The novel AML stem cell associated antigen CLL-1 aids in discrimination between normal and leukemic stem cells

Short title: CLL-1 is an AML stem cell associated antigen

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Abstract

In CD34 positive AML the malignant stem cells reside in the CD38 negative compartment. We have shown before that the frequency of such CD34+CD38- cells at diagnosis correlates with minimal residual disease (MRD) frequency after chemotherapy and with survival. Specific targeting of CD34+CD38- cells might thus offer therapeutic options.

Previously we found that C-type lectin-like molecule-1 (CLL-1) has high expression on the whole blast compartment in the majority of AML cases. We now show that CLL-1 expression is also present on the CD34+CD38- stem cell compartment in AML (77/89 patients). The CD34+CLL-1+ population, containing the CD34+CD38-CLL-1+ cells, does engraft in NOD/SCID mice with outgrowth to CLL-1 positive blasts. CLL-1 expression was not different between diagnosis and relapse (n=9). In remission, both CLL-1 negative normal and CLL-1 positive malignant CD34+CD38- cells were present. A high CLL-1 positive fraction was associated with quick relapse.

CLL-1 expression is completely absent both on CD34+CD38- cells in normal (n=11) and in regenerating bone marrow controls (n=6). This AML stem cell specificity of the anti-CLL-1 antibody under all conditions of disease and the leukemia-initiating properties of CD34+CLL-1+ cells, indicate that anti-CLL-1 antibody enables both AML-specific stem cell detection and possibly antigen-targeting in future.
**Introduction**

Despite high dose chemotherapy only 30-40% of patients with acute myeloid leukemia (AML) survive, which is mainly due to relapse of the disease.\(^1\) AML is generally regarded as a stem cell disease. However there is debate whether normal stem cells undergoing leukemogenic mutations is the only option to explain leukemogenesis. Alternatively, leukemogenic mutations occurring at a later developmental stage, resulting in stem-cell like behaviour, might be an alternative or additional option.\(^2\)-\(^4\) For CD34 positive AML, several authors have shown that leukemic stem cells are present in the CD34+CD38- compartment.\(^5\);\(^6\) It has been proven *in vitro* that these stem cells are more resistant to chemotherapy, compared to the progenitor CD34+CD38+ cells.\(^7\) *In vivo*, after chemotherapy, the residual malignant CD34+CD38- cells are thought to differentiate to a limited extent, producing leukemic cells with an immunophenotype, which usually reflects that at diagnosis. Sensitive techniques allow early detection of small numbers of these differentiated leukemic cells, called minimal residual disease (MRD) which eventually cause relapse of the disease.\(^8\) Since in this concept the stem cell is the origin of MRD and relapse, stem cell targeted therapy would be of potentially high benefit for AML patients. Moreover, early detection of leukemic stem cells after chemotherapeutic treatment might offer prognostic value in predicting relapse of the disease. Different options for stem cell identification and/or targeted therapy have been described such as anti-CD123, anti-CD44 and anti-CD33, but all have some (potential) disadvantages, including expression on normal stem cells and/or non-hematological tissues.\(^9\)-\(^11\) Since the bone marrow of a (chemotherapy-) treated patient cannot be considered as normal, it is extremely important to study whether after treatment normal stem cells in such regenerating bone marrow remain negative.
for the antigen of interest. So far, this has not been examined for CD33, CD44 and CD123.

In this paper we focus on the newly discovered antigen CLL-1, which we have described to be present on the majority of CD34 positive as well as CD34 negative AML cases. In peripheral blood both monocytes and granulocytes show some CLL-1 expression, while it is absent in other tissues. The intracellular domain of CLL-1 contains both an immunotyrosine-based inhibition motif as well as an YXXM motif, suggesting a role for CLL-1 as a signaling receptor. Phosphorylation of immunotyrosine-based inhibition motif-containing receptors on a variety of cells leads to inhibition of activation pathways via recruitment of the protein tyrosine phosphatases SHP-1, SHP-2, and SHIP. The YXXM motif, on the other hand, encompasses a potential SH2 domain-binding site for the p85 subunit of phosphatidylinositol 3'-kinase, an enzyme implicated in cellular activation pathways. Whether these properties can be translated to CLL-1 is still unknown.

