Concomitant aberrant overexpression of *RUNX1* and NCAM in regenerating bone marrow of myeloid leukemia of Down's syndrome

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Background and Objectives. Myeloid leukemia of Down's syndrome (ML-DS) has characteristic biological features (e.g. expression of the truncated GATA1s), which are different from those of non-DS childhood acute myeloid leukemias (AML). The objective of this study was to investigate factors predisposing to the development of ML-DS.

Design and methods. We analyzed 134 bone marrow specimens from 64 children with ML-DS and non-DS AML during chemotherapy and 7 specimens from DS children without leukemia, who did not receive any chemotherapy, The specimens were analyzed by multiparameter flow cytometry and quantitative reverse transcriptase polymerase chain reaction for transcription factors involved in hematopoiesis.

Results. Samples taken from children with ML-DS in complete remission during chemotherapy aberrantly expressed CD56 (NCAM) at the surface of monocytic and granulocytic cells. Compared to non-DS AML cases, children with ML-DS had a statistically significant higher proportion of $CD56^+$ cells in the $CD33^+$ fraction: 71%±6% vs. 4%±1% ($p<0.00001$). A significant decrease of the amount of $CD33^+/CD56^+$ cells was observed during and after maintenance therapy. An increased number of $CD33^+/CD56^+$ cells was also present (>85%) in children with DS who did not receive chemotherapy, but showed a left-shift (due to infection), compared with DS children without left-shift (<10% $CD33^+/CD56^+$ cells). Within the $CD33^+/CD56^+$ fraction, *RUNX1* was overexpressed more than 5-fold ($p<0.02$) compared to $CD33^+/CD56$- cells, whereas there were no differences regarding *GATA1*, *SPI1*, *ERG* or *ETS-2* levels.

Interpretation and Conclusions. The combined overexpression of *RUNX1* and *NCAM* during stress hematopoiesis in children with DS might be a key factor in the development of overt leukemia and/or in the growth advantage of the malignant GATA1s clone in ML-DS.

Key words: acute myeloid leukemia, Down's syndrome, RUNX1, NCAM, leukemogenesis.

Haematologica 2006; 91:1473-1480

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Children with Down's syndrome (DS), characterized by constitutional trisomy 21, have a 150-fold increased risk of developing acute myeloid leukemia (AML) before the age of 4-5 years.[1] As this type of leukemia – characterized by megakaryoblastic/erythroblastic features associated with mutations in exon 2 of the transcription factor *GATA1* – is unique to individuals with DS, it has been proposed that it is classified as *myeloid leukemia of DS* (ML-DS).[2] The blast cells of this type of leukemia have been studied in detail by immunophenotyping[3,4] and microarray analysis.[5-7] About 10% of neonates with DS exhibit a transient myeloproliferative disease (TMD), in which blast cells in the peripheral blood the same morphological and immunological features as those of ML-DS blasts,[3] typically accompanied by clinical signs such as hyperleukocytosis, thrombocytopenia, anemia or liver disease.[8] Interestingly, in most cases TMD disappears spontaneously, whereas ML-DS requires intensive chemotherapy. However, due to a higher sensitivity to cytostatic drugs,[9] the outcome of ML-DS is greatly superior to that of non-DS AMKL (overall survival: 91% vs. 64%).[10] Mutations in the transcription factor *GATA1*, resulting in translation into a shorter isoform of the protein (GATA-1s), are detectable in virtually all cases of TMD and ML-DS, but are absent during remission and in children with non-DS AML.[11-15] In a recent study, Ahmed *et al.* demonstrated GATA1 mutations in neonates with DS who subsequently developed a TMD and/or ML-DS, indicating that these mutations are acquired *in utero*.[16] Neural cell adhesion molecule 1 (NCAM, CD56) – a member of the immunoglobulin superfamily – is a membrane-bound glycoprotein that plays a role in cell-cell and cell-matrix adhesion. This antigen, usually expressed on natural-killer (NK) cells, is also found in a subset of $CD3^+$ cytotoxic T-cells and a small population of $CD4^+$. Furthermore, it has been found to be expressed in various hematopoietic neoplasms, including about 20% of

