

Interleukin-18 directly activates T-bet expression and function via p38 mitogen-activated protein kinase and nuclear factor- κ B in acute myeloid leukemia-derived predendritic KG-1 cells

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Abstract

The leukemic cell line KG-1 was isolated from a patient with acute myeloid leukemia and is regarded a cellular model of human dendritic cell progenitors. The T helper type 1 cytokine interleukin (IL)-18 has been shown to induce the maturation of these cells towards a dendritic phenotype and, moreover, is able to mediate IFN γ production in this model. Because T-box expressed in T cells (T-bet) is considered to be of paramount importance for dendritic cell function, the effects of IL-18 on this transcription factor have been investigated in the current study. Here, we show that activation of KG-1 cells by IL-18 induces T-bet mRNA and protein within 4 to 6 h of incubation. This hitherto unrecognized function of IL-18 was suppressed by the inhibition of p38 mitogen-activated protein kinase activity and nuclear factor- κ B function. Blockage of translation by cycloheximide, usage of neutralizing antibodies, and the inability of IFN γ to mediate significant p38 mitogen-activated protein kinase activation in KG-1 cells clearly revealed that activation of T-bet was not via autocrine IFN γ . T-bet function was evaluated by short interfering RNA technology. Notably, specific suppression of T-bet induction impaired secretion of IFN γ by KG-1 cells under the influence of IL-18. Therapeutic application of IL-18 has the potential to profoundly affect the biology of acute myeloid leukemia predendritic cells such as KG-1 cells. Under these conditions, activation of

T-bet may play a key role in processes that have the potential to correct the T helper type 1 deficiency associated with leukemia-mediated immunosuppression. [Mol Cancer Ther 2007;6(2):723–31]

Introduction

Interleukin (IL)-18 is a proinflammatory T helper type 1 (Th1)-like cytokine that is secreted primarily by monocytes/macrophages and dendritic cells upon activation of the innate immune system. In fact, this cytokine seems to be located at a rather proximal position within the proinflammatory cytokine cascade and plays a pivotal role in the generation of efficient immunity against various bacterial and viral infections (1–9). A prominent function of IL-18 is its crucial role as an efficient costimulus for IFN γ production, particularly in synergism with IL-12 or T cell receptor stimulation. Besides T cells and natural killer cells, human dendritic cells (DC) are also capable of potently releasing IFN γ under the influence of IL-18 (3, 10). IL-18 biological activity seems to be controlled by an IFN γ -induced negative feedback loop mediated by the IL-18 opponent IL-18-binding protein (11). Interestingly, it has recently been shown that IL-18 was able to induce the maturation of human monocyte-derived dendritic cells with regard to biochemical as well as functional characteristics (12).

The transcription factor T-box expressed in T cells (T-bet; Tbx21) has been identified as chief determinant of Th1 lineage commitment and Th1-like immune responses in general (13, 14). In fact, ectopic expression of T-bet in highly polarized human Th2 cells lead to the strong induction of IFN γ , which is a prominent Th1 signature cytokine (15). A recent genetic approach further underscored the relevance of T-bet for Th1/Th2 decisions in humans (16). Interestingly, T-bet is up-regulated in patients with Crohn's disease, an inflammatory disorder considered to be driven by Th1-like cytokines. Spontaneous IFN γ production by *ex vivo*-cultured lamina propria mononuclear cells obtained from these patients was actually associated with high levels of T-bet expression (17). T-bet-deficient mice display drastically increased susceptibility for severe infection with *Salmonella* (18) or *Mycobacterium tuberculosis* (19). These observations again emphasize important functions of this transcription factor in Th1-dependent immunity. Recent data also indicate that T-bet activation is a key determinant of dendritic cell function. T-bet is potently induced by IFN γ in human monocytes and myeloid dendritic cells (20). Interestingly, dendritic cells from T-bet-deficient mice have severely impaired capabilities to produce IFN γ (21).

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The KG-1 cell line was isolated from a patient with acute myeloid leukemia (AML) and displays characteristics of predendritic cells. Activation of KG-1 cells by specific cytokines including IL-18 is actually sufficient to initiate the process of maturation towards a clear dendritic phenotype (12, 22). In order to further broaden the current knowledge on the biology of human dendritic cells, and in particular, leukemic AML-DC, we studied the expression of T-bet in this cell type. Specifically, the effects of the IFN γ -inducing factor IL-18 on T-bet are investigated herein.

Materials and Methods

Chemicals

SB203580, SB202190, and I κ B kinase (IKK) inhibitor VII (IKK-VII) were from Calbiochem-Novabiochem (Bad Soden, Germany). Cycloheximide, nigericin, and the phorbol ester TPA were from Sigma (Taufkirchen, Germany). IFN γ was obtained from TEBU/Peptrotech (Frankfurt am Main, Germany), whereas IL-18 was obtained from R&D Systems/MBL (Wiesbaden, Germany). An IFN γ -neutralizing antibody (NA/LE quality) was purchased from BD Bioscience/PharMingen (Heidelberg, Germany).

