Targeting C-type lectin-like molecule-1 for antibody-mediated immunotherapy in acute myeloid leukemia

by Xiaoxian Zhao, Shweta Singh, Cecile Pardoux, Jingsong Zhao, Eric D. Hsi, Arie Abo, and Wouter Korver

Haematologica 2009 [Epub ahead of print]
doi:10.3324/haematol.2009.009811

Publisher's Disclaimer.
E-publishing ahead of print is increasingly important for the rapid dissemination of science. Haematologica is, therefore, E-publishing PDF files of an early version of manuscripts that have completed a regular peer review and have been accepted for publication. E-publishing of this PDF file has been approved by the authors. This paper will now undergo editing, proof correction and final approval by the authors. Please note that during this production process changes may be made, and errors may be identified and corrected. The final version of the manuscript will appear both in the print and the online journal. All legal disclaimers that apply to the journal also pertain to this production process.

Haematologica (pISSN: 0390-6078, eISSN: 1592-8721, NLM ID: 0417435, www.haematologica.org) publishes peer-reviewed papers across all areas of experimental and clinical hematology. The journal is owned by the Ferrata Storti Foundation, a non-profit organization, and serves the scientific community with strict adherence to the principles of open access publishing (www.doaj.org). In addition, the journal makes every paper published immediately available in PubMed Central (PMC), the US National Institutes of Health (NIH) free digital archive of biomedical and life sciences journal literature. Haematologica is the official organ of the European Hematology Association (www.ehaweb.org).

Support Haematologica and Open Access Publishing by becoming a member of the European Hematology Association (EHA) and enjoying the benefits of this membership, which include free participation in the online CME program.
Targeting C-type lectin-like molecule-1 for antibody-mediated immunotherapy in acute myeloid leukemia

Xiaoxian Zhao,1 Shweta Singh,1 Cecile Pardoux,2 Jingsong Zhao,2 Eric D. Hsi,1 Arie Abo,2 and Wouter Korver1,3

1Department of Clinical Pathology, Cleveland Clinic, Cleveland, OH, USA, and 2Nuvelo, Inc. San Carlos, CA, USA

ABSTRACT

Background

C-type lectin-like molecule-1 (CLL-1) is a transmembrane receptor expressed on myeloid cells, AML blasts and leukemic stem cells. To validate the potential of CLL-1 as a therapeutic target for AML, we generated a series of monoclonal antibodies (mAbs) against the extracellular domain of CLL-1 and used them to extend the expression profile analysis of AML cells and to select cytotoxic mAbs against AML cells in preclinical models.

Design and Methods

CLL-1 expression was analyzed in AML cell lines and myeloid derived cells from AML patients and healthy donors. Anti-CLL-1 antibody-mediated in vitro cytotoxic activity (CDC/ADCC) against AML blasts/cell lines and in vivo anti-cancer activity in a mouse xenograft model was assessed. Internalization of CLL-1 mAbs upon receptor ligation was also investigated.

Results

CLL-1 was expressed in 86.5% (45/52) of AML cases but not in ALL blasts (n=5). Its expression on AML CD34+/CD38– stem cells was detected in 54.5% (12/22) of cases. Selected anti-CLL-1 mAbs could mediate dose-dependent complement dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC) specifically against AML-derived cell lines. Exogenous expression of CLL-1 in HEK293 cells resulted in cells susceptible to anti-CLL-1 mAb-mediated killing. Furthermore, anti-CLL-1 mAbs demonstrated strong CDC activity against freshly isolated AML blasts (15/16 cases, 94%). CLL-1 mAbs were also efficiently internalized upon binding to CLL-1 in HL-60 cells. Moreover, a lead chimeric CLL-1 mAb reduced the tumor size in xenograft mice implanted with HL-60 cells.

Conclusions

Our results demonstrate that targeting CLL-1 with specific cytotoxic mAbs is an attractive approach which could lead to novel AML therapeutics.

Key words: C-type, lectin-like molecule-1, immunotherapy, acute myeloid leukemia.


