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ORIGINAL ARTICLE

The novel function of CD82 and its impact on BCL2L12 via AKT/STAT5 signal pathway in acute myelogenous leukemia cells

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The aim of this study was to explore the biological functions of a tetraspanin family protein CD82 expressed aberrantly in chemotherapy-resistant $CD34^+/CD38^-$ acute myelogenous leukemia (AML) cells. Microarray analysis of patient-isolated $CD34^+/CD38^-$ AML cells revealed that the levels of anti-apoptotic protein BCL2L12 were downregulated after CD82 depletion by specific short hairpin RNA (shRNA). Western blot analysis indicated that BCL2L12 was aberrantly expressed in patient-isolated AML cells and AML cell lines. Furthermore, CD82 blockade by a specific antibody downregulated BCL2L12 in parallel with dephosphorylation of signal transducer and activator of transcription 5 (STAT5) and AKT, whereas pharmacological inhibition of STAT5 and AKT activation decreased BCL2L12 expression in leukemia cells. In addition, shRNA-mediated downregulation of BCL2L12 increased the levels of cleaved caspase-3 and suppressed proliferation of leukemia cells, impairing their engraftment in immunodeficient mice. Taken together, our results indicate that CD82 regulated BCL2L12 expression via STAT5A and AKT signaling and stimulated proliferation and engrafting of leukemia cells, suggesting that CD82 and BCL2L12 may be promising therapeutic targets in AML.

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INTRODUCTION

Acute myelogenous leukemia (AML) is the most common form of acute leukemia affecting adults[1] and is initiated and maintained by a subset of self-renewing leukemia stem cells.[2] $CD34^+/CD38^-$ AML cells have been shown to fulfill the criteria for leukemia stem cells *in vivo*,[3,4] and recent studies on severely immunocompromised mice have found that even $CD34^-$ or $CD38^+$ AML cells were able to reconstitute AML.[5,6] $CD34^+/CD38^-$ AML cells are refractory to conventional chemotherapeutic agents such as cytarabine and anthracyclines that interfere with DNA synthesis and induce apoptosis primarily in replicating cells.[7]

The BCL2 family of proteins comprises pro-apoptotic members BAX, BAD, BID and BCL-Xs and anti-apoptotic members BCL-2, BCL-xL and BCL-W.[8] Bcl2-like protein 12 (BCL2L12) is a newly identified member of the BCL2 family encoded by the *BCL2L12* gene located on chromosome 19p13.3 between the *IRF3* and *PRMT1/HRMT1L2* genes and close to the *RRAS* oncogene;[9,10] it has 13 distinct transcripts resulting from alterative gene splicing.[11] BCL2L12 contains a highly conserved BH2 domain, a BH3-like motif and a proline-rich region, and its classical BCL2L12 isoform 1 is expressed in various tissues including the breast, thymus, prostate, fetal liver, colon, placenta, pancreas, small intestine, spinal cord, kidney and bone marrow.[12,13]

Cytoplasmic BCL2L12 has been shown to block post-mitochondrial apoptosis in glioblastoma[14] via inhibition of caspase-3/7 activation, conferring apoptotic resistance and a tendency to necrosis in glial cells.[15] BCL2L12 does not affect cytochrome *c* release or apoptosome-driven caspase-9 activation, but instead inhibits post-mitochondrial apoptosis signaling in glioblastoma at the level of effector caspase activation.[16] Nuclear BCL2L12 interacts with the p53 tumor suppressor protein and inhibits p53-dependent apoptosis. Thus, BCL2L12 is a multi-functional protein that blocks apoptosis and induces necrosis via interaction with cytoplasmic and nuclear apoptotic pathways in glioblastoma cells.[14–17]

Notably, the neural cell adhesion molecule CD56 activates nuclear translocation of nuclear factor-κB that increases levels of BCL2L12 in AML cells. Interestingly, high levels of CD56 associates with poor prognosis of AML.[18] Moreover, BCL2L12 overexpression is associated with poor disease outcome in AML patients.[19] High levels of BCL2L12 mRNA correlate with advanced clinical stage and shorter overall survival in patients with solid tumors and hematological malignancies, including chronic myeloid leukemia and chronic lymphocytic leukemia.[19–23] These observations suggested that BCL2L12 may be chemotherapy response marker and chemotherapy target in various types of malignancies. However, the role of BCL2L12 in the maintenance of leukemia stem cells and AML resistance to conventional treatment has not been studied.

We recently compared protein expression profiles of $CD34^+/CD38^-$ and $CD34^+/CD38^+$ cells freshly isolated from AML patients using isobaric tags for relative and absolute quantitation (iTRAQ) and detected aberrant CD82 expression in $CD34^+/CD38^-$ AML cells.[24] CD82 was found to negatively regulate matrix metalloproteinase 9 and promote the adherence of these cells to fibronectin in bone marrow microenvironment. Downregulation of CD82 has been shown to inhibit colony-forming ability of $CD34^+/CD38^-$AML cells, suggesting that CD82 supports $CD34^+/CD38^-$ cell survival.[24] However, the mechanism underlying CD82 pro-survival activity in AML cells remains obscure.