We now demonstrate that CD34+CLL-1+ cells of AML patients showed engraftment in NOD/SCID mice indicating that CLL-1 is expressed on the leukemic stem cell population in these patients. Also, we found CLL-1 to be present on AML and absent on normal CD34+CD38- cells at different time points of disease/treatment, which holds potential to serve as a tool to detect residual leukemic CD34+CD38- cells after therapy and as a possible target for therapy.
Materials and methods

Patient and control samples

The AML clinical protocols and the biological studies were approved by the scientific research committee and the medical ethical committee of the VU University Medical Center, Amsterdam, The Netherlands. Leukemic cells of 89 patients presenting with CD34 positive AML at our institute were obtained after informed consent, at diagnosis and after chemotherapeutic treatment. CD34 positive AML was defined as samples with a CD34% >1 since we have previously shown that in samples with <1% CD34+ cells, these CD34+ cells are in general of normal origin. In 16 cases bone marrow (BM) was not available at diagnosis and peripheral blood (PB) was used instead. After chemotherapeutic treatment only BM was used. Relapse AML BM samples were obtained from 9 patients. Diagnosis of patients was based on morphology using FAB classification, immunophenotyping and cytogenetics. Control normal bone marrow (NBM) was obtained from 11 patients undergoing cardiac surgery after informed consent. Control regenerating bone marrow (RBM) was obtained from three patients with acute lymfoblastic leukemia, one patient with non-Hodgkin’s lymphoma, one patient with completely CD34 negative AML and one patient with CLL-1 negative AML. Mobilized peripheral blood (MPB) was obtained from six non-AML patients after G-CSF stimulation.

Patient characteristics are shown in table 1. AML samples were analyzed freshly (n=52) or after storage in liquid nitrogen (n=37). For expression studies after chemotherapy and in controls fresh material was used. For relapse studies (n=9) in four cases frozen-thawed material was used. NOD/SCID mice repopulation experiments were performed using frozen-thawed material. Procedures used in this study have previously been validated for the use of both fresh and frozen-thawed
material. In fresh samples red blood cells were lysed using a 10 minutes red blood cell lysis on ice with 10 ml lysisbuffer (155 mM NH₄Cl, 10mM KHCO₃, 0.1mM Na₂EDTA, pH 7.4) and washed twice with phosphate-buffered-saline (PBS) with 0.1% human serum albumin (HSA) added. Frozen samples were prepared using a Ficoll gradient (1.077 g/ml) (Amersham Biosciences, Freiburg, Germany) and subsequent red blood cell lysis. Cells were then frozen in RPMI (Gibco, Paisley, UK) with 20% heat-inactivated fetal bovine serum (FBS) (Greiner, Alphen a/d Rijn, The Netherlands) and 10% DMSO (Riedel-de Haen, Seelze, Germany) in isopropanol-filled containers and subsequently stored in liquid nitrogen. When needed for analysis, cells were thawed and suspended in pre-warmed RPMI with 40% FCS at 37°C. Cells were washed and enabled to recover for 45 minutes in RPMI with 40% FCS at 37°C. Cells were washed again and suspended in PBS with 0.1% HSA.

**FACS analysis of CD34+CD38- cells**

Procedures have been described in detail before. In short, fresh cells were incubated with monoclonal antibodies for 15 minutes at room temperature, washed once in PBS containing 0.1% HSA and analyzed by flow cytometry. Monoclonal antibody combinations contained fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinyl chlorophyllin (PerCP) or allophycocyanin (APC) labeled monoclonal antibodies. Anti-CD34 FITC, anti-CD34 PerCP, anti-CD45 PerCP, anti-CD45 APC, anti-CD38 APC, anti-CD123 PE, anti-CD7 PE, anti-CD19 PE, anti-CD33 FITC, anti-CD33 APC and Via-Probe (7AAD) were all from BD Biosciences, anti-CD34 FITC was in part of the samples from Immunotech (Marseille, France) and annexinV FITC from Nexins Research (Kattendijke, The Netherlands). Anti-CLL-1 and isotype controls (GBS and DNP) were from Crucell (Leiden, The Netherlands).
When frozen-thawed cells were used, annexinV FITC was included in the majority of samples to gate out apoptotic/dead cells before stem cell assessment. In the remaining samples this was done by Syto16 (Molecular Probes, Eugene, OR) together with 7AAD in one tube, which enabled to gate out apoptotic/dead cells. The scatter properties of the viable cells were then used in the tube containing CLL-1.

PBS was used as a negative control, since for these specific antibodies isotype controls offered the same results. Data acquisition was performed using a FACScalibur (BD Biosciences) equipped with an argon and red diode laser and analysis was performed using Cellquest software (BD Biosciences).

Blasts were identified by CD45dim/low side scatter characteristics according to Lacombe taking into account that the CD34+CD38- population is a minor population.