Cultivation of KG-1 Cells, HL-60 Cells, and THP-1 Cells

The human AML-DC cell line KG-1 was obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Cells were cultivated in RPMI 1640 supplemented with 100 units/mL of penicillin, 100 μ g/mL of streptomycin, and 10% heat-inactivated FCS (Life Technologies, Eggenstein, Germany). For experiments, cells were routinely seeded at a density of 6×10^6 cells/2 mL in six-well polystyrene plates (Greiner, Frickenhausen, Germany) using the aforementioned medium. AML-HL-60 cells (kindly provided by Prof. Steinhilber, Institute of Pharmaceutical Chemistry, Johann Wolfgang Goethe-University, Frankfurt am Main, Germany) were maintained and stimulated as described for KG-1 cells. Monocytic THP-1 cells were obtained from the German Collection of Microorganisms and Cell Cultures. Cells were maintained and stimulated in the aforementioned supplemented RPMI 1640 culture medium (plus 10 mmol/L HEPES) using polystyrene flasks (Greiner). For experiments, 2 or 1 mL of cell suspensions were seeded into six-well or 24-well polystyrene plates, respectively (Greiner), at 0.5×10^6 cells/mL. All incubations of cells were done at 37°C and 5% CO $_2$. Cell viability was determined by the trypan blue dye exclusion assay.

Detection of Cytokines by ELISA

Levels of IFN γ (BD Bioscience/PharMingen), IL-12 (p70; Biosource, Solingen, Germany), and IL-18 (MBL/Biosource) in cell-free culture supernatants were determined by ELISA according to the instructions of the manufacturer. The latter IL-18 ELISA specifically quantifies mature IL-18 processed by caspase-1. Passive release of pro-IL-18 (e.g., due to cell necrosis) was not detected by this assay.

Detection of Human IL-18, T-bet, p38, and pp38 Mitogen-Activated Protein Kinase, Inhibitor of κ B α , and β -Actin by Western Blot Analysis

Cells were harvested using lysis buffer [150 mmol/L NaCl, 1 mmol/L CaCl $_2$, 25 mmol/L Tris-Cl (pH 7.4), 1% Triton X-100, supplemented with protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), DTT, Na $_3$ VO $_4$, phenylmethylsulfonyl fluoride (each 1 mmol/L), and NaF (20 mmol/L)]. Unless otherwise indicated, 50 μ g of total protein per lane were used. Antibodies and SDS-PAGE conditions: IL-18, 15% SDS-PAGE, polyclonal antibody R&D Systems; T-bet, 10% SDS-PAGE, monoclonal antibody (Santa Cruz Biotechnology, Heidelberg, Germany), to detect T-bet and β -actin (monoclonal antibody; Sigma) on the same blot, the blot was cut in half; inhibitor of κ B α (I κ B α), 12% SDS-PAGE, polyclonal antibody (Santa Cruz Biotechnology); phospho-p38 mitogen-activated protein kinase (MAPK; T180, S182), 12% SDS-PAGE; for detection of total p38 MAPK blots were stripped and reprobed. Both p38 MAPK antibodies were rabbit polyclonal (Cell Signaling, Frankfurt am Main, Germany).

Analysis of IFN γ , RelB, and Glyceraldehyde-3-Phosphate Dehydrogenase by Standard PCR

After RNA isolation (peqGold TriFast; Peqlab, Erlangen, Germany), 1 μ g of total RNA was transcribed using hexameric primers and Moloney virus reverse transcriptase (Applied Biosystems, Darmstadt, Germany). The following sequence was done for each PCR reaction: 95°C for 10 min (1 cycle); 95°C for 30 s, 60°C [RelB, glyceraldehyde-3-phosphate dehydrogenase (GAPDH)] or 50°C (IFN γ) for 30 s, and 72°C for 1 min (with variable number of cycles); and a final extension phase at 72°C for 7 min. The numbers of cycles for GAPDH, IFN γ , and RelB were 23, 30, and 37, respectively. To study IFN γ /RelB/GAPDH expression, the following primers were used: IFN γ , forward, 5'-AGTTA-TATCTTGGCTTTTCA-3'; reverse, 5'-AGTCAGTTACCGA-ATAATTA-3'; RelB, forward, 5'-CATCCTGGACCACT-TCTTGC-3'; reverse, 5'-GAACATGTTGCTGCCAGAAAG-3'; GAPDH, forward, 5'-ACCACAGTCCATGCCATCAC-3', reverse, 5'-TCCACCACCCTGTTGCTGTA-3'. Amplicon length was 363, 355, and 452 bp for IFN γ , RelB, and GAPDH, respectively.

Transfection of KG-1 Cells

Either T-bet-directed short interfering RNA (siRNA; 5'-GGAAGUUUCAUUUGGAAAdTdT-3'; Ambion, Cambridgeshire, United Kingdom; 0.4 μ g) or control siRNA (Silencer Negative Control siRNA #1, Ambion; 0.4 μ g) were electroporated using 5×10^6 KG-1 cells in 200 μ L of RPMI 1640 with a pulse of 230 V and 500 μ F (Gene pulser II; Bio-Rad, Munich, Germany). All conditions without siRNA or control-siRNA were mock-transfected by electroporation under the same conditions. After electroporation, the vitality of cells was determined by trypan blue. Thereafter, equal amounts of vital cells were seeded in six-well polystyrene plates (Greiner). After 5 h of incubation in culture medium, cells were stimulated with IL-18 (10 ng/mL) for 6 h and harvested thereafter.

