on 1.5% agarose gels.

IL-6 secretion

2x10$^6$ cells were plated in 1 ml RPMI 1640 containing 10% FCS and treated with anti-IL-6 antibodies as indicated. After 24 hrs, cell-free supernatants were obtained by centrifugation of the suspension. IL-6 protein levels were measured using the commercially available Enzyme Linked Immuno Sorbent Assay according to the manufacturer’s recommendations (CLB, Amsterdam, The Netherlands)

Statistics

For IL-6 secretion, experiments were done in triplo and differences between groups were tested for significance using the two-tailed T-test. Differences with p<0.05 were considered significant.
Results

Constitutive and non-IL-6-inducible STAT3 tyr705 and ser727 phosphorylation and DNA binding in AML cells

To assess the phosphorylation status of STAT3 in AML cells, blasts of 20 untreated patients were cultured in RPMI 1640 containing 10% FCS and either left unstimulated or stimulated with 10 ng/ml IL-6 for 15 min. Total cell extracts were Western blotted and STAT3 was visualized using specific antibodies against phosphorylated STAT3 on tyr705 and ser727. Of 20 patients under investigation, 5 (25%) showed constitutive STAT3 tyrosine and serine phosphorylation, which was not further upregulated by stimulation with IL-6 (Fig.1; no 1, 11, 14, 18 and 19). 15 patients showed normal transient IL-6-induced STAT3 phosphorylation patterns with similar kinetics as in HepG2 cells (Fig.1). Interestingly, the expression levels of STAT3 varied strongly amongst the AML samples. Also, there was a strong variation in IL-6-induced STAT3 phosphorylation levels; in AML cases with constitutive STAT3 activation the degree of phosphorylation was relatively low (Fig.1).

Fig.1 Constitutive and non-IL-6 inducible STAT3 phosphorylation patterns are observed in 25% of the investigated AML cases. AML cells and HepG2 cells were plated in 12-wells culture plates, either unstimulated or stimulated with 10 ng/ml IL-6 for 15 min (AML) or for 5-60 min (HepG2), and equal amounts of total cell lysates were subjected to SDS-PAGE followed by Western blot analysis. Phosphorylated STAT3 was visualized using phosphospecific antibodies recognizing tyr705 and ser727 phosphorylated STAT3. As a control, equal amounts of total cell lysates were Western blotted using antibodies against STAT3 (K-15).
Nuclear extracts were isolated from 3 AML samples (no 17, 18 and 19, Fig.2) and STAT3 DNA binding was studied on the IRE from the ICAM-1 promoter. The AML cells characterized by IL-6-induced STAT3 tyr705 phosphorylation also demonstrated an IL-6-induced STAT3 DNA binding. Superhift analysis demonstrated that the complexes bound to the IRE in response to 10 ng/ml IL-6 comprise of STAT3, but not of STAT1. In contrast, stimulation with 10 ng/ml IFN-γ strongly induced STAT1 DNA binding. Constitutive STAT3 tyr705 correlated well with constitutive DNA binding which could not be further upregulated by IL-6. Finally, Western blotting experiments using antibodies recognizing tyrosine701 phosphorylated STAT1 demonstrated no constitutive or IL-6-induced STAT1 phosphorylation (data not shown). Taken together, these data demonstrate that approximately 25% of the AML patients are characterized by a constitutive activation of STAT3, which is not inducible by IL-6.

Constitutive STAT3 activation is correlated with high IL-6 secretion levels

To further investigate the nature of the constitutive STAT3 activation in AML cells, IL-6 protein levels were determined in cell-free supernatants after 24 hrs of culture. Patients characterized by constitutive STAT3 phosphorylation and DNA binding demonstrated high levels of spontaneous IL-6 protein secretion (946-23667 pg/ml), whereas AML cells with IL-6-inducible STAT3 activation showed low or no detectable IL-6 protein levels (Table 1).
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Treatment of AML cells with anti-IL-6, but not anti-IL-1, restores STAT3 inducibility by IL-6