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Here, we performed a functional and mechanistic analysis of the role of CD82 in AML cells and severely immunocompromised mice to elucidate its biological functions.

MATERIALS AND METHODS

Sample collection and isolation of $CD34^+/CD38^-$ and $CD34^+/CD38^+$ AML cells

Each study participant provided informed written consent, and the study was approved by the Kochi University Institutional Review Board (Nankoku, Japan). Leukemia cells were isolated from the patients with AML ($n = 28$, Table 1) classified according to the World Health Organization classification system as minimally differentiated AML (case 3), AML without maturation (cases 1, 7, 13 and 17), AML with maturation (cases 2, 11, 14, 18, 24, 27 and 28), acute myelomonocytic leukemia (cases 4, 12 and 25), acute monocytic leukemia (cases 15, 16 and 21), AML with myelodysplasia changes (cases 5, 6, 8, 9, 19 and 23) and therapy-related AML (cases 10, 20, 22 and 26). $CD34^+/CD38^-$ and $CD34^+/CD38^+$ cells were purified by magnetic cell sorting using the CD34 MultiSort kit and CD38 MicroBead kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), as previously described.[24]

Isolation of $CD34^+/CD38^-/CD82^-$ and $CD34^+/CD38^-/CD82^+$ AML cells and RT-PCR

Leukemia cells were stained with fluorescent-labeled antibodies fluorescein isothiocyanate-anti-CD34 (IM1870; Beckman Coulter, Brea, CA, USA), allophycocyanin-anti-CD38 (cat. no. 303509; BioLegend, San Diego, CA, USA) and phycoerythrin-anti CD82 (cat. no. 342104; BioLegend) and separated by cell sorting using a FACS Aria II instrument (BD Biosciences, Heidelberg, Germany). RNA was extracted using the CellAmp Direct RNA Prep Kit for reverse transcriptase-PCR (RT-PCR; Takara, Shiga, Japan) and reverse transcription was performed using the One Step PrimeScript RT-PCR Kit (Takara). Amplification was conducted using a StepOne plus system (Life Technology, CA, USA) at the following conditions: 42 °C for 5 min, 95 °C for 10 s, and 40 cycles at 95 °C for 5 s, 55 °C for 30 s and 72 °C for 1 min. The *18S* gene was used as internal control. PCR primers are listed in Table 2.

Cell culture

Acute monocytic leukemia cell line MOLM13 carrying an internal tandem duplication (ITD) of the juxtamembrane domain of FLT3 (FLT3/ITD) was kindly provided by Dr Yoshinobu Matsuo (Fujisaki Cell Center, Okayama, Japan).[25] Kasumi-1 cells carrying Asn822Lys c-Kit mutation were a kind gift from Dr Asou (Hiroshima University, Hiroshima, Japan). Leukemia cell lines HL60, NB4, U937 and THP-1 and FLT3/ITD-expressing MV4-11 were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Cell viability was determined after Trypan blue staining by light microscopy.

Pharmacological inhibition

The phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 was purchased from LC Laboratories (Woburn, MA, USA), and the Janus-associated kinase 2 inhibitor AZ960 was synthesized by AstraZeneca R&D (Osaka, Japan).[26] MOLM13 and Kasumi-1 cells were treated with LY294002 (1, 5 and 10 μM) or AZ960 (1, 2 and 5 μM) for 48 h before the analysis of protein expression by western blotting.

Microarray hybridization

Total RNA was extracted using the single-step Trizol RNA extraction kit (Invitrogen, Carlsbad, CA, USA) according to the protocol, concentrated by isopropanol precipitation and column-purified using the QIAGEN RNeasy Mini Kit (QIAGEN, Venlo, Holland). RNA concentration was determined using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) and RNA quality was evaluated using the Agilent 2100 Bioanalyzer expert software (Agilent Technologies, Santa Clara, CA, USA). RNA samples with the RNA integrity number $\geqslant 6.0$ were considered of a sufficient quality for gene expression profiling.

Cy3 and Cy5 dyes were used to label control and experimental complementary DNA (250 ng) according to the protocol of Miltenyi Biotec. Cy3- and Cy5-labeled complementary DNA was combined and hybridized for 17 h at 65 °C to the Agilent Whole Human Genome Oligo Microarrays $8 \times 60K$ using a hybridization chamber and oven (Agilent Technologies). The microarrays were washed once with the Agilent Gene Expression Wash Buffer 1 for 1 min at room temperature followed by a second wash with preheated (37 °C) Agilent Gene Expression Wash Buffer 2 for 1 min. The last washing step was performed with acetonitrile. After vigorous washing, the hybridized microarrays were scanned using an Agilent DNA microarray scanner (Agilent Technologies). The resulting images were analyzed using the Rosetta Resolver gene expression data analysis system (Rosetta Biosoftware, Kirkland, WA, USA) (National Center for Biotechnology Information (NCBI); GSE64527).

