Stat5 tetramer formation is associated with leukemogenesis

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Summary

Activation of Stat5 is frequently found in leukemias. To study the mechanism and role of Stat5 activation, we introduced a constitutively activated Stat5a mutant, cS5^F, into murine bone marrow (BM) cells. BM transplantation with cS5^F-transfected cells caused development of multilineage leukemias in lethally irradiated wild-type or nonirradiated Rag2$^{-/-}$ mice. The leukemic cells showed strongly enhanced levels of cS5^F tetramers but unchanged cS5^F dimer levels in a DNA binding assay. Moreover, Stat5a mutants engineered to form only dimers, but not tetramers, failed to induce leukemias. In addition, Stat5 tetramers were found to accumulate in excess compared to dimers in various human leukemias. These data suggest that Stat5 tetramers are associated with leukemogenesis.

Introduction

Gene transcription through the Jak-Stat pathway mediates cytokine and growth factor functions, with important consequences for cellular proliferation, differentiation, and survival (Levy and Gilliland, 2000; O'Shea et al., 2002). Persistent Stat1/3/5 activation is frequently found in cancer. Stat1 activation is a negative regulator of proliferation, whereas Stat3/5 activation is associated with cancer progression, defining Stat3/5 as a molecular target for therapeutic intervention (Bromberg, 2002; Buettner et al., 2002). Stat5a and Stat5b double-deficient knockout mice (Stat5$^{-/-}$) have multiple hematopoietic defects. Stat5$^{-/-}$ cells display impaired cytokine-mediated proliferation and survival of hematopoietic progenitors, and T, NK, erythroid, and myeloid cells (Bunting et al., 2002; Kieslinger et al., 2000; Moriggl et al., 1999a; Teglund et al., 1998). The responsible Stat5-dependent cytokine pathways are pleiotropic (O'Shea et al., 2002).

Most signal transduction events are controlled by gene expression or require kinase cascades and adaptor molecules to phosphorylate and activate nuclear transcription factors (Brivanlou and Darnell, 2002). In contrast, Stat proteins are highly and constitutively expressed, facilitating their rapid activation. Cancer cells often become cytokine- and growth factor-independent through autocrine factor synthesis or through persistent activation of key signaling molecules, like deregulated tyrosine kinase or tyrosine phosphatases (Brivanlou and Darnell, 2002; O'Shea et al., 2002). This can cause persistent Stat activation. A well-known example of Stat5 activation is signaling through the Bcr-Abl tyrosine kinase in chronic myelogenous leukemia (CML; Buettner et al., 2002). Other genetic changes leading to Stat5 activation in leukemias include Flt-3 or c-Kit mutations, which recruit Stat5 to the respective receptors and thereby lead to persistent Stat5 activation (Mizuki et al., 2003; Taketani et al., 2004). In addition, Stat5 activation is found in

SIGNIFICANCE

Stats, particularly Stat3 and Stat5, are gaining increasing attention as essential players in the formation of leukemias and solid tumors. Stats are rarely mutated but frequently overexpressed and hyperactivated, often by alterations in upstream signaling pathways. We provide evidence that a Stat5a mutant, which forms enhanced levels of stable tetramers but otherwise behaves like wt Stat5, caused multilineage leukemias. Furthermore, our studies show that tetramerization is essential for leukemia development in mice. In addition, human leukemic samples displayed strong Stat5 tetramer formation. Our results suggest that disruption of Stat5 tetramer formation could represent a therapeutic concept to treat leukemia.

carcinomas of the breast, prostate, ovary, head, and neck (Bromberg, 2002; Buettner et al., 2002).

Recently, several mouse models showing transgenic Stat5 overexpression have been established. Stat5 activation was found to be a proliferation- and/or survival-promoting event in neoplastic cells (Iavnilovitch et al., 2002; Kelly et al., 2003; Tsuruyama et al., 2002). In other studies, Stat5 function was analyzed through the use of a constitutively active (ca) Stat5 mutant, which carried two engineered point mutations (H_{299}→R and S_{711}→F, called caStat5a1*6 and referred here to $cS5^{RF}$; Onishi et al., 1998). Expression of the $cS5^{RF}$ mutant in various cell lines showed its heterodimerization to endogenous Stat5 (Lee et al., 2001; Nosaka et al., 1999; Onishi et al., 1998; Santos et al., 2001; Schwaller et al., 2000).

Stat5 proteins contain several domains with different functional properties (Figure 1A). The N-terminal tetramer formation domain (TD) is of major interest for this study. Stat N termini have regulatory functions such as receptor association, phosphatase binding, and nuclear translocation. They modulate oligomerization, tyrosine dephosphorylation, and nuclear accumulation (Meyer et al., 2004; Ota et al., 2004; Vinkemeier et al., 1996, 1998; Xu et al., 1996). Stat tetramerization is thought to increase specificity among Stat family members. Stat3 tetramerization was shown to occur on the α2-macroglobulin promoter, where multiple transcription factors assemble to build up an efficient transcription enhanceosome (Lerner et al., 2003). Similarly, tetramerization has been observed for Stat4 in the IFN-γ transcriptional regulatory region (Xu et al., 1996). Tetramer formation of Stat5 was analyzed on various transcriptional regulatory regions (John et al., 1999; Kim and Leonard, 2002; Meyer et al., 1997; Soldaini et al., 2000; Verdier et al., 1998). Stat tetramers result in more stable DNA binding complexes than dimers through increased contact points with DNA, which also allow binding to weak affinity sites (Lerner et al., 2003; Soldaini et al., 2000). Stabilized tetramers increase binding site occupancy to a threshold required for transcriptional activity. The greater degree of flexibility in DNA sequence by Stat tetramer recognition was suggested to widen target gene spectra (John et al., 1999; Meyer et al., 1997).

We studied the role of persistent Stat5a activation using a $cS5^{F}$ mutant, which closely mimicked wt Stat5 function. Our data show that enhanced Stat5 tetramers are present in human leukemias, and that they are the relevant Stat5 DNA binding complexes to induce leukemia.

Results

Characterization of a constitutively active Stat5a mutant

The introduction of the original $cS5^{RF}$ mutant into wt BM induced myeloid hyperplasia (Schwaller et al., 2000). We failed, however, to complement $Stat5^{-/-}$ hematopoietic phenotypes with $cS5^{RF}$. Thus, it remained unclear to what extent $cS5^{RF}$ functionally resembles wt Stat5. To dissect which point mutation of the originally described $cS5^{RF}$ molecule can both substitute for wt Stat5a (S5) function and induce hyperplasia, we constructed the respective single Stat5 point mutants (Figure 1A). We termed them $S5^{R}$ and $cS5^{F}$, carrying the single point mutations H_{299}→R or S_{711}→F. The $cS5^{F}$ mutant converts Stat5a to a constitutively active mutant, and as a control for $cS5^{F}$, we replaced S_{711}→A for a closer structural resemblance to $cS5^{F}$ and termed it $S5^{A}$.

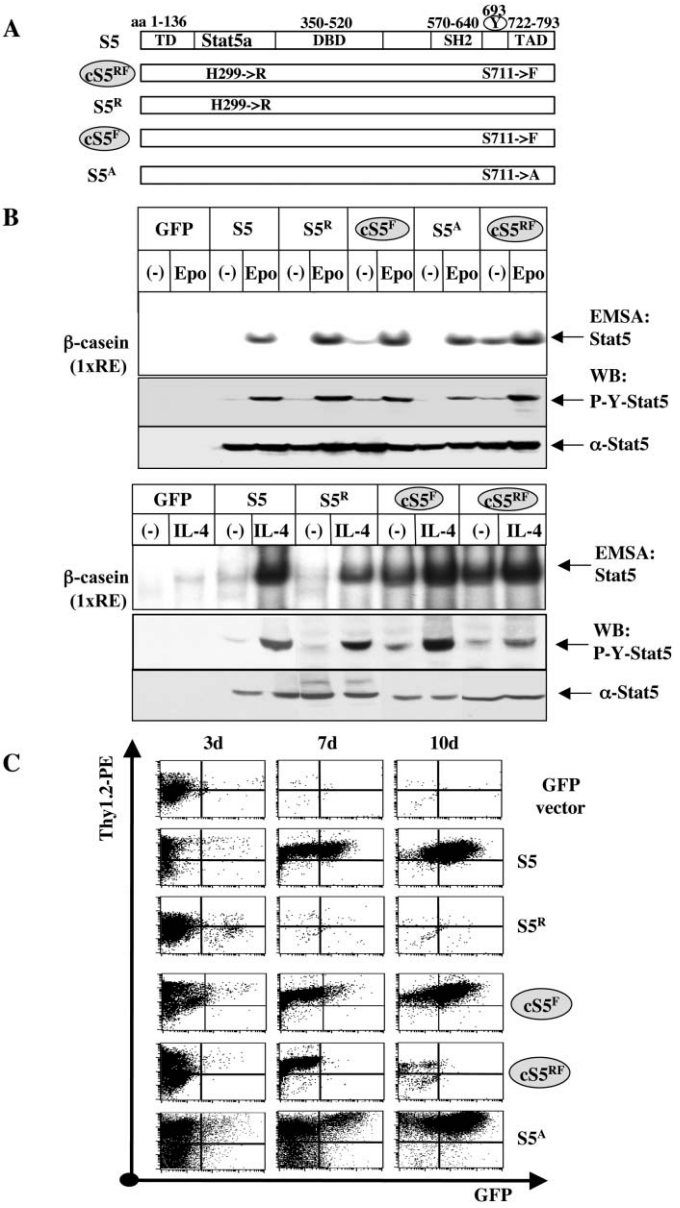


Figure 1. Biochemical and functional characterization of constitutively active Stat5a mutants

A: Schematic diagram of Stat5a and mutant derivatives. Amino acid positions of different functional domains and point mutations are indicated. Gray circles highlight the caStat5 mutants.