Since AML cells characterized by constitutive STAT3 activation also spontaneously secreted high levels of IL-6 protein into the medium, we questioned whether IL-6 might be responsible for the constitutive STAT3 activation. Subsequently, AML cells (no 1, 11, 14, 18, 19) were cultured for 24 hrs in the absence and presence of anti-IL-6, washed, followed by IL-6 stimulation for 15 min. Pretreatment with anti-IL-6 resulted in an inhibition of the basal tyr705 and ser727 phosphorylation of STAT3. A representative experiment is shown in fig.3A. In addition, STAT3 tyr705 and ser727 phosphorylations could now be induced by treatment with IL-6 to 18.4-fold and 6.8-fold induction, respectively.

To further study the kinetics of STAT3 tyr705 phosphorylation upon longer stimulation of IL-6, two AMLs with and without a constitutive STAT3 activation were

Fig.3 Treatment with anti-IL-6 but not anti-IL-1 blocks constitutive STAT3 phosphorylation and restores IL-6 inducibility. (A) AML cells (no 19) were cultured in 12-well plates and pretreated with either anti-IL-6 (1:1000) or left untreated. After 24 hrs, cells were washed and either left unstimulated or stimulated with 10 ng/ml IL-6 for 15 min. (B) AML cells (no 18 and 20) were cultured in 12-well plates and stimulated with 10 ng/ml IL-6 for several time periods as indicated. After 24 hrs, cells were treated with anti-IL-6 (1:1000) where indicated for 15 or 60 min. Equal amounts of total cell lysates were subjected to SDS-PAGE followed by Western blot analysis as described in (A). (C) AML cells (no 18) were cultured in 12-well plates were either pretreated with anti-IL-1 (1:250) overnight or left untreated. After 24 hrs, cells were washed and stimulated with 10 ng/ml IL-6 for 15 min as indicated. Equal amounts of total cell lysates were Western Blotted as described in (A).
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treated with IL-6 for 15 min until 24 hrs (Fig. 3B). IL-6 induced a quick and strong upregulation of STAT3 tyr705 phosphorylation in AML20, which returned to low levels after 60 min. In AML18, IL-6 did not induce STAT3 tyr705 phosphorylation within 24 hrs. Furthermore, the time-course of the action of anti-IL-6 was investigated in both cases after 24 hrs of stimulation with IL-6. As depicted in Fig.3B, treatment of 15 min with anti-IL6 already reduced tyr705 phosphorylation, which was completely reduced to low basal levels upon 60 min of treatment with anti-IL-6. These data indicate that constitutive STAT3 phosphorylation can quickly be downregulated by treating the cells with anti-IL-6.

Previously, it has been demonstrated that IL-1 is an important cytokine involved in the IL-6 production by AML cells1,41,42. Thus, the constitutive secretion of IL-6 might be due to a spontaneous secretion of IL-1. To investigate this possibility, AML cells (n=3) with spontaneous phosphorylation of STAT3 were cultured for 24 hrs in the presence or absence of anti-IL-1. A typical example is depicted in Fig.3C. Pretreatment with anti-IL-1 for 24 hrs did not reduce the spontaneous tyrosine phosphorylation nor restore the IL-6 inducibility of STAT3 phosphorylation indicating that IL-1 is not responsible for the spontaneous IL-6 protein secretion in the investigated AML cells.

Fig.4 High IL-6 secretion levels correlate with high SOCS1 and SOCS3 expression levels. AML cells (no 11 and 7) were cultured in 12-well plates and stimulated for 1 hr with IL-6 (lanes 2, 5, 8 and 11) or with anti-IL-6 for 2 hrs (lanes 3, 6, 9 and 12). In lanes 1-6, cells were cultured in RPMI1640 for 24 hrs prior to stimulation; in lanes 7-9, AML no 7 was cultured in the supernatant of AML no 11, and in lanes 10-12 AML no 7 was cultured in the supernatant of AML no 11 which was depleted of IL-6 by adding anti-IL-6. Total RNA was isolated and 3 g RNA was reverse transcribed with M-MuLV Reverse Transcriptase and cDNAs were used in a PCR reaction using specific primers for SOCS-1, SOCS-3 or β2-μglobulin as a control.
Constitutive STAT3 activation and high IL-6 protein secretion levels correlate with high expression levels of the STAT inhibitors SOCS-1 and SOCS-3