Analysis of T-bet, GATA-3, and GAPDH by Real-time PCR

During real-time PCR analysis, changes in fluorescence were caused by the Taq-polymerase degrading the probe that contains a fluorescent dye (FAM used for T-bet or GATA-3, VIC used for GAPDH) and a quencher (TAMRA). For T-bet (HS00203436m1), GATA-3 (HS00231122m1), and GAPDH (4310884E) predeveloped assay reagents were obtained from Applied Biosystems. The assay mix was purchased from Invitrogen (Karlsruhe, Germany). Real-time PCR was done using the AbiPrism 7700 Sequence Detector (Applied Biosystems) as follows: one initial step at 50°C for 2 min and 95°C for 2 min was followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Detection of the dequenched probe, calculation of threshold cycles (Ct values), and further analyses of these data were done by the Sequence Detector software. Relative changes in gene expression of the signals of the target transcript versus those of untreated controls, normalized to GAPDH, were quantified by the $2^{-\Delta\Delta C_t}$ method.

Statistics

Data are shown as mean \pm SD and are presented as picograms per milliliter, as fold induction compared with unstimulated control, as the percentage of IL-18 alone, or as the percentage of control. Data were analyzed by unpaired Student's *t* test on raw data using Sigma Plot (Jandel Scientific).

Results

Induction of IFN γ by IL-18 in KG-1 Cells

Previous work identified IL-18 as potent stimulus for IFN γ production by KG-1 cells (23). Here, we confirm mRNA induction and IFN γ secretion under the influence of IL-18 by standard PCR and ELISA, respectively. Detailed short-term kinetic analysis revealed that IFN γ becomes detectable by ELISA in cell culture supernatants after \sim 4 h of incubation (Fig. 1A and B). The observed induction of IFN γ was not dependent on autocrine IL-12 because this cytokine was not induced by IL-18 in these cell cultures (data not shown). Incubation of KG-1 cells with IL-18 (10 ng/mL) for up to 32 h only modestly affected cell viability ($12.0\% \pm 2.1\%$ reduction of viability by IL-18 versus unstimulated control after 32 h, $n = 4$, this trend did not reach statistical significance). We also observed the induction of the transcription factor RelB by IL-18 (10 ng/mL, 4 h; data not shown) confirming that IL-18 is able to initiate processes that are associated with KG-1 cell differentiation towards a dendritic phenotype (24). To investigate a potential role for endogenous IL-18, Western blot analysis was done and revealed that KG-1 cells express clearly detectable levels of pro-IL-18, which are at least similar to those seen in monocytic THP-1 cells (Fig. 1C). Treatment with IL-18 (10 ng/mL) did not modulate the expression of pro-IL-18 in either cell type (data not shown). The microbial toxin nigericin is a well characterized potent stimulus for pro-IL-18 processing by caspase-1 and subsequent secretion of biologically active mature IL-18 (2, 6).

Interestingly, experiments shown in Table 1 revealed that KG-1 cells display a pronounced defect in IL-18 secretion. Therefore, autocrine stimulation of cells by endogenous IL-18 is highly unlikely in this cellular system.

IL-18 Induces T-bet mRNA and Protein Independent of Autocrine IFN γ

Real-time PCR analysis revealed the induction of T-bet mRNA expression by IL-18, which gained significance after 4 h of exposure (Fig. 2A). Compared with IL-18 (10 ng/mL), the induction of T-bet by IFN γ (20 ng/mL) was relatively modest (2.7 ± 1.2 -fold induction after a 6-h incubation period, $n = 4$; a similar weak induction was also observed at earlier time points; data not shown). However, in the same set of experiments, a significant and robust increase of T-bet mRNA expression was mediated by IL-18 at 10 ng/mL after 6 h of stimulation (9.3 ± 3.1 -fold induction, $n = 4$; $P < 0.005$ compared with unstimulated control). In contrast to T-bet, its immunoregulatory counterpart GATA-3 (25) remained unchanged in KG-1 cells even after 6 h of activation by IL-18 (Fig. 2A, *inset*). Figure 2B shows that mRNA induction of T-bet by IL-18 translated well into increased levels of cellular T-bet protein as detected by Western blot analysis. In order to investigate whether the induction of T-bet by IL-18 is via autocrine IFN γ , two experimental approaches were pursued. First, the expression of T-bet was analyzed under blockage of translation using cycloheximide. Figure 2C shows that IL-18 achieved significant induction of T-bet even in the presence of cycloheximide. In these same experiments, cycloheximide lead to complete suppression of IFN γ secretion back to the very low levels that are also seen in unstimulated KG-1 cells (Fig. 3C, *inset*). Second, IL-18 induction of T-bet was not effected by coincubation of KG-1 cells with a neutralizing anti-IFN γ antibody (Fig. 2D).

Induction of T-bet by IL-18 in KG-1 Cells is Dependent on p38 MAPK and Nuclear Factor- κ B Activation

Activation of the p38 MAPK pathway is considered to be a hallmark of IL-18 signal transduction in the human system (26–28). Therefore, we investigated the involvement of this kinase by pharmacologically suppressing its activity using the compound SB203580. p38 MAPK inhibition markedly reduced the expression of T-bet in KG-1 cells activated by IL-18. This modulatory effect was evident at both the mRNA (Fig. 3A) and protein (Fig. 3B) levels. Similar results were obtained by using the alternative p38 MAPK inhibitor SB202190 (data not shown). In agreement with these data, a rapid and clear increase in the cellular content of the phosphorylated form of p38 MAPK, indicative of its activation, could be observed upon IL-18 stimulation (Fig. 3C). Interestingly, IFN γ itself was unable to mediate a significant activation of p38 MAPK in these cells (Fig. 3D). In a further step to characterize biochemical actions of IL-18 in the KG-1 cell system, functional consequences of p38 MAPK blockage with regard to IFN γ secretion were investigated. As shown in Fig. 3E, the addition of SB203580 substantially inhibited the release of IFN γ under the influence of IL-18. To test whether blockage of the p38 MAPK pathway was also able to affect nuclear