B: DNA binding activity (EMSA; β-casein RE), P-Y-Stat5 (WB), and subsequent total Stat5 analysis by Western blotting. The $cS5^{F}$ and $cS5^{RF}$ mutants bound to DNA due to persistent tyrosine-phosphorylation in absence of cytokine activation. Upper panel: 293T cells were transfected with the Stat constructs indicated, together with the EpoR, and stimulated with Epo (50 U/ml) for 30 min or left untreated. Lower panel: $Stat5^{-/-}$ T cell lymphoma lines expressing the Stat5 proteins indicated were stimulated with IL-4 (50 U/ml) for 30 min or left untreated.

C: Rescue of $Stat5^{-/-}$ primary T cells in vitro. Splenic T cells from $Stat5^{-/-}$ mice cannot proliferate in vitro in response to α-CD3 (1 μg/ml) and IL-2 (500 U/ml), but they regained proliferation capacity upon retroviral rescue with S5, $S5^{A}$, or $cS5^{F}$. Proliferating T cells ($Thy1.2^{+}$) become increasingly GFP^{+} after 3, 7, and 10 culture days. Shown are representative examples from 3 individual experiments with similar results.

Two different cell systems were employed to functionally compare Stat5a mutants to wt Stat5a. First, respective retroviral vectors were transiently transfected into 293T cells, together with the erythropoietin receptor (EpoR). Secondly, the same constructs were transduced into a transformed Stat5$^{-/-}$ cytokine-independent T cell lymphoma line and sorted for high GFP expression. Transfected 293T cells and mutant-expressing T cell lines (expressing the endogenous IL-4 receptor) were stimulated with Epo or IL-4. In both cell systems, bandshift assays and Western blotting revealed that cS5^{F} and cS5^{RF} were persistently activated in absence of exogenous cytokine stimulation, in contrast to S5, S5^{R}, and S5^{A} (Figure 1B). DNA binding specificity of Stat5a and mutant derivatives was verified on β-casein (binding of Stat5/6), IGκ light chain-ϵ (Stat6), and SIEm67 binding sites (Stat1/3/4; Figure 1B and data not shown).

Next, we asked whether the mutants could complement peripheral T cell proliferation like Stat5a in Stat5$^{-/-}$ T cells. We have shown that Stat5$^{-/-}$ T cells fail to proliferate upon IL-2 or IL-4 stimulation independent of T cell receptor activation, but proliferation can be regained upon retroviral transduction of S5 into Stat5$^{-/-}$ T cells (Moriggl et al., 1999a, 1999b; Wang et al., 2000). Interestingly, S5, cS5^{F}, and S5^{A} were able to rescue T cell proliferation, while S5^{R} or cS5^{RF} failed to do so (Figure 1C). In particular, the mutant cS5^{RF} was unable to rescue Stat5$^{-/-}$ T cells, probably due to the S5^{R} mutation (Figure 1C). The expression level of the Stat5a protein variants was moderate and ∼3× enhanced in primary T cells. cS5^{F} and Stat5a-transduced Stat5$^{-/-}$ T cells maintained a physiological IL-2 dose response for proliferation (data not shown).

Transplantation systems define cS5^{F} as a powerful oncogene

To analyze the leukemogenesis of the caStat5 mutants, BM cells were infected with respective retroviral constructs, analyzed for infection efficiency by FACS (∼5%), and injected into lethally irradiated wt or nonirradiated Rag2$^{-/-}$ mice. Mice were fully reconstituted at 4 weeks posttransplantation. Mice grafted with cells carrying the cS5^{F} mutation developed leukemia after 4 weeks, while no disease was seen in S5-transplanted controls. A highly elevated WBC, blast cell morphology in the BM, splenomegaly, lymphadenopathy, and massive liver infiltration manifested leukemia by hematopoietic blasts. Histopathology revealed similar alterations in all cS5^{F}-transplanted mice, but with varying latency depending on the transplant systems. The cS5^{F}-induced leukemia first evolved as early as 4 weeks posttransplant in lethally irradiated wt mice, which did not survive 8 weeks posttransplantation. In contrast, transplantation of S5, S5^{R}, S5^{A}, or the GFP vector did not induce leukemia up to 10 months. cS5^{F} transplantation induced leukemias within one month in all (n = 8 mice) lethally irradiated (1 Gy) wt mice, but not in nonirradiated Rag2$^{-/-}$ mice (6 month latency, ∼70% mice affected). Sublethal γ irradiation (0.7 Gy) of wt mice again prolonged cS5^{F} disease onset in all mice analyzed (n = 6 mice, data not shown). A second cS5^{F} mutant (including a C-terminal FLAG, termed cS5^{F}-FLAG) was similarly leukemogenic when compared to cS5^{F}. Lymphoid and myeloid blasts densely infiltrated the BM of diseased mice (Figure 2A). Moreover, mice developed splenomegaly (∼10× enlargement), with disrupted splenic architecture (Figure 2A). Liver sections showed massive infiltration by leukemic cells. Lymphoid and myeloid cells (Figure 2B and immunophenotyping by FACS, data not shown) densely infiltrated the portal liver fields of diseased cS5F-transplanted mice.

One report showed that the cS5^{RF} mutant caused myeloid hyperproliferation in the presence of Stat5a (Schwaller et al., 2000), but when Stat5$^{-/-}$ BM was transduced with this mutant and transplanted, no disease occurred (R.M., unpublished data). This raised the question of whether the cS5^{F} mutant can cause leukemia independent of an endogenous Stat5 heterodimerization partner. Thus, Stat5$^{-/-}$ BM was transduced with cS5^{F} and transplanted into lethally irradiated wt mice. Although leukemia onset was delayed (6 months), the evolving leukemia was comparable to that induced by transplantation of cS5^{F}-transduced wt BM. These results define cS5^{F} as oncogenic, independent of wt Stat5 as a heterodimerization partner.

FACS surface marker analysis manifests development of a multilineage leukemia

Leukemic cells from cS5^{F}-transplanted animals and empty GFP-vector-transplanted controls were subjected to lineage marker analysis by FACS, testing for GFP^{+} cells from blood, spleen, and BM (Figure 3). FACS was either done by testing cells for the lineage marker plus GFP, or by gating (see black histogram plots in Figures 3A–3C) and analyzing GFP^{+} cells for lineage marker expression. The erythroid marker Ter119, the myeloid markers Gr-1/Mac-1, the B and T lymphocyte markers CD19 and Thy-1.2, and combinations of CD31, CD34, and Sca-1 with c-Kit, typical for immature multipotent cells or HSCs, were analyzed (Figure 3). The majority of GFP^{+} BM cells were Gr-1 and Mac-1 double positive myeloid cells, and ∼20% of GFP^{+} BM cells were of B or T lymphoid origin (Figure 3B). Most notably, we obtained cS5^{F}-leukemia-specific fractions of GFP^{+} BM cells, which expressed marker combinations typical for primitive multipotent cells, suggesting that cS5^{F} induced the HSCs or immature multipotent progenitors to give rise to multilineage leukemia. These cS5^{F}-GFP^{+} BM cells from diseased mice were expanded in vitro and retained an immature HSC surface marker phenotype (F. Gouilleux and R.M., unpublished data). GFP^{-} cells showing FACS surface markers similar to GFP^{+} cells were not enriched, pointing to a cell-autonomous advantage of cS5^{F}-transduced cells. Furthermore, the leukemia latency in nonirradiated Rag2$^{-/-}$ mice or upon transplantation of Stat5$^{-/-}$ BM (Figure 2) could indicate that cS5^{F} also integrated into HSC/multipotent progenitors. The majority of leukemic cells in the blood and the spleen represented T (Thy1.2), B cells (CD19), and erythroblasts (Ter119, Figures 3B and 3C). FACS analysis of leukemic mouse blood (without red blood cell lysis) revealed a high fraction of erythroid GFP^{+}/Ter119^{+} cells or >3× elevated GFP^{+} platelet/megakaryocyte numbers (data not shown). Finally, livers from diseased mice also contained abnormally high levels of multiple hematopoietic cell types, as verified by FACS analysis (data not shown). We conclude that cS5^{F}-transplanted mice develop multilineage leukemia originating from primitive stem cell-like progenitors.