cases of AML, particularly M5, M7, M2 with t(8;21) and M3.[17-20] CD56 is commonly expressed on blast cells of ML-DS and TMD.[3] NCAM is related to the hematopoiesis-supporting capacity of stromal cells and thereby has a role in the maintenance of hematopoietic stem cells.[21] The runt-related transcription factor 1 (*RUNX1, AML1, PEBP2*), localized on the long arm of chromosome 21 (21q22.3), encodes the α subunit of the transcription factor PEBP2/CBF and is essential for the establishment of fetal liver hematopoiesis. RUNX1 is one of the most frequent targets of chromosome translocations associated with leukemia.[22] Two different transcript variants encoding different isoforms have been found for this gene. In contrast to acquired trisomy 21 in adult AML, in which RUNX1 point mutations are frequent, in children with congenital trisomy 21 mutations of RUNX1 do not play a significant role in the development of hematologic disorders.[23,24] *RUNX1* is important for early granulocytopoiesis[25] and is required for maturation of megakaryocytes and differentiation of T and B cells, but not for maintenance of hematopoietic stem cells in adult hematopoiesis.[26] Although it has been suggested that an increased dosage of wild-type *RUNX1* could play a role in DS-related leukemias,[15,27] we and others could not confirm that there was overexpression of *RUNX1* in blast cells of children suffering from ML-DS or TMD.[5,28] It has been recently shown that there is a physical interaction between the zinc finger domains – and not the N-terminus – of *GATA1* and the Runt domain in *RUNX1*, suggesting that their interaction and synergy are retained in ML-DS.[29] During normal neutrophil differentiation, a downregulation of *RUNX1* and GATA1 takes place, whereas the expression of SPI1 increases.[30]

The aim of this study was to investigate factors predisposing to the development of ML-DS. Therefore, we analyzed regenerating bone marrow (BM) specimens of children who were treated for their ML-DS within the AML-BFM 98 trial and compared these specimens to remission BM specimens from patients with non-DS AML. We focused on the expression of surface antigens and the gene expression profile of transcription factors that are involved in hematopoiesis in different cell populations.

Design and Methods

Patients

We studied the occurrence of CD33/CD56 coexpressing cells in 134 follow-up BM specimens from 64 children (27 with DS, 37 without DS) with AML in complete remission who were treated according to the AML-BFM-98-study. The children without DS consisted of two groups: the first group was selected for French-American-British (FAB) subtype and included cases with M7 (n=13) and M6 (n=3) AML, because of the highly similar morphological and immunological features of the blast cells from these forms of AML and ML-DS (apart from GATA1s). The second group consisted of children with FAB M2 (n=11) and M5 (n=10), who were selected to represent genetically homogeneous subgroups of sporadic AML (Table 1). Quantitative real-time polymerase chain reaction (RQ-RT-PCR) was performed on 19 specimens of FACS sorted cells ($CD33^+/CD56^+$ and $CD33^+/CD56^-$) derived from BM of children with and without DS.

Table 1. Patients studied for the occurence of $CD33^+/CD56^+$ cells during chemotherapy.

	ML-DS	*non-DS AML*
Number of patients	27	37
Number of follow-ups	58	76
Gender M/F	11/16	18/19
Age at diagnosis [median (range)]	1.8 (0.7-6.5)	4.1 (0.3-17.7)
M2	–	11
M5	1	10
M6	–	3
M7	–	13
ML-DS	26	–

Additionaly, BM or peripheral blood specimens from seven children with DS who did not receive any chemotherapy treatment were studied by flow cytometry for the occurrence of CD33/CD56 coexpressing cells. These BM aspirations were performed to exclude a leukemic disorder as a differential diagnosis. BM specimens were obtained after informed consent from each patient and/or the patient's guardian. All investigations had been approved by the local ethics committees and were in accordance with an assurance filed with and approved by the Department of Health and Human Services.