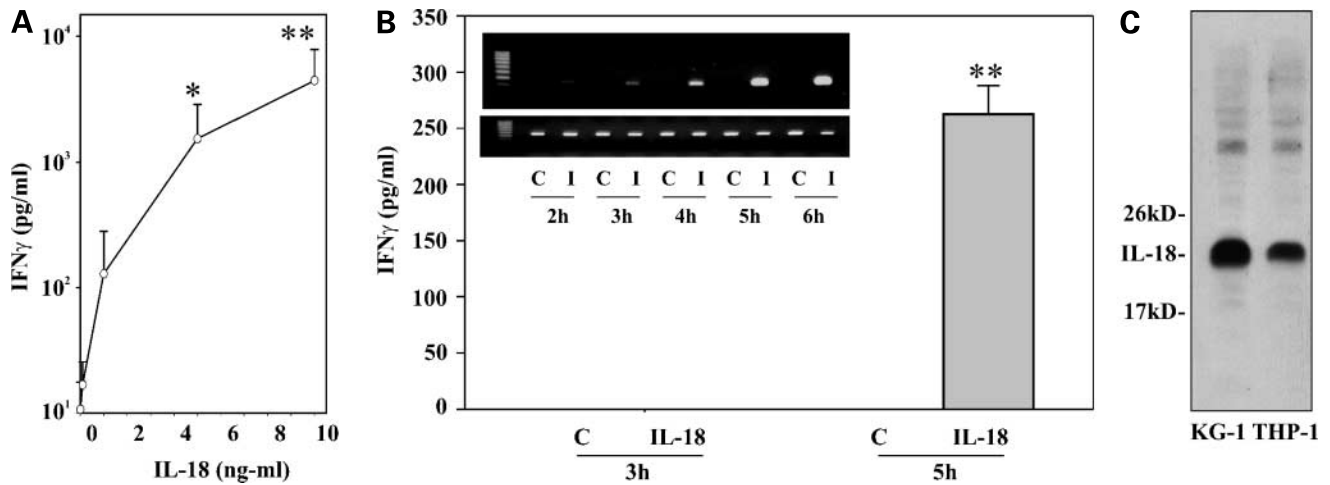


Figure 1. IL-18 mediates the production of IFN γ by KG-1 cells. **A**, KG-1 cells were either kept as unstimulated controls or were stimulated with the indicated concentrations of IL-18. After 30 h, IFN γ release was determined by ELISA. Points, means; bars, SD ($n = 7$); *, $P < 0.05$; **, $P < 0.01$ compared with unstimulated control. **B**, KG-1 cells were kept as unstimulated controls or were stimulated with IL-18 (10 ng/mL) for either 3 or 5 h. Thereafter, IFN γ release was determined by ELISA. Column, mean; bars, SD ($n = 3$); **, $P < 0.01$ compared with unstimulated control. Inset, KG-1 cells were either kept as unstimulated controls or were stimulated with IL-18 (10 ng/mL) for the indicated time periods. Thereafter, cells were harvested and IFN γ mRNA was evaluated by standard PCR analysis. **C**, expression of IL-18 in unstimulated KG-1 cells was evaluated by Western blot analysis. Unstimulated THP-1 cells served as positive controls for IL-18 expression.

factor- κ B (NF- κ B) activation in response to IL-18, I κ B α degradation, a read-out for NF- κ B activation, was evaluated in KG-1 cells exposed to SB203580 or SB202190. Figure 3F shows that I κ B α degradation, and thus NF- κ B activation, was not influenced by the p38 MAPK pathway. Previously, activation of NF- κ B was identified as being a prerequisite for IL-18-induced IFN γ production in KG-1 cells (29). Therefore, the effects of IL-18 on T-bet were investigated in the context of NF- κ B blockade. For that purpose, cells were exposed to IKK-VII. Treatment with this agent efficiently suppressed the degradation of I κ B and thus NF- κ B activation in IL-18-activated KG-1 cells (data not shown). Within a 4.75-h incubation period, no signs of toxicity were observed in cultures incubated with IKK-VII at 50 μ mol/L. Interestingly, inhibition of NF- κ B was associated with impaired T-bet induction by IL-18 (Fig. 3G).

Silencing of T-bet in KG-1 Cells by Using siRNA Technology Impairs IL-18-Induced IFN γ Production

To study the effect of T-bet on the production of IFN γ in response to IL-18, the induction of this transcription factor

was silenced by use of the siRNA approach. After having established an electroporation protocol for KG-1 cells that provided the necessary effectiveness for achieving sufficient suppression of T-bet protein, three independent experiments were done. Reduction of IL-18-induced T-bet protein expression (Fig. 4A) was, in all cases, associated with reduction of IFN γ secretion in these same experiments (Fig. 4B), indicating that, in fact, T-bet contributes to IFN γ production in KG-1 cells. In the course of the current study, we also investigated the alternative AML cell line HL-60. In contrast to KG-1 cells, these cells did not show up-regulation of T-bet expression in response to IL-18. Lack of T-bet induction was associated with lack of IFN γ secretion by HL-60 cells, further underscoring the pivotal role of T-bet in IL-18-induced IFN γ production (data not shown).