Persistent Stat5 activation causes enhanced Stat5 tetramer formation

All Stat5a mutants maintained DNA binding specificity and were supershifted by antibodies against N- or C-terminal epitopes of Stat5a (Figure 4A). To rule out that slight structural changes in mutant dimers would alter DNA binding specificity, we performed DNA binding site selection assays (Selex), but did not

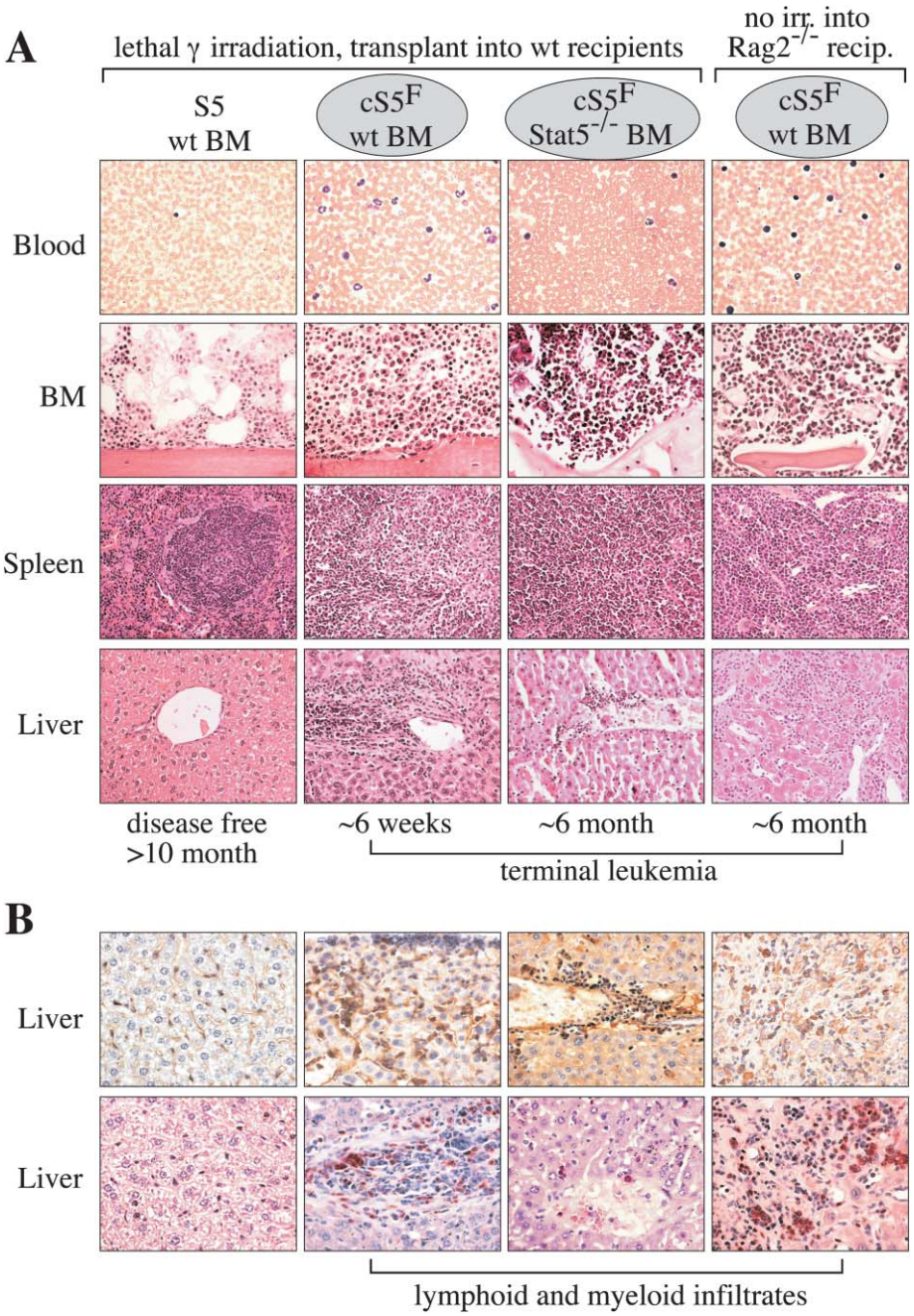


Figure 2. Histopathology and myeloid or T cell marker analysis

A: BM from wt or Stat5$^{-/-}$ was retrovirally transduced with Stat5a or cS5^{F} and transplanted into lethally irradiated wt mice (wt BM: S5, n = 8 mice or cS5^{F}, n = 8 mice; Stat5$^{-/-}$ BM: S5, n = 4 mice [not shown] or cS5^{F}, n = 4 mice). In addition, nonirradiated Rag2$^{-/-}$ mice were employed (for Stat5a, n = 4 mice, data not shown; or cS5^{F}, n = 4 mice). The cS5^{F}-transplanted animal groups developed leukemia with distinct latency. S5 transplanted mice do not develop leukemia and have a normal histology. Mice transplanted with cS5^{F}-transduced cells developed leukemia with elevated numbers of lymphocytes, myeloid lineage cells, and immature precursors in PB. The spleen germinal center architecture is disrupted in cS5^{F}-grafted mice, and splenomegaly developed. The cS5^{F}-transduced BM is filled with blast-like cells. In addition, the portal tracts as well as parts of cS5^{F} liver parenchyma are diffusely infiltrated by hematopoietic blasts. Note the significant delay (>4× longer) in onset of disease when cS5^{F} is transplanted into Rag2$^{-/-}$ mice or transduced into Stat5$^{-/-}$ BM.

B: Diseased livers were examined for the presence of hematopoietic cells using immunostaining for α-CD3 (T cell surface marker, brown staining, upper) or esterase (myeloid marker, red staining, lower). The cS5^{F}-transplanted mice show highly positive immunostaining in contrast to controls. Four mice were analyzed from each transplant group with similar histology.

observe significant DNA consensus site differences between wt and mutant Stat5 proteins (see Figure 4B and Supplemental Table S1). Importantly, several Stat5 target genes contain multiple high-affinity Stat5 binding sites to one another in their transcriptional regulatory region, and we give sequence examples of the Stat5 response elements in the Supplemental Data (if spaced less than 70 bp, they are given in sequence context). We selected three Stat5 tetramer elements from natural promoters, which had 2 closely spaced (3 to 11 base pairs) response elements (CIS-AB, CIS-CD, CD25), and used a β-casein-2x high-affinity Stat5 tetramer element to analyze mutant tetramer formation compared to wt Stat5a (see Supplemental Data). Transiently transfected EpoR-293T cells were stimulated with Epo to activate Stat5a or mutant derivatives. First, we verified DNA binding activity with the β-casein dimer site. Stat5a and mutant Stat5a extracts were added to the DNA binding reactions in rising amounts as indicated in Figure 4C. Second, the CIS-CD and IL-2Rα tetramer elements only allowed the formation of a slow migrating Stat5a tetramer complex (see below), whereas the tetramer elements CIS-AB and β-casein-2× allowed both tetramer and dimer binding (Figure 4D). Specificity of the observed complexes was verified by supershift analysis (Figure 4D). Next, the adjusted mutant extracts were loaded onto continuously running native PAGE gels. Cold probes were added after achieving a saturated binding reaction, displacing dimers within minutes. Tetramers, however, required more than

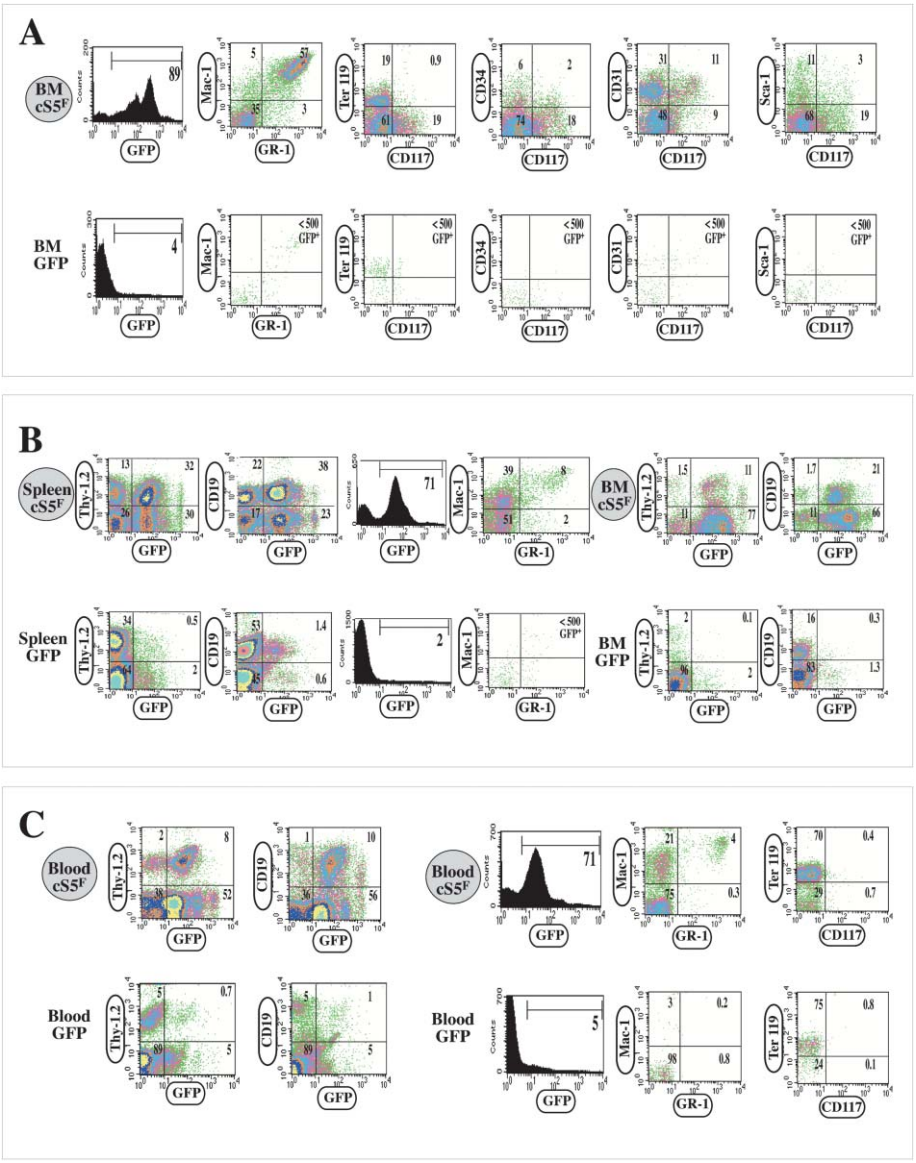


Figure 3. FACS analysis of leukemic cS5^F-cells defines a multilineage leukemia

Cells from BM (**A** and **B**), spleen (**B**), and PB (**C**) from cS5^F and control transplanted animals (empty GFP vector, GFP) were subjected to FACS analysis, using markers and combinations typical for erythroid (Ter119/CD117) myeloid, (GR-1/Mac-1) T (Thy-1.2), and B cells (CD19) and multipotent cells (CD117, CD31, CD34, Sca-1). Cells were analyzed after gating for GFP$^+$ cells, showing GFP fluorescence intensity distributions (black histograms), and percentages of GFP$^+$ cells were gate-selected as indicated in the histogram plots (**A–C**). Alternatively, a live gate was applied to analyze cells double-positive for GFP and the marker chosen (**B** and **C:** lymphoid markers in BM, spleen, or PB). Here, GFP$^+$ empty vector control cells contained >14-fold reduced numbers of GFP$^+$ cells and showed FACS surface marker patterns distinct from those of the cS5^F-transduced leukemic cells. Four cS5^F-transplanted mice were analyzed at 5 weeks post-transplant with ~15× elevated WBC, versus two GFP-transplanted mice with normal WBC. The relative FACS marker expression in the four different cS5^F-transplanted mice was similar.

