

C-Type Lectin-Like Molecule-1: A Novel Myeloid Cell Surface Marker Associated with Acute Myeloid Leukemia

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ABSTRACT

Acute myeloid leukemia (AML) has a poor prognosis due to treatment-resistant relapses. A humanized anti-CD33 antibody (Mylotarg) showed a limited response rate in relapsed AML. To discover novel AML antibody targets, we selected a panel of single chain Fv fragments using phage display technology combined with flow cytometry on AML tumor samples. One selected single chain Fv fragment broadly reacted with AML samples and with myeloid cell lineages within peripheral blood. Expression cloning identified the antigen recognized as C-type lectin-like molecule-1 (CLL-1), a previously undescribed transmembrane glycoprotein. CLL-1 expression was analyzed with a human anti-CLL-1 antibody that was generated from the single chain Fv fragment. CLL-1 is restricted to the hematopoietic lineage, in particular to myeloid cells present in peripheral blood and bone marrow. CLL-1 is absent on uncommitted CD34⁺/CD38⁻ or CD34⁺/CD33⁻ stem cells and present on subsets of CD34⁺/CD38⁺ or CD34⁺/CD33⁺ progenitor cells. CLL-1 is not expressed in any other tissue. In contrast, analysis of primary AMLs demonstrated CLL-1 expression in 92% (68 of 74) of the samples. As an AML marker, CLL-1 was able to complement CD33, because 67% (8 of 12) of the CD33⁻ AMLs expressed CLL-1. CLL-1 showed variable expression (10–60%) in CD34⁺ cells in chronic myelogenous leukemia and myelodysplastic syndrome but was absent in 12 of 13 cases of acute lymphoblastic leukemia. The AML reactivity combined with the restricted expression on normal cells identifies CLL-1 as a novel potential target for AML treatment.

INTRODUCTION

Despite important advances in the therapy of acute myeloid leukemia (AML), the majority of patients still die from their disease as a consequence of treatment-resistant relapses (1). Therefore, alternative strategies are needed to complement the currently used chemotherapy treatment protocols. In past clinical trials, monoclonal antibody-based immunotherapy targeting leukocyte-specific antigens such as CD33, CD45, and CDw52 has been evaluated for the treatment of leukemia (2, 3). Whereas therapeutic trials using unmodified antibodies have thus far generated limited success, a calicheamicin-conjugated humanized anti-CD33 antibody (Mylotarg) has yielded a 30% overall response rate in Phase II studies (4). On the basis of its activity and safety profile, Mylotarg was approved for the treatment of CD33-positive AML in relapsed patients >60 years of age. Notwithstanding these encouraging results, the current antibody-based immunotherapies for the treatment of relapsed AML still have limitations with respect to the antigens that are targeted. Consequently, there is a need to identify novel cell surface antigens on AML blasts that can be exploited for this purpose.

Monoclonal antibodies recognizing differentiation antigens dis-

played on the cell surface of AML blasts and their hematopoietic progenitor cells have been used to identify leukemic cells (5, 6). Examples are antigens such as CD34, CD10, CD13, CD19, CD7, CD20, and CD33 that are expressed on specific subsets of normal hematopoietic cells. Besides their value in the diagnosis of leukemias, differentiation antigens restricted to the earliest stages of AML blasts may have therapeutic potential, because outgrowth of AML occurs from these cells (7). Similar to CD33, a novel target antigen for AML is preferably not expressed on hematopoietic stem cells. This allows the *in vivo* ablation of antigen-bearing leukemic stem cells and normal cells followed by the re-establishment of normal hematopoiesis through the remaining normal stem cells. Ideally, the absence of the target antigen on committed normal progenitor cells would also limit the period of hypoplasia after treatment.

Phage display antibody technology permits the selection of human antibody fragments from large naïve libraries that have been assembled *in vitro* from the genetic elements encoding antibodies (8). Initially, this technology was applied to select phage-antibodies displaying desirable specificities by panning on purified, solid phase bound antigens. Subsequently, more complex substrates including intact eukaryotic cells were used to select phage antibodies. Selections on whole cells can be extended by using an excess of absorber cells with the aim of removing unwanted specificities from the library. Our laboratory has pioneered and optimized such a competitive selection strategy on intact eukaryotic cells using complex mixtures with an excess of absorber cells by combining phage display technology with flow cytometry (9). This combined technology allows the isolation of phage antibodies recognizing novel cell surface-expressed molecules and epitopes irrespective of the immunogenicity of the target antigen.

In the present study we have used our phage display technology to identify novel cell surface antigens on AML blasts. We describe the identification and the initial characterization of C-type lectin-like molecule (CLL-1), a previously undescribed surface antigen differentially expressed on malignant and normal myeloid cells.

MATERIALS AND METHODS

Cells, Patients, and Antibodies. PER.C6 cells were cultured in DMEM, 10% fetal bovine serum, and 10 mmol/L MgCl. HL60 and U937 cells were cultured in RPMI 1640, 10% fetal bovine serum, and 2 mmol/L L-glutamine. THP1 cells were cultured in RPMI 1640, 10% fetal bovine serum-heat inactivated, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, and 0.00035% β-mercapto-EtOH. 293T cells were cultured in DMEM, 10% fetal bovine serum, and 0.4 mmol/L L-glutamine. All of the cells were cultured at 37°C, 10% CO₂.

Peripheral blood buffy coats were obtained from the Sanquin Blood Supply Foundation (Leiden, The Netherlands). Leukemia cells were obtained from bone marrow or peripheral blood of newly diagnosed AML, chronic myelogenous leukemia (CML), myelodysplastic syndrome, and acute lymphoblastic leukemia patients after informed consent. AML was classified according to the French-American-British (FAB) classification. Myelodysplastic syndrome patients had low-grade disease, and acute lymphoblastic leukemia patients had either B-lineage acute lymphoblastic leukemia or T-lineage acute lymphoblas-

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tic leukemia. Normal bone marrow samples were obtained after informed consent from patients undergoing cardiovascular surgery.

Monoclonal antibodies used in this study included the following: isotype control IgG1, isotype control IgG1-phycoerythrin, CD14-FITC, CD16-FITC, CD19-FITC, CD32-FITC, CD33-APC, CD56-phycoerythrin, and CD64-FITC (PharMingen, Alphen a/d Rijn, The Netherlands); CD33-PC5 (Immunotech, Mijdrecht, The Netherlands); CD3-FITC, CD7-FITC, CD19-FITC, CD33-APC, CD34-FITC, CD34-phycoerythrin, CD34-PerCP, CD34-APC, CD38-APC, CD44-FITC and CD45-PerCP, and CD45-APC (Becton Dickinson, Alphen a/d Rijn, The Netherlands); and CD5-FITC, CD13-FITC, and goat-anti-mouse-IgG-FITC (Dako, Glostrup, Denmark).