**CD34 positive cell selection**

CD34+ cells were selected for the CFU-assays from NBM and for the FISH analysis of a sample from an AML patient in complete remission. After Ficoll separation and red blood cell lysis, mononuclear cells were incubated in PBS containing 5mM EDTA and 0.1% HSA for 30 minutes at room temperature with CD34 Reagent (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer’s procedure. Cells were washed and allowed to flow through a positive selection column in a magnetic field (Automacs, Miltenyi Biotec). After two rounds of selection CD34+ cells were collected. Purity was checked using flow cytometry and was above 95% in all cases.
**FACSsorting of CD34+ cells**

FACSsorted cells were used for CFU-assays, FISH analysis and NOD/SCID mice experiments. Cells were incubated for 15 minutes at room temperature with Via-Probe (7-Amino-Actinomycin D, 7AAD), anti-CD34 FITC, anti-CD38 APC and anti-CLL-1 PE and washed in PBS containing 0.1% HSA. Cells were subsequently sorted using a FACS Vantage cell sorter with Turbo Sort upgrade (BD Biosciences) equipped with an argon (377G) and a helium-neon (127) laser, both from Spectra-Physics (Mountain View, CA, USA) or a FACSaria equipped with solid-state lasers (red, blue and violet, BD Biosciences). Cells were sorted based on viability (7AAD negative), CD34 positive expression, CD38 positive expression for the CFU-assays and absence of CD38 for the FISH analysis, and CLL-1 expression. Purity of the sorted populations was above 95%.

**Transplantation of AML blasts in non-obese diabetic/severe combined immunodeficiency mice**

Engraftment of diagnosis CD34+CLL-1+ cells was studied in non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice (Charles River Laboratories, Wilmington, MA). The mice were irradiated with 3.5 Gy using a linear accelerator, delivering a 15 MV photon beam; tungsten jaws and multi-leafs collimated and shaped the irradiation field. The beam is calibrated, i.e. the output for well-defined standard irradiation geometry is adjusted to a well-measured signal of the monitor chamber. The monitor chamber is the system in the accelerator controlling with high accuracy the dose-delivery to the mice.

Cells used were from three patients; samples were selected for CD34 expression, CLL-1 expression on the CD34+CD38- cells and engraftment in NOD/SCID mice. Patient 1 had AML with FAB M2 and Flt3 ITD, patient 2 with FAB
M1 and Flt3 ITD and patient 3 with FAB M6, Flt3 ITD and +i(8)(q10)x2. CD34+CLL-1+ cells (3.5-8 x10^6) were injected in the lateral tail vein. The mice were sacrificed after 6 weeks in accordance with the institutional animal research regulations or at onset of clinical symptoms. BM was isolated from two femurs per mouse. Chimerism was determined using flow cytometric detection of human CD45 expression. Engrafted cells were analyzed for myeloid origin (CD33 positive and CD19 negative), for malignant origin (presence of the leukemia-associated phenotype) and for CLL-1 expression.

**FISH analysis**

FISH analysis for t(8;21) was performed using LSI ETO/AML1 from Vysis (IL, USA) on FACS-sorted populations of a patient sample after chemotherapeutic treatment. Procedures have been described before.15

**CFU-assays**

For CFU assays, cells obtained after CD34 isolation and FACS-sorting, were cultured at 37°C in 5% CO2, in a humidified incubator, in semi-solid medium containing α-methylcellulose (Methocult GF H4434, StemCell Technologies, Vancouver, CA). The number of colonies was evaluated after 14 days culture in semi-solid medium.20 CFU-MK assays were performed as described before.21

**Statistical analyses**

Statistical analysis of the data was performed using the SPSS 9.0 software package (SPSS, Chicago, IL, USA).

Mann-Whitney was used for CLL-1 expression in BM controls and AML samples. Wilcoxon signed-rank and Spearman correlation analysis were used for the analysis of the diagnosis-relapse samples. Spearman correlation analysis was also
used for the correlation between the frequency of CLL-1 positive CD34+CD38- cells and MRD frequency.