30 min for partial displacement. All mutant extracts displayed a similar DNA binding activity compared to Stat5a extracts on the CIS-AB site (Figure 4E). The CIS-AB site cannot be regarded as a loading control of Stat5-tetramers. Only the dimer activity adjustment allows for comparison of different extracts on tetramer sites (Figures 4C and 4E). The mutant cS5^F proteins showed more stable tetramer formation and affinity on the CIS-CD, β-casein-2×, and IL-2Rα sites, as compared to Stat5a. Differences in tetramerization were most striking on the β-casein-2× site, on which Stat5a did not form a stable tetramer (Figure 4E).

cS5^F-rescued Stat5$^{-/-}$ T cells retain Stat5 target gene specificity in response to IL-2

We used the Stat5$^{-/-}$ T cell rescue assay to determine the regulation of IL-2 regulated mRNA expression in Stat5-deficient T cells, complemented by mutant Stat5 (Figure 1C). Primary GFP$^+$ T cell cultures were established, and mRNAs were isolated from S5-, cS5^F-, S5^A-complemented Stat5$^{-/-}$ or wt T cells for controls. Peripheral T cells lacking Stat5 failed to enter the cell cycle and died in presence of cytokines. We focused on Stat5 target genes important for cell-cycle progression or survival in T cells, like c-*Myc*, Bcl-x$_L$, or D-type cyclins (Lord et al., 2000; Moriggl et al., 1999a, 1999b). Moreover, we hybridized for the expression of the cytokine oncostatin M (Osm), the IL-2Rα chain, and members of the Socs gene family (CIS, Socs-1, Socs-3) as important regulators for cytokine signaling and known Stat5-regulated mRNAs. Thus, we performed Northern blot analysis of mRNAs isolated from Stat5$^{-/-}$ T cells rescued by Stat5a, cS5^F, or S5^A upon IL-2 deprivation and IL-2 restimulation, in comparison to wt T cells. An aliquot of the stimulated cells was used for an EMSA to control for the starvation and restimulation with IL-2 (Figure 5A). cS5^F-rescued T cells had activated Stat5a proteins even after 18 hr of IL-2 deprivation. The control cells lost Stat5a activation and downregulated cyclin D2, cyclin D3, and c-*Myc* mRNAs upon IL-2 withdrawal (Figure 5). cS5^F-rescued T cells showed impaired downregulation of

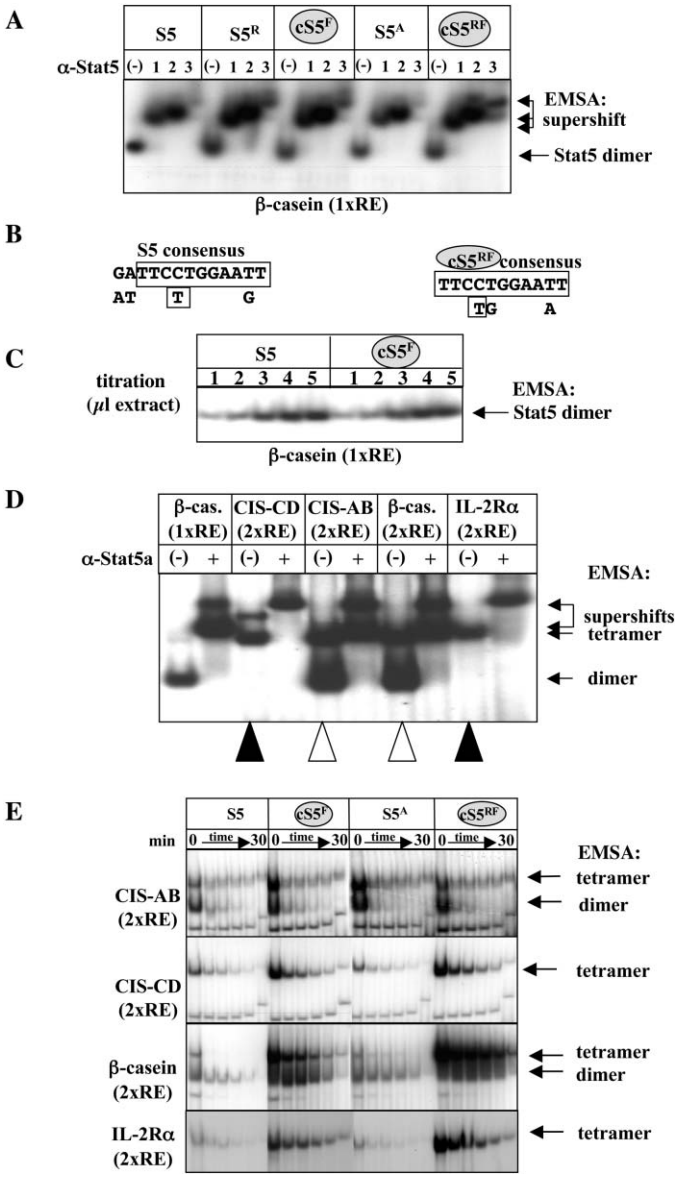


Figure 4. The cS5^F mutant exhibits unchanged dimers, but enhanced tetramer formation

A: Stat5a containing extracts from 293T cells stimulated with Epo (50 U/ml; 30 min) were subjected to EMSA using the β-casein site. Three different antisera for supershift analysis were used (Stat5 epitope: 1 = N20, N-terminal; 2 = C17, C-terminal; 3 = α-Stat5a, C-terminal; Wang et al., 2000).

B: Selex analysis revealed a closely related consensus sequence recognized by Stat5a or cS5^{RF} (see Supplemental Table S1).

C: Stat5a or cS5^F were stimulated with Epo and equal activities were loaded to estimate equal dimer activity before analyzing the extracts in tetramer assays.

D: Dimer or tetramer formation assays with Stat5a extracts on various Stat5 response elements, allowing dimer (β-casein) or tetramer binding (CIS-AB, CIS-CD, β-casein2×, IL-2Rα). The tetramer elements show either dimer and tetramer formation (white arrowheads) or tetramer formation only (black arrowheads). C17 antisera were used for supershift analysis.

E: Tetramer formation of the indicated Stat5a derivatives was analyzed with 293T cell extracts stimulated with Epo using four different tetramer DNA binding elements (see Supplemental Data). Stat5a and mutant derivatives bound to the CIS-AB site similarly. Tetramers were more stable toward competition by cold DNA as compared to dimers. The absence of cold DNA (0) represents the saturated binding reaction. This was followed by a 100× cold DNA competition for the following times: 1, 5, 10, 20, or 30 min. Note that cS5^F and cS5^{RF} form tetramers with enhanced DNA binding activity and stability toward competition by cold DNA on the CIS-CD, β-casein-2×, and IL-2Rα elements. S5 and the control mutant S5^A failed to do so. Representative blots of two individual experiments are shown.

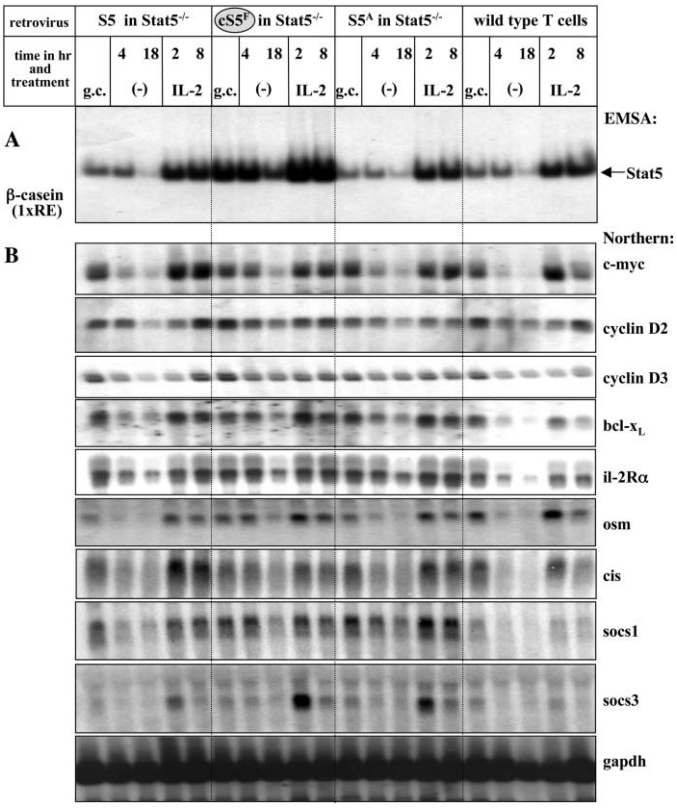


Figure 5. Analysis of cytokine-dependent mRNA expression of complemented Stat5$^{-/-}$ T cells

A: Stat5$^{-/-}$ T cells were rescued by transduction with Stat5a, cS5^F, or S5^A, and wt T cells served as a control. Cells were taken after 2 weeks growing cultures (g.c.), and before starvation, they were washed 3× in T cell media without IL-2. Cell aliquots were taken after 4 or 18 hr of IL-2 starvation (−), after which IL-2 was added back for 2 or 8 hr to restimulate Stat5a signaling. Extracts were isolated and introduced to EMSA assays.