Phage Selection. Antibody fragments were selected using a semisynthetic antibody phage display library described previously (10) that was rescued using the C-terminal helper phage (11). An aliquot of phage library (500 μL , 10^{13} colony-forming units) was blocked and presubstrated three times with peripheral blood leukocytes in serum containing medium (RPMI 1640). Subsequently, 4×10^6 HL60 cells were mixed with phage library for 2 hours at 4°C . After removal of unbound phages, the cells were pelleted, and the attached phages were eluted and rescued as described (11). A second round of selection was performed using an aliquot of the rescued phage sublibrary that was incubated with 5×10^6 thawed primary AML blasts (90% $\text{CD}33^+\text{CD}34^+$ blasts, FAB M0). After extensive washing, the cell mixture was subjected to flow cytometry, and 9×10^5 cells were sorted on the basis of low side scatter combined with CD34-phycoerythrin (Becton Dickinson) staining using a FACSVantage flow cytometer (Becton Dickinson). The remaining attached phages were eluted from the $\text{CD}34^+$ cells and rescued, and monoclonal phage preparations were generated and analyzed for binding to HL60 cells and AML blasts as described previously (11). From the selected single chain Fv fragments, clone SC02-357 plasmid DNA was isolated, and nucleotide sequences were determined as described (11).

Expression Cloning. We set out to identify the antigen recognized by SC02-357 using an expression cloning strategy as described previously (12), with minor modifications. A cDNA library prepared from human bone marrow (Invitrogen, Breda, The Netherlands) was transfected into 293T cells using the Lipofectamine (Life Technologies, Inc., Breda, The Netherlands) or Fugene (Roche, Almere, The Netherlands) reagent according to protocols provided by the manufacturer. Forty-eight hours after transfection the cells were harvested and stained with a saturating amount of SC02-357 phage antibody as described (11). To avoid the selection of 293T cells expressing Fc-receptor-encoding cDNAs, the transiently transfected cells were costained with FITC-conjugated antibodies recognizing CD16, CD32, and CD64. Single-positive transfected cells, displaying bright SC02-357 phage antibody staining (phycoerythrin), were collected using flow cytometry for retrieval of plasmid DNA. After four rounds of selection, plasmid pools and, subsequently, single colonies were selected that were able to confer SC02-357 reactivity.

Reverse Transcription-PCR analysis. For PCR analysis of human CLL-1 transcripts, the following primers were used: forward 5'-GTGATGATGTC-CAAACATGGC-3' and reverse 5'-GATTGATGCCTCATGCCTC-3' (Proligo Primers and Probes), which yields a PCR product of 365 bp. PCR was performed with TITANIUM TaqPCR kit (Becton Dickinson) according to the manufacturer's recommendations. Five μL of each human tissue cDNA (Becton Dickinson) was used as template in a 50- μL PCR reaction containing 1 μL 50 \times deoxynucleotide triphosphates (10 mmol/L each), 5 μL of 10 \times TITANIUM TaqPCR buffer, 1 μL of 50 \times TITANIUM Taq Polymerase, and 1 μL of each primer (10 $\mu\text{mol/L}$). PCR reactions were carried out in a Biometra T3 thermocycler by incubating reactions for 1 minute at 94°C followed by 25 cycles for 30 s at 94°C , 1 minute at 60°C , and a 5-minute extension time at 68°C . The integrity of the cDNA was assessed by PCR by using β -actin primers under identical conditions.

Immunohistochemistry. For immunoperoxidase staining of frozen sections bivalent single-chain Fv fragments produced by *Pichia Pastoris* were used. Usage of the bivalent single-chain Fv fragments format has the advantage over the human IgG1 format in that background staining due to endogenous human IgG present in tissues does not need to be circumvented. To express the bivalent single-chain Fv fragments in *P. Pastoris*, an expression vector was constructed encoding a single-chain Fv fragment fused to the hinge region of mouse IgG3 and the myc- and His-tags in the pPICZaB plasmid (Invitrogen). Bivalent single-chain Fv fragments were purified from yeast supernatant by

immobilized metal affinity chromatography using NiNTA agarose (Qiagen, Venlo, The Netherlands).

Frozen sections of human tissues were cut, mounted, and dried at room temperature. The sections were blocked with 50 mmol/L sodium azide containing 0.035% H_2O_2 for 20 minutes followed by blocking with PBS containing 4% bovine serum albumen (BSA) before incubation with the antihuman CLL-1 bivalent single-chain Fv fragments for 60 minutes at room temperature. Hereafter, the sections were fixed with 4% formaldehyde for 30 minutes. Bound bivalent single-chain Fv fragments were visualized using the 9E10 anti-myc monoclonal antibody (mAb) followed by Envision antimouse IgG amplification reagent (Dako) and incubation with 3,3'-diaminobenzidine. The sections were counterstained with hematoxylin. As controls, all of the sections were stained with no bivalent single-chain Fv fragments and with a bivalent single-chain Fv fragment recognizing human CD46 (positive control), respectively.

Generation of Human IgG1. The human anti-CLL-1 IgG1 mAb was generated as described (13). The variable regions of the single-chain Fv fragments were recloned in separate vectors for IgG heavy and light chain expression. To that purpose, VH and VL regions were PCR amplified using designated primers to append restriction sites and restore complete human frameworks. The resulting construct encoding the anti-CLL-1 human IgG1 heavy chain was transiently expressed in combination with the κ light chain construct in PER.C6 cells. IgG1 antibody was purified over protein A and size-exclusion columns. The human anti-CLL-1 and control IgG1 mAb were labeled with phycoerythrin (IQ Products, Groningen, The Netherlands and Molecular Probes, Leiden, The Netherlands).

Flow Cytometry of Normal Bone Marrow, AML, CML, Myelodysplastic Syndrome, and Acute Lymphoblastic Leukemia. For the expression analysis of CLL-1 on normal $\text{CD}34^+$ progenitor/stem cell subsets, normal bone marrow cells were preincubated with 50 $\mu\text{g/mL}$ rabbit IgG for 30 minutes on ice to block Fc receptors. CD34-FITC, CLL-1-phycoerythrin, CD45-PerCP, and CD38-APC mAbs were added. After 15 minutes, red blood cells were lysed with ammonium chloride (BD Pharm lyse, Becton Dickinson), and cells were washed twice with PBS/0.1% BSA. Data acquisition was performed using a FACSCalibur and analyzed using Cellquest software (Becton Dickinson). $\text{CD}34^+$ cells were gated using the ISHAGE gating procedure (14).