©2010 Ferrata Storti Foundation. This is an open-access paper.
Introduction

AML is the most common acute leukemia affecting adults and its incidence increases with age. AML has a poor prognosis, primarily due to relapses with conventional chemotherapy regimens. The overall 5-year leukemia-free survival rate is only 25-35% and even lower in patients > 60 years of age. Alternative therapeutic strategies, like applying FLT3 tyrosine kinase inhibitors in combination with chemotherapy in therapeutic strategies, like applying FLT3 tyrosine kinase inhibitors in combination with chemotherapy in patients positive for FLT3 mutation, are currently being evaluated.13

Therapies using monoclonal antibodies (mAbs) to eliminate leukemic cells provide a promising targeted approach for the treatment of AML. Gemtuzumab ozogamicin (Mylotarg®) is a toxin-conjugated monoclonal antibody which binds to CD33 on myeloid leukemia blasts and is approved for treatment of patients over 60 years old with relapsed AML. Its overall response rate is about 30% in clinical studies.57 Although these therapeutic strategies have made significant progress in the clinic in recent years, novel treatment approaches are still needed.14

Human CLL-1 (also known as MICT or CLEC12A), is a type II transmembrane glycoprotein and member of the large family of C-type lectin-like receptors involved in immune regulation. CLL-1 has previously been identified from myeloid derived cells by four different groups.3,4 The intracellular domain of CLL-1 contains an immunotyrosine-based inhibition motif (ITIM) and a YXXM motif. Phosphorylation of ITIM-containing receptors on a variety of cells results in inhibition of activation pathways through recruitment of protein tyrosine phosphatases SHP-1, SHP-2 and SHIP.12 The YXXM motif has a potential SH2 domain-binding site for the p85 subunit of PI-3 kinase,13 which has been implicated in cellular activation pathways, revealing a potential dual role of CLL-1 as an inhibitory and activating molecule on myeloid cells. Indeed, association of CLL-1 with SHP-1 and SHP-2 has been demonstrated experimentally in transfected and myeloid derived cell lines.

CLL-1 has a restricted expression pattern in hematopoietic cells, and is found in particular in myeloid cells derived from peripheral blood and bone marrow, as well as in the majority of AML blasts.8 Furthermore, a recent study indicated that CLL-1 is also present on the majority of leukemic stem cells in the CD34+/CD38- compartment in AML but absent on CD34+/CD38+ cells in normal and in regenerating bone marrow controls, which aids in discrimination between normal and leukemic stem cells.14

In the present study, we describe the generation of specific monoclonal antibodies against CLL-1 and used these mAbs to provide detailed expression analyses of normal and AML samples as well as functional studies of this target for potential immunotherapy in vitro and in vivo assays.

Design and Methods

Antibody generation

Hybridomas were generated using standard protocols.15 In brief, 6-week old Balb/c mice were immunized with 100 g of the purified recombinant protein in Freund’s adjuvant. After 5 biweekly boosts in incomplete Freund’s, titers were assessed and a fusion of spleen cells was performed 5 days after a last boost in saline. Sp2/0 cells were used as fusion partner. Hybridomas were selected and supernatants from the resulting clones were screened by ELISA and FACS. mAbs were purified using standard protein A columns (GE Healthcare, Piscataway, NJ, USA).

ELISA and FACS screen of hybridoma supernatants for binding to CLL-1

ELISA for binding to CLL-1 was performed using standard techniques.16 The secondary goat anti-mouse Ig-HRP antibody was from Bio-Rad (Hercules, CA, USA) (#170-6516) and TMB substrate from KPL (Gaithersburg, VA, USA) (#50-76-03). Plates were read on a Spectramax plate reader (Molecular Devices, Sunnyvale, CA, USA). Flow cytometry was performed using standard protocols.17 Secondary antibody was from BD Pharmingen (San Diego, CA, USA) (goat anti-mouse PE-conjugated, #550589) and. Cells were analyzed using an Automated Microsampler (Cytek, Fremont, CA, USA) attached to a FACScalibur (Becton Dickinson, San José, CA, USA).

Chimeric mAb generation

RNA was isolated from hybridoma fusion cells expressing the anti-IREM-1 mAb of interest. Using standard RACE/RT-PCR techniques, the heavy and light variable regions were cloned into two separate expression vectors in fusion with cDNA encoding for human IgG1 constant regions. The resulting plasmids were cotransfected into CHO cells and stable cell lines were selected secreting full-length chimeric mAbs.

Real-time binding analysis

Surface plasmon resonance was carried out on a Biacore (Piscataway, NJ, USA) system. mAbs were diluted to 2 µg/mL and then captured on the biosensor surface using an anti-mouse mAb. Antigen was diluted to a starting concentration of 46 nM and tested for binding to the mAb samples using a 3-fold dilution series. Each of 5 concentrations was tested twice except the highest concentration which was tested 5 times in total, two times with a short dissociation of 300 seconds and then 3 times with a dissociation of 60 minutes. The data sets from the long dissociation experiments were globally fit with the shorter association experiments to determine binding constants for the interactions. The analysis was carried out in HBS, pH 7.4 buffer at 25°C.18

Expression of CLL-1 by quantitative flow cytometry

QIFIKIT (K0078 from Dako) was used for the quantitative determination of receptor number per cell, according to manufacturer’s instructions. Before use,
cells were blocked for 10 min in 100 µl of 10% heat inactivated human serum.