SOCS (suppressor of cytokine signaling) proteins represent a family of negative regulators of cytokine signaling which probably switch off the cytokine signal by binding to JAK proteins via SH2 domains, thereby inhibiting the activation of STATs\(^{17-19}\). To investigate the IL-6-induced upregulation of SOCS-1 and SOCS-3 in AML cells, we prepared cDNA of unstimulated or IL-6 stimulated cells and performed RT-PCR using specific primers for SOCS-1 and SOCS-3. SOCS-1 and -3 mRNA levels were low in unstimulated AML cells that were characterized by IL-6-inducible STAT3 phosphorylation, and were quickly upregulated by IL-6 (Fig.4, lanes 4 and 5). In contrast, AML cells characterized by high IL-6 secretion levels and constitutive STAT3 phosphorylation patterns showed high basal levels of SOCS-1 and SOCS-3, while exogenous added IL-6 did not further enhance the expression (Fig.4, lanes 1 and 2). Similar results were obtained in RT-PCR analyses for two other AML cases with constitutive STAT3 activation (no 1 and 14, data not shown). Surprisingly, treatment of these AML cells with anti-IL-6 for 2 hrs did not result in reduced SOCS-1 and SOCS-3 mRNA levels (Fig.4, lane 3) suggesting that other cytokines were secreted by these AML cells able to induce the expression of SOCS-1 and SOCS-3. To underscore this possibility, the cell free supernatant of AML 11 with a constitutive STAT3 activation was collected and added to AML 7 without a constitutive STAT3 activation. As demonstrated in Fig.4, culturing AML 7 cells in this supernatant resulted in high and non-IL-6-inducible levels of SOCS-1 and SOCS-3 expression (Fig.4; lanes 7-9), even when it was depleted of IL-6 by applying saturating amounts of anti-IL-6 to the supernatant (Fig.4, lanes 10-12). Taken together, these data indicate that in AML cells characterized by high IL-6 secretion levels and constitutive STAT3 phosphorylation SOCS-1 and SOCS-3 expression is disturbed.

Discussion

Although the exact function of IL-6-induced STAT3 signaling in hematopoietic cells is not well defined, it has been suggested that IL-6 plays an important role in the proliferation and survival of early hematopoietic progenitor cells\(^{43,44}\). Also, IL-6 signaling results in gene expression patterns important for lineage restricted
differentiation along the myeloid and lymphoid lineages\textsuperscript{45-48}. Particularly, STAT3 activation has been implicated in macrophage differentiation\textsuperscript{49}. However, the effects of IL-6 on the growth and survival of AML cells are variable. In the majority of cases growth supportive effects of IL-6 are described especially in conjunction with additional cytokines, while in a minority of cases a growth inhibitory effect is noticed\textsuperscript{50-54}. Previously, a constitutive STAT3 activation has been demonstrated in AML and is described in 15-20\% of the cases\textsuperscript{33-36}. In the present study it is demonstrated that (1) in 25\% of the investigated AML cases spontaneous phosphorylation of tyr705 and serine 727 is observed which is not further inducible by IL-6; (2) the constitutive STAT3 phosphorylation is related to the autocrine secretion of IL-6; and (3) that a constitutive non-IL-6-inducible STAT3 activation pattern in AML is correlated with increased expression levels of SOCS1- and SOCS-3.