Diagnosis

Diagnoses and classifications were established according to the criteria of the FAB[31-33] group by the reference laboratory of the AML-BFM studies in Muenster and were reviewed by an expert group of independent hematologists. The diagnoses of M0 and M7 subtypes were always confirmed by immunological methods. Cytogenetic and molecular genetic data were obtained from the reference laboratory of the AML-BFM study (*J. Harbott, Giessen*).

Flow cytometry/FACS sorting

Four-color flow-cytometry was performed using anticoagulated BM samples. After incubating the BM samples with monoclonal antibodies for 15 minutes, erythrocytes were lysed for 7 minutes using Versa Lyse™ (Beckman Coulter). Afterwards, the specimens were washed twice with 2 mL phosphate buffered saline (pH 7.4) and centrifuged (5 minutes, 20° C, 600 g) to remove excess anti-

bodies and lysed red blood cells. Specimens were measured using the FC-500™ (Beckman Coulter), analyzing at least 30,000 events.

A wide antibodies based on a CD33/CD34 backbone including fluorescence conjugated myeloid markers CD13-PE (SJ1D1; Immunotech), CD15-FITC (MMA; Becton Dickinson), CD33-APC (D3HL60.251; Immunotech) and HLA-DR-FITC (L243, Becton Dickinson), lymphoid markers CD7-PE (8H8.1; Immunotech), CD10-FITC (ALB2; Immunotech), CD19-FITC (J4.119; Immunotech), CD56-PE (NCAM 16.2; Becton Dickinson), the activation and proliferation marker CD38-PE (HB7; Becton Dickinson) as well as the progenitor-associated markers CD34-PC7 (581; Immunotech) and CD117-FITC (95C3; Immunotech) was applied. Syto 16 (Molecular Probes) was used to stain nucleated cells, enabling debris and not completely lysed erythrocytes to be excluded from analysis. Cell populations were sorted using standard techniques and the FACS Vantage™ cell sorter (Becton Dickinson). BM specimens were labeled with CD33-PC5, CD56-PE and CD15-FITC antibodies. Cells were sorted according to their forward and sideward scatter and the expression levels of the labeled antigens. The mean purity of isolated cell populations was 93% (range 83-99%).

RQ-RT-PCR

Total RNA was isolated from FACS-sorted cells with the RNeasy Kit (Quiagen, Hilden, Germany) according to the manufacturer`s instructions. Depending on the amount of RNA, 0.15-2 μg total RNA was reverse transcribed into cDNA using SuperScript™ II Reverse Transcriptase (Invitrogen). Gene expression levels of seven hematopoietic transcription factors (GATA1, GATA2, RUNX1, ERG, ETS2, SPI1 and NCAM) were analyzed by RQ-RT-PCR. For the first six of these genes, expression levels were determined by the LightCycler™ 2.0 System (Roche Applied Science) using SYBR green and gene-specific primer kits (Search-LC, Germany). The expression levels of NCAM were quantified in the 7300 Real-Time PCR System (Applied Biosystems, Germany) using Quantitect Primer Assay and Quantitect SYBR Green RT-PCR™ kits (Qiagen, Germany). Expression levels were calculated either by determining copy numbers according to specific standard curves and normalizing them to the expression level (Search-LC Kits) of the house-keeping gene cyclophilin B or by relative quantification using the $2^{-\Delta\Delta CT}$ method (Quantitect Kit).[34]

Results

Abnormal coexpression of CD56+ cells in the CD33+ fraction in children with ML-DS during chemotherapy

Children with ML-DS in complete remission during chemotherapy (determined by morphology and multiparameter flow cytometry) aberrantly expressed CD56 (NCAM) at the surface of myeloid cells. These $CD33^+/CD56^+$ cells could be further separated by their forward-/sideward scatter features into monocytes and granulocytes (Figure 1). The CD56-positive myelocytes did not differ morphologically from normal ($CD56^-$) monocytic or granulocytic cells. Apart from CD56, their immunophenotype did not differ from normal monocytes ($CD33^{++}$, $CD13^{++}$, $CD15^+$, $CD36^{++}$, $CD14^{++}$, $CD65w^{++}$) or granulocytes ($CD33^+$, $CD13^{++}$, $CD15^{++}$, $CD14^+$, $CD65w^+$).