AML cells were cryopreserved in liquid nitrogen after Ficoll-Isopaque density gradient isolation (Pharmacia, Uppsala, Sweden) and lysis of remaining red blood cells. Validation of the use of cryopreserved material showed no false-positive or false-negative staining of the mAbs used. Upon thawing, 2 to 4×10^5 cells were preincubated with rabbit IgG, and CLL-1-phycoerythrin, CD45-PerCP, and CD33-APC mAbs were added and incubated for 30 minutes. The cells were washed twice with PBS/1% BSA and were stained with annexin V-FITC (Caltag, Burlingame, CA) according to instructions provided by the manufacturer for exclusion of dead and apoptotic cells. For final analysis, blast cells were gated based on low side scatter *versus* $\text{CD}45^{\text{dim}}$ expression in the Annexin V-negative population. A sample was considered positive for CLL-1 or CD33 if $>20\%$ of the cells expressed the antigen (compared with the control sample). The relative mean fluorescence intensity was calculated by dividing the mean fluorescence intensity of the specific antibody by the control sample.

For CML, myelodysplastic syndrome, and acute lymphoblastic leukemia a similar approach was applied. Four color panels were used (sequence indicates FITC/phycoerythrin/PerCP or 7-AAD/APC): for CML the main combination was $\text{CD}34/\text{CLL-1}/7\text{-AAD}/\text{CD}33$; for myelodysplastic syndrome the main combinations were CD7 or CD5 or $\text{CD}13/\text{CLL-1}/\text{CD}34/\text{CD}38$ and CD7 or CD5 or $\text{CD}13/\text{CLL-1}/7\text{-AAD}/\text{CD}34$, in which CD7, CD5, and CD13 were used to define aberrant marker expression on the $\text{CD}34^+$ cells to additionally substantiate the malignant character of the $\text{CD}34^+$ cells; and for acute lymphoblastic leukemia the main combinations were: $\text{CD}7/\text{CLL-1}/7\text{-AAD}/\text{CD}45$ for T-lineage acute lymphoblastic leukemia and $\text{CD}19/\text{CLL-1}/7\text{-AAD}/\text{CD}45$ for B-lineage acute lymphoblastic leukemia. All of the combinations were also applied using the isotype control instead of CLL-1.

Antigen Density Analysis. The CLL-1 cell surface density was analyzed by quantitative flow cytometry (Qifikit, DAKO). To this purpose a chimeric anti-CLL-1 antibody containing a mouse IgG1 constant region was constructed and produced. The use of this chimeric human-mouse anti-CLL-1 antibody allowed quantification against calibrated microbeads having different densities of mouse IgG1 molecules on their surface. Upon preincubation with rabbit IgG, saturating amounts of the purified chimeric anti-CCL-1 antibody, control

IgG1 or mouse anti-CD33 antibody was used to stain myeloid leukemic cell lines, peripheral blood leukocytes, or AML patient samples, followed by goat-antimouse-IgG FITC staining (DAKO). Data acquisition was performed using a FACSCalibur, and the data were analyzed according to the Qifikit instructions.

Internalization. Internalization of CLL-1 upon antibody binding was quantified by flow cytometry as was described previously with some modifications (15, 16). U937 cells were preincubated with rabbit IgG and were loaded with the phycoerythrin-labeled anti-CLL-1 IgG1 antibody or with a FITC-labeled anti-CD44 antibody (negative control). Unbound antibody was removed, and aliquots of the cells were resuspended in 50 μ L of medium and incubated at either 4°C (no internalization) or 37°C for different time points to allow internalization. After extensive washing, cell surface-bound CLL-1-IgG complexes were stripped from the cells using 50 μ L Qiagen protease (Qiagen) at 7.5 AU/mL for 1 hour at 4°C. Cells were washed twice with ice-cold PBS-1% BSA, and samples were analyzed by flow cytometry. The fluorescence at $t = 0$ in the absence of protease treatment was normalized to 100%.

RESULTS

Selection of Single-Chain Fv Fragments Recognizing AML. In the current study we combined phage display antibody technology and flow cytometry to identify antibodies that recognize potential novel cell surface antigens on AML blasts. To this purpose HL-60 cells were incubated with a semisynthetic phage display library of human single-chain Fv fragments, and the phages that bound to the cells were isolated. A second round of selection was performed by incubating the rescued sublibrary with primary AML blasts followed by isolating the CD34⁺ cells with attached phages by cell sorting. Upon analysis of monoclonal phage antibody preparations, 7 unique single-chain Fv fragments were additionally characterized using FACS on a panel of primary AML samples and peripheral blood (Table 1). From this panel of single-chain Fv fragments SC02-357 was selected for target identification on the basis of its broad AML recognition and lack of reactivity with peripheral blood erythrocytes and platelets.

Identification of CLL-1. By performing expression cloning using a human bone marrow cDNA library we isolated the cDNA encoding the antigen recognized by SC02-357. After four rounds of selection, a cDNA clone of 1492 bp (7H10-A) was obtained that induced SC02-357 phage antibody staining upon transfection in 293T cells (Fig. 1A). A GenBank search revealed that the 7H10-A cDNA insert was nearly identical to an mRNA sequence encoding the human CLL-1 protein (Accession no. NM_138337). Apart from the first stretch of 39 nucleotides, full homology was observed between 7H10-A and the deposited CLL-1 mRNA. The *CLL-1* gene is localized within the NK gene complex on the telomeric region of chromosome 12. Alignment of the 7H10-A cDNA clone with a bacterial artificial chromosome containing a part of human chromosome 12 (Accession no. AC091814) explained the difference between the 7H10-A cDNA and the deposited CLL-1 mRNA (Accession no. NM_138337). The first stretch of nucleotides of the 7H10-A cDNA

that did not match the deposited CLL-1 mRNA was identical to one region in the bacterial artificial chromosome clone, whereas the subsequent nucleotide sequence of the cDNA annealed to another region that was separated by 20 kb of nonhomologous intronic DNA (Fig. 1B). Because the 5 prime region of the deposited CLL-1 cDNA (NM_138337) that does not match 7H10-A is completely homologous to this second intron-exon boundary in the bacterial artificial chromosome clone, it is clear that the deposited cDNA clone contains intronic DNA and, therefore, reflects a partially or alternatively spliced mRNA. The 7H10-A cDNA obtains an open reading frame encoding a 275-amino acid (aa) type II transmembrane protein (Fig. 1C). The predicted polypeptide has an intracellular region of 53 aa, a 23-aa transmembrane region, and a 199-aa extracellular domain. The presence of several characteristic structural motifs in the extracellular region indicates that this protein is a member of the C-type lectin superfamily. Interestingly, the 7H10-A cDNA contains an open reading frame that extends the deposited CLL-1 protein with an extra 10 amino acids at its NH₂ terminus, adding a YXXM motif to the intracellular domain of CLL-1. In addition, the cytoplasmic domain contains an I/VXYXXL immunotyrosine-based inhibition motif. Alignment of the single extracellular carbohydrate recognition domain of CLL-1 with other C-type lectin receptors indicated that CLL-1 bears the highest homology to CLEC-1 (26% identity at the amino acid level; ref. 17).