**Cell lines and Western Blotting**

All cells were purchased from ATCC (Manassas, VA, USA) and maintained in their recommended medium. Lysates were prepared and 25 g of total lysate was analyzed by Western blotting. Secondary antibody (goat anti-mouse IgG-HRP, BioRad #170-6516) was used and membranes were developed using Pierce's (Rockford, IL, USA) ECL western blot substrate (#32209).

**Flow cytometry staining of normal human peripheral blood, bone marrow, from AML, MDS and ALL patient samples**

Clinical samples were obtained from patients diagnosed at the Cleveland Clinic and the Stanford Cancer Center after informed consent. For the expression of CLL-1 on patient samples or normal bone marrow aspirate, four color staining with CLL-1 Alexa488/CD34PE-CD45PerCP/CD38APC was performed. For normal blood specimen, Immunophenotyping was performed with CLL-1 Alexa488/CD14-16PE/CD45PerCP/CD38APC panel. IgG-Alexa488 was used as control for all of the above tests. Expression of CD33 was analyzed with CD33FITC/CD34PE-CD45PerCP/CD38APC panel and IgG-FITC was used as control. Detailed staining process was described previously.® Blasts were gated based on low side scatter versus CD45 dim expression. A sample was considered positive for CLL-1 or CD33 if the ratio of the geometric mean fluorescence intensity of stained sample and that of isoform IgG control (medium fluorescence intensity MFI) >2 and >20% of the cells expressed the antigen compared with the control sample. All antibody used for flow cytometry were from BD Biosciences except for CLL-1 Alexa488 and IgG Alexa488 which were self-prepared using Invitrogen’s Alexa Fluor 488 monoclonal antibody labeling kit according to the instructions.

**Tissue microarray staining of CLL-1**

Immunohistochemical staining of tissue microarray slide was performed using a BenchMark XT platform (Ventana Medical System, Inc., Tucson, AZ, USA), including on-site deparaffinization without antigen retrieval. Mouse monoclonal antibody of CLL-1 were diluted to 2.5 µg/ml and incubated on section for 1h. Detection was performed using Ultraview DAB kit (Ventana).

**CDC assay**

Fresh peripheral blood or bone marrow aspirate from AML patients was used for blast preparation via Ficoll-Paque gradient separation. For most AML cases, enriched blasts accounted more than 85% of total population based on flow cytometry assay. Cell lines were used at log phase of growth. 105 cells were suspended in 50 µL complete RPMI media and plated in a 96-well plate, 50 µL of 2× antibody/IgG isotype, made up in same medium was added to each well and the plates left at RT for 15 min. 2.5 µL of freshly prepared baby rabbit complement (CL3441; Cedarlane labs, Burlington, NC) was added to respective wells followed by incubation at 37°C for 1h. After equilibrating plates to RT, Cell viability was analyzed using Cell Titer Glo (G7571; Promega, Madison, WI, USA) and luminescence was measured via Victor 1420 Multilabel Counter (Perkin Elmer Life Science). Triple test was conducted for each group and data were normalized to complement + isotype.

**Antibody dependent cell cytotoxicity (ADCC)**

Effector cells

Human NK cells were isolated from buffy coat (purchased from Stanford blood center, Palo Alto, CA) by negative selection using Rosette Sep NK cell enrichment cocktail from Stem Cell Technologies, according to manufacturer’s instructions. Target cells: Specific lysis of target cells was determined by using a standard 4hr 51Cr release assay in a 96 well plate format as previously described.° Target: effector ratio of 1:40 was typically used. No pre-incubation step of effector cells and antibody was performed. % Lysis was calculated using the following standard equation:

\[
\frac{((\text{TEST}-\text{BGD})/(\text{Max}-\text{BGD})) \times 100}{\text{where TEST is sample release of } 51\text{Cr}, \text{BGD is spontaneous release and Max. is Triton X mediated release}}
\]