Discussion

In the present study, the effects of IL-18 on T-bet expression and function were investigated in human predendritic KG-1 cells. By inducing a translational block using cycloheximide and by coincubation with an IFN γ -neutralizing antibody, we show that IL-18 directly induces T-bet mRNA and protein. This newly described property may determine the functional characteristics of IL-18 in this cellular context. T-bet function is shown by siRNA technology. In fact, to the best of our knowledge, we also show for the first time that activation of endogenous T-bet is able to at least partially determine IFN γ production by human DC-like cells. Because NF- κ B has been previously shown to be essential for IL-18-induced IFN γ production in KG-1 cells (29), we suggest that both IL-18-inducible transcription factors, NF- κ B and T-bet, cooperate to mediate efficient

Table 1. KG-1 cells display a defect in the secretion of IL-18

	Control	Nigericin	TPA/Nigericin
THP-1	2.4 \pm 1.8	265.5 \pm 73.6*	374.3 \pm 162.6*
KG-1	2.5 \pm 1.6	1.0 \pm 1.4	13.3 \pm 5.3 [†]

NOTE: KG-1 cells or THP-1 cells were either kept as unstimulated control or were stimulated with nigericin (20 μ mol/L) with or without TPA at 50 ng/mL. Cells were preincubated with TPA for 4 h. After the adjacent 1 h incubation period, the levels of IL-18 in cell-free supernatants were determined by ELISA. Data are expressed as means \pm SD in pg/mL ($n = 3$).

* $P < 0.01$ compared with unstimulated controls.

[†] $P < 0.05$ compared with unstimulated controls.

IFN γ production in this cellular system. This view is further underscored by the observation that T-bet induction by IL-18 is suppressed in the context of NF- κ B inhibition. In contrast to T-bet, GATA-3, a determinant of Th2-like cytokine responses (25) was not influenced by IL-18. The present data shows that IL-18 has the potential to drive broad Th1-like cytokine responses even in the absence of its partner, IL-12. Notably, IL-12 production was not detectable in culture supernatants obtained from IL-18-stimulated KG-1 cells.

The p38 MAPK signaling pathway has been identified as pivotal for the activation of human cells by IL-18 (26–28). The present data supports this assumption. IL-18 potently activated p38 MAPK in KG-1 cells and suppression of kinase activity by SB203580 or SB202190 impaired T-bet induction. Thus, the inability of IFN γ to efficiently activate p38 MAPK in KG-1 cells again proved that IL-18-induced T-bet was not via autocrine IFN γ . These observations

complement recent data indicating that p38 MAPK determines T-bet expression in murine CD4⁺ T cells (30) and B cells (31), respectively. Because SB203580 potently suppressed IL-18-induced IFN γ in the current study and p38 MAPK is regarded to be essential for full activation of the human IFN γ promoter (32), we propose a signaling complex involving p38 MAPK, NF- κ B, and T-bet that mediates robust IFN γ production by IL-18-activated KG-1 cells.

Due to potent tumor-suppressive effects detected in animal studies (33–36), IL-18 (ibixotekine) is currently being evaluated in phase II clinical trials for the treatment of immunologically sensitive cancers, specifically, melanoma and renal cell cancer. Previously, a phase I study revealed that IL-18 could be given safely to patients with advanced cancer (37). Notably, activation of dendritic cells by IL-18 has been proven successful in preclinical cancer models (34, 35). The anticancer properties of IL-18 seem to