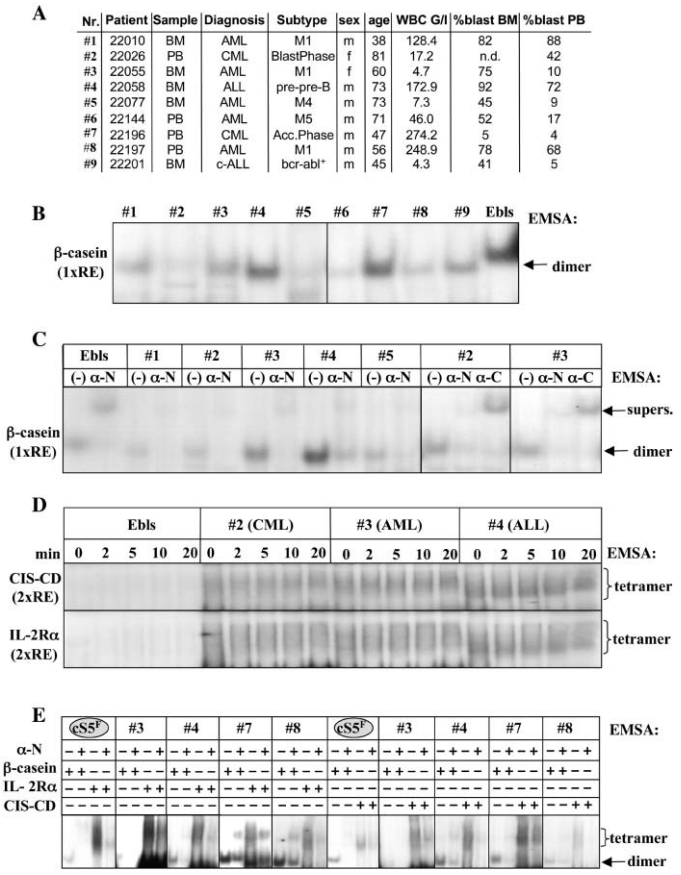
B: Total RNA was isolated, followed by Northern blot using probes specific for Stat5 target genes or gapdh as loading control. Two individual experiments yielded similar results.

mRNAs for c-*Myc*, Bcl-x$_L$, cyclin D2, cyclin D3, IL-2Rα, Osm, CIS, or Socs-1 upon IL-2 deprivation (Figure 5B). Cyclin D3 mRNA upregulation occurs only after 8 hr of IL-2 readdition in wt T cells (data not shown), whereas S5^A- or cS5^F-rescued Stat5$^{-/-}$ T cells displayed enhanced cyclin D3 levels even after 2 hr of IL-2 readdition. Differences of magnitude in mRNA induction are seen in the regulation of CIS, which is less activated upon IL-2 restimulation in cS5^F-rescued T cells than in the three control groups. Socs-1 mRNA induction was transiently enhanced in cS5^F-, S5^A-, or S5-rescued T cells, but Socs-1 and Socs-3 activation were not significantly expressed in wt T cells. We conclude that target gene specificity was retained when cS5^F was introduced to Stat5$^{-/-}$ T cells. In general, S5- or S5^A-rescued T cells displayed a stronger mRNA upregulation after

IL-2 restimulation. As a consequence, cS5^F-rescued T cells did not undergo a G1 cell cycle arrest upon IL-2 deprivation (R.M., unpublished data).

Leukemic Stat5a proteins from patient samples display enhanced tetramer formation

To demonstrate relevance for human leukemias, we screened samples of primary cells obtained from 58 patients with leukemias and 6 controls (BM or peripheral blood, PB), and analyzed Stat5-tetramers. Stat5 activation was detectable in roughly 25% of the leukemic samples independent of the type of leukemia, but not in normal human BM or PB. Nine different leukemia samples were analyzed in detail (Figures 6A and 6B). As a control, we used human erythroblasts (Ebls; Leberbauer et al., 2005) stimulated with Epo (100 u/ml). Control and leukemic extracts were adjusted to equal dimer DNA binding activity (data not shown). We verified Stat5 specificity by supershift analysis with N-terminal antisera for integrity of the tetramerization domain (Figure 6C). The N-terminal rabbit polyclonal antisera results in partial supershift and DNA binding inhibition, as can be seen upon comparison with the C-terminal antisera supershift (Figure 6C, right). Some patient samples contain various shorter Stat5 proteins as recognized by a faster migration in the DNA binding assays (Figures 6B and 6C). The normalized extracts were then tested for tetramers using the four tetramer binding DNA elements as described above. Results from the exclusive tetramer DNA binding sites CIS-CD and IL-2Rα are shown in Figure 6D. No Stat5 tetramer formation was observed in Ebls, even when 10$\times$ more Ebls extract was loaded (see Supplemental Data). However, we found strong Stat5 tetramers and stability under competition kinetics independent of the leukemia type (CML, AML, and ALL; Figure 6D). Supershifts with N- or C-terminal antisera were carried out (see Supplemental Data). Stat5 tetramer formation was enhanced in the 9 leukemic patient samples investigated. Antibody supershift reactions were performed to further characterize the stoichiometry of dimers versus tetramers in patient extracts. cS5^F-transfected 293T cell extracts (see above) served as controls (Figure 6E), since Ebls extracts did not form tetramers (Figure 6D). The quantities of dimer and tetramer DNA binding response elements were equalized and incubated with extracts with and without N-terminal antisera (Figure 6E). Stat5 tetramer formation resulted in stronger and larger DNA binding complexes than dimer DNA binding complexes. Although there were strong differences between the individual patient samples, the above analysis allows the conclusion that a fraction of human leukemic patient extracts display persistent Stat5 dimer and accelerated Stat5 tetramer activity, independent of the leukemia cell type.



Nr.	Patient	Sample	Diagnosis	Subtype	sex	age	WBC G/l	%blast BM	%blast PB
#1	22010	BM	AML	M1	m	38	128.4	82	88
#2	22026	PB	CML	BlastPhase	f	81	17.2	n.d.	42
#3	22055	BM	AML	M1	f	60	4.7	75	10
#4	22058	BM	ALL	pre-pre-B	m	73	172.9	92	72
#5	22077	BM	AML	M4	m	73	7.3	45	9
#6	22144	PB	AML	M5	m	71	46.0	52	17
#7	22196	PB	CML	Acc.Phase	m	47	274.2	5	4
#8	22197	PB	AML	M1	m	56	248.9	78	68
#9	22201	BM	c-ALL	bcr-abl$^+$	m	45	4.3	41	5

Figure 6. Human leukemia samples display strong Stat5 tetramerization

A: Primary human leukemic blast cells were obtained from BM or PB of five patients with AML, two patients with pre-pre-B or Bcr-Abl$^+$ ALL, and two patients with CML in blast or accelerated phase (all patients' first diagnosis and untreated). Informed consent was obtained prior to PB donation or BM puncture.

B: Human Ebls were cultivated and stimulated with Epo (100 U/ml) for 30 min. Extracts from Ebls and leukemic patient samples were loaded on the β-casein dimer site. Note the different migration of DNA binding complexes.

C: Specificity of the complexes was verified with an N- (N20) or C-terminal Stat5 antisera (C17).

D: Tetramer competition was carried out as described in Figure 4D. The Epo-stimulated Ebls extracts did not form tetramers, in contrast to the three leukemia patient samples on the CIS-CD and IL-2Rα tetramer response elements. Specificity of the slow migrating tetramer complexes was verified on tetramer DNA binding sites using N20- and C17-terminal supershift analysis (see Supplemental Data).

E: Stoichiometry of dimer versus tetramer Stat5 DNA binding complexes was analyzed, and specificity was controlled by N-terminal Stat5 antisera (N20) of four individual leukemia extracts on the CIS-CD and IL-2Rα tetramer DNA binding response elements. cS5^F extracts from 293T cells served as a control.

Stat5a tetramer formation is essential for induction of multilineage leukemias

We performed a genetic approach to address the questions of whether Stat5a tetramers are essential for normal Stat5 function and whether cS5^F tetramers are required for multilineage leukemia onset. Two Stat mutants were described as defective in tetramerization: (1) N-terminal deletion of a stable domain of 136 amino acids and (2) a point mutation of a conserved tryptophane residue (W_{37}) in the polar interface mutated to alanine (John et al., 1999; Vinkemeier et al., 1998). Both mutations abolish Stat5a tetramerization (data not shown). Hence, they were introduced into cS5^F (Figure 7A). Transient transfection of the mutants into 293T cells, together with the EpoR, led to an Epo-inducible complex when extracts were analyzed using the β-casein site. Surprisingly, deletion of the N-terminal domain of wt Stat5a (S5ΔN) rendered S5ΔN constitutively active due to tyrosine phosphorylation (Figures 7B and 7C). Thus, the Stat5a N terminus has a regulatory role in preventing Stat5 activation in absence of cytokines. The W_{37}→A mutation was only constitutively active in combination with the cS5^F mutation (Figures 7A–7C).