Even though $CD33^+/CD56^+$ cells are also detectable in remission BM specimens from non-DS AML cases, children with ML-DS have a statistically significantly higher proportion of $CD33^+/CD56^+$ cells in the $CD33^+$ fraction: 71%±6% vs. 4%±1%, $p<0.00001$ (Figure 2).

The occurrence of CD33+/CD56+ cells is associated with stress hematopoiesis in children with DS

We wanted to determine whether the occurrence of $CD33^+/CD56^+$ cells in regenerating BM of children with ML-DS was treatment-dependent. We, therefore, studied the relationship between time-points and the size of the CD33/CD56 population. Using a time-dependent variance analysis, we found that there was a significant decrease ($p=0.02$) in the size of the $CD33^+/CD56^+$ population over time: i.e. lower values during and after maintenance therapy (BM samples 7 and 8), than during the period of intensive chemotherapy (BM samples 2 to 5) (Figure 3). For both groups (ML-DS and non-DS AML), morphological studies showed significantly increased proportions of myeloid and erythroid lineage progenitor cells (>50% each), an indicator of stress hematopoiesis, during the period of intensive chemotherapy than during and after maintenace therapy. The levels at the latter timepoints were in the same range as those of normal control BM samples.

$CD33^+/CD56^+$ cells were also present at high levels (>85%) in neonates and children with DS who did not receive any chemotherapy, whose hemogram showed a left-shift accompanied by an increased white blood count (WBC) due to increased hematopoiesis (for instance as a consequence of severe infections and/or sepsis). In contrast, $CD33^+/CD56^+$ cells in DS children with a normal differential blood count (without any sign of leukemic blasts or increased hematopoiesis with a left-shift) accounted for less than 10% of all myeloid cells (Table 2).

RUNX1 is overexpressed in CD33+/CD56+ cells

Using RQ-RT-PCR, we confirmed the overexpression of NCAM in DS $CD33^+/CD56^+$ cells compared to its expression in DS $CD33^+/CD56^-$ cells (122±46 vs. 1.4±0.4, $p=0.04$) and non-DS $CD33^+/CD56^-$ cells (122±46 vs. 10.6±4.8, $p=0.02$). Non-DS $CD33^+/CD56^+$ cells show a 1.6-fold higher NCAM expression compared to non-DS $CD33^+/CD56^-$ cells, reflecting the lower expression of NCAM in non-DS $CD33^+/CD56^+$ cells, as determined by flow cytometry (Figure 4A). We also analyzed the level of RUNX1 mRNA in the