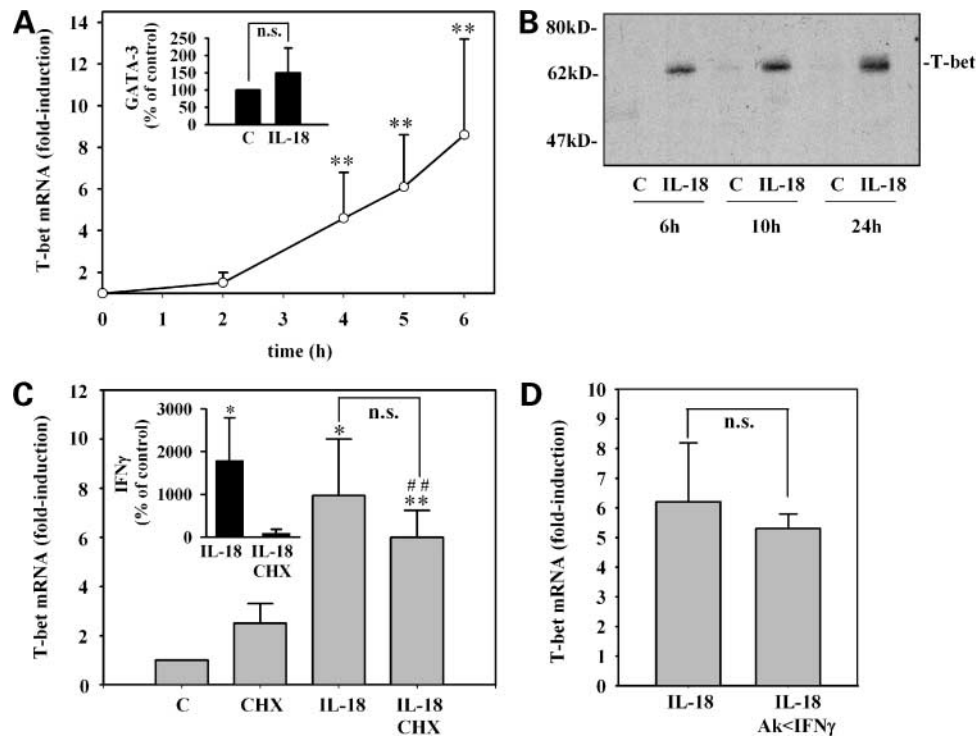


Figure 2. IL-18 directly induces T-bet expression in KG-1 cells. **A**, KG-1 cells were kept as unstimulated controls or were stimulated with IL-18 (10 ng/mL). After the indicated time periods, T-bet mRNA expression was evaluated by real-time PCR analysis. T-bet mRNA was normalized to that of GAPDH. Points, mean fold induction compared with unstimulated control; bars, SD ($n = 7-9$ for 4, 5, and 6 h of stimulation; $n = 4$ for 2 h of stimulation); **, $P < 0.01$ compared with unstimulated control. Inset, KG-1 cells were kept as unstimulated controls or stimulated with IL-18 (10 ng/mL). After 6 h, GATA-3 mRNA expression was evaluated by real-time PCR analysis. GATA-3 mRNA was normalized to that of GAPDH. Data are expressed as the percentage of unstimulated control \pm SD ($n = 3$). **B**, KG-1 cells were either kept as unstimulated control or were stimulated with IL-18 (10 ng/mL) for the indicated time periods. Thereafter, T-bet protein expression was evaluated by Western blot analysis. One representative of three independently performed experiments is shown. **C**, KG-1 cells were kept as unstimulated controls or stimulated with cycloheximide (10 μ g/mL), IL-18 (10 ng/mL), and cycloheximide (10 μ g/mL) plus IL-18 (10 ng/mL). In these experiments, cycloheximide was added to the cultures 2 h before the onset of stimulation with IL-18. After a stimulation period of 5 h, T-bet mRNA expression was evaluated by real-time PCR analysis. T-bet mRNA was normalized to that of GAPDH. Columns, mean fold induction compared with unstimulated control; bars, SD ($n = 6$); *, $P < 0.05$; **, $P < 0.01$ compared with unstimulated control; #, $P < 0.01$ compared with cycloheximide alone. Inset, in these same experiments, IFN γ concentrations were determined in culture supernatants by ELISA. Columns, mean percentage of unstimulated controls; bars, SD ($n = 6$); *, $P < 0.05$ compared with unstimulated controls; IFN γ concentrations for the cycloheximide plus IL-18 condition were equal to those of unstimulated controls. **D**, KG-1 cells were stimulated with IL-18 (10 ng/mL) in the presence or absence of anti-IFN γ neutralizing antibody (10 μ g/mL). After 5 h, T-bet mRNA expression was evaluated by real-time PCR analysis. T-bet mRNA was normalized to that of GAPDH. Columns, mean fold induction compared with unstimulated controls; bars, SD ($n = 3$).

be mediated by Th1-like cytokines, through the inhibition of angiogenesis primarily via the production of IFN γ -dependent antiangiogenic mediators like CXCL9 and CXCL10, and by enhancing Fas/Fas-L-dependent apoptosis (38, 39). Recent data indicates that AML can be regarded as a malignancy of DC precursors. Because appropriate DC function and Th1 type of immune responses are key to effective adaptive tumor immunity, insufficient T cell responses including impaired proliferation and Th1 cytokine production are plausible hematologic characteristics seen in patients with AML. In fact, bacterial infections are frequent in these patients, even before leukemia is diagnosed (40–43). Recently, a modest (~2-fold) increase of IL-18 mRNA was detected by standard PCR in bone marrow mononuclear cells of patients with AML (44).

However, information on IL-18 protein levels is lacking and the pathophysiologic relevance of that observation has to be substantiated. A crucial role for T cell immunity has been proposed for the control of leukemic malignancies. Interestingly, an IL-18 gene transfer approach showed promising results in a murine model of lymphoid leukemia (45).

Notably, the function of IL-18 in cancer apparently displays Janus-faced characteristics (46) and is largely determined by the potential of this cytokine to amplify the production of the anticancer cytokine IFN γ . Under conditions in which IL-18 is able to mount an efficient IFN γ response, the cytokine is likely protective. If not, the proinflammatory functions of IL-18 prevail with induction of variables that may even enhance cancer growth. These