We then addressed the ability of the mutants to dimerize or tetramerize, using the CIS-AB tetramer and dimer, and the

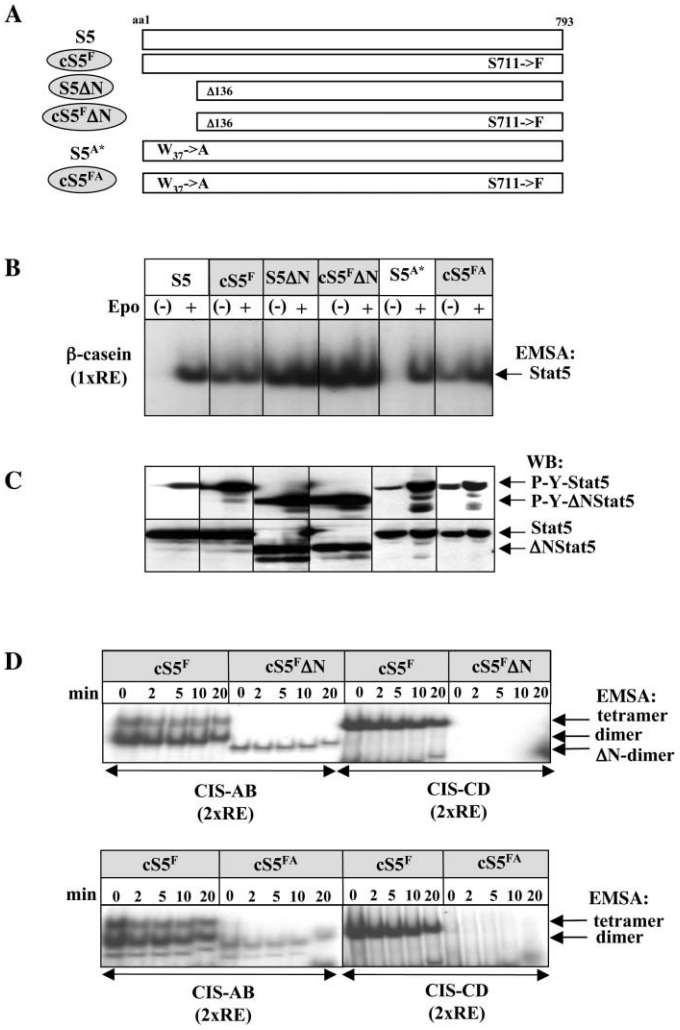


Figure 7. Biochemical characterization of tetramer-deficient Stat5a mutants

A: Schematic diagram of mutant derivatives of Stat5a and cS5^F, either lacking the tetramerization domain or carrying a point mutation at amino acid position 37 (W→A).

B: DNA binding activity on the β-casein RE. Retroviral vectors expressing Stat5a and mutant proteins listed in **A** were cotransfected with the EpoR into 293T cells, which were then stimulated with Epo (50 U/ml, 30 min).

C: Tyrosine phosphorylation and expression analysis with extracts as described in **B**. Mutants cS5^F, cS5^{FA}, and cS5^{F}ΔN, but also S5ΔN, bound DNA due to persistent tyrosine phosphorylation in absence of cytokine activation (highlighted by gray fields).

D: Tetramer analysis using extracts from 293T cells stimulated with Epo. Extracts were adjusted for equal dimer binding activities and analyzed in tetramer DNA binding assays as described in Figure 4C. On the CIS-AB element, cS5^F and the tetramer-deficient cS5^{FA} or cS5^{F}ΔN derivatives showed similar dimer binding, while tetramer binding was lost in the mutants, also seen for the tetramer-only binding CIS-CD element. Similar results were obtained using β-casein2× and CD25 tetramer elements (not shown).

CIS-CD tetramer DNA binding elements. While the parental cS5^F behaved like expected and formed tetramers, both the S5^{F}ΔN and cS5^{FA} were unable to bind to CIS-CD and bound CIS-AB only as a dimer (Figure 7D). The faster migrating S5^{F}ΔN-CIS-AB complex resulted from N-terminal truncation (Figure 7D, upper panel). Similar results were obtained with the S5ΔN or S5^{A*} mutants. The results were confirmed using the β-casein-2× and the CD25 tetramer DNA binding elements (data not shown).

Next, we performed BM transplantations with the tetramerization-deficient mutants (Figure 7A) and corresponding controls (Figure 1A), including the cS5^F-FLAG mutant (see above). Both cS5^F and cS5^F-FLAG engrafted mice developed multilineage leukemia with comparable latency and a highly elevated WBC (Figures 8A and 8B). In contrast, mice engrafted with BM cells expressing the above-described tetramerization-deficient mutants stayed disease-free for more then 10 months posttransplantation. All transplanted mice were analyzed by routine WBC and FACS analysis up to 10 months after injection (Figure 8B and data not shown). While the cS5^F and cS5^F-FLAG-transplanted animals developed typical multilineage leukemia (see Figures 2 and 3), histological analysis of mice engrafted with the three tetramer-deficient caStat5 mutants failed to reveal major histological abnormalities, despite the fact that these Stat5 proteins were persistently active (Figure 7). These experiments prove that enhanced Stat5a tetramer formation is essential for leukemogenesis.

The cS5^F-transduced leukemic BM cells promote factor-independent colony growth

One characteristic feature of leukemic cells is that their cytokine requirement for survival and proliferation in vitro is decreased. BM was isolated six weeks after transplantation, and cells analyzed for colony formation in response to various factor combinations (IL-3, IL-6, oncostatin M and/or SCF, see Experimental Procedures). No significant differences in colony formation was seen in cS5^F-transduced BM cells compared to respective Stat5a- or wt BM cells, when exposed to high or limiting (10-fold reduced) cytokine levels (data not shown). Similarly, IL-3-dependent colony formation was comparable (Figure 8C, left panel), emphasizing that the cS5^F mutant transduced BM cells were still responsive to cytokines. However, cS5^F-transduced, but not S5 or control BM cells, gave rise to factor-independent colonies, which remained viable and increased in size for 3 weeks. Cytospins and FACS analysis showed that the factor-independent colonies were >70% GFP/Mac-1/Gr-1-positive, identifying them as monocyte-like cells (Figure 8C, right panel). Moreover, continuously proliferating GFP$^+$ cell lines could be generated from the cS5^F-transduced BM cells in the presence of SCF (data not shown).

Discussion

Increasing evidence points to an essential role for persistent activation of Stat3 and Stat5 in the formation of leukemias or solid tumors (Levy and Gilliland, 2000; Bromberg, 2002; Yu and Jove, 2004). We reconstituted mice with bone marrow cells expressing a constitutively active Stat5a point mutant (cS5^F). This mutation increased both the abundance and the stability of Stat5 tetramers as compared to S5, and converted Stat5 into an oncogene able to induce multilineage leukemia. Several N-terminal mutations engineered to abolish tetramers while leaving dimers intact failed to induce multilineage leukemia. Importantly, enhanced Stat5 tetramer formation was also observed in human leukemia. Taken together, our study identifies Stat5 tetramers as a driving force of Stat5-induced leukemia and postulates that interference with Stat5 tetramers may represent a new approach for targeting leukemia cell growth.

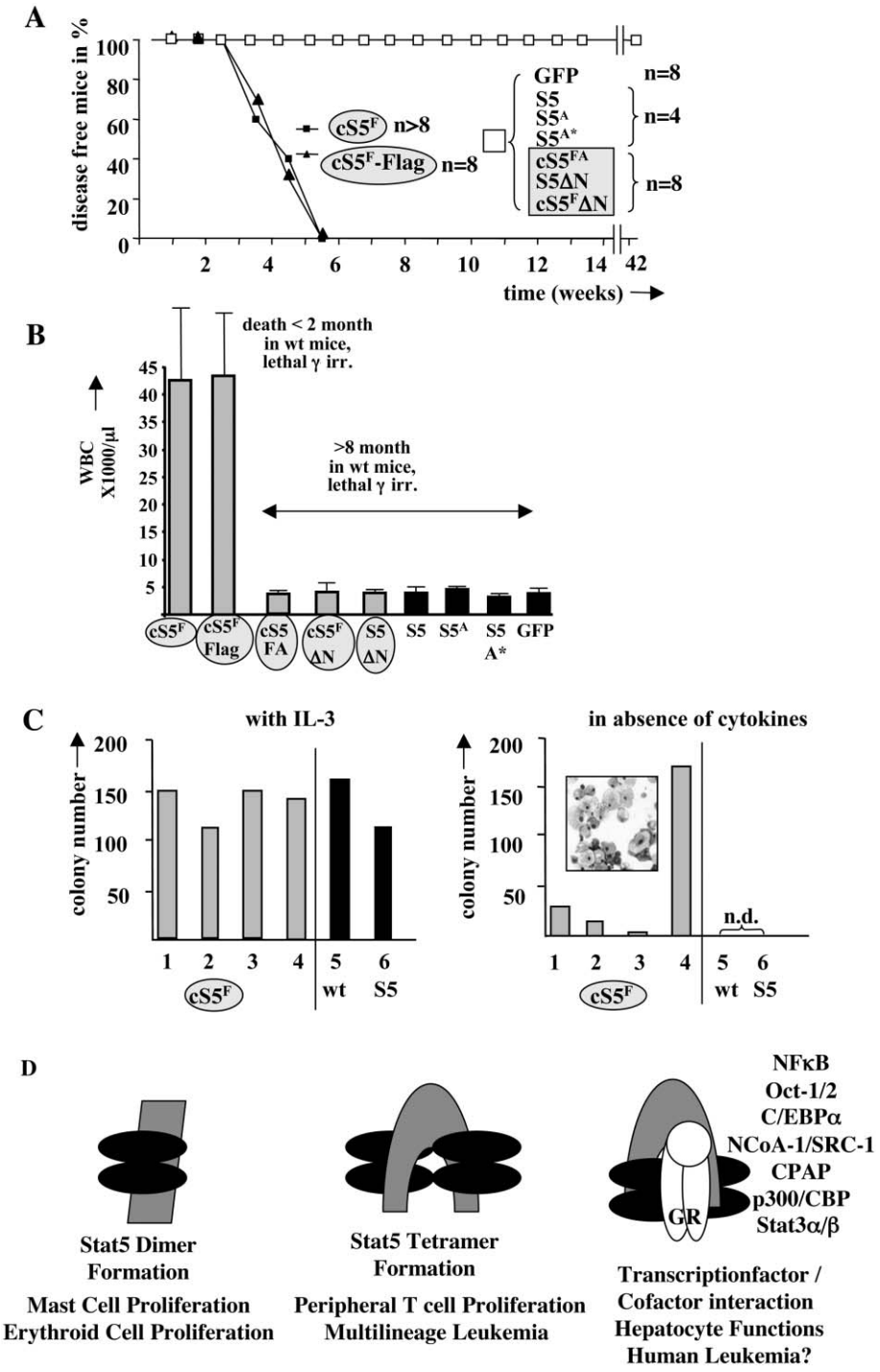


Figure 8. Leukemia depends on a functional cS5^F tetramer supporting factor-independent colonies