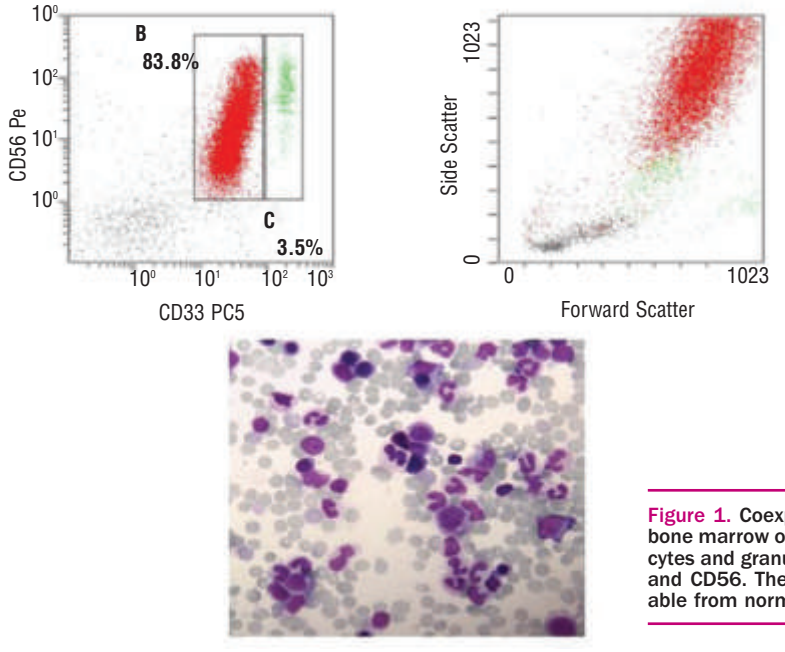

Figure 1. Coexpression of CD33 and CD56 in regenerating bone marrow of a child treated for ML-DS. Virtually all monocytes and granulocytes show aberrant coexpression of CD33 and CD56. These cells are not morphologically distinguishable from normal monocytes and granulocytes.

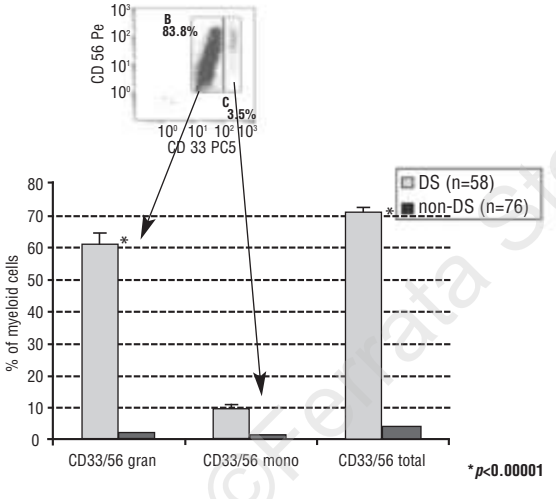

Figure 2. CD33⁺/CD56⁺ cells are also detectable in regenerating BM of children with non-DS AML, however at a significantly lower level.

CD33⁺/CD56⁺ of children with and without DS. The expression of RUNX1 was more than 5 times higher in the CD33⁺/CD56⁺ cells than in the CD33⁺/CD56⁻ ones (p<0.02). Expression within the CD33⁺/CD56⁺ and CD33⁺/CD56- cells was similar in children with and without DS (Figure 4B).

Regarding the chromosome 21 encoded transcription factors ERG and ETS2, there were no significant differences in the levels of mRNA expression between DS CD33⁺/CD56⁺ and CD33⁺/CD56⁻ cells. Furthermore, there were no statistically significant differences between the analyzed cell populations regarding either *GATA1*, which was expressed at low levels as expected in monocytic and granulocytic cells, or SPI1, which showed high levels of expression that are characteristic of granulocytes and monocytes.

Discussion

In the course of performing minimal residual disease studies in childhood AML, we found that, during intensive chemotherapy, children with ML-DS exhibit aberrant populations of CD56-coexpressing myelocytes (granulocytes and monocytes). These cells do not show any signs of being leukemic blasts either by morphology or by multi-color flow cytometry. Functionally, they are not different from their normal counterparts. As normal hematopoietic cells do not coexpress CD56 on myeloid cells, the combination of CD33⁺/CD56⁺/CD34⁺/⁻ is commonly regarded as a sensitive and specific leukemia-associated immunophenotype (LAIP) for monitoring minimal residual disease.[35-37] We are the first group to describe that the aberrant coexpression of CD33 and CD56 in regenerating bone marrow is common in children with ML-DS and is not associated with poor outcome. This prompted us to systematically screen (a) remission marrow from non-DS children during their treatment for AML, and (b) marrow from DS children without hematologic malignancies for the occurrence of CD33⁺/CD56⁺ cells. Interestingly, we found that the appearance of CD33⁺/CD56⁺ cells in individuals with DS is strongly correlated to stress hematopoiesis, either due to regeneration during inten-