CLL-1 Expression in Normal Tissues. PCR analysis of a panel of human cDNAs obtained from normal tissues revealed expression of CLL-1 transcripts in peripheral blood leukocytes and in spleen (Fig. 2A). Protein expression of CLL-1 within human tissues was analyzed by indirect immunohistochemistry on frozen tissue using purified bivalent SC02-357 single-chain Fv fragments. Among 36 tissue types analyzed, specific CLL-1 staining was only observed in spleen (Table 2). This CLL-1 expression within spleen was detected in tissue granulocytes, a finding that is consistent with the observed tissue distribution of CLL-1 at the mRNA level.

Flow cytometry analysis of a panel of tumor cell lines of both hematopoietic and nonhematopoietic origin using the human anti-CLL-1 IgG1 antibody revealed that expression of CLL-1 was restricted to a subset of tumor cell lines of myeloid origin: HL-60, THP-1, and U937 (data not shown). Within peripheral blood leukocytes the anti-CLL-1 antibody specifically recognized granulocytes and monocytes (Fig. 2B). The anti-CLL-1 antibody did not recognize CD3⁺ T lymphocytes, CD19⁺ B lymphocytes, and CD56⁺ natural killer cells. In contrast, we reproducibly observed a population of 2% to 5% of CD3⁻CD19⁻CD56⁻ cells within the lymphocyte gate (based on forward scatter and side scatter) that did display CLL-1 reactivity (data not shown). Subsets of dendritic cells were analyzed by staining with CD14, CD16, and CD33 antibodies. Accordingly, a more mature dendritic cell population can be distinguished as a CD33^{bright}CD14⁻CD16⁻ population, whereas a precursor dendritic cell population can be seen as a CD33^{dim}CD14⁻CD16⁻ population (18). As shown in Fig. 2B, the anti-CLL-1 IgG1 antibody stains both the mature and the precursor dendritic cell subsets within peripheral blood.

Within normal bone marrow, lymphoid and myeloid subpopulations were segregated on the basis of side scatter properties and CD45 staining. Granulocytes (CD45^{dim} and high side scatter) as well as monocytes (CD45^{dim} and side scatter^{dim}) stained with the CLL-1 antibody, thus mirroring the staining profile in peripheral blood (data not shown).

CD34 expressing progenitor and stem cells were tested for CLL-1 reactivity by initially gating the CD34⁺ cells and subsequently co-analyzing CLL-1 and CD33 or CD38 staining. Fig. 2C shows that only a minority of the CD34⁺ cells displayed reactivity with the

Table 1 Reactivity of selected scFv on primary AML samples and peripheral blood

| | AML | | | | Peripheral blood | | | | | |
|----------|-----|----|----|----|------------------|-----|---------|---------|-----------|--------------|
| | M1 | M1 | M2 | M4 | RBC | PLT | B cells | T cells | Monocytes | Granulocytes |
| SC02-357 | + | + | + | + | - | - | - | - | + | + |
| SC02-361 | s+* | + | - | s+ | - | - | - | - | + | - |
| SC02-366 | s+ | - | - | s+ | - | - | - | - | - | - |
| SC02-378 | s+ | s+ | s+ | s+ | - | - | - | - | s+ | + |
| SC02-395 | + | + | + | s+ | - | s+ | s+ | s+ | + | + |
| SC02-396 | s+ | + | - | s+ | - | - | - | - | s+ | + |
| SC02-397 | s+ | s+ | s+ | s+ | - | - | - | - | + | + |

Abbreviations: scFv, single-chain Fv fragment; PLT, platelets.

* Subpopulation of analyzed sample displays scFv reactivity.

