

Characterization of Two Novel mAbs Recognizing Different Epitopes on CD43

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JL1, a specific epitope on CD43, is a potential biomarker for the diagnosis of acute leukemia. Although qualitative assays for detecting leukemia-specific CD43 exist, there is a need to develop quantitative assays for the same. Here, we developed two novel monoclonal antibodies (mAbs), 2C8 and 8E10, recognizing different epitopes on CD43. These clones are capable of pairing with YG5, another mAb against JL1 epitope, because they were selectively obtained using sandwich ELISA. Antigens recognized by 2C8 and 8E10 were confirmed as CD43 by western blotting using the CD43-hFC recombinant protein. When expression on various leukemic cell lines was investigated, 2C8 and 8E10 displayed a disparity in the distribution of the epitope. Enzyme assays revealed that these mAbs recognized a sialic acid-dependent epitope on CD43. Using normal thymus and lymph node paraffin-embedded tissues, we confirmed a difference in the epitopes recognized by the two mAbs that was predicted based on the maturity of the cells in the tissue. In summary, we developed and characterized two mAbs, 2C8 and 8E10, which can be used with YG5 in a sandwich ELISA for detecting leukemia-specific CD43.

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INTRODUCTION

CD43 is a major sialoglycoprotein found on human leukocytes, is a member of the Mucin family of glycoproteins, is glycosylated with sugar chains that consist of O-glycan and sialic acid (1,2), and it has a molecular weight of 120~130 kDa. Due to the sialic acid present at the end of O-glycan chains, CD43 is also called leucosialin or sialophorin, and is mainly expressed in T cells and myeloid cells, and in some cases, on the membrane and in the cytoplasm of B cells (1-3). Most lymphocytes express CD43, a molecule known to control the activity and movement of white blood cells (1,5,6). The frequent use of various antibodies against CD43 has already demonstrated its function in aggregating blood cells (7), and has revealed its role in the activity and proliferation of blood cells (4,6). The CD43 epitope has been researched extensively in studies using various leukemia cell lines. The antibodies against CD43 developed so far are known to recognize an epitope that is influenced by sialic acid, as evidenced by assays utilizing neuraminidase. In addition, all the antibodies against CD43 have also been shown to recognize a glycoprotein epitope, since the binding is disturbed when a glycoprotease from *Pasteurella haemolytica* is used (1).

The CD43 epitope can be partially exposed or hidden during blood cell differentiation. JL1, one such CD43 epitope, has a specific distribution across stages of blood cell diffe-

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Abbreviations: ELISA, enzyme-linked immunosorbent assay; mAb, monoclonal antibody; ALL, acute lymphoblastic leukemia; AML, acute myeloid lymphoma; CHO, chinese hamster ovary

rentiation. It is expressed in cells at multiple stages, including at the early stage of blood cell differentiation (lymphoid precursor cells) and at a later stage of differentiation of myeloid lineage cells (myelomonocytic-lineage cells) (8,9). Hematopoietic pluripotent stem cells do not express JL1 (8,10). In other words, JL1 is not expressed on the stem cells, is transiently expressed in the mid-stage differentiated blood cells, and it shows restricted expression in the peripheral blood cells (11).

YG5 is a mAb against JL1 of IgG isotype that recognizes the CD43 antigen (9). It has been reported that YG5 shows immunoreactivity in 75% of acute lymphoblastic leukemia (ALL) patients (>92% in T-ALL patients) (9). YG5 demonstrates positive reactivity in T and B cell lines as well as in myeloid leukemia cell lines but is negative in the normal peripheral blood (12). Diagnosis of leukemia using YG5 has shown 87% accuracy in approximately 200 leukemia patients, including ALL and acute myeloid lymphoma (AML), and this diagnostic rate is higher than the leukemia diagnosis rate (76%) for diagnosis using other antibody against CD43 (13). In addition, YG5 can be internalized into cell cytoplasm after its binding to the antigen, thus having a potential to be used in the immunotherapy against leukemia by conjugating cytotoxic compounds to the antibody and enhancing the cytotoxicity (13). Therefore, YG5 has been suggested as a promising candidate for therapy and diagnosis of AML and ALL (11-13).