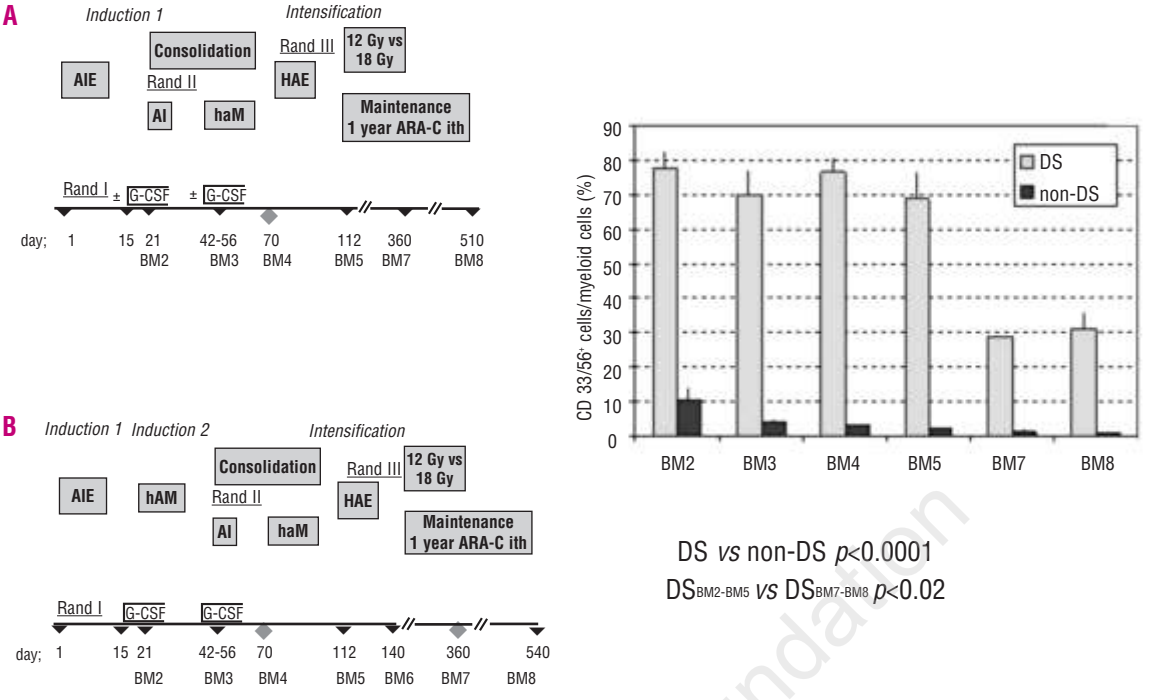

Figure 3. CD33/56 measurements in bone marrow aspirates were correlated to the pre-defined timepoints during treatment in the AML-BFM studies. As children with ML-DS do not receive a second induction course, maintenance treatment starts earlier, so that timepoint BM6 does not exist in these children. (AIE: Ara-C [cytosine arabinoside], idarubicin, etoposide, HAM: high-dose Ara-C, mitoxantrone, AI: Ara-C, idarubicin, haM: medium-high dose Ara-C, mitoxantrone, HAE: high-dose Ara-C, etoposide, G-CSF: granulocyte colony-stimulating factor, Rand: randomization). A. Treatment schedule for ML-DS. B. Treatment schedule AML-BFM 98 study (excl. ML-DS).

sive chemotherapy or as a consequence of severe infections or sepsis. In addition, we were able to show that the population of $CD33^+/CD56^+$ cells decreases significantly over time during maintenance treatment, which is less BM suppressive than chemotherapy during the intensive phases of treatment. By quantitative RT-PCR we also validated the increased NCAM expression at the level of mRNA. During chemotherapy, there were significantly lower proportions of $CD33^+/CD56^+$ cells in non-DS children than in DS children, suggesting qualitative differences in the cell populations of DS and non-DS children during stress hematopoiesis. In an attempt to find possible explanations for the excessive expression of NCAM in DS individuals with increased hematopoiesis, we analyzed the expression of transcription factors that are involved in hematopoiesis and/or leukemogenesis in ML-DS. We did not find differences in the expression levels of *GATA1*, *GATA2*, *SPI1*, *ERG* or ETS2 between $CD33^+/CD56^+$ and $CD33^+/CD56^-$ cells. The most important finding was the elevated expression of *RUNX1* in $CD33^+/CD56^+$ cells. The recently detected *RUNX1* binding site within the NCAM promoter[38] might explain this observation.