P-values <0.05 were considered to indicate a statistically significant difference.
Results

CLL-1 expression of CD34+CD38- cells in AML at diagnosis and in normal bone marrow

Previously we have shown that C-type lectin-like molecule-1 (CLL-1) is present on leukemic blasts at diagnosis in the majority of AML cases (68 out of 74). Now CD34+ AML samples at diagnosis were analyzed for CLL-1 expression in the CD34+CD38- stem cell compartment. Representative examples of CLL-1 staining on CD34+CD38- cells are depicted in figure 1. In 77 out of 89 CD34+ AML cases the CD34+CD38- cells showed a positive shift (compared to the isotype control) for CLL-1; since in part of the cases these shifts were small, overall (n=89) a median CLL-1 expression of 33% was found for the CD34+CD38- compartment (Fig. 2). CLL-1 expression in the CD34+CD38- compartment was found throughout all FAB subtypes studied. CLL-1 expression on the CD34+CD38- cells did neither correlate with any AML subtype nor with prognosis in our patient cohort. Although in NBM samples the CD34+CD38+ progenitor population was partly CLL-1 positive, the CD34+CD38- cells were CLL-1 negative (Figures 1G and 1H and Figure 2). To conclude, CLL-1 is specifically expressed on AML CD34+CD38- cells and not on NBM CD34+CD38- cells.

CLL-1 expressing CD34+ cells contain leukemia-initiating stem cells

NOD/SCID transplantation experiments were performed to show that the CD34+CLL-1 positive compartment contains leukemic stem cells. Of the 89 AML patients we had cells for NOD/SCID experiments from 10 patients. The total blast population from these patients was transplanted in NOD/SCID mice, which resulted in engraftment of human cells in 3/10 patients. From these three AML patients (with a CD34+CD38- compartment that was homogeneously positive for CLL-1) the
CD34+CLL-1+ cell population was FACSorted and injected into sub-lethally irradiated NOD/SCID mice. After 6 weeks the mice were sacrificed. The engrafted CD45 positive cells were of myeloid origin, i.e. CD33 positive and CD19 negative, had a leukemia-associated-immunophenotype and showed CLL-1 expression on all blasts (Figures 3A-3I). Engraftment of human cells was found for samples of all three patients and in all mice (Fig. 4). Therefore we can conclude that the CD34+CLL-1+ population of these three patients does contain CLL-1-expressing leukemic stem cells.

**Expression of CLL-1 in regenerating bone marrow and mobilized peripheral blood**

Both for possible future therapeutic use and for specific detection of malignant stem cells it is of utmost importance that during and after treatment, the non-malignant CD34+CD38- normal hematopoietic stem cells should remain CLL-1 negative. To establish that, remission bone marrow of both non-AML patients and CD34 negative or CLL-1 negative AML patients was investigated. No CLL-1 expression on the CD34+CD38- stem cell compartment was observed (Fig. 2). Furthermore, since G-CSF mobilization is part of the several current AML treatment protocols, CLL-1 expression was determined in G-CSF mobilized blood. The CD34+CD38- population was almost completely CLL-1 negative in all samples (Fig 2).

To conclude, normal CD34+CD38- cells remain CLL-1 negative in BM recovering after chemotherapy and in PB after mobilization with growth factors.

**Identity of CLL-1 positive and negative normal bone marrow progenitors**

Our previous finding that CLL-1 is expressed on part of the CD34+CD38+ progenitor population in NBM\(^{12}\), was confirmed in 11 additional samples: median
expression 40%, ranging from 26-60%. Next the nature of the CD34+CD38+CLL-1 positive and CD34+CD38+CLL-1 negative subpopulations in normal BM was determined by FACSsorting and subsequent application of different colony assays. Results are depicted in figure 5. Most CFU-GM grew out of the CD34+CD38+CLL-1 positive population (second pair of rows). All monocytic colonies originated from the CLL-1 positive fraction, whereas the granulocytic colonies originated only in part from the CLL-1 positive fraction (data not shown). These observations are in accordance with granulocytes and monocytes being CLL-1 positive.12 In contrast, erythroid colonies (CFU-E) and megakaryotic colonies (CFU-Mk) originated from the CD34+CD38+CLL-1 negative compartment. Therefore, targeting CLL-1 positive cells using anti-CLL-1 based therapy would most likely only affect granulocyte recovery.

**CLL-1 expression of AML CD34+CD38- cells at diagnosis versus relapse**

For reliable assessment during treatment/disease of the AML stem-cell compartment using CLL-1, its expression should be stable in the course of the disease. Paired diagnosis/relapse samples of 9 AML patients covering a large range of CLL-1 expression, showed no significant differences (p=0.9) and were significantly correlated (R=0.7, p=0.04); median expression at diagnosis was 34% (range 8-91%) versus 42% (range 5-92%) at relapse.