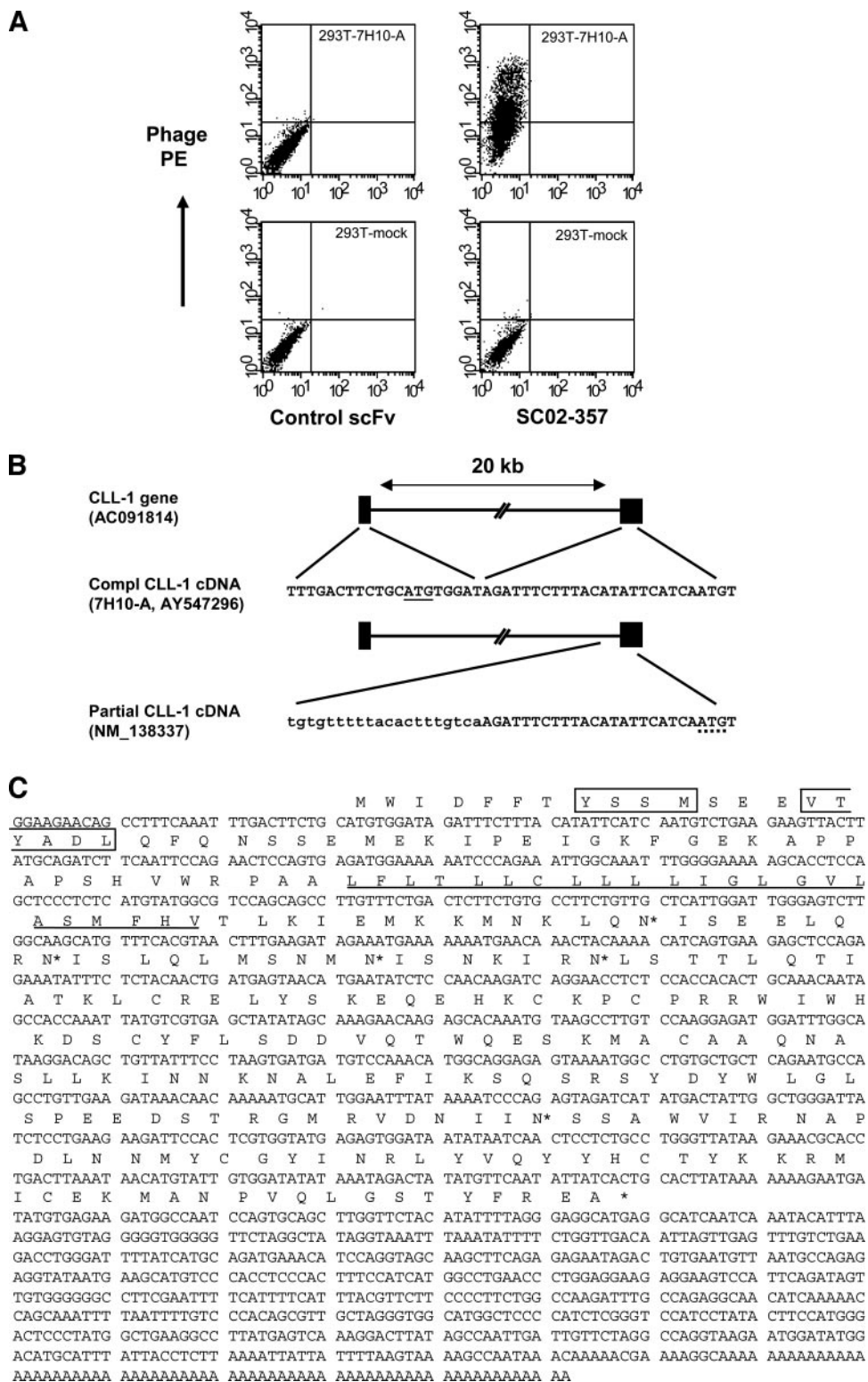


Fig. 1. Phage antibody SC02-357 recognizes CLL-1. A, transfection of 7H10-A plasmid confers SC02-357 reactivity. 293T cells were transiently transfected with 7H10-A plasmid or with a control plasmid (*mock*). Forty-eight hours after transfection the cells were stained with SC02-357 or with a control phage antibody. B, genomic organization of part of the *CLL-1* gene. Alignment of the 7H10-A cDNA and the deposited CLL-1 cDNA with a bacterial artificial chromosome clone containing part of chromosome 12 is shown. ■, exons within the bacterial artificial chromosome clone. Lower-case nucleotides, the 5' prime stretch of the deposited CLL-1 mRNA that does not match 7H10-A. The start codon of the complete CLL-1 cDNA is underlined, whereas the proposed start codon of the partial CLL-1 cDNA is marked with a dotted line. C, nucleotide and predicted amino acid sequence of human CLL-1. (GenBank accession no. AY547296). YXXM and ITIM motifs in the intracellular domain are indicated with boxes, the predicted transmembrane region is underlined, and potential N-linked glycosylation sites are depicted with * (PE, phycoerythrin)

CLL-1 antibody, in particular the cells with the highest CD33 or CD38 expression that presumably represent granulocyte precursors. Moreover, dim expression of CD38 and CD33, presumably representing monocyte precursors, corresponded to absence of CLL-1 staining. Most importantly, in 4 bone marrow samples, CD34⁺CD38⁻ stem cells lacked the antigen (example in Fig. 2C), whereas 40% (39–44%) of the CD34⁺CD38⁺ compartment was CLL-1⁺. In all of the cases analyzed also CD34⁺CD33⁻ cells did not express CLL-1.

CLL-1 Expression in AML. To determine the expression of CLL-1 in AML we analyzed a panel of *de novo* AML samples by flow cytometry. Representative examples of CLL-1 and CD33 expression on AML blasts are shown in Fig. 2D. As summarized in Table 3, the CLL-1 antigen was expressed on >20% of blast cells in 68 of 74 AML samples (92%) and was effective for the detection of AML samples throughout the different FAB subtypes analyzed. CD33 expression was found in 62 of 74 AML samples (84%). The currently