The *a priori* presence of three copies of *RUNX1* in individuals with DS might be the prerequisite for the abnormal hematopoiesis under the condition of stress: as we

Table 2. Expression of $CD33^+/CD56^+$ cells in neonates and children with DS who did not receive chemotherapy.

Patient	*Gender*	*Age*	*Source*	*Leukocytes [1/μL]*	*Neutrophils [%]*	*Comment*	*CD33/56 cells [%]*
M.F.	M	16 years	BM	n.a.	78	left shift (sepsis)	98
J.S.	M	5 months	BM	65500	67	left shift (pneumonia)	100
J.N.	M	1 day	PB	36300	48	left shift (sepsis)	87
P.D.	F	5 days	BM	n.a.	49	normocellular, representative	5
S.P.	M	13 days	BM	n.a.	69	normal differential blood profile	9
F.W.	M	9 days	PB	n.a.	28	normal differential blood profile	0
S.M.	M	1 day	PB	n.a.	n.a.	n.a.	5

BM: bone marrow; PB: peripheral blood; WBC: white blood count; n.a.: not available.

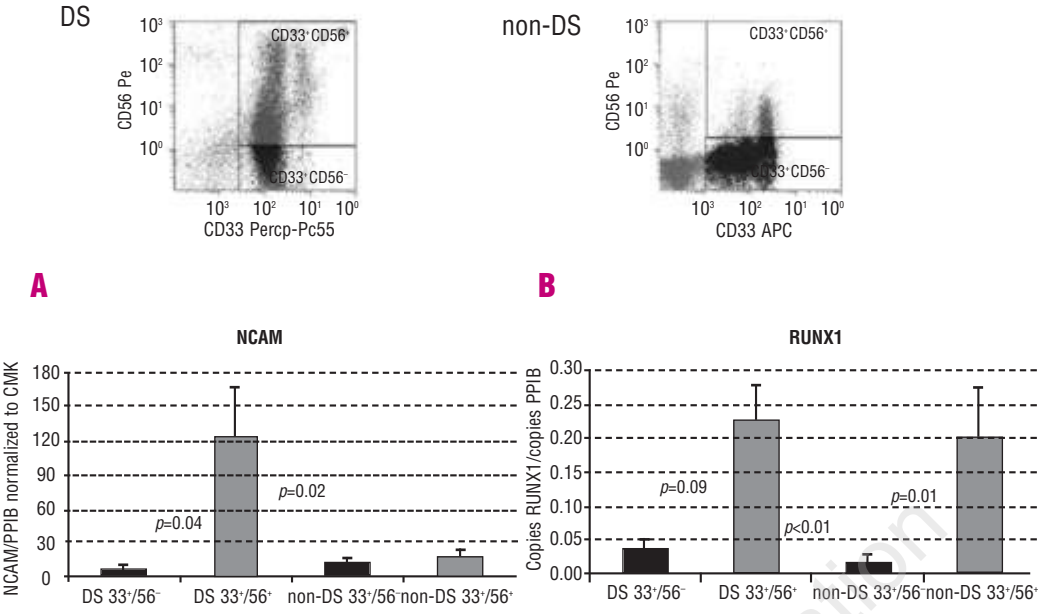

Figure 4. RQ-RT-PCR showed NCAM overexpression in CD33$^+$/CD56$^+$ cells (A), confirming the observed overexpression of NCAM by flow-cytometry. Moreover, a greater than 5-fold overexpression of RUNX1 was observed in the CD33$^+$/CD56$^+$ subclone of cells (B).

