AML1/ETO inhibits AML1/CCAAT-enhancer binding protein-α mediated activation of the CD11c promoter and represses CD11c expression in HL60 cells

The translocation t(8;21)(q22;q22) creates the AML1/ETO-protein that interferes with normal myeloid differentiation. CD11c, a myeloid differentiation marker, was identified as new target for AML1/ETO-dependent gene repression. The C-terminus of ETO may mediate this effect by recruitment of histone deacetylases and/or direct interaction with other transcription factors, e.g. AML1 and C/EBPα.

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The translocation t(8;21)(q22;q22) rearranges the AML1 gene on chromosome 21q22 and the ETO gene on chromosome 8q22 and creates the chimeric gene product AML1/ETO.[1] AML1 contains a runt homology domain (RHD) which mediates binding to the DNA consensus sequence TGYGGT and complexes with core binding factor beta (CBFβ) to regulate target gene transcription.[2] Many AML1-regulated genes have been implicated in myeloid differentiation, including interleukin-3, granulocyte-macrophage colony-stimulating factor, myeloperoxidase and neutrophil elastase.[1] The ETO protein contains a *nervy* homology domain which interacts with nuclear co-repressors N-CoR and Sin3A to recruit an active histone deacetylase (HDAC).[3] The AML1/ETO fusion protein retains the RHD of AML1 and the ETO sequences that mediate interaction with nuclear co-repressors.[1] AML1/ETO may function as a repressor of AML1-dependent transcriptional activation.[4] In addition, AML1/ETO can interfere with other myeloid transcription factors such as CCAAT-enhancer binding protein-α (C/EBPα), which is essential for normal granulocytic differentiation.[5] To analyze the influence of AML1/ETO on myeloid differentiation further, we studied its effect on the expression of the β_2-integrin CD11c.[6] CD11c was selected because its expression is upregulated during differentiation and its promoter contains binding sites for AML1 and C/EBPα.[6,7]

The effects of AML1/ETO on CD11c mRNA and protein expression were studied in HL60 cells transfected with ALM1/ETO. A deletion mutant of AML1/ETO lacking the C-terminal binding site for N-CoR and mSin3 (AE/bam) and the empty vector pcDNA3 were used as controls. In addition, cells were incubated with the histone deacetylase inhibitor, trichostatin A (TSA). The effects of AML1b, C/EBPα, AML1/ETO and the deletion mutant AE/bam on the CD11c promotor were investigated in transient reporter co-transfection assay.

There was no difference in the percentage of CD11c positive cells between wildtype HL60 (72±7%), pcDNA3 clones (65±10%) or pcAE/bam clones (60±10%). In contrast, clones transfected with AML1/ETO signficantly reduced CD11c expression to 13±11% (Figure 1A). In parallel, CD11c mRNA levels of cells transfected with pcDNA3 or pcAE/bam could be compared to those of wildtype HL60 cells (Figure 1B). AML1/ETO positive clones showed an approximately 80% reduction of CD11c mRNA. TSA treatment of AML1/ETO positive clones increased CD11c surface expression from 13±11% to 61±11% (Figure 1C) and CD11c mRNA expression (Figure 1D). TSA had no effect on wildtype HL60 and the control cells.