A: Kaplan-Meier plot of Stat5a or mutant derivatives used in this survival study (n ≥ 4 for each group). Only cS5^F- or cS5^F-FLAG-transplanted mice (n ≥ 8 for each group) died within ~7 weeks posttransplantation, while mice transplanted with Stat5a or the tetramer-deficient mutants remained disease-free for up to 10 months after transplantation.

B: Elevated WBC counts are seen in cS5^F-grafted mice, but not in wt Stat5a or tetramer-deficient Stat5a mutant transplanted mice (see **A**).

C: BM from wt mice or Stat5a- (2 mice analyzed) or cS5^F-transplanted mice (4 mice analyzed) were isolated 5 weeks after transplantation, seeded into methylcellulose, and analyzed for IL-3-dependent or factor-independent colony formation. Inset: May-Grünwald-Giemsa-stained cytospin from isolated, factor-independent colonies, containing GFP/Mac-1/Gr-1 triple-positive cells with a monocyte-like morphology. Typical data from three individual experiments yielding identical results are shown.

D: Schematic model for the functional Stat5 complexes in hematopoietic cells. Stat5 monomers (black ellipse) can form a dimer (left) or tetramer (middle and right) on DNA (gray area). Stat5 tetramer formation is essential for peripheral T cell proliferation and multilineage leukemia development, but tetramerization is dispensable for myeloid mast or erythroid cell proliferation (K.D.B. and R.M., manuscript in preparation). Other transcription or cofactors like the GR dock to Stat5 to enhance transcription, but they might use Stat5 tetramers as a signaling platform in leukemias (right).

Enhanced Stat5 tetramer formation is essential for multilineage leukemia

The single point mutation (S_{711}→F) in cS5^F rendered Stat5 constitutively active and allowed the formation of stable tetramers on a variety of binding sites. S5 and the S5^A control mutant (S_{711}→A) lacked constitutive activity and formed tetramers to a lower extent on a restricted number of DNA elements (Figure 4E and data not shown). While S5 and S5^A rescued proliferation of Stat5$^{-/-}$ T cells, they failed to induce multilineage leukemia. Moreover, Stat5a mutations at the N terminus designed to abolish tetramer formation also failed to induce leukemias or to rescue Stat5$^{-/-}$ T cell proliferation, despite the persistent activation of S5ΔN proteins. These observations indicate that the enhanced tetramer formation of cS5^F is the essential feature responsible for leukemogenesis.

Stat tetramers have more contact points with DNA than dimers, and they form more stable DNA complexes with enhanced binding to "weak" binding sites (Soldaini et al., 2000;

Zhang and Darnell, 2001). Tetramer formation may therefore increase binding site occupancy on weak sites to a threshold required for transcriptional activity which, together with the greater degree of flexibility in DNA sequence tetramer recognition, was suggested to widen target gene spectra (John et al., 1999; Meyer et al., 1997). Thus, tetramerization increases specificity and selectivity among Stat family members, as has been described for the CD25 enhancer (Meyer et al., 1997). Putative Stat5 tetramer target genes include D-type cyclins, Bcl-x_L, Osm, CD25, CIS, Socs-2, als, and igf-1, all exhibiting two to four high-affinity Stat5 binding sites in their transcriptional regulatory units (see Supplemental Table S2). Stat tetramers have a different protein surface accessible to different proteins compared to dimers (Vinkemeier et al., 1998). This may allow selective recruitment of transcription factors or coactivators by Stat5 tetramers. The binding of activated Stat5 to the tetramer elements in the transcriptional regulatory regions of spi2.1, β-casein, igf-1, or als led to recruitment of the GR in absence of GR-DNA binding (see model Figure 8D; Stoecklin et al., 1997; Tronche et al., 2004).

Two recent reports show that the N-terminal domain of Stat4 has functions in oligomerization, dephosphorylation, and nuclear accumulation (Meyer et al., 2004; Ota et al., 2004). These observations raise the issue of whether other functions of Stat5 besides tetramer formation are affected in the S5ΔN, $S5^F$ΔN, $S5^{A*}$, or $S5^{FA*}$ mutants. However, the deletion of the Stat4 N terminus resulted in a lack of cytokine inducibility, and the mutation of the conserved W_{37} residue to alanine led to misfolding of Stat1 or Stat4 (Meyer et al., 2004; Ota et al., 2004). This was not the case for Stat5, since both N-terminal Stat5a mutants showed normal tyrosine phosphorylation, dimer formation, and dimer binding to DNA (Figure 7C). Moreover, both $S5^F$ΔN and $S5^{FA}$ rescued cytokine-dependent cell expansion of primary $Stat5^{-/-}$ mast cells, again indicating that these mutants show largely normal biological activity (K.D.B. and R.M., unpublished data). Further evidence comes from a recently published report on the generation of $Stat5^{Null}$ mice (Cui et al., 2004). These mice display an aggravated phenotype compared to the originally described hypomorphic $Stat5^{-/-}$ mice (Cui et al., 2004; Teglund et al., 1998). The hypomorphic $Stat5^{-/-}$ express N-terminally truncated Stat5 proteins to those we have characterized here with the S5ΔN mutant. Apparently, the S5ΔN mutant is able to partially compensate the complete loss of Stat5a and Stat5b in a cell lineage-specific manner (see model, Figure 8D).

$cS5^F$ induces multilineage leukemia in the absence of endogenous Stat5 proteins

We also show that $cS5^F$ induces multilineage leukemia in the absence of wt Stat5a, i.e., in $Stat5^{-/-}$ BM cells. This strongly supports our concept that the essential function of $cS5^F$ in leukemogenesis is enhanced Stat5 tetramer formation on promoter elements where wt Stat5 forms predominantly dimers. $cS5^{RF}$ mutant phenotypes may also be interpreted as a consequence of heterodimers or heterotetramers formed between mutant and wt Stat5 proteins in cells containing wt Stat5. The $cS5^{RF}$ mutant showed constitutive activation and enhanced tetramers similar to $cS5^F$ (Figures 1 and 4). $cS5^{RF}$ caused myeloid hyperproliferation upon transplantation of wt BM (Schwaller et al., 2000), but it failed to induce both $Stat5^{-/-}$ T cell proliferation and leukemia in $Stat5^{-/-}$ bone marrow. The $cS5^{RF}$ mutant also promoted IL-3-independent Ba/F3 cell proliferation, while IL-3 readdition to $cS5^{RF}$-expressing Ba/F3 cells induced apoptosis (Nosaka et al., 1999). These responses are unexpected and hard to explain, since wt Stat5 promotes cytokine-dependent survival and cell-cycle progression (Bunting et al., 2002; Kieslinger et al., 2000; Moriggl et al., 1999b). $cS5^F$ causes the same events in a cytokine-independent fashion (data not shown). Other studies described a role for $cS5^{RF}$ in γ-δ T cell generation (Lee et al., 2001), despite the presence of γ-δ T cells in $Stat5^{-/-}$ mice (Moriggl et al., 1999b). We therefore speculate that the $cS5^{RF}$ mutant has additional, cell type-specific functions as a heterodimer partner of endogenous wt Stat5.

Another important question is whether $cS5^F$ and S5 differ in their DNA binding specificity. DNA binding site selection revealed that both use essentially the same consensus DNA binding site. Analysis of >10 Stat5 target genes for IL-2-dependent activation in $Stat5^{-/-}$ T cells confirmed that $cS5^F$ and S5 induce the same target genes. Moreover, we did not observe any significant upregulation of c-*fos* mRNA expression, a known target gene of Stat3 in T cells, or mRNA expression of Socs-2, a gene predominantly controlled via Stat5 in hepatocytes (data not shown). The most striking difference was the kinetic of target gene mRNA downregulation after IL-2 withdrawal. Loss of gene activation occurred more slowly in $cS5^F$-transduced $Stat5^{-/-}$ T cells. This observation is consistent with cell cycle analysis performed in $cS5^F$-transduced $Stat5^{-/-}$ T cells. A significant fraction of $cS5^F$-transduced $Stat5^{-/-}$ T cells remained in the S-G2-M phases of the cell cycle upon cytokine starvation, similar to growing culture conditions. In contrast, wt T cells strictly depended on IL-2 and arrested in G1 or died upon IL-2 deprivation (R.M., unpublished data).

What is the target cell of $cS5^F$-induced multilineage leukemia?