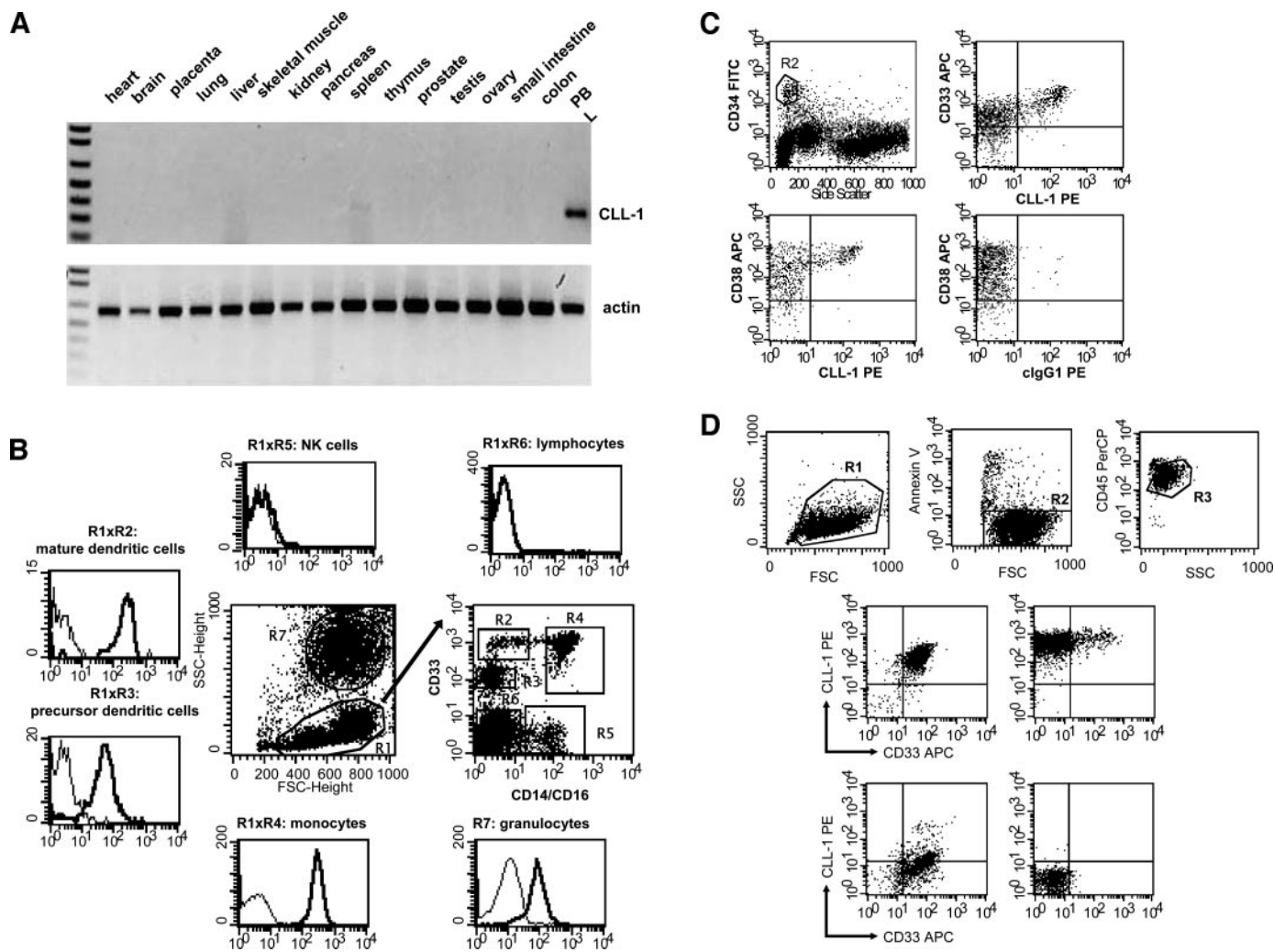


Fig. 2. Expression of CLL-1 in normal tissues and in AML. **A**, PCR amplification of CLL-1 transcripts from cDNA obtained by reverse transcription of RNA from normal human tissues. **B**, flow cytometric analysis of CLL-1 expression on peripheral blood leukocytes. Leukocytes were divided in subsets based on forward scatter and side scatter analysis. Subsequently the gated lymphocytes were divided on the basis of CD33-PC5 and CD14/CD16-FITC staining, respectively. In the panels displaying the selected subsets, CLL-1- PE staining was analyzed compared with control human IgG1-PE. One representative experiment of six is shown. **C**, flow cytometric analysis of CLL-1 expression on CD34⁺ cells within normal bone marrow. CD34⁺ cells were gated according to the ISHAGE protocol, using a CD45, side scatter, and CD34-based gating strategy, but for convenience only the CD34/side scatter gate is shown. Subsequently, on the gated CD34⁺ cells (in R2) CLL-1-PE expression was analyzed in combination with CD38-APC or CD33-APC expression. One representative experiment of four is shown. **D**, flow cytometric analysis of CLL-1 expression on AML. First three dot plots show the gating of viable AML blasts. Initially, cells were gated based on forward and side scatter properties (R1). Subsequently, viable annexin V negative cells were gated (R1xR2). Finally, viable AML blasts were selected based on low side scatter versus CD45^{dim} expression (R1xR2xR3). In the lower four dot plots CLL-1 and CD33 expression on four representative R1xR2xR3-gated AML blast cell populations are depicted. (PE, phycoerythrin; NK, natural killer)

analyzed sample set included 12 CD33-negative AML samples of which 8 of 12 samples (67%) displayed CLL-1 expression (Table 3). Of the 6 CLL-1-negative samples, 2 were reactive with the anti-CD33 antibody. Thus, only 4 of 74 samples were negative for both CD33 and CLL-1.

Analysis of the CLL-1 antigen density using a quantitative flow cytometry assay showed that ~6 to 17 × 10³ CLL-1 molecules per cell were present on the myeloid leukemic cell lines HL-60, U937, and THP-1, whereas peripheral blood monocytes and granulocytes expressed on average 10 × 10³ and 4 × 10³ molecules per cell, respectively (Table 3). In primary AML samples CLL-1 was expressed on average at 7 × 10³ molecules per cell (n = 26). No clear differences were observed in CLL-1 expression in the different FAB subtypes that were analyzed. As shown in Table 3 the expression levels of both CLL-1 and CD33 appear to be equal among the different FAB subtypes with the exception of FAB M3. In acute promyelocytic leukemia (FAB M3) CD33 expression may be higher as compared with CLL-1.

Table 2 Expression of CLL-1 protein in normal tissues

| Tissue | CLL-1 | Tissue | CLL-1 |
|-----------------|-------|------------------|-------|
| Adrenal gland | - * | Esophagus | - |
| Aorta | - | Ovary | - |
| Bladder | - | Pancreas | - |
| Cerebellum | - | Parathyroid | - |
| Cerebrum | - | Peripheral nerve | - |
| Cervix | - | Pituitary gland | - |
| Colon | - | Placenta | - |
| Coronary artery | - | Prostate | - |
| Duodenum | - | Salivary gland | - |
| Endometrium | - | Skin | - |
| Fallopian tube | - | Spinal cord | - |
| Heart | - | Spleen | ± † |
| Ileum | - | Stomach | - |
| Kidney | - | Testis | - |
| Liver | - | Thyroid | - |
| Lung | - | Tonsil | - |
| Lymph node | - | Ureter | - |

* No CLL-1 staining within tissue was observed; the integrity of each tissue and staining procedure was confirmed using a positive control bivalent single-chain Fv fragment recognizing CD46.

† Scattered CLL-1+ granulocytes were observed.

Table 3 *CLL-1 and CD33 expression on normal myeloid cells and primary AML samples*

| FAB | CLL-1 | | | CD33 | | |
|--------------|------------------|---------------------|----------------------|------------------|-------------------|------------------------|
| | % positive cases | % positive blasts * | Density † | % positive cases | % positive blasts | Density † |
| M0 | 80 (4‡/5§) | 82 ± 27 | 4143; 5543 | 80 (4/5) | 75 ± 20 | 3182; 30539 |
| M1 | 85 (11/13) | 77 ± 22 | 5659 ± 4661 (n = 5) | 77 (10/13) | 68 ± 29 | 6108 ± 4769 (n = 5) |
| M2 | 92 (12/13) | 74 ± 17 | 3604 ± 2554 (n = 3) | 85 (11/13) | 79 ± 26 | 5222 ± 1923 (n = 4) |
| M3 | 100 (2/2) | 96, 76 | 4087 | 100 (2/2) | 100, 100 | 28413 |
| M4 | 100 (9/9) | 82 ± 23 | 8384 ± 2600 (n = 6) | 89 (8/9) | 80 ± 23 | 10580 ± 4512 (n = 5) |
| M4eo | 100 (2/2) | 99, 33 | Not tested | 100 (2/2) | 71, 98 | Not tested |
| M5 | 100 (9/9) | 82 ± 21 | 11092 ± 3913 (n = 4) | 89 (8/9) | 97 ± 4 | 19325 ± 13601 (n = 3) |
| M5a | 100 (8/8) | 83 ± 21 | 7842 ± 4363 (n = 3) | 100 (8/8) | 72 ± 34 | 5813; 12219 |
| M5b | 100 (5/5) | 90 ± 8 | 10188 | 100 (5/5) | 96 ± 4 | 6531 |
| M6 | 67 (2/3) | 74, 60 | Not tested | 67 (2/3) | 98, 88 | Not tested |
| RAEB-t | 100 (2/2) | 21, 60 | Not tested | 0 (0/2) | NA | Not tested |
| Unclassified | 67 (2/3) | 100, 82 | 6039 | 67 (2/3) | 99, 35 | 5295 |
| Total AML | 92 (68/74) | 74 ± 27 | 7600 ± 3998 (n = 26) | 84 (62/74) | 81 ± 24 | 12876 ± 12838 (n = 24) |
| HL60 | NA | NA | 6041 ± 3239 (n = 4) | NA | NA | 19650; 45449 |
| U937 | NA | NA | 17314 ± 3292 (n = 4) | NA | NA | 9390; 30831 |
| THP1 | NA | NA | 6638; 5213 | NA | NA | 57990 |
| Monocytes | NA | NA | 9318; 10431 | NA | NA | 4485; 11769 |
| Granulocytes | NA | NA | 3203; 5408 | NA | NA | 577; 2049 |

B. Frequency distribution of CLL-1 and CD33 on AML

| Total AML | CD33 ⁺ | CD33 ⁻ | |
|--------------------|-------------------|-------------------|----|
| CLL-1 ⁺ | 60 | 8 | 68 |
| CLL-1 ⁻ | 2 | 4 | 6 |
| | 62 | 12 | 74 |

* Average ± SD of % blast cells in positive samples; in case the sample size is <3 the individual values are depicted.