**CLL-1 expression of CD34+CD38- cells in remission bone marrow**

Subsequently the possibility to discriminate CLL-1 positive malignant CD34+CD38- cells from CLL-1 negative normal CD34+CD38- cells in remission bone marrow at different stages of treatment was investigated. Figures 6A, 6C and 6E show representative flowcytometry pictures of three patients with high expression of CLL-1 on CD34+CD38- cells at diagnosis.
Patient one (figure 6A and 6B) reached complete remission (CR) with resulting low frequency (0.01%) of minimal residual disease (MRD) as measured using leukemia-associated phenotype (LAP) expression, which is prognostically highly favorable\(^8\). In line with this, CLL-1 expression was not present on the CD34+CD38- compartment (Fig. 6B). Patient two (figure 6C and 6D) reached CR, but with relatively high MRD frequency (0.89%). This patient quickly relapsed (within one month). CD34+CD38- cells remained largely CLL-1 positive (Fig. 6D), showing that the majority of BM stem cells were of malignant origin. Patient three (figure 6E and 6F) reached CR after the first cycle of chemotherapy. The MRD frequency at that time point was 0.82% which decreased to a prognostically favorable 0.02% after the second cycle of chemotherapy. Despite this, the patient relapsed within 6 months. In contrast to MRD, the fraction of CLL-1 positive cells remained high: figure 6F shows the first cycle with 72% CLL-1 expression on the CD34+CD38- cells. Since this patient was diagnosed with t(8;21) we were able to FACSsort the CD34+CD38- cells after the first cycle and to show by FISH analysis that, in very good agreement with figure 6F, these were 80% positive for t(8;21). Quantitative PCR for t(8;21) confirmed these results (data not shown). The malignant nature of the CD34+CD38-CLL-1+ population was further confirmed by aberrant expression of the NK-cell marker CD56 (72%) on CD34+CD38- cells. The CD34+CD56+ immunophenotype has been shown to be a leukemia-associated phenotype and is used for MRD detection,\(^8,22-24\) and malignant stem cell detection.\(^25\)

Figure 7 shows representative examples of follow-up analysis for two patients with a different course of the disease. Patient one reached CR which lasts now for three years. Figure 7A shows that this patient had high CLL-1 expression on the CD34+CD38- cells at diagnosis, but this expression decreased after chemotherapy.
and remained absent; also the frequency of the CD34+CD38-CLL-1+ cells within the WBC compartment (Fig.7B), which is a measure for the amount of CLL-1+ CD34+CD38- cells, diminished and remained undetectable in time. Patient two reached CR, but relapsed after the second course of chemotherapy. CLL-1 was continuously expressed on the CD34+CD38- compartment, indicating that this compartment remained predominantly malignant at all time points analyzed (Fig.7C). Also the frequency of the CLL-1 positive CD34+CD38- cells within the WBC compartment remained high and even increased towards relapse (Fig.7D). These figures are representations of similar patients who had a good remission (total n=4) or a fast relapse (total n=4).

These results indicate that, using CLL-1, malignant CD34+CD38- cells can indeed be detected in remission bone marrow and can be discriminated from the normal CD34+CD38- compartment. In a series of 13 patients the putative possible role in prognosis was studied: in 8/9 cases with a quick relapse i.e. median 6 months after diagnosis (range 3-11 months), CLL-1 expression on the whole stem cell compartment was high (median 62%, range 27-91%). This is in contrast to the patients still in remission (longest 33 months), for which CLL-1 positive stem cells were very low or absent in 4/5 cases (median 0%, range 0-2%). Moreover we were able to show that, irrespective of the time point in disease/treatment, the frequency of CD34+CD38- cells within the WBC compartment significantly correlated with the frequency of MRD cells in these AML patients (n=44 time points from 15 patients, R=0.7, p<0.001). MRD detection serves as the golden standard for risk assessment during treatment/disease, since it is a strong independent predictor for survival in AML patients as we and others have shown before. The prognostic impact of MRD frequency in the present patient cohort has been published by us before.
Discussion

In the present study we show that CLL-1 is a marker of the malignant CD34+CD38- stem cell compartment in the majority of CD34 positive AML patients. Transplantation of the CD34+CLL-1+ cells, which putatively contain the CD34+CD38- stem cells capable of initiating leukemia, resulted in the development of leukemia in NOD/SCID mice in the three patients analyzed, with recovery of the diagnosis CLL-1 expression. CLL-1 expression turned out to be specific for leukemic CD34+CD38- cells, since it is absent both on normal CD34+CD38- resting bone marrow cells, on CD34+CD38- cells in treated patients with other diseases and in CD34+CD38- cells obtained after G-CSF stimulation from non-AML patients. CLL-1 expression on the AML cells is likely stable with no difference found between samples at diagnosis and relapse.