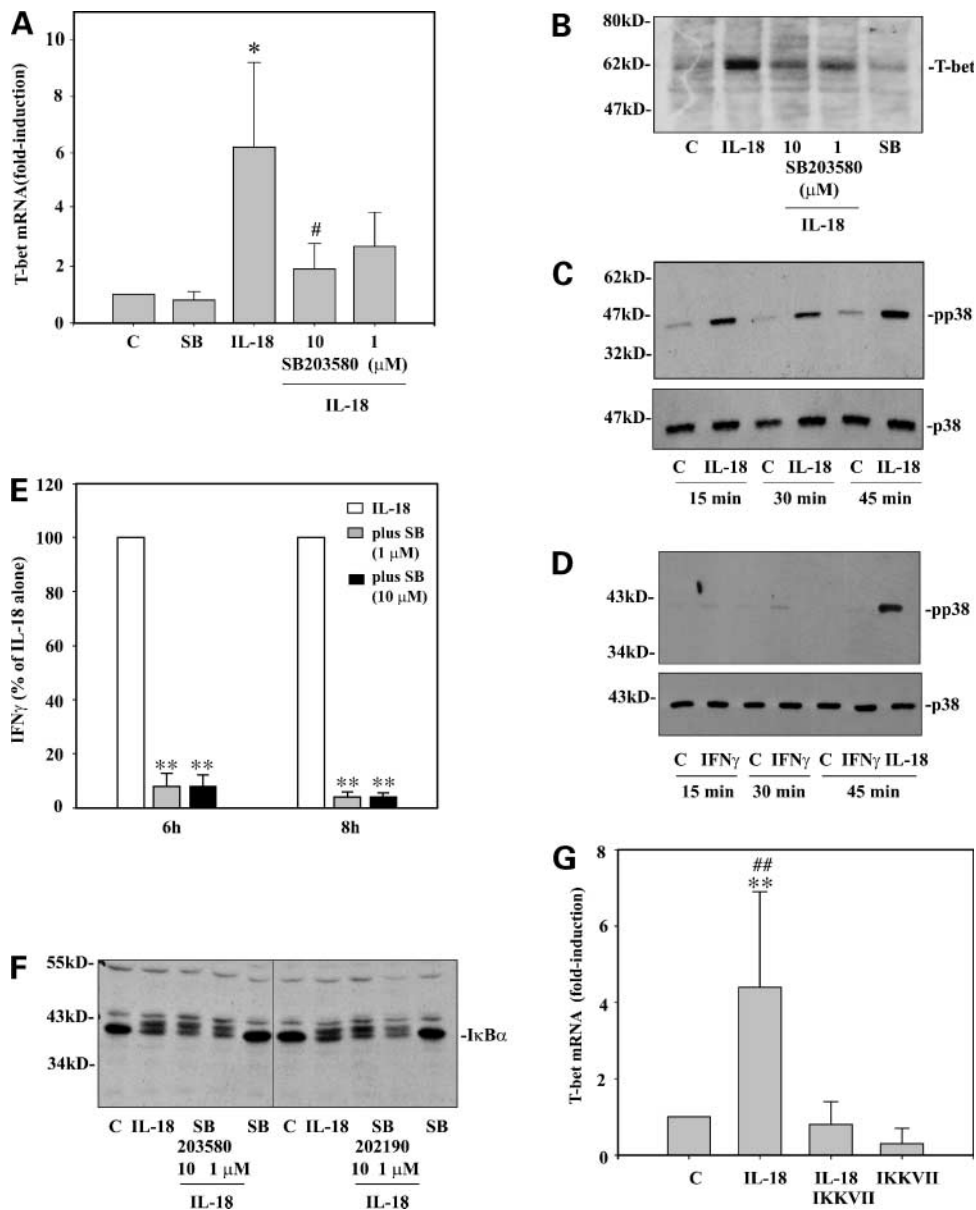
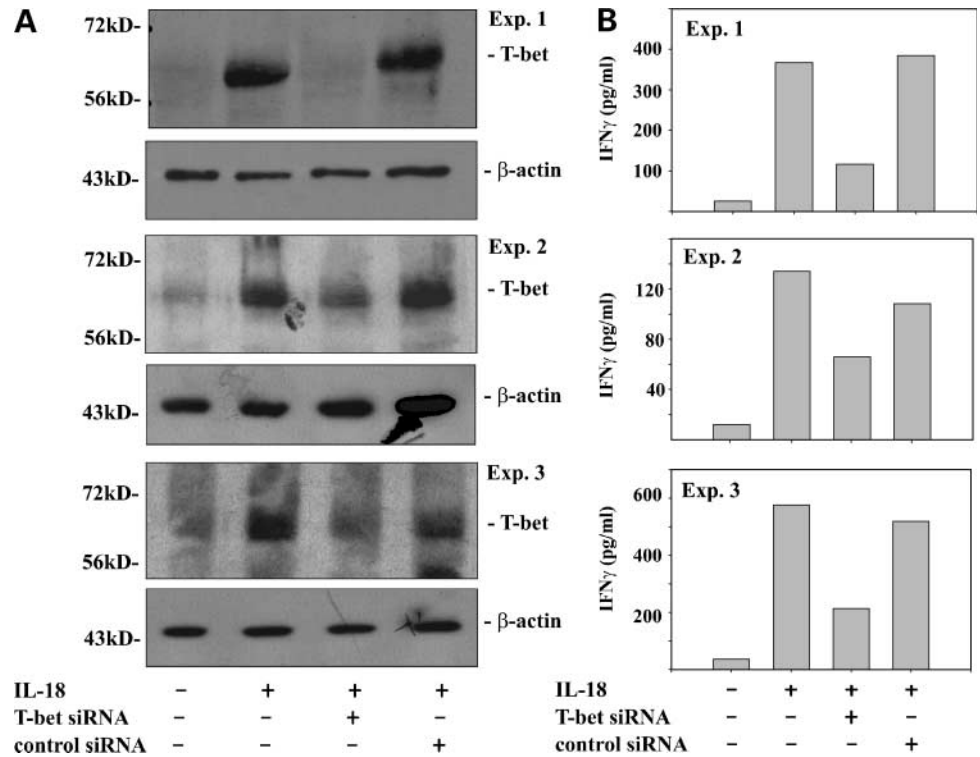