† CLL-1 and CD33 antigen density on different cell types as determined by quantitative flow cytometry. Depicted is the mean ± SD (n = x); in case the sample size is <3 the individual values are depicted.

‡ Number of positive cases; a sample was considered positive if >20% of the blast population stained with the CLL-1 or CD33 antibody.

§ Number of cases tested.

Table 4 *CLL-1 expression in CML, MDS, and ALL*

| Type of leukemia | Initial blast % * | CLL-1 expression † (% of blasts) ‡ |
|----------------------------|-------------------|---------------------------------------|
| CML 1 | 10 | 8 |
| CML 2 | 9 | 12.2 |
| CML 3 | 4 | 35.7 |
| CML 4 | 5 | 55.8 |
| MDS 1 | 0.7 | 10.8 |
| MDS 2 | 0.7 | 26.4 |
| MDS 3 | 0.5 | 26.9 |
| MDS 4 | 3.2 | 29.7 |
| MDS 5 | 0.8 | 49.5 |
| T-ALL (n = 5) | 86 (63–99) § | 0 (–0.9 to +8.8) § |
| B-ALL (n = 7) | 67 (29–82) § | 0.08 (–1.2 to 7.0) § |
| B-ALL (n = 1) ¶ | 69 | 99 |
| Normal bone marrow (n = 4) | | 32 (28–33) § |

Abbreviations: MDS, myelodysplastic syndrome; ALL, acute lymphoblastic leukemia.

* For CML and MDS in original bone marrow sample, for ALL in Ficoll-purified samples.

† Corrected for IgG control. Expression was established using the panels described in Materials and Methods section, “Flow cytometry of normal bone marrow, AML, CML, MDS and ALL.”

‡ For CML and MDS blasts are the CD34⁺ cells.

§ Median (range).

¶ Exceptional case of B-ALL: all blasts are CLL-1 positive.

CLL-1 Expression in CML, Myelodysplastic Syndrome, and Acute Lymphoblastic Leukemia. In CML and myelodysplastic syndrome CLL-1 expression patterns were quite similar to those found in normal CD34⁺ cells, which have been shown before (Fig. 2C). However, in contrast to CD34⁺ cells in normal bone marrow, the malignant CD34⁺ cells in both malignancies showed a large variation in the percentage of positive cells (Table 4). In acute lymphoblastic leukemia, blasts were completely negative in 7 of 8 B-lineage acute lymphoblastic leukemia and 5 of 5 T-lineage acute lymphoblastic leukemia cases. One B-lineage acute lymphoblastic leukemia sample was positive for CLL-1 in all of the blasts (Table 4); no correlation was found with expression of myeloid markers (data not shown).

Internalization of CLL-1. To analyze whether antibody-mediated cross-linking of CLL-1 on the cell surface could result in internalization of the antigen, *in vitro* studies were performed using the myeloid leukemic cell line U937. Antibody binding assays at 37°C showed that the fluorescent labeled anti-CLL-1 antibody had disappeared from the cell surface and became associated with a protease-resistant compartment over time (Fig. 3A). After 3 hours of incubation ~50% of the fluorescent anti-CLL-1 antibody was internalized, whereas a control antibody that recognized CD44 remained protease sensitive during this period (Fig. 3B).

DISCUSSION

In an effort to find cell surface targets expressed on AML blasts for use as potential therapeutic targets, we identified CLL-1 as a new

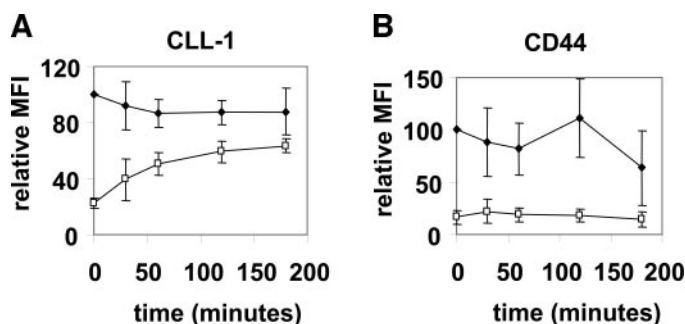


Fig. 3. Internalization of CLL-1 upon antibody-mediated ligation. U937 cells were loaded with CLL-1 (A) or CD44 (B) and were incubated at 37°C for several time points. Hereafter, the cells were (open symbols) or were not (closed symbols) subjected to protease treatment prior to flow cytometry analysis. In the absence of protease treatment the fluorescence represents both cell surface and intracellular staining. The fluorescence measured after protease treatment represents solely intracellular CLL-1 staining. Each data point represents the average of three separate experiments. The fluorescence at t = 0 in the absence of protease treatment was normalized to 100%. Bars, ±SD.

antigen expressed on malignant and normal myeloid cells. To our knowledge this is the first time that a leukocyte antigen is discovered using phage display technology that was not identified previously using antibodies obtained via mouse hybridoma technology. Apparently, the subtractive selection strategy that can be performed *in vitro* allows the selection of phage antibodies against CLL-1 that are not easily obtained from mouse B cells upon immunization with myeloid cells. In addition, this finding demonstrates that a novel disease-associated molecule can be identified using phage display technology, whereas this antigen had not been found previously via genetic profiling approaches (19, 20).