CLL-1 expression thus can be detected specifically on AML CD34+CD38- cells present after chemotherapy in AML patients in complete remission. In this way CLL-1 expression may serve as marker for quantification of minimal residual stem cell disease. The observation of quick relapses preceded by the presence of AML stem cells, holds the promise that such may offer clinically highly relevant information additional to classic immunophenotypical MRD detection.8,22-24 Moreover, these results show that CLL-1 is a potential target for anti-leukemia stem cell therapy in remission bone marrow with AML stem cells still present. Since thrombocytopenia is a major side effect of most therapies in the majority of AML patients, and thus often a dose-limiting factor, it is of importance that CLL-1 is absent on megakaryocytic progenitors.

How would CLL-1 perform compared to CD123, CD33 and CD44? CD123 has been reported to be a stem cell specific marker in AML.26 An advantage is the high
staining intensity of CD34+CD38- cells observed in the majority of AML\textsuperscript{26}, which we were able to confirm (median expression 98\% ranging from 5 to 100\% in 36 cases). The median expression on NBM CD34+CD38- cells (n=5) was higher compared to CLL-1 (14.9\% ranging from 0-18.8\%), which was also found by others.\textsuperscript{11} To explore potential therapeutic use, a toxin-labeled IL-3 has been developed to target a functional IL3-receptor (consisting of both CD123 and CD131) and is currently being examined in (pre-) clinical studies.\textsuperscript{27} However, important control experiments, including possible staining of normal CD34+CD38- cells in regenerating bone marrow (RBM) of patients treated with chemotherapy, have not yet been reported. Since we have found high (median 60\%) CD123 expression on non-AML regenerating bone marrow CD34+CD38- cells in five such cases (not shown), this issue needs serious attention. In sharp contrast, we did not detect CLL-1 on RBM CD34+CD38- cells.

CD33 is also expressed on CD34+CD38- stem cells of AML samples at diagnosis (median 80\%, range 26-100\%, n=13, unpublished). In contrast to anti-CD123 antibody and similar to anti-CLL-1 antibody, internalisation of anti-CD33 occurs upon binding to the receptor.\textsuperscript{28} However, CD33 is not specific for AML stem cells: expression has been shown on normal stem cells.\textsuperscript{11} In our own experience too, CD33 is highly expressed on normal CD34+CD38- stem cells: both in resting NBM (median 84\%, range 16-100\%, n=9) and in RBM (61\%, 79\% and 95\% in 3 different BM samples). These observations might offer part of the explanation for the considerable hematological toxicity in patients undergoing anti-CD33 therapy,\textsuperscript{29} part of which is certainly due to its effect on normal CD33 positive progenitors\textsuperscript{30,31}, and, maybe even more important, on CD34+CD38- stem cells in normal bone marrow.\textsuperscript{11}
CD44 has recently been described as a target on leukemic CD34+CD38-stem cells. It was shown that the activating antibody H90 results in differentiation of cells and in a major reduction of engraftment in NOD/SCID mice. However, CD44 expression is also weakly expressed on normal CD34+CD38- cells and on more differentiated hematopoietic cells. Regenerating bone marrow was not evaluated. Moreover, the different CD44-isoforms are expressed on many different tissues.

An alternative approach to specifically target leukemic stem cells is inhibition of the proteasome. Normal CD34+ progenitor/stem cells do not express NF-κB, whereas CD34+CD38-CD123+ cells in AML do. The proteasome inhibitor MG-132, a well-known inhibitor of NF-κB, has been proposed for stem cell targeted therapy. In vitro there is synergism between this NF-κB inhibitor and a conventional chemotherapeutic agent, idarubicin. Also, Parthenolide, has been proposed to selectively target leukemic stem cells while sparing normal hematopoietic cells.

Internalization has been described for CLL-1 after incubation with the antibody. However it is unlikely that the anti-CLL-1 antibody as such will have anti-leukemic effect, since Moab binding to the CLL-1 positive HL60 cells had no effect on cell proliferation using a tritium-thymidine assay, while culturing normal CD34 positive cells in a long-term culture system in the presence of anti-CLL-1 antibody has no effect on CFU-E and CFU-GM colony formation (data not shown). Also, CLL-1 labeling of cells did not influence engraftment of AML CD34+ cells in NOD/SCID mice (not shown). It is thus unlikely, although still unknown, that induction of antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity via CLL-1 will be an effective targeting mechanism. However, the putative function of CLL-1 in signal transduction might offer possibilities that can be translated in therapeutic applications. Another promising therapeutic strategy would be to couple
a toxic moiety to the anti-CLL-1 antibody: if such a complex is internalized after antigen-binding this would result in cell death after antigen-mediated uptake.