Figure 4. Suppression of T-bet induction by siRNA impairs IL-18–induced IFN γ release. KG-1 cells were transfected with either siRNA directed against T-bet or with control-siRNA. In addition, KG-1 cells were mock-transfected for control conditions or IL-18 (10 ng/mL) stimulations that were done in the absence of siRNA or control-siRNA. **A**, after 6 h, cells were harvested (three independent experiments; *Exp. 1–3*) and T-bet expression was evaluated by Western blot analysis. β -Actin was assessed on the same blot by cutting the blot in half. Eighty micrograms per lane instead of 50 μ g/lane of total protein lysates were used for the Western blot (*Exp. 3*). **B**, IFN γ concentrations in culture supernatants of this same set of experiments were determined by ELISA and are expressed as picograms per milliliter.



include adhesion molecules (46), proangiogenic IL-8 (47), and matrix metalloprotease-9 (48, 49). Therefore, the application of recombinant IL-18 seems to be a promising therapeutic option in those patients with leukemia in which this cytokine is able to establish a robust cancer-suppressive Th1/IFN γ –associated cytokine cascade. The associated IFN γ production may actually be able to counteract the aforementioned tumor-promoting activities of IL-18. In fact, IFN γ is able to down-regulate both IL-8 and matrix

metalloprotease-9 in the human system (50, 51). The beneficial effects of IL-18 may also include a correction of the antiapoptotic microenvironment characteristic for AML (52). Interestingly, T-bet up-regulates granzyme B and perforin production by natural killer cells (14). Without doubt, the activation and maturation of leukemic cells towards a dendritic phenotype by IL-18 may have the potential to significantly improve anticancer immunity in patients with AML. Direct induction of T-bet by IL-18 may

Figure 3. Induction of T-bet and IFN γ by IL-18 is dependent on the activation of p38 MAPK and NF- κ B. **A**, KG-1 cells were kept as unstimulated controls or were incubated with SB203580 (10 μ mol/L), IL-18 (10 ng/mL), and IL-18 (10 ng/mL) in combination with the indicated concentrations of SB203580. After a stimulation period of 4 h, T-bet mRNA expression was evaluated by real-time PCR analysis. T-bet mRNA was normalized to that of GAPDH. *Columns*, mean fold induction compared with unstimulated controls; *bars*, SD ($n = 4$, except SB203580 alone: $n = 3$); *, $P < 0.05$ compared with unstimulated control; #, $P < 0.05$ compared with IL-18 alone. In all experiments, SB203580 or SB202190 were added to the cultures 3 h before the onset of stimulation with IL-18. **B**, KG-1 cells were either kept as unstimulated controls or were stimulated with IL-18 (10 ng/mL), SB203580 (10 μ mol/L), and with IL-18 in combination with the indicated concentrations of SB203580. After 6 h, T-bet protein expression was evaluated by Western blot analysis. One representative of four independently performed experiments is shown. **C**, KG-1 cells were kept as unstimulated controls or were stimulated with IL-18 (10 ng/mL) for the indicated time periods. Thereafter, p38 MAPK phosphorylation was evaluated by Western blot analysis using a phosphospecific antibody. After stripping, the same blot was stained using an antibody specific for total p38 MAPK. One representative of three independently performed experiments is shown. **D**, KG-1 cells were kept as unstimulated controls or were stimulated with IFN γ (10 ng/mL) for the indicated time periods. Stimulation with IL-18 (10 ng/mL, 45 min) served as a positive control for efficient activation of p38 MAPK. After the respective stimulation periods, p38 MAPK phosphorylation was evaluated by Western blot analysis using a phosphospecific antibody. After stripping, the same blot was stained using an antibody specific for total p38 MAPK. One representative of three independently performed experiments is shown. **E**, KG-1 cells were stimulated with IL-18 (10 ng/mL) for 6 or 8 h in the presence or absence of the indicated concentrations of SB203580, respectively. Thereafter, IFN γ release was determined by ELISA. *Columns*, mean percentage of IL-18 alone; *bars*, SD ($n = 4$); **, $P < 0.01$ compared with IL-18 alone. **F**, KG-1 cells were either kept as unstimulated controls or were stimulated with IL-18 (10 ng/mL), SB203580 (*left*, 10 μ mol/L), SB202190 (*right*, 10 μ mol/L), and with IL-18 (10 ng/mL) in combination with the indicated concentrations of either SB203580 or SB202190. After 45 min, I κ B α expression was evaluated by Western blot analysis. One representative of three independently performed experiments is shown. All conditions in the experiments evaluating effects of SB202190 (*right*) were adjusted to a final DMSO concentration of 0.08%, which is the vehicle for SB202190 at 10 μ mol/L. For SB203580, the water-soluble form was used. **G**, KG-1 cells were kept as unstimulated controls or were incubated with IL-18 (10 ng/mL), IKK-VII (50 μ mol/L), and IL-18 (10 ng/mL) in combination with IKK-VII (50 μ mol/L). KG-1 cells were preincubated with IKK-VII for 45 min. After a stimulation period of 4 h, T-bet mRNA expression was evaluated by real-time PCR analysis. T-bet mRNA was normalized to that of GAPDH. *Columns*, mean fold induction compared with unstimulated control; *bars*, SD ($n = 4$); **, $P < 0.01$ compared with unstimulated control; ##, $P < 0.01$ compared with IL-18 plus IKK-VII.

prove to be of particular relevance for early events related to AML-DC maturation, and thus, for potential immunotherapeutic AML treatment protocols based on the pharmacological application of IL-18.

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