A shortened form of CLL-1 that was obtained via database mining has been described recently as myeloid inhibitory C-type lectin-like receptor (21). In contrast to our data, low levels of myeloid inhibitory C-type lectin-like receptor RNA expression were reported in heart, colon, lung, and placenta using Northern blot analysis. The absence of a CLL-1 signal in these tissues in our reverse transcription-PCR analysis might be explained by the differences in stringency of the methods that were used. From the combined expression data analyzing both mRNA and protein expression in our study, we conclude that the CLL-1 antigen is a pan-myeloid antigen that is not expressed on normal primitive CD34⁺CD38⁻ or CD34⁺CD33⁻ stem cells and only on a minor portion of the CD34⁺CD38⁺ or CD34⁺CD33⁺ progenitor cells. Strikingly, the CLL-1 antigen is abundantly expressed on myeloid cells in bone marrow and peripheral blood, but the antigen could not be detected on resident cells within tissues. The only tissue in which CLL-1 expression was spleen, where CLL-1⁺ granulocytes were observed. Whereas peripheral blood monocytes and dendritic cells are clearly positive, no CLL-1 staining is observed in tissue macrophages or tissue dendritic cells. A similar loss of antigen expression upon migration of myeloid cells into peripheral tissues has been observed for the CD33 antigen, which is abundantly expressed in bone marrow and peripheral blood but is absent on resident macrophages and dendritic cells in peripheral tissues (22).

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 (23). It was shown recently that the immunotyrosine-based inhibition motif of CLL-1 in the context of a chimeric receptor was capable of recruiting SHP-1 and SHP-2 upon receptor cross-linking (21).

The YXXM motif, on the other hand, encompasses a potential SH2 domain-binding site for the p85 subunit of phosphatidylinositol 3'-kinase (24), an enzyme implicated in cellular activation pathways. Whether the interaction of CLL-1 with its ligand leads to cellular activation or immunomodulation may depend on the level of phosphorylation of both motifs as well as on the efficiency of recruitment of SHP-1/2 and p85. Besides its role in phosphatidylinositol 3'-kinase recruitment, the YXXM motif has been demonstrated to function as an internalization motif (25, 26). Therefore, this motif might be responsible for the observed internalization of the CLL-1 receptor upon antibody-mediated cross-linking. Endocytosis via tyrosine-based motifs has been described for other C-type lectins on antigen presenting cells that mediate antigen uptake (27). It is unlikely that CLL-1 is involved in the uptake of carbohydrate-containing complexes, because CLL-1 lacks the conserved residues that are responsible for Ca²⁺-mediated carbohydrate binding (28). Instead, internalization of CLL-1 upon ligand encounter may rather act as a switch to terminate receptor-mediated signaling.

Upon analysis of a panel of primary *de novo* AML samples we observed that 92% of AML were CLL-1 positive, clearly indicating

the potential for CLL-1 as a novel target for AML. In most cases the bulk of the population of blast cells showed no clear separation in populations with negative and positive blasts. In the current sample set 82% of the analyzed AML samples was positive for CD33, a figure that is comparable with that of other published studies where the percentage of CD33-positive AML ranges from 75% to 92% (29–31). Interestingly, CLL-1 and CD33 complement each other as diagnostic markers and (potential) therapeutic targets, because 33% of the CLL-1 negative AML samples express CD33, and 67% of the CD33 negative samples express CLL-1 (Table 3). Moreover, when the two diagnostic markers are combined, 95% (70 of 74) of the AML samples could be identified.

Expression patterns in other malignancies, *i.e.*, CML and low-grade myelodysplastic syndrome, contrast to AML and have clearly separated subpopulations that are either negative or positive, like normal bone marrow CD34⁺ cells, with one remarkable phenomenon: whereas CLL-1 expression on normal CD34⁺ cells is highly reproducible, expression in both CML and myelodysplastic syndrome varies widely. These aberrant expression patterns warrant additional investigation. Up to now no correlation with other properties such as expression of other markers is evident.

The lack of expression of CLL-1 on normal hematopoietic stem cells and the relative low expression on CD34⁺ progenitor cells position the antigen as a potential therapeutic target. This finding allows *in vivo* targeting and eradication of antigen-bearing leukemic cells and the subsequent re-establishment of normal hematopoiesis through the remaining normal stem cells. Moreover, even the majority of the more committed normal CD34⁺ cells would be spared in the case of CLL-1 targeting, with only the most mature CD38^{high} or CD33^{high} cells being eradicated. As compared with CD33 targeting, the latter eradicates all of the CD33⁺ progenitors and leaves only the CD33⁻ population that includes the stem cells, unaffected.

CLL-1 may be exploited as a target antigen in AML in several ways. Targeting of the antigen on leukemic blasts might directly result in tumor cell death as a consequence of antigen-mediated signaling. We addressed this potential route by incubation of the anti-CLL-1 antibody with CLL-1⁺ myeloid leukemic cell lines *in vitro*. However, this did not result in the induction of apoptosis (data not shown). Another way of antibody-mediated targeting of CLL-1⁺ AML uses antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity. The efficacy of these immune effector mechanisms strongly depends on the density of the target antigen that is displayed on the surface of the tumor cell. For CD33 it was shown that its density was a limiting factor prohibiting significant induction of antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity (30). In our studies we observed a CLL-1 antigen density that was comparable with CD33 and with published CD33 expression data (32, 33). Therefore, we do not envision that induction of antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity via CLL-1 will be an effective targeting mechanism. Another therapeutic strategy involves the targeting of a toxic moiety to CLL-1-bearing cells that results in cell death after antigen-mediated uptake. Preferably, the target-antibody complex is internalized upon antigen binding, thus minimizing extracellular release of the toxic compound. Our studies showed that CLL-1 efficiently internalizes upon antibody engagement (Fig. 3), which positions CLL-1 as a suitable target antigen for toxin-conjugated antibody therapy. Similar to Mylotarg, a calicheamicin-conjugated humanized anti-CD33 antibody, therapeutic efficacy in elderly relapsed AML patients may be achieved using toxin-conjugated antibodies that target CLL-1.

Lastly, in preliminary experiments with AML samples with a high CLL-1 expression and containing CD34⁺ cells, we observed CLL-1 positivity both on the CD34⁺CD38⁺ population and the CD34⁻CD38⁻

population. Because the latter has been shown to contain the leukemic stem cell (34), CLL-1, in contrast to antibodies directed at other lineage-restricted markers like CD33, may even target the leukemic stem cell, potentially offering more chance on cure. Moreover, this might be of use in diagnostic phenotyping, establishing new prognostic factors. Because the normal CD34⁺CD38⁻ subpopulation is CLL-1 negative, it would additionally allow us to include CLL-1 in the diagnosis of minimal residual disease in the stem cell compartment. At present, minimal residual disease is diagnosed using the whole leukemic population (35).

In summary, we have identified a novel C-type lectin that is broadly expressed on AML blasts as well as on normally differentiated myeloid cells. The absence of expression of CLL-1 on normal hematopoietic stem cells and the relative low expression on CD34⁺ progenitor cells might improve the selective targeting of AML blast cells resulting in a reduced bone marrow cytotoxicity. Our studies showed that CLL-1 clearly complements CD33 as a marker for AML. Therefore, CLL-1 is an attractive target for AML that may also therapeutically complement CD33 in the treatment of AML.

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