% Specific lysis has effector control subtracted

**Internalization by immunofluorescence microscopy and flow cytometry**

For immunofluorescence microscopy experiments, CLL-1 expressing HEK 293 cells were seeded onto 12-mm glass coverslips coated with poly-L-lysine (Sigma-Aldrich, St. Louis, MO, USA). The next day, the cells were preincubated in PBS-10% human serum (HS) for 15 min before incubation for 1 hour at 4°C with 10 µg/ml of Alexa-488-conjugated mAb 1075.7 or Alexa-488 control isotype antibody in PBS-2.5% HS. After three washes with ice-cold FACS buffer (PBS-1% BSA), cells were incubated at 37°C (in 5% CO2 and air) in PBS-5% FBS for up to 2 hours to allow internalization. Cells were then washed with ice-cold PBS, fixed with 4% paraformaldehyde and mounted onto slides with Prolong Gold antifade reagent with DAPI (Invitrogen, Carlsbad, CA). The stained specimens were examined using a Zeiss Axiovert 200 microscope with X40 oil objective (Zeiss, Thornwood, NY, USA). For flow cytometry experiments, HL-60 cells were preincubated with ice-cold PBS-10% HS, followed by incubation for 40 min at 4°C with saturating amounts of Alexa-488-conjugated mAb 1075.7, PE-labeled anti-CD44 antibody (negative control, clone 515, BD Biosciences, San Jose, CA) or their corresponding isotype control antibodies. After three washes in ice-cold FACS buffer, cells were resuspended in 100 µL of RPMI-10% PBS-PS and incubated either at 4°C (no internalization) or at 37°C for different time points. After washing, cell-surface-bound antibody complexes were stripped from the cells using 50 µL of Qiagen protease (QI, Qiagen #19155) at 5 AU/ml in cold PBS for 30 min at 4°C. Cells were...
washed three times with ice-cold FACS buffer with 0.02% NaN3, fixed in PBS-1% formaldehyde and analyzed by flow cytometry using the FACS Calibur system. The background mean fluorescence intensity (MFI) determined with cells incubated with the labeled isotype control antibodies was subtracted for each time point. The specific MFI at T0 in the absence of protease treatment was normalized to 100% and the relative specific MFI for each time point was calculated using the formula: \(100\% \times \frac{\text{specific MFI Ab} + \text{QP Tx}}{\text{specific MFI Ab} - \text{QP Tx}}\).

**HL-60 Xenograft**

The xenograft HL-60 model in BALB/c scid mice was carried out as described. The animal study protocols were reviewed and approved by the Institutional Animal Care and Use Committee according to governmental guidelines for animal welfare. All of the mice were acclimatized before use. Xenografts were allowed to establish to an average size of 50-100 mm3, after which mice were randomized into various conditional groups (10 animals per group). mAbs were given to each mouse at designated dose via intraperitoneal injection at a frequency of twice a week. Each mouse was measured for tumor size using a caliper on alternate days. Animal body weight and any sign of morbidity were also closely monitored. The mAb treatment lasted for two to three weeks at which point mice were euthanized, tumor xenografts were extirpated, weighed, and correlated with the tumor size measurement. Statistical analysis was done using a log-rank (Mantel-Cox) test.

**Results**

**Generation of mAbs against CLL-1**

A comprehensive expression analysis of putative cell surface proteins allowed us to identify several genes encoding for receptors that were differentially expressed in AML disease samples. By using this approach we identified a member of the C-type lectin-like receptor family, CLL-1, which showed a myeloid-restricted expression pattern. To further characterize the cell surface expression and function of CLL-1, we generated recombinant protein spanning the extra-cellular domain of CLL-1 and used it to immunize mice to generate mAbs. We generated ~500 hybridoma clones recognizing CLL-1 protein by ELISA and 275 of these mAbs recognize cell surface expression of CLL-1 on the myeloid cell line HL-60. To select lead mAbs we determined affinity, isotype and cytotoxic activity. Lead mAbs (21.16 and 1075.7) were selected and further characterized and subsequently used in detailed expression analysis and functional studies.

**Affinity of lead CLL-1 mAbs**

After confirming the binding of lead mAbs to CLL-1 on the surface of myeloid cells, the affinity of the mAbs for CLL-1 was measured using surface plasmon resonance. Anti-CLL-1 mAbs were captured using anti-mouse Fc Ab and recombinant extra-cellular domain of CLL-1 was allowed to bind to the mAb at various concentrations. The kinetic rate constants (ka and kd) of this reaction were determined and used to calculate the KD values of our lead mAbs, 21.16 (15.3 nM) and 1075.7 (0.36 nM).