Currently, antibody-antigen interaction-based assays are commonly used for the diagnosis of leukemia and other cancers (14,15). The diagnosis of diseases using immunoreactivity can confirm abnormal protein expression, in addition to providing morphological information that potentiates more accurate and objective diagnosis of the disease (14). Enzyme-linked immunosorbent assay (ELISA) is one method that utilizes the antibody-antigen reaction that is simple to perform, and is relatively low cost. With the advantage of being capable of analyzing multiple samples in a single test, it is the preferred method for comparative analysis (16,17). This method is often used when the amount of antigen is limited, due to its high sensitivity that can enable the quantification of the sample even in low concentrations (16,17). However, the sandwich ELISA method requires two antibodies recognizing two different epitope sites on the same antigen (16). In this study, YG5 was used to screen for antibodies that recognize different epitope sites on CD43 for the purpose of developing an immunodiagnostic assay for detecting leukemia-specific CD43. The results yielded two mAb clones, 2C8 and 8E10,

which can be effectively used as pairing antibody in a sandwich ELISA assay with YG5.

MATERIALS AND METHODS

Cells and reagents

The leukemic cell lines CEM7, Jurkat, IM9, Ramos, Raji, Daudi, HL60, Kasumi, K562, Reh, THP-1, and KG1 were obtained from American Type Culture Collection. The leukemic cell lines were maintained in Roswell Park Memorial Institute medium (RPMI) that was supplemented with 10% fetal bovine serum (FBS). Human paraffin tissues were collected from the Department of Pathology, Chungbuk National University Hospital.

Generation of hybridomas

Six-week-old Balb/c mice were immunized with 10^7 CEM7 cells at 4-2-2 week intervals for 2 months. Test bleed was performed in CEM7 by flow cytometry. We induced cell fusion of splenocytes from immunized mice by using a mouse myeloma cell line, SP2/0. The hybrid cells were selected using the HAT medium.

Selection of mAbs using Sandwich ELISA

To select for a mAb that can be paired with YG5, the culture supernatants of hybrid cells were harvested and tested using indirect sandwich ELISA. Anti-JL1 chimeric antibody was used as the capture antibody, and the CEM7 cell lysate was used as an antigen. The culture supernatants from hybrid cells and anti-mouse F_c specific antibody were used as first and second detector antibodies. The selected hybrid clones were named 2C8, 8E10, and 8B6. Three clones were subcloned by limiting dilution, and the culture supernatants of the clones were tested for antibody production. From these, the final hybridoma clones, 2C8-2-6 and 8E10-1-23, were selected.

Production of CD43-hFC recombinant protein

Total RNA was purified from the CEM7 cells using the Easy-BLUE RNA Extraction kit (iNtRON Biotechnology, Daejeon, Korea). Primers were designed using the sequence of known human CD43 extracellular region. The sequence was amplified using sense primer (5'-GGT ACC AAG CTT AGC ACA ACA GCA GTG CAG-3') and antisense primer (5'-AGA TCT GAT ATC TCG TGA GTT CTC ATC TGG-3'), and *Hind*III and *Eco*RV restriction enzyme sites were introduced (underlined). The amplicons were purified using QIAquick gel extraction

kit (Qiagen, USA), and confirmed by sequencing. The DNA products were digested using *Hind*III and *Eco*RV and ligated into the human pSec Tag expression vector (Invitrogen, USA), which contains the human Fc fragment. The vector was transfected into Chinese hamster ovary (CHO) cells using Effectene transfection reagent. After 3 days, the culture supernatant was tested by both conventional and sandwich ELISA assays.

Western blot analysis

To analyze the antigens recognized by 2C8 and 8E10 mAbs, purified CD43-hFc recombinant protein was separated on an 8% SDS-PAGE gel, and the protein was transferred to a nylon membrane. The membranes were blocked in 5% skimmed milk and incubated with YG5, 2C8, 8E10, or DFT-1 mAb at 4°C overnight. The membrane was washed 3 times with PBST and incubated with peroxidase-conjugated secondary antibody for 1 h. After washing in PBST, the bands were visualized via enhanced chemiluminescence.

Flow cytometry

Various leukemic cell lines were incubated with the purified YG5, 2C8, 8E10, and DFT-1 mAbs at 4°C for 30 min. Thereafter, the cell lines were washed with PBS and incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody at 4°C for 20 min. After washing, the cells were fixed with 1% paraformaldehyde, and analyzed by flow cytometry (FACScalibur, BD, USA).