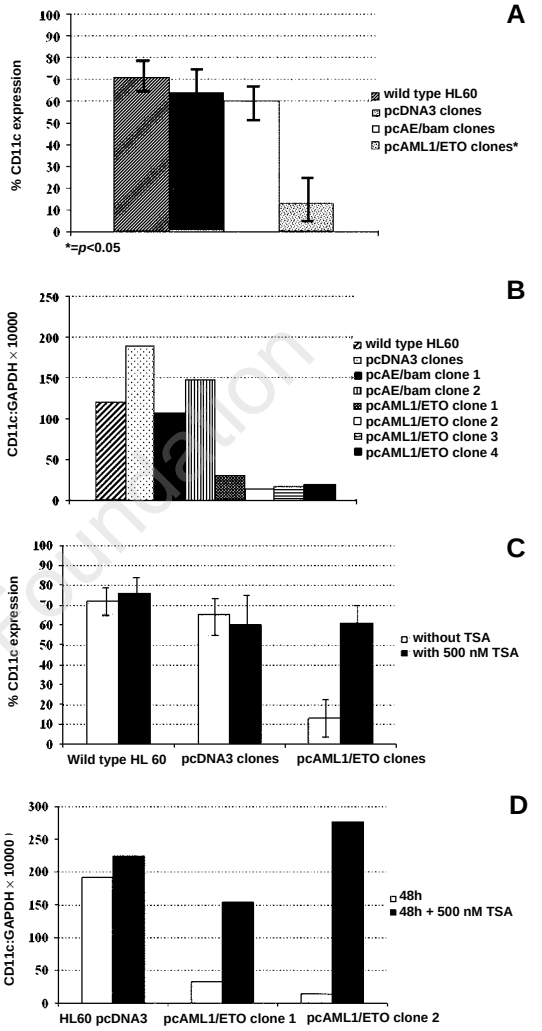
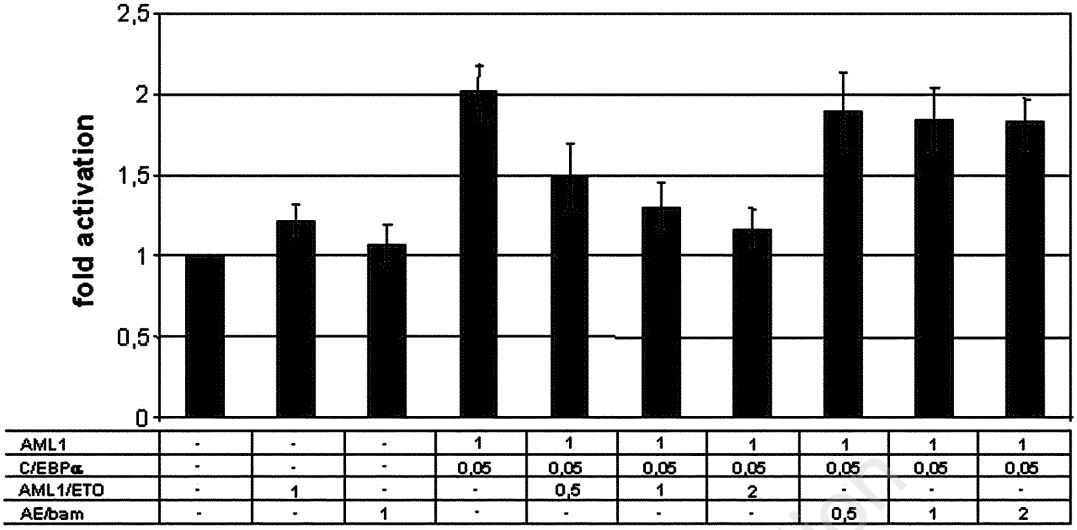


Figure 1. Analysis of CD11c surface and mRNA expression in wild type HL60 and cells transfected with various vector constructs analyzed by FACS and real time reverse transcription polymerase chain reaction (RT-PCR). HL60 cells were transfected with 5mg of plasmid DNA of pcAML1/ETO or pcAE/bam by electroporation. The empty pcDNA3 vector was used as a control. RNA extraction, cDNA synthesis and real time RT-PCR for AML1/ETO and GAPDH as a control, were performed as described.[10] A: CD11c surface expression was significantly lower in the HL60 cells transfected with AML1/ETO than in cells after control transfections. B: Whereas wild type HL60 cells, pcDNA3 clones and pcAE/bam clones showed a relatively high CD11c mRNA expression, the CD11c mRNA was markedly reduced in all pcAML1/ETO clones. C: Effect of TSA on CD11c surface expression. While 48-hour incubation in the presence of 500 nM TSA markedly upregulated CD11c surface expression in the pcAML1/ETO clones, TSA had no effect on the control cells. D: Effect of TSA on CD11c mRNA expression. Two representative pcAML1/ETO clones and a control clone transfected with pcDNA3 are shown. Only in the pcAML1/ETO clones did TSA stimulation lead to a marked upregulation of CD11c mRNA expression.

B



A

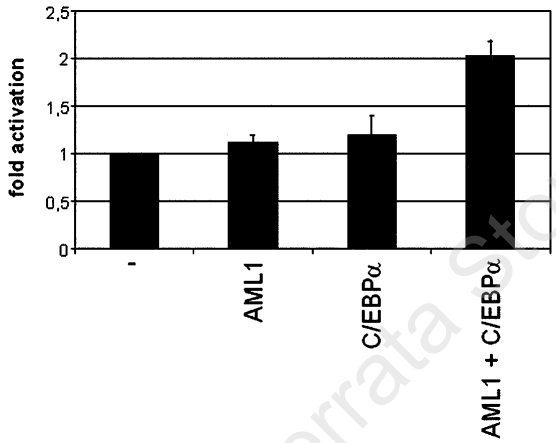


Figure 2. Effect of AML1/ETO on the CD11c promoter in a luciferase reporter assay. Human embryonic kidney cells (HEK 293) were transfected using the calcium phosphate transfection method (Promega protocols). The pRL-CD11c plasmid (1 μg/well) was transfected together with TK-β-Gal (1 μg/well) as an internal transfection control and different amounts of pcAML1, pcAML1/ETO, pcAE/bam and pcC/EBPα. After 72 hours of incubation cells were lysed with a buffer containing Triton-X-100 (f.c 0.1%), DTT (f.c. 1 mM) and glycerol (f.c. 15%). The luciferase activity was determined in a 10-second measurement period after adding the substrate luciferin to the lysates. All experiments were carried out in triplicate. A: co-transfection of the cells with AML1b and C/EBPα led to synergistic activation of the CD11c promoter. B: activation of the CD11c promotor by AML1b and C/EBPα was repressed by AML1/ETO. The AE/bam deletion construct had no effect. The numbers on the x-axis indicate the micrograms of transfected DNA.