**Expression of CLL-1 in myeloid cells**

We first surveyed the expression of CLL-1 in a wide variety of AML cell lines derived from AML patients of different AML subtypes. Various expression levels of CLL-1 were detected in myeloid cell lines by either flow cytometry or Western blotting analysis. Receptor number ranged between 0 and 100,000 CLL-1 molecules per cell as determined by quantitative flow cytometry (Online Supplementary Figure 1A). CLL-1 expression was not detected in cell lines from lymphoid origin (results not shown).

A comprehensive expression analysis of putative cell surface proteins allowed us to identify several genes encoding for receptors that were differentially expressed in AML disease samples. By using this approach we identified a member of the C-type lectin-like receptor family, CLL-1, which showed a myeloid-restricted expression pattern. To further characterize the cell surface expression and function of CLL-1, we generated recombinant protein spanning the extra-cellular domain of CLL-1 and used it to immunize mice to generate mAbs. We generated ~500 hybridoma clones recognizing CLL-1 protein by ELISA and 275 of these mAbs recognize cell surface expression of CLL-1 on the myeloid cell line HL-60. To select lead mAbs we determined affinity, isotype and cytotoxic activity. Lead mAbs (21.16 and 1075.7) were selected and further characterized and subsequently used in detailed expression analysis and functional studies.

After confirming the binding of lead mAbs to CLL-1 on the surface of myeloid cells, the affinity of the mAbs for CLL-1 was measured using surface plasmon resonance. Anti-CLL-1 mAbs were captured using anti-mouse Fc Ab and recombinant extra-cellular domain of CLL-1 was allowed to bind to the mAb at various concentrations. The kinetic rate constants (ka and kd) of this reaction were determined and used to calculate the KD values of our lead mAbs, 21.16 (15.3 nM) and 1075.7 (0.36 nM).

**Expression of CLL-1 in myeloid cells**

We first surveyed the expression of CLL-1 in a wide variety of AML cell lines derived from AML patients of different AML subtypes. Various expression levels of CLL-1 were detected in myeloid cell lines by either flow cytometry or Western blotting analysis. Receptor number ranged between 0 and 100,000 CLL-1 molecules per cell as determined by quantitative flow cytometry (Online Supplementary Figure 1A). CLL-1 expression was not detected in cell lines from lymphoid origin (results not shown).

It was previously reported that CLL-1 is expressed on the cell surface of myeloid lineages(9). We used our specific CLL-1 mAbs to further analyze the expression of CLL-1 in peripheral blood leukocytes and bone marrow and extended expression analysis in AML patient samples (Online Supplementary Figure 1B). AML samples from the peripheral blood and bone marrow of 52 AML patients resembling a variety of AML subtypes were obtained and analyzed (summarized in Table 1). CLL-1 expression was detected in 86.5% of AML cases (45 of 52) with a 23-99% (mean 69.6%) range of positive blasts, and a 100% incidence in the myelomonocytic and monocytic AML subtype (21 of 21). Immunohistochemical CLL-1 staining of bone marrow clots in a tissue microarray AML panel showed positive staining in 37 out of 38 cases (Online Supplementary Figure 1C). In contrast, CLL-1 expression was not detected in blasts from ALL patients (n=5, data not shown). Next, we analyzed CLL-1 expression in the CD34+/CD38- stem cell compartment. Sufficient events of CD34+/CD38- leukemia stem cells were collected from 22 AML cases and CLL-1 was detected on stem cells in 12 of these cases (54.5%, summarized in Online Supplementary Figure 1D). CD34+ progenitor cells from negative lymphoma staging bone marrow samples (n=8) showed 2 cases expressed CLL-1 (23% and 59% positive cells). We also tested CD34+/CD38+ bone marrow derived stem cells from 5 morphological normal samples and one case had > 20% cells positive for CLL-1.

Additionally, we surveyed CLL-1 expression in either bone marrow aspirate or peripheral blood from 13 myelodysplastic syndrome patients and blasts of 6 cases were positive (46.2%) with a 21-85% (mean 49.3%) range of positive blasts (data not shown). Among the 13 MDS cases, one refractory anemia and all five RAEB-1 cases were negative for CLL-1 expression, while 2 of 3 RAEB-2 cases were positive, one MDS/MPD, one therapy related MDS, one MDS 5q(-) syndrome and one MDS unclassifiable case were all positive. CD34+/CD38- cells from 2 MDS patients (therapy related MDS and MDS 5q- syndrome) were negative for CLL-1.