Neuraminidase treatment

K562 cells in the concentration of 3×10^6 were harvested and washed with PBS. Cells were treated with neuraminidase

(0.08 U in 200 μ l total volume) and incubated at 37°C for 30 min, and washed with PBS after the incubation. Following the wash, the cells were stained with YG5, 2C8, 8E10, and DFT-1, and analyzed using a flow cytometer.

Immunohistochemistry

After the antigen retrieval step, human paraffin-embedded tissues of thymus and lymph node were incubated with YG5, 2C8, 8E10, and DFT-1 at 4°C overnight. Tissues were washed with TBS and incubated with biotinylated anti-mouse immunoglobulin at room temperature for 10 min. Following the washes, the tissues were incubated with streptavidin-horse-radish peroxidase at room temperature for 10 min. The reaction was visualized using 3'-diaminobenzidine.

RESULTS

Development of 2C8 and 8E10 mAbs

About 1000 hybridomas were developed after immunization of Balb/c mice with 10^7 CEM7 leukemic cell line. 16 high expression clones were selected at the fusion screening. We selectively obtained clones that paired with YG5 using sandwich ELISA by using YG5 as a pairing antibody. On the first screening, clones 2C8, 8E10, and 8B6 were selected (Fig. 1A). Subsequently, the final clones 2C8-2-6 and 8E10-1-23 (designated hence forth as 2C8 and 8E10, respectively) were established by two consecutive limiting dilutions (Fig. 1B). These two antibodies were deemed suitable for use as a detector antibody in a sandwich ELISA, when YG5 was used as the capture antibody.

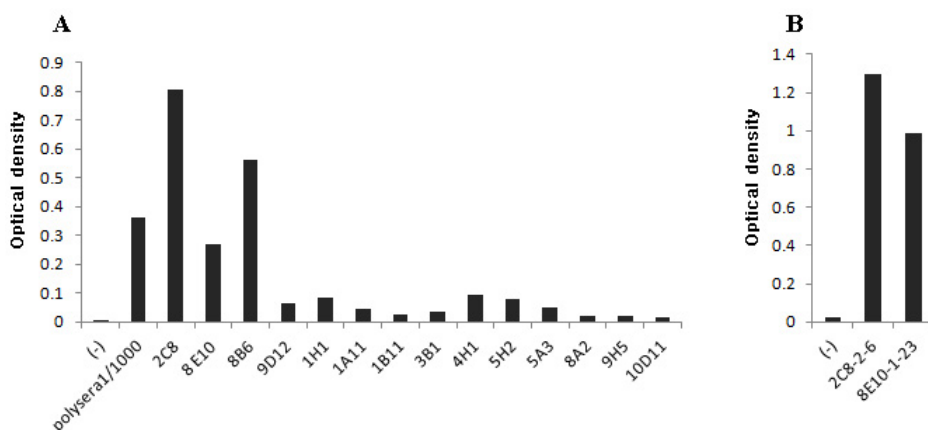


Figure 1. Selection of YG5 pairing mAb to detect JL1 by sandwich ELISA. Sandwich ELISA was used to screen for clones, 2C8, 8E10, and 8B6, which resulted in a high OD (A). The final clones, 2C8-2-6 and 8E10-1-23, were selected in an experiment by limiting dilution (B). Both antibodies were suitable for use as a detector antibody in sandwich ELISA, when YG5 was used as the capture antibody.

Generation of recombinant CD43-hFC protein

In order to identify the antigen recognized by 2C8 and 8E10, CD43-hFC recombinant protein was generated by amplifying the DNA fragment of human CD43 extracellular region, and ligating the construct into pSec Tag expression vector (Invitrogen, USA), which contains the human F_c fragment (Fig. 2A). This vector was then transfected into CHO cells, and the protein levels in the media were confirmed by sandwich ELISA using anti-human F_c antibody as the capture and detection antibody, 3 days after the start of the culture (Fig. 2B). Purified CD43-hFC recombinant protein was also detected by conventional ELISA, where YG5 was used as the detector antibody (Fig. 2C).