Anti-CLL-1 antibody mediated therapy might be effective in CD34- AML as well, since CLL-1 expression is high in blast cells of CD34- AML (median 96%, range 15-100%, n=11, including three AML FAB M3). In the absence of a CD34+CD38-compartment, the side population (SP) defined by Hoechst staining is the most likely candidate to contain leukemic stem cells, which we indeed found in 6/9 CD34- AML patients. Preliminary results indicate that CLL-1 is indeed expressed on SP cells of these patients in 6/6 (median 64%, range 46-100%). Also, other myeloid malignancies, like MDS and CML, show CLL-1 expression on the CD34 positive cells.\(^{12}\)

In conclusion, CLL-1 is a marker that is expressed on AML CD34+CD38- stem cells and not on normal CD34+CD38- cells under all conditions of treatment and disease. In three patients tested we found that CD34+CLL-1+ cells repopulate in sub-lethally irradiated NOD/SCID mice, indicating that they contain leukemia-initiating cells. The use of anti-CLL-1 for the detection of minimal residual disease would offer an attractive approach, additional to the established MRD frequency assessments, both as a prognostic marker and to guide time points for therapeutic intervention. Moreover, these unique properties might be exploited for the development of effective antibody conjugates for stem cell directed therapy.
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Anna van Rhenen designed part of the research, performed main part of the experiments, data analysis, wrote the paper
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Sonja Zweegman guided part of the research and reviewed the manuscript
Gert J. Ossenkoppele responsible for availability of clinical samples, reviewed the manuscript
Gerrit Jan Schuurhuis designed and guided the main part of the study, main reviewer of the manuscript
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Table 1 Patient characteristics

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<tr>
<td>No. of patients</td>
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<td>Age at diagnosis, years, mean (range)</td>
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<tr>
<td>M6</td>
<td>3 (3)</td>
</tr>
<tr>
<td>Refractory anemia with excess blasts</td>
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<tr>
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<tr>
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Figure 1 Examples of CLL-1 expression in AML samples at diagnosis and in normal bone marrow

After labelling of the cells with the appropriate antibody combinations, the CD45dimCD34+CD38- cells were identified by a Boolean gating strategy (see Materials and Methods, not shown) with subsequent detection of isotype and CLL-1 expression. For the CD34 negative AML (Figure 1E-1F) only blast gating (CD45dim) was performed. Figures 1A-1F show isotype and CLL-1 expression in AML samples. The percentages in the lower right quadrant indicate CLL-1 expression as a percentage of the CD34+CD38- compartment, in the lower left quadrant the percentage CLL-1 negative cells within the CD34+CD38- compartment is shown. Figure 1B shows an example of CLL-1 expression on CD34+CD38- cells close to the median. Figure 1D offers an example of a high CLL-1 expression. Figure 1F shows a representative example of CLL-1 expression in a CD34 negative AML sample. Finally figure 1H shows the absence of CLL-1 expression on CD34+CD38- cells of normal bone marrow. Note that CLL-1 is expressed on part of the progenitor population.
Figure 2. CLL-1 expression on CD34+CD38- stem cells in AML, normal bone marrow (NBM), regenerating bone marrow (RBM) and G-CSF mobilized peripheral blood (MPB)

After labelling of the cells with the appropriate antibody combinations, the CD34+CD38- cells were identified by a Boolean gating strategy (as described in Materials and Methods) and subsequently CLL-1 expression was determined. The percentage of CLL-1 expression on CD34+CD38- cells for every individual patient and control are depicted with a symbol. In NBM median CLL-1 expression was 0% (range 0-11%, n=10), in RBM 0% (range 0-5%, n=6) and in mobilized peripheral blood (MPB) 0.6% (range 0-3.7%, n=6). The CD34-positive FAB M3 samples of this study showed CLL-1 expression on the CD34+CD38- cells of 17, 83 and 89% respectively.
Figure 3. Flow cytometry of engrafted cells in NOD/SCID mice

FACS sorted CD34+CLL-1+ cells from three different AML patients were transplanted in NOD/SCID mice. Six weeks after transplantation the mice were sacrificed and engraftment of human cells in these mice was studied using human anti-CD45 labelling. The engrafted cells were analysed for myeloid origin (CD19 negative and CD33 positive, data not shown), leukemia-associated phenotype and CLL-1 expression. Patient numbers are the same as in figure 4.