We next compared the expression pattern of CLL-1 with the myeloid antigen CD33. As previously reported, monocytes expressed the highest levels of CD33,
while lower expression was observed in granulocytes, and no expression could be detected in lymphocytes regardless whether the specimens were obtained from healthy controls or AML patients.\(^2\) CLL-1 had a similar expression pattern as CD33 in normal fresh peripheral blood cells (Online Supplementary Figure 2A). Similar to CLL-1, CD33 was detected in AML blasts of 44/47 (93.6%) cases tested in this study, while in the CD34\(^+\)/CD38\(^–\) cell population of the five cases tested, 2 and 3 cases exhibited > 20% cells positive for CD33 and CLL-1 expression, respectively (Online Supplementary Figure 2B).

These studies confirm the restricted expression of CLL-1 in cells from myeloid origin, in the majority of AML blasts and in a subset of CD34\(^+\)/CD38\(^–\) leukemia stem cells, highlighting the potential of CLL-1 as a target for antibody-based therapeutics in the treatment of myeloid leukemia.

Specific CDC activity of mAbs against CLL-1

To assess the therapeutic potential of anti-CLL-1 mAbs we measured their cytotoxic activity. We first characterized CDC activity against the AML derived cell line OCI-AML-5 which expresses CLL-1 as seen by Western blotting and flow cytometry analysis. Lead mAbs against CLL-1 displayed a dose-dependent CDC activity and eliminated over 80% of target cells at doses of 100 ng/mL and higher (results not shown). To determine whether the cytotoxic activity detected with anti-CLL-1 mAbs was mediated through a direct interaction with CLL-1 on the cell membrane, CDC assays were performed against a surrogate cell line. The CLL-1 negative HEK293 cell line was transfected and stably expressing CLL-1. CDC activity with CLL-1 mAb was detected against the HEK293-CLL-1 cells but not against the HEK293 parental line (Figure 1A), indicating that the CDC activity is mediated specifically through CLL-1. In addition, the Burkitt lymphoma B cell line CA46, which does not express CLL-1 was insensitive to cytotoxicity induced by anti-CLL-1 mAbs (Figure 1A).

To further explore the therapeutic potential of CLL-1 mAbs, we performed CDC assays on fresh AML blasts obtained from AML patients. In these ex vivo experiments, the cytotoxic activity of the mouse monoclonal antibody against human CLL-1 was observed in a dose-dependent manner against 15 of 16 (94%) CLL-1 positive samples tested, with lysed cells ranging between 25 and 85%. In contrast, ALL blasts lacking CLL-1 were insensitive to CDC mediated activity (data not shown). To further advance lead CLL-1 mAbs for AML therapeutics, two chimeric monoclonal antibody constructs were generated by fusing the 1075.7 and 21.16 variable sequences to human IgG1 constant region sequences.

| Table 1. CLL-1 expression in blasts of AML, MDS and ALL. |
|-----------------|-----------------|-----------------|
| **AML subtype** | **Positive sample (n)** |
| AML with CBFβ/MHY11 | 3/3 |
| AML with PML/RARα | 1/2 |
| AML with 11q23 abnormality | 2/2 |
| AML with multilineage dysplasia | 4/4 |
| AML minimally differentiated | 1/1 |
| AML without maturation | 8/11 |
| AML with maturation | 6/8 |
| Acute myelomonocytic leukemia | 6/6 |
| Acute monocytic leukemia | 15/15 |
| MDS | 6/13 |
| ALL | 0/5 |

Figure 1. Complement-dependent cytotoxicity (CDC) and Antibody-dependent cellular cytotoxicity (ADCC) activity of CLL-1 mAbs. (A) CDC assay using Human Embryonic Kidney 293 cells stably expressing CLL-1 compared to wild-type HEK293 cells and lymphoma CA46 cells. Expression of CLL-1 on 293 parental and transfectants is shown in the histograms in the top left corner (B) Ex vivo CDC activity of chimeric mAb 1075.7 against freshly isolated AML blasts. (C) Antibody-Dependent Cellular Cytotoxicity (ADCC) activity of anti-CLL-1 chimeric mab 1075.7 against HL-60 cells.
Recombinant chimeric mAbs were expressed in and purified from CHO cells and the activities were compared between the mouse and chimeric mAbs. Similarly to their mouse counterparts, both chimeric mAbs were capable of mediating dose-dependent CDC killing activity against freshly isolated AML blasts, as tested in a total of 5 samples expressing CLL-1. The EC50s from a representative experiment with chimeric mAbs 1075.7 and 21.16 were 158.5 ng/ml and 2056 ng/ml, respectively (Figure 1B).