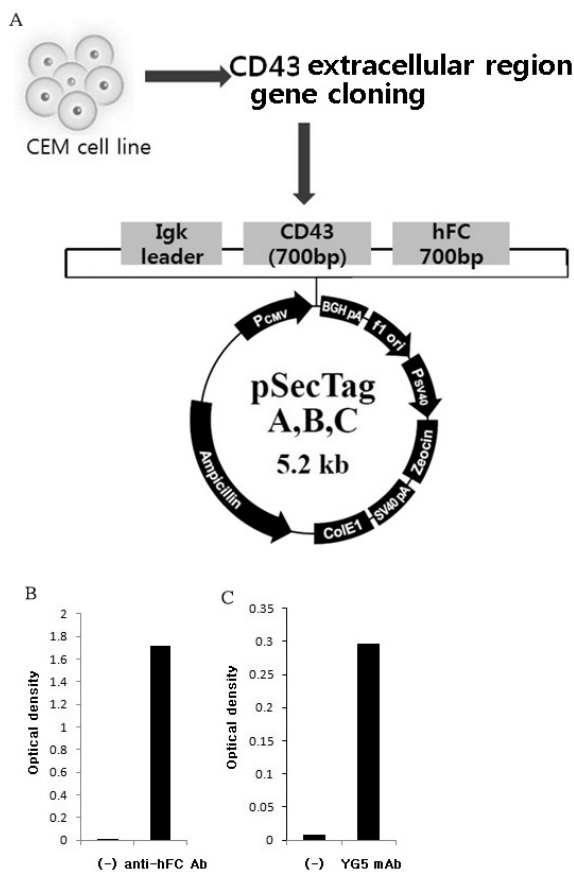


Figure 2. Generation of recombinant CD43-hFC protein. To identify the antigen recognized by 2C8 and 8E10, CD43-hFC recombinant protein was generated. Schematic representation of gene cloning strategy (A). Protein levels confirmed by sandwich ELISA using anti-human F_c antibody as the capture and detector antibody (B). Purified CD43-hFC recombinant protein was detected by conventional ELISA, when YG5 was used as the detector antibody (C).

Identification of antigen recognized by 2C8 and 8E10 by western blot analysis

To study the antigen recognized by 2C8 and 8E10, purified CD43-hFC recombinant protein was analyzed by western blot. YG5, 2C8, 8E10, and DFT-1 mAbs revealed a single band at approximately 150 kDa when run under non-reducing conditions, supporting that 2C8 and 8E10 recognize the CD43 antigen (Fig. 3). CD43-hFC recombinant protein was detected at 50~60 kDa by YG5, 2C8, and DFT-1 mAbs, except 8E10 mAb under reducing conditions. Therefore, 8E10 mAb recognized a conformational epitope in contrast to 2C8 mAb.

2C8 and 8E10 recognize two different epitope sites on CD43

In order to profile the expression pattern of CD43 epitopes recognized by 2C8 or 8E10, flow cytometric analyses were performed using various leukemic cell lines and normal peripheral blood cells (Table I). The cell lines CEM, Jurkat, IM9, and K562 showed strong labeling with both 2C8 and 8E10, while the extent of immunoreactivity was different for Reh and Kasumi cells among the two mAbs (Table I).

The treatment of cells with neuraminidase can cleave sialylated carbohydrate moieties. To further characterize the dependency of binding of these two mAbs in the presence or absence of sialic acid, K562 cells were treated with neuraminidase to identify the epitope specificity. Upon treatment with neuraminidase, 2C8, 8E10, and DFT-1 mAbs showed reduced immunoreactivity (Fig. 4). On the other hand, the YG5 demonstrated enhanced immunoreactivity. These results sug-

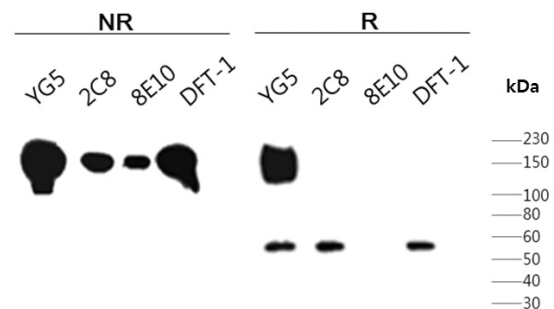


Figure 3. Identification of antigen recognized by 2C8 and 8E10 by western blot analysis. CD43-hFC recombinant protein was separated on 8% SDS-PAGE gel and detected using YG5, 2C8, 8E10, and DFT-1 mAbs at approximately 150 kDa under non-reducing conditions. CD43-hFC recombinant protein was detected at 50~60 kDa by all the anti-CD43 mAbs except 8E10 mAb under reducing conditions. NR: non reducing condition R: reducing condition.