While the CD11c promoter was only minimally activated by transfection with AML1b or C/EBPα alone, co-transfection with AML1b and C/EBPα consistently induced synergistic CD11c promoter activation (Figure 2A). This activation was suppressed by AML1/ETO in a dominant negative way (Figure 2B), while again pcAE/bam had no effect on the promotor activity.

Recent data suggest that AML1/ETO can interfere with AML1-dependent gene expression via the recruitment of HDAC by ETO.[3,4] We identified CD11c, a myeloid differentiation marker, as another target for AML1/ETO-dependent gene expression. Several lines of evidence have been provided which support the hypothesis that the repression of CD11c expression is mediated via the *nervy* homology region of ETO. A deletion construct lacking the binding domains for the repressor complex had no effect on CD11c mRNA and protein expression. In addition, stimulation of AML1/ETO-transfected cells by the histone deacetylase inhibitor TSA reversed the ETO-dependent CD11c suppression.

Furthermore, AML1/ETO-dependent inhibition of the AML1b and C/EBPα activation of the CD11c promotor was found to depend on the presence of the C-terminus of AML1/ETO. Combined data confirm that the Zn-finger domain of ETO is necessary for AML1/ETO-mediated gene repression. These results agree with previous reports showing that AML1/ETO blocks activation of the NP-3 promoter by AML1b and C/EBPα and that its C-terminal part is crucial for this effect. This interference is most probably due to a direct interaction between AML1/ETO and C/EBPα.[8,9]

Taken together, CD11c was identified as a new target for AML1/ETO-mediated gene repression. This effect is most likely mediated via recruitment of HDAC by the C-terminal part of ETO and/or an interference of AML1/ETO with AML1b-C/EBPα-mediated activation of the CD11c promoter.

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Early changes in bone marrow morphology induced by thalidomide in patients with refractory myeloma

Bone marrow morphology and the number of CD34+ cells were evaluated in 17 patients with refractory multiple myeloma at the start of therapy with low-dose thalidomide and after 3 months. All responding patients showed an evident increase of cellularity, reappearance of erythroblasts and myeloid precursors in various phases of differentiation, and an increase of megakaryocytes. Nine of the ten responders also had increase of bone marrow CD34+ cells.

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Although high-dose therapy produces high response rates and overall survival in multiple myeloma (MM), recurrence of disease usually develops and options of salvage therapy are limited.[1,2] Thalidomide has proven to be very effective in MM[3,4] through several biological pathways.[5,6] We have previously reported that clinical and hematologic recovery can be observed even in patients who obtain less response.[7] Moreover, we described the early morphologic changes in two refractory MM patients treated with thalidomide.[8]

Based on these observations, we performed this study on a cohort of patients with refractory MM treated with thalidomide in order to evaluate the morphologic bone marrow changes and to estimate the variation of bone marrow CD34+ cells.

The 17 patients included in the study were refractory to at least 2 previous lines of therapy, which in 6 patients had been one or two autologous bone marrow transplantations. Thalidomide was given at a dose of 100 mg/day and escalated to 200 mg/day in case of resistance. Four patients, because of severe disease-related symptoms, also received dexamethasone 20 mg/day for two days every two weeks for the first two months. Response to therapy was assessed according to the criteria of the European Bone Marrow Transplantation Group.[9] A minimum of three months of uninterrupted therapy was necessary to evaluate the response. The clinical and laboratory characteristics of the 17 evaluable patients recorded at the start of thalidomide were: median age 55 (37-67) years; 13 IgG, 2 IgA, 2 Bence Jones; 1 stage I, 4 stage II, 12 stage III. The evaluation of the bone marrow morphology was performed on May-Grünwald Giemsa smears before and after three months of thalidomide. Bone marrow smears were evaluated by three independent investigators, and by an external morphologist. Percentages of erythroblasts, myeloid precursors, and plasma cells were calculated on a minimum of 500 cells. Cytometric evaluation of bone marrow CD34+ cells was performed as previously reported.[10]

Thalidomide was well tolerated and side effects were always mild. After three months of thalidomide, 10 out of 17 patients (59%) had achieved a response (6 partial, 1 minor, 3 stable disease), and 7 (41%) had progressed. Table 1 describes the morphologic changes in detail. As shown, an increase of bone marrow cellularity was evident in 8 of the 10 responders. A striking increase in megakaryocyte number accompanied by reappearance of myeloid precursors, very rare in pre-therapy smears, at various stages of differentiation was observed in all responding patients. The percentage of erythroblasts was significantly higher after therapy, increasing from a mean value of 3.21% to 10.14% ($p<0.0001$). An increase of the bone marrow eosinophils was also noted. The bone marrow CD34+ cells, evaluated as absolute numbers and percentages, showed a parallel increase in 9 of 10 responding patients (Figure 1), from a mean of 134.4±194/mcrL (0.6%) before starting therapy to 462± 335/mcrL (1.8%) after thalidomide ($p=0.02$ and $p=0.007$, respectively). By contrast, in non-responding patients bone marrow cellularity very often appeared reduced. Similarly, in these