$cS5^F$-induced multilineage leukemia is characterized by abundant, immature myeloid/erythroid GFP^+ cells in the BM and massive infiltrates of myeloid and lymphoid cells in peripheral organs. This suggests that HSC or primitive multipotent progenitor cells are the target cells of $cS5^F$ for leukemia induction. The fact that $cS5^F$ induces leukemia in $Stat5^{-/-}$ BM with ~6× prolonged latency supports this idea, since $Stat5^{-/-}$ HSC do not respond efficiently to cytokines and thus, the retrovirus can not efficiently transduce $Stat5^{-/-}$ HSC (Bunting et al., 2002). In addition, $Stat5^{-/-}$ HSC have ~10× reduced capacity to repopulate the hematopoietic system under competitive engraftment conditions (Bunting et al., 2002). Importantly, enforced hematopoiesis as experimentally mimicked by lethal γ irradiation is beside the presence of endogenous Stat5 an additional driving force for $cS5^F$ induced multilineage leukemias. We did not observe any latency difference when we used $Stat5^{+/-}$ versus wt bone marrow (data not shown), but we observed increased latency when sublethal instead of lethal γ irradiation was chosen. Lethal γ irradiation followed by transplantation mimicked enforced hematopoiesis, and genetic changes led to cytokine release and Stat5 activation that might accelerate leukemogenesis. The ~6× shorter disease onset in lethally irradiated wt mice as compared to nonirradiated $Rag2^{-/-}$ mice supports the above hypothesis for leukemia promoting factors under enforced hematopoiesis, and overall, $cS5^F$ is a potent protooncogene.

Are Stat5 tetramers targets for selective intervention in human cancer?

Hyperactivation of Stat5 is a frequent event in various leukemias (AML, ALL, CML, and HTLV-1). Stat5 activation was also shown to be important for head and neck, prostate, and breast carcinomas (Nevalainen et al., 2004; Ren et al., 2002; Xi et al., 2003a, 2003b). Our analysis of human leukemic patient samples and the murine transplant experiments suggests a pathophysiologically relevant signaling function for Stat5 tetramers in leukemia. Tetrameric Stat5 may rarely be the consequence of mutations in human leukemia, but it could result from a persistent Stat5 activation caused by overexpressed or mutated constitutively active receptor tyrosine kinases (i.e. c-Kit and Flt3; Stirewalt and Radich, 2003). Furthermore, Stat5 tetramer complexes from leukemic extracts migrated slower then activated Stat5a tetramers from 293T cells or Epo-stimulated Ebls, which did not form significant Stat5 tetramer complexes (compare Figures 4D, 4E, 6D, and 6E and Supplemental Data). Probably due to the use of N-terminal antisera, Stat5 tetramer complexes showed an unusual behavior in supershift experiments, which had to be used to probe for the integrity of the tetramerization domain of Stat5 and to detect different C-terminal splice variants of Stat5 in leukemia (Figures 6B and 6C). The reasons for the observed incomplete Stat5 tetramer supershifts are currently unclear. Unfortunately, the N-terminal antisera employed are the only source specific to the native N terminus of Stat5 (we noted batch variations in quality). Thus, improved antisera able to recognize distinct variants of Stat5 DNA binding complexes are required for future human cancer research.

Our current work describes the expression of N-terminal variants of Stat5 in vivo, and such proteins can still interact with endogenous Stat5 or other transcription factor complexes (K.D.B., R.M., and M. Kerenyi, unpublished data). It is also tempting to speculate that leukemic Stat5 tetramer complexes recruit tetramer-specific Stat5-protein binding partners, which enhance Stat5 tetramerization. One candidate protein is the glucocorticoid receptor (GR), which docks to Stat5 as a cofactor independent of its own DNA binding ability (Figure 8D; Lerner et al., 2003; Tronche et al., 2004). In addition, several other transcription factors, chromatin regulators, or cofactors were shown in the past to use DNA-bound Stat5 as a signaling platform, as illustrated in Figure 8D.

Our data from the murine transplant models indicate that the specific disruption of Stat5 tetramers may be an innovative approach to inhibit growth of neoplastic cells. Whether the same holds true for human leukemias is presently unknown. It may be an attractive concept, since human leukemia samples contained significant Stat5 tetramer levels. Moreover, we found that incubation of primary CML cells with the Bcr/Abl tyrosine kinase inhibitor STI571 (clinically used to target leukemia cell growth) results in downregulation of Stat5 activity, and a parallel decrease in Stat5 tetramer formation (R.M., unpublished data). In this regard, it is also noteworthy that peptides from the Stat3 or Stat5 tetramerization domain were found to inhibit breast cancer cell growth (Primiano et al., 2003).

Experimental procedures

Human patient samples, animals, primary cell isolation, cultivation, and retroviral infection

Splenic T or BM cells were isolated from 6- to 12-week-old wt mice with the same genetic background as the Stat5$^{-/-}$ mice (Moriggl et al., 1999b; Teglund et al., 1998). BM was harvested from both hind limbs of either Stat5$^{-/-}$ or wt males. Freshly isolated BM cells were preactivated for 48 hr in medium containing IL-3 (25 ng/ml), IL-6 (50 ng/ml), and SCF (200 ng/ml), and consequently cocultured on irradiated (1.5 Gy) semiconfluent ecotropic producer cell lines for 48 hr in the presence of 6 μg/ml polybrene. Lethally irradiated wt female mice (1 Gy) or nonirradiated Rag2$^{-/-}$ female recipients (C57B/L6) were reconstituted with the transduced BM by tail vein injection (4×10^6 cells). Transplanted mice were checked for disease onset, upon which they were analyzed. Primary human leukemic blast cells were isolated from BM or PB using Ficoll and washed in ice-cold PBS supplemented with complete protease inhibitors (Boehringer) and phosphatase blockers (1 mM NaF, 1 mM Na_3VO_4, 10 mM β-glycerophosphate).

Cytokine stimulation, Western blotting, and DNA binding assays

Naive splenic T cells were stimulated with α-CD3 (145.2C11; Pharmingen) and IL-2 (500 U/ml; R&D Systems) and expanded in presence of IL-2. Cell purity was determined by FACS. At the indicated times, the cells were collected, added to excess ice-cold PBS, and processed to lysis. 293T cells were transfected by calcium phosphate precipitation with the murine pXM-EpoR expression vector (4 μg/transfection) and/or Stat5a (4 μg/transfection) or mutant derivatives in the retroviral pMSCV-IRES-EGFP vector. Sample preparation, electrophoresis, and transfers were as described (Moriggl et al., 1999b). Stat5 Y-phosphorylation was detected by α-P-Y-Stat5ab (#71-6900; Zymed), and reprobed with α-Stat5ab (aa 451–649; BD Transduction Laboratories). Bandshift assays were performed for Stat5 dimers or for tetramers (Vinkemeier et al., 1996). 20 μg of extracts were analyzed using blunt-ended annealed oligonucleotides. C17 and N20 supershift reagents were purchased from Santa Cruz.

Northern blot analysis

RNA was isolated as described (Moriggl et al., 1999b) and murine DNA probes for cyclin D2 (1.2 kb EcoRI), D3 (1.7 kb EcoRI), c-*Myc* (1 kb SacII/XbaI), Bcl-x_L (0.2 kb BamHI), Osm (0.6 kb of Acc. D31942: nt26–588), CD25 (0.5 kb of Acc. M30856: nt451–980), CIS (0.9 kb EcoRI/XbaI), Socs-1 (EcoRI/XbaI), and Socs-3 (0.8 kb EcoRI) genes were used, with gapdh (1 kb PstI) as the loading control.

Generation of retroviral packaging cell lines and description of Stat5$^{-/-}$ T cell lines

The Stat5a-pMSCV-IRES-GFP vector was used, and all mutants were PCR-mutagenized and sequenced (Moriggl et al., 1999b). Ecotropic, replication incompetent, retroviral producer cell lines were based on the gpE+86 system. GFP$^+$ FACS-sorted mass populations or single clones gave identical leukemia onset. Producer cell lines had high viral titers, in the range of $\sim 10^6$ particles/ml. The MuMoMTV T cell lymphoma cell line was obtained after transformation with the murine moloney leukemia virus isolated from a T cell lymphoma arising 6 month post infection from Stat5$^{-/-}$ mice. Retroviral constructs were transduced and sorted 2× for stable GFP expression (>98%).

Mouse PB hematology, FACS, and histology

Tissues were fixed in 4% formaldehyde/PBS, paraffin-embedded, sectioned, and stained with hematoxylin-eosin. BM analysis was performed with paraffin and acrylate-embedded specimens. Esterase and α-CD3 staining (DAKO) were performed. Blood was obtained from the tail vein, and WBCs were determined using an automated cell counter (SYSMEX K-1000; TAO Medical Electronics). PB smears were stained with a HEMA3 xanthene/thiazine dye set (Fisher Scientific). Cell suspensions were preincubated with CD16/CD32 antibodies to prevent nonspecific Fc receptor binding. Thereafter, 5×10^5 cells were stained with antibodies conjugated with fluorescent markers (Pharmingen) and analyzed by FACS (Becton Dickinson Calibur, Cell Quest Software).

In vitro transformation assays

BM was harvested from mice, washed 2× in PBS, and plated in methylcellulose (M3231, StemCell Technology) at a density of 1×10^5 cells/ml. Cytokines were used individually or in combinations: IL-3 (25 ng/ml), IL-6 (50 ng/ml), oncostatin M (20 ng/ml), and SCF (200 ng/ml).

Supplemental data

Supplemental data for this article can be found at http://www.cancercell.org/cgi/content/full/7/1/87/DC1/.

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