showed, there is an imbalance towards more immature hematopoietic cells. Therefore, the anticipated down-regulation of RUNX1 expression during normal neutrophil development[30] might be delayed. This relatively increased RUNX1 expression might induce (through promoter binding) an elevated NCAM level that again supports the maintenance of hematopoietic stem cells[21] resulting in a self-inducing system of increased hematopoiesis. The co-operation between NCAM, with its hematopoiesis-supporting capacity, and RUNX1, which is responsible for early granulocytopoiesis is likely to produce an environment conducive to increased growth of granulocytes. This proposed mechanism is even more likely to occur in DS individuals - due to the three copies of RUNX1 - giving rise to the observed coexpression of RUNX1 and NCAM in granulocytes and monocytes. Overexpression of NCAM has also been reported in non-hematopoietic cells of mouse embryos with trisomy 16,[39] indicating its generally critical role in the pathophysiology of humans with trisomy 21. The observed overexpression of RUNX1 during particular stages of hematopoiesis in individuals with DS might explain its role in leukemogenesis in ML-DS: it has been shown recently that RUNX1 promotes proliferation of megakaryocytic progenitors and downregulates terminal differentiation of megakaryocytes.[40] The fact that fetuses with DS show increased megakaryopoiesis (*unpublished data; personal communication from I. Roberts, London, UK*) confirms the proposed higher turnover of megakaryocytic progenitors. This phenomenon might result clinically in the often described thrombocytosis in infants with DS.[41] There are, however, also reports of transient thrombocytopenia in neonates with DS,[42] which must be put into perspective because of the high incidence of congestive heart failure (15/25) and furthermore the possibly undetected cases of TMD in this cohort. This would be in line with the hypothesis, published by Greaves *et al.*,[43] that leukemia-specific genetic alterations could arise in any type of proliferating cell but they are only likely to gain a clonal advantage in the context of a particular developmental pathway.

The fact that *RUNX1* is not overexpressed in the leukemic blast cell population of ML-DS and TMD[5,44] does not necessarily exclude that it has a role during leukemogenesis. On the one hand, the final *mature* blast cell with megakaryocytic features is not typically the cell in which the malignant transformation has taken place; more likely, as proposed by Bonnet and Dick,[45] leukemogenesis seems to be a multistep process starting from a leukemic stem cell that undergoes hierarchical differentiation into leukemic blasts, as known from normal hematopoiesis. On the other hand, even though RUNX1 seems not to be relevant for the proliferation of *mature* blast cells, it might play a decisive role in the growth advantage of the leukemic clone – most likely expressed by the generation of GATA1s. For the malignant transformation to ML-DS we propose that trisomy 21 can be assumed as both a predisposition and a first event[46] (class II mutation, according to the model proposed by Gililand *et al.*).[47] For the development of overt leukemia, the here described phenomenon of combined

RUNX1 and NCAM overexpression during increased hematopoiesis (in the context of stress) in individuals with DS might be an important requirement for the selection of the malignant GATA1s clone during leukemogenesis: spontaneous mutation of GATA1 is more likely to occur during increased turnover of megakaryocytic progenitors – due to the overexpression of RUNX1 – than under normal hematopoietic conditions. We hypothesize that at least three events are required to occur within a certain time frame for leukemogenesis: (i) trisomy 21, (ii) acquired mutation of GATA1 and (iii) increased and qualitatively different hematopoiesis with overexpression of RUNX1.

CL: conception and design of the study, analysis and interpretation of data, writing of the manuscript; J-HK: conception of the study, interpretation of data; KW: acqusition and analysis of data; MK: acqusition and analysis of data; UP: conception and design of the study; DR: conception and design of the study and final approval of the manuscript. The authors declare that they have no potential conflicts of interest.

Funding: this work was supported by the Madeleine Schickedanz Kinderkrebs-Stiftung.

Manuscript received May 17, 2006. Accepted August 30, 2006.

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