**ADCC activity of CLL-1 mAb**

Antibody-dependent cellular cytotoxicity (ADCC) is an important mechanism of cancer cell cytotoxicity in vivo. To evaluate the ability of anti-CLL-1 chimeric antibody to elicit ADCC activity, HL-60 cells were labeled with 51Cr and incubated with freshly isolated human NK cells in the presence of chimeric mAb. A dose-dependent ADCC-mediated lysis was observed when using chimeric mAb 1075.7, whereas an isotype control mAb had no detectable activity (Figure 1C). Nearly 50% of cells were lysed with an EC50 of 20 ng/mL. These results indicate that CLL-1 mAbs are potent and selective cytotoxic agents in vitro and in vivo against CLL-1 expressing cells and exhibit potential as targeted anticancer agents, possibly through a combination of multiple mechanisms.

**Internalization of CLL-1**

While the CLL-1 chimeric mAbs were effective in cytotoxic assays, we also considered a toxin conjugation approach to inflict cell death upon antigen-mediated toxin uptake. To investigate the potential for a CLL-1 antibody-drug conjugate we first determined whether CLL-1 mAbs trigger receptor internalization. HEK293 cells stably expressing CLL-1 were stained with fluorescently labeled mouse mAb 1075.7Ab, incubated for the indicated times at 37°C, fixed and examined using fluorescence microscopy. Similarly to their mouse counterparts, both chimeric mAbs were capable of mediating ADCC killing activity against freshly isolated AML blasts, as tested in a total of 5 samples expressing CLL-1. The EC50s from a representative experiment with chimeric mAbs 1075.7 and 21.16 were 158.5 ng/ml and 2056 ng/ml, respectively (Figure 1B).

**ADCC activity of CLL-1 mAb**

Antibody-dependent cellular cytotoxicity (ADCC) is an important mechanism of cancer cell cytotoxicity in vivo. To evaluate the ability of anti-CLL-1 chimeric antibody to elicit ADCC activity, HL-60 cells were labeled with 51Cr and incubated with freshly isolated human NK cells in the presence of chimeric mAb. A dose-dependent ADCC-mediated lysis was observed when using chimeric mAb 1075.7, whereas an isotype control mAb had no detectable activity (Figure 1C). Nearly 50% of cells were lysed with an EC50 of 20 ng/mL. These results indicate that CLL-1 mAbs are potent and selective cytotoxic agents in vitro and in vivo against CLL-1 expressing cells and exhibit potential as targeted anticancer agents, possibly through a combination of multiple mechanisms.

**Internalization of CLL-1**

While the CLL-1 chimeric mAbs were effective in
2B and results not shown). These data confirm the internalization of CLL-1 upon engagement with anti-CLL-1 mAbs and the potential of designing an antibody-drug conjugate for this target.

**Anti-tumor activity of CLL-1 mAb in mouse xenograft model**

To further evaluate the potential in vivo anti-tumor effect of the 1075.7 chimeric anti-CLL-1 mAb was tested in immunodeficient mice bearing HL-60 xenografts. HL-60 cells were inoculated into the flank of SCID mice and treated with chimeric mAb 1075.7 after tumors had established. These rapidly growing tumors were measured bi-weekly and a dose-dependent delay in tumor growth was observed in mice treated with chimeric CLL-1 mAb compared to mice treated with isotype control chimeric mAb (Figure 3A). Tumor growth delay was calculated using the end point set at 2000 mm$^3$. The time to endpoint in days (TTE) for control chimeric human IgG1 (5.0 mg/kg) was 15.7 days while that of chimeric 1075.7 significantly increased to 20.2 days ($p<0.05$). At a dose of 0.5 mg/kg, chimeric 1075.7 also demonstrated a detectable delay of TTE (17.5 days). In terms of tumor growth delay (TGD) as percentage compared to the saline-treated control group, chimeric 1075.7 treated group delayed tumor growth by 38% and 19.1% at doses of 5 and 0.5 mg/kg, respectively (Figure 3B). These data validate anti-CLL-1 mAb1075.7 as an inhibitor of myeloid leukemia cell growth in vivo.

**Discussion**

Selective expression of a cell surface antigen on target cells provides opportunity for antibody based therapy both for leukemia and solid tumors. Here, we identified CLL-1 as a potential receptor expressed on normal myeloid and leukemia blasts. We generated a series of mAbs against CLL-1 and selected two lead mAbs with high affinity and cytotoxic activity. Flow cytometry and western blot analysis of CLL-1 in normal and patient samples revealed an expression pattern restricted to cells from myeloid origin. We generated murine and chimeric mAbs against CLL-1, demonstrated their specificity and evaluated their anti-cancer activity against AML cell lines and primary blasts in various in vitro and ex/in vivo models.