In Figure 3A-E the results of engrafted cells in one mouse out of three, transplanted with cells from patient two are shown. Figure 3A shows CLL-1 expression on CD34+ cells at diagnosis (the CD34 negative cells in this sample were also CLL-1+, not shown). Figure 3B shows aberrant expression of CD7 on part of the leukemic CD34+ cells at diagnosis. Figure 3C shows the CD45 positive human population within the white blood cell compartment of the mouse. Figure 3D shows that the human cells that grew out of the transplanted CD34+CLL-1+ cells are all CLL-1 positive, similar to the whole blast compartment at diagnosis. Fig. 3E shows that similar to diagnosis, part of the cells again show aberrant CD7 expression.

Figures 3F and 3H show the human CD45 positive population within the white blood cell compartment of mice transplanted with cells from patients three and one, respectively. CLL-1 expression on engrafted cells is shown in figures 3G and 3I, respectively.
Figure 4 Engraftment of CD34+CLL-1 positive AML blasts in NOD/SCID mice

FACSsorted CD34+CLL-1+ cells from three different AML patients were transplanted in NOD/SCID mice. Six weeks after transplantation the mice were sacrificed and engraftment of human cells in these mice was studied using human anti-CD45. In this figure the overall results are depicted on a logarithmic scale; every symbol represents the percentage engraftment of human CD45 positive cells in one mouse. In a mouse transplanted with the whole CD34+ population from patient 2, which included the CD34+CD38-CLL+, but also a clear CD34+CD38-CLL-1- population (15% of the CD34+CD38- population), outgrowth of CD45dimCD33+CD19- AML cells was likewise accompanied by outgrowth of CD45dimCD33-CD19+ cells (18% of human cells), which are presumably normal (data not shown).36
Figure 5. Colony-forming capacity of CLL-1 defined CD34 subpopulations in normal bone marrow.

CD34+CD38+CLL-1- and CD34+CD38+CLL-1+ subpopulations were purified from NBM cells. Cell input was 2500 cells/ml for CFU-E/CFU-GM. Cell input for CFU-MK was 5000 cells/ml. The number of colonies was determined after 14 days of culture. The black bars show the output of the CD34+CD38+CLL-1+ population and the gray bars of the CD34+CD38+CLL-1- population. The error bars show the standard deviation. Two (CFU-Mk) and three (BFU-E/CFU-GM) independent experiments were performed, each in duplicate.
Figure 6. Examples of CLL-1 expression on CD34+CD38- cells in diagnosis and follow-up samples.

Gating was performed based on CD45dim/SSC characteristics and CD34 positive expression. Afterwards CD38 and CLL-1 expression were determined. Similar to figure 1, percentages shown concern the CLL-1 positive and negative populations within the CD34+CD38- compartment. Figures 6A and 6B show an example of a patient who remained in continuous remission. This patient showed high CLL-1 expression on CD34+CD38- cells at diagnosis (Fig.6A) but not after chemotherapy (Fig.6B). Note the similarity with CLL-1 expression on normal bone marrow CD34+CD38- cells in figure 1H. Figures 6C and 6D show a diagnosis and MRD picture, as 6A and 6B, but now for a patient with a large proportion of CD34+CD38- cells being CLL-1 positive in the MRD sample (Fig.6D). Figures 6E (diagnosis) and 6F (after first course of chemotherapy) show CLL-1 expression on CD34+CD38- cells of an AML patient diagnosed with t(8,21). In the remission sample the malignant character of the CD34+CD38-CLL-1+ population could be confirmed using FISH technique.
Figure 7 Stem cell parameters in AML patients with a different course of the disease.

Cells were analyzed at diagnosis and at different time points during follow-up of two AML patients. Patient one is depicted in figures A and B, patient two in figures C and D. Figures A and C show malignant CD34+CD38- cells (defined by CLL-1) as a fraction of the total CD34+CD38- population. The solid line represents the background expression in RBM for CLL-1. Figures B and D show the frequency of CLL-1 positive CD34+CD38- cells as percentage of the total WBC count. D is diagnosis; R is Relapse; 1st is after first course of chemotherapy; 2nd and 3rd is after the second and third course of chemotherapy, respectively.

Patient one reached complete remission after the first course of chemotherapy and is in continuous complete remission now for three years. Percentage of CLL-1 expression on the total CD34+CD38- compartment declined rapidly (Fig.7A) as did the frequency of CD34+CD38-CLL-1+ cells (Fig.7B).

Patient two reached complete remission after the first course of chemotherapy; however a relapse occurred already after the second course of chemotherapy. Although CR was reached, there was a continuous expression of CLL-1 on the CD34+CD38- cells (Fig.7C). Also, the frequency of CD34+CD38-CLL-1+ cells did not decrease after chemotherapy, but even increased (Fig.7D).
The novel AML stem cell associated antigen CLL-1 aids in discrimination between normal and leukemic stem cells

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