IL-6-induced STAT3 transactivation involves phosphorylation of both tyr705 and ser727 residues. Tyrosine phosphorylation allows STAT dimerization, translocation to the nucleus and binding to target gene promoters. Although it is still unclear how STAT3 ser727 phosphorylation is linked to transcriptional activation, it has been clearly demonstrated in many cases that this residue is essential for maximal STAT3 transcriptional potential\textsuperscript{14,15}. It has been speculated that STAT ser727 phosphorylation allows binding of specific cofactors, thus coupling the RNA polymerase II machinery to STAT transcription factors. In blast cells of AML patients, we find a constitutive STAT3 DNA binding and phosphorylation on both tyr705 and ser727 in approximately 25\% of the cases, demonstrating that STAT3 is not only constitutively bound to target gene promoters, but is also constitutively initiating gene transcription. Recently, it has been demonstrated that STAT3 plays a key role in G1 to S phase cell-cycle transition through upregulation of cyclins D2, D3 and A, and cdc25A, and the concomitant downregulation of p21 and p27\textsuperscript{37}. Thus, the constitutive STAT3 activation might affect proliferation and cell survival leading to a growth advantage over normal cells. Indeed, a constitutive STAT3 activation leads to higher cell survival rates and to a less susceptibility for cytostatic agents (J.J.Schuringa, unpublished observations). Similar results have been described in multiple myeloma whereby the constitutive STAT3 activation is associated with an upregulation of BcL-xL and the concomitant prevention of apoptosis\textsuperscript{55}, although the cause of the constitutive STAT3 activation might be different for AML cells. Furthermore, Bromberg et al. demonstrated that a constitutive active STAT3 mutant, in which two cysteine residues within the C-terminal loop of the SH2 domain are substituted, induces cellular transformation and strongly upregulates the expression of cyclin D1, BcL-xL and c-myc\textsuperscript{56}. These data suggest that
a constitutive STAT3 activation might contribute by multiple mechanisms to the malignant phenotype of cells.

Strikingly, in the investigated AML cells the constitutive STAT3 phosphorylation is caused by the autocrine secretion of IL-6 since treatment of anti-IL-6 resulted in a restored IL-6 inducibility of STAT3 tyr705 and ser727 phosphorylation. Therefore, we conclude that gp130 downstream signaling is normal in the investigated AMLs and that the constitutive STAT3 activation is not linked to oncogene activation as has also been described in cells transformed by Src, Abl, and various other oncoproteins and tumor viruses\textsuperscript{24}. The cause of the constitutive expression of IL-6 in the selected number of AML cases is not elucidated, but it seems not be caused by IL-1. Previous studies have demonstrated that spontaneous IL-6 secretion was linked to NF-κB DNA binding which could not be blocked by anti-IL1, suggesting an aberrant function or triggering of the IκB kinases or related proteins\textsuperscript{6,57,58}.

Also, the negative feedback loop of SOCS-1 and SOCS-3 expression, which is normally quickly upregulated by IL-6, is constitutively activated in the AML cases with constitutive STAT3 phosphorylation. In these AML cases, SOCS expression was relatively high, and IL-6 did not further upregulate SOCS mRNA levels. In line with the elevated SOCS-1 and SOCS-3 expression, the levels of STAT3 phosphorylation were lower as compared to the IL-6-induced phosphorylation levels in AML cells without a constitutive STAT3 activation. Surprisingly, treating constitutive AMLs with anti-IL-6 did not result in reduced SOCS-1 and -3 mRNA levels, while STAT3 tyr705 and ser727 phosphorylation levels were reduced to basal levels. The discrepancy between both findings seems to be related to the fact that additional cytokines also regulate SOCS expression. It has been demonstrated that, amongst IL-6, many other cytokines and growth factors can upregulate the expression of SOCS-1 and SOCS-3, including LIF, IL-4, IFN-γ and G-CSF\textsuperscript{18,19,21}. However, it is intriguing that after treatment with anti-IL-6, the IL-6-induced STAT3 phosphorylation is totally restored despite of the persistent expression of SOCS-1 and SOCS-3. These findings suggest that the functional activity of the negative feedback loop is not only determined by the degree of expression but might also depend on post-translational modification.

In conclusion, the data demonstrate that the autocrine and/or paracrine secretion of IL-6 by the AML cells causes the constitutive activation of STAT3, which might have important consequences for the growth and survival characteristics of AML cells.
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References


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