Table I. Immunoreactivity of four different anti-CD43 mAbs on leukemic cell lines and normal peripheral blood cells

Cell line	Origin	YG5	2C8	8E10	DFT-1
		(% gated)			
CEM7	T cell	++++	++++	++++	++++
Jurkat	T cell	++++	++++	++	++++
IM9	B cell	++++	++++	++++	++++
Ramos	B cell	+	—	—	+++
Raji	B cell	—	++	++	+
Daudi	B cell	—	—	—	—
HL60	Myeloblast	+++	+	+	++++
Kasumi	Promyeloblast	—	++	+++	++++
K562	Lymphoblast (CML)	—	++++	++++	++++
Reh	Lymphoblast (non B, non T)	—	—	+	++++
THP-1	Monocyte (AML)	++	+++	+++	++++
KG1	Myeloblast (AML)	—	++	++	++++
Normal bonemarrow	Bone marrow	+	—	++	++++
PBL		—	++++	++++	++++

(% gated = —; <10%, +; 10~25%, ++; 25~50%, +++; 50~75%, ++++; 75~100%)

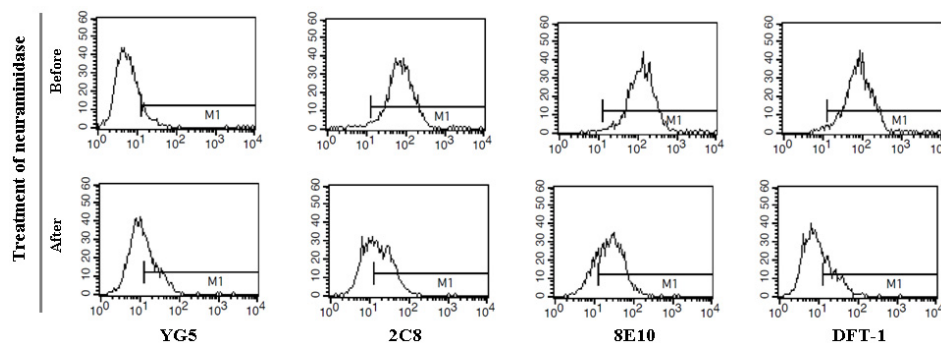


Figure 4. Characterization of CD43 epitopes recognized by anti-CD43 mAbs. Treatment with neuraminidase reveals epitopes that are sensitive to the treatment. The epitope recognized by YG5 mAb was neuraminidase-independent, but the epitopes recognized by 2C8, 8E10, and DFT-1 mAbs were neuraminidase-sensitive.

gest that 2C8 and 8E10 recognize a neuraminidase-sensitive epitope, unlike YG5.

To demonstrate the tissue distribution pattern of antigens recognized by YG5, 2C8, 8E10, and DFT-1, immunohistochemical staining was performed on human thymus and lymph node tissues (Fig. 5). YG5 labeled the cortex of thymus strongly, where many immature T cells are present, whereas no immunoreactivity was detected in the lymph node. On the contrary, the medulla of thymus and the lymph node, where mature T cells reside, were strongly labeled with 8E10 and DFT-1. 2C8 displayed weak immunoreactivity in the cortex of the thymus and the lymph node.

DISCUSSION

To establish a leukemia-specific CD43 sandwich ELISA assay, a YG5 pairing antibody needed to be developed. The objective of the current study was to develop a mAb that can recognize a new epitope on CD43 to establish a sandwich ELISA method with YG5 that can be used in the detection of leukemia-specific CD43. As result, two mAbs, 2C8 and 8E10, were developed and their characteristics were analyzed.