Consistent with previous previously published work, we confirmed that CLL-1 is expressed on the cell surface of the majority of malignant AML blasts (45/52, 86.5%) as well as in the majority of the CD34+/CD38- leukemia stem cells (12/22, 54.5%). Based on our flow cytometry assays, there is a high incidence of CLL-1 expression in more mature acute myelomonocytic/monocytic leukemia (21/21, 100%, Table 1). The immunohistochemical staining of bone marrow clots in a tissue microarray AML panel further demonstrated that CLL-1 is an abundant cell surface protein in AML blasts (37/38, 97.3%). Our preliminary studies also indicated that CLL-1 is expressed in blasts of a subset of MDS cases (6/13, 46.2%), a disease with few effective treatment options, while 6 MDS cases of RA and RAEB-1 (<9% blasts) were all negative for CLL-1. Further studies are needed to address whether CLL-1 has a different expression pattern in lower risk MDS (<5% marrow blasts) compared to higher risk MDS and whether its expression changes with the progression of MDS to AML. A further analysis of a large number of MDS samples will be required to fully elucidate the CLL-1 expression levels in MDS stem cells.

The expression profile of CLL-1 as presented here and in previous reports, highlight the potential of CLL-1 as a target for antibody-mediated therapy against AML blasts and possibly CD34+/CD38- leukemic stem cells. We demonstrate for the first time direct cytotoxic and anti-cancer activity of anti-CLL-1 mAbs in functional studies using in vitro, ex vivo and in vivo models. Therefore, CLL-1 has potential as a novel therapeutic target for antibody-mediated immunotherapy.

Multiple mechanisms, including CDC and ADCC, may be involved in antibody-mediated targeted immunotherapy in vivo. One report described the density of CD33 as a limiting factor prohibiting significant induction of ADCC and CDC activity. While anti-CD33 induced cytotoxicity has been demonstrated, our novel chimeric anti-CLL-1 mAbs described here appear capable of mediating dose-dependent ADCC and CDC activity towards blasts freshly isolated from all five AML cases tested. Our mouse and chimeric CLL-1 mAbs have shown CDC activity in 15 of 16 (94%) AML blasts derived from patients classified by various AML subtypes.

Interaction of antigen-antibody may cause altered downstream signaling and induce target cells to undergo apoptosis. This has been described for rituximab and anti-CD33 mAbs as an alternative mechanism contributing to the efficacy of antibody-mediated therapy. Although anti-CLL-1 mediated CDC and ADCC activity were reported here, it is currently unclear whether engagement of CLL-1 with antibody could alter cell signaling that may further contribute to anti-cancer activity against malignant blasts. Since the intracellular domain of CLL-1 contains an immunotyrosine-based inhibition motif (ITIM) and a YXXM motif, it is reasonable to assume that CLL-1 could serve as an inhibitory signaling molecule. The impact of CLL-1/antibody binding and consequent signaling alteration in AML blasts remains to be addressed.

In addition to CDC, ADCC and other potential direct cytotoxic activities, conjugation of CLL-1 mAbs to a toxin or drug may represent an alternative strategy for therapeutic intervention in AML. We observed internalization within 30 minutes of exposure of cells to CLL-1 mAbs. Intracellular signaling motifs in CLL-1 include an ITIM, which has been identified as required for internalization of CD33 and a YXXM motif, which has been shown to be involved in lysosomal targeting of receptors. Therefore, these motifs could be important for the internalization of CLL-1 we have observed in AML cells.

In the in vivo xenograft model described here, we treated established HL-60 tumors with anti-CLL-1 chimeric mAbs and observed a significant tumor growth delay of up to 38% compared to control treat-
ments. Further work is needed to fully understand the potential of anti-leukemic mAbs against CLL-1. Other animal models using cells with higher CLL-1 levels may better represent patients with (myelo)monocytic leukemia and lead to better efficacy of anti-CLL-1 mAbs. Combination treatment with chemotherapeutic agents or a toxin-conjugated mAb could also enhance the anti-cancer activity. Taken together, we have shown that the CLL-1 receptor is expressed in AML blasts and majority of leukemia stem cells and have selected CLL-1 cytotoxic mAbs that potentially could be developed for AML therapeutics.

References


Authorship and Disclosures

XZ, SS, CP, JZ and WK performed experiments; XZ, EDH, AA and WK designed study and wrote and reviewed the manuscript; XZ and EDH are consultants for Nuvelo, Inc. SS, CP, JZ, AA and WK are employed by Nuvelo, Inc.

The authors reported no potential conflicts of interest.