To develop an antibody for a specific epitope, it is important to begin with establishing an effective strategy. For the screening strategy at the antibody development step, sandwich ELISA method was employed, where YG5 was used to coat the bottom of the ELISA plate. This strategy is highly effective since it enables the confirmation of antibody pairing

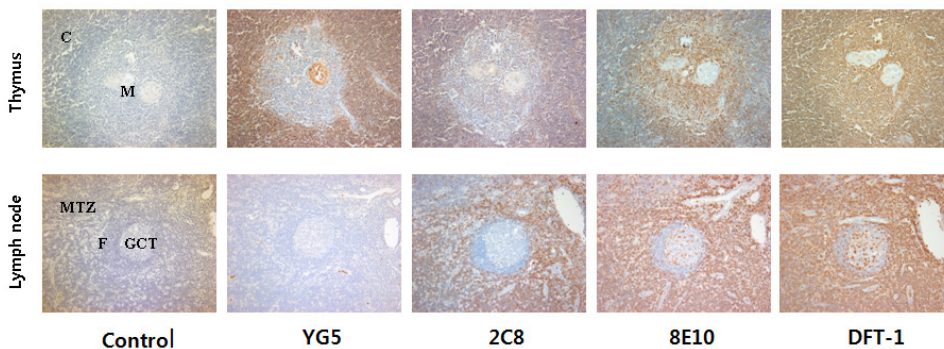


Figure 5. The tissue distribution pattern of antigens recognized by anti-CD43 mAbs. YG5 showed strong immunoreactivity in the cortex of thymus, but lymph node remained unstained. 8E10 and DFT-1 displayed strong immunoreactivity on the mature T cell zone of the lymph node. In contrast, 2C8 displayed weak immunoreactivity in the cortex of thymus and the lymph node ($\times 200$). C: cortex, M: medulla, MTZ: mature T cell zone, F: follicle, GCT: germinal center of T cell.

in the sandwich ELISA from the start of screening stage for the positive hybridoma clones.

Antibodies developed by this strategy were expected to recognize a new epitope on CD43 and to be used as detection antibodies in the CD43 sandwich ELISA assay. As expected, we were able to confirm using CD43-hFC that the epitope recognized by 2C8 and 8E10, which showed strong positive response in the sandwich ELISA, was CD43. The DNA sequence for CD43-hFC recombinant protein was acquired from CD43 DNA that has been obtained from the RNA of CEM leukemic cell line, and was developed by cloning into a plasmid vector containing hFc. In addition, a stable cell line producing CD-43-hFC antigen was also established. It is crucial that the two mAbs targeting the antigen used to establish sandwich ELISA be different in the epitope site recognized by them. The two mAbs developed in this study displayed differential expression among various leukemic cell lines, also supporting that they differ in the antigen recognition site.

To determine the characteristics of the epitope recognized by the two mAbs, the immunoreactivity was verified by flow cytometry using immunofluorescent labeling of cells that are treated for elimination of sialic acid residues. Results revealed that the two mAbs recognizing the epitope sites are sensitive to the elimination of sialic acid. In comparison, YG5 showed no sensitivity to the elimination of sialic acid residues. Using paraffin embedded thymus and lymph node sections from normal patients, we also confirmed that there is a difference in the sites recognized by the two mAbs, which was predicted according to the maturity of the cells in the tissue. The medulla of thymus and the lymph node, where mature T cells reside, were strongly labeled with 8E10. 2C8 displayed weak immunoreactivity in the cortex of thymus and the mature T cell zone of lymph node. In contrast, YG5 labeled the cortex of thymus strongly, where many immature T cells are present,

whereas no immunoreactivity was detected in the lymph node. These results confirm that the epitope recognized by 8E10 is expressed in the most mature cells whereas the epitope recognized by 2C8 is expressed in the semi-mature cells. This was in contrast to the expression of JL1, which is exclusively expressed in immature cells. Therefore, 2C8 and 8E10 mAbs are two distinct mAbs that do not recognize leukemia-specific epitope on CD43, but can be paired with YG5.

In the current study, we developed and characterized two mAbs 2C8 and 8E10 recognizing a novel epitope site on CD43, which can be paired with YG5 to be used in a sandwich ELISA. Through this work, we provided the groundwork and generated reagents for establishing a sandwich ELISA technique that enables quantitative and qualitative assessment of leukemia-specific CD43.

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CONFLICTS OF INTEREST

The authors declare no financial conflict of interest.

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