Western blotting

Total cell proteins or nuclear protein were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (30 μg of total cell protein per lane) and western blot analysis was performed as described previously[24] using the primary antibodies against phospho(p)-STAT5, p-AKT, AKT, caspase-3, cleaved caspase-3, poly ADP-ribose polymerase (Cell Signaling Technology, Beverly, MA, USA), STAT5, CD82, BAX, p53 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), BCL2L12 and GAPDH (Abcam, Cambridge, UK). Band intensities were quantified using the ImageJ software (Wayne Rasband, NIH, Bethesda, MD, USA).

Flow cytometry analysis

The level of CD82 in leukemia cells was assessed by flow cytometry using phycoerythrin-anti CD82 (BioLegend).

RNA isolation and real-time RT-PCR

Total RNA was extracted from leukemia cells and reverse transcribed according to the manufacturer's instructions (Takara). Real-time RT-PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK) in a StepOne plus system at the following conditions: 95 °C for 10 min and 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The expression of the *18S* gene was used for normalization. The primers are listed in Table 2.

Lentiviral and plasmid vectors and cell transfection

Short hairpin RNA (shRNA) specific to human *BCL2L12* was designed according to the human *BCL2L12* gene sequence (NCBI accession number NM_138639). Human CD82-specific shRNA was synthesized based on the human *CD82* transcript variant 2 (NCBI accession number NM_001024844). The control, BCL2L12, and CD82 shRNA lentiviral vectors, designed to coexpress GFP, were purchased from GeneCopoeia (Rockville, MD, USA). Lentiviral shRNA particles were produced in 293FT packaging cells (Invitrogen) using the Lenti-Pac FIV Expression Packaging Kit (GeneCopoeia). Three types of CD82 shRNA-expressing lentivirus particles were mixed and used for cell transduction in serum-free medium. After overnight incubation, fresh 10% fetal bovine serum-supplemented medium containing 1 μg/ml puromycin was added for 7 days. CD82 cDNA was purchased from the Mammalian Gene Collection (BC000726, GE Healthcare, Little Chalfont, Buckinghamshire, UK) and was used as the template for PCR. STAT5A cDNA (NM_003152) was synthesized by TAKARA BIO Inc. (Shiga, Japan). Gene products were cloned into the pLenti6.3/V5-TOPO vector (Invitrogen). The product was cloned into the pLenti6.3/V5-TOPO vector (Invitrogen). Lentiviral particles were produced using the viral power packaging system (Invitrogen) and transduced into MOLM13 cells as previously described.[24] After 72 h, blasticidin (10 μg/ml; Invitrogen) was added to select for stably transduced cells. MOLM13 cells were transiently transfected with the Gag-AKT expression vector[27] using the Fugene transfection reagent (Promega, Madison, WI, USA).

CD82 antibody binding

The binding of human anti-CD82 monoclonal antibody (53H5) (Santa Cruz Biotechnology) to cell surface of leukemia cells was examined by microscopy (OLYMPUS FV1000-D, Tokyo, Japan). The phycoerythrin-conjugate secondary antibody was obtained from BioLegend.

Apoptosis assay

Cellular apoptosis was assessed by the release of cytochrome *c* from mitochondria into cytosol using the Cytochrome *c* Releasing Apoptosis Assay Kit (BioVision, Milpitas, CA, USA) according to the manufacturer's instructions.

Table 1. Patients' characteristics

Pt. no.	*Sex*	*Age*	*WHO*	*Cytogenetics*	*FLT3 mutation*
1	M	68	AML without maturation	Normal	ITD
2	F	81	AML with maturation	Normal	WT
3	M	60	minimally differentiated AML	Normal	WT
4	M	78	Acute myelomonocytic leukemia	68, XX, -Y, +1, +4, -5, +6, +8, -9, -10, +11, -13, -15, -16, -17, +18, +19, -21, +22 [2]/ 70, idem, +2, -6, +7, +20 [16]	NA
5	M	62	AML with myelodysplasia changes	Normal	WT
6	M	63	AML with myelodysplasia changes	43, XY, add(3)(p11), -5, -5, +6, -7, +8, add(11)(q23), add(11)(q23), -12, add(12)(p11.2), -15, -17, -19, -22, +mar1, +mar2, +mar3 [2]/44, idem, -mar3, +mar4, +mar5 [11]	NA
7	F	84	AML without maturation	NA	NA
8	M	77	AML with myelodysplasia changes	46, XY, der(2)t(2;5)(q21;q13)ins(2;?)(q21;?), del(5)(q?), del(7)(q11.2), del(11)(q?) [4]/ 45, idem, add(X)(q22), -3, add(6)(p23), add(8)(q24), add(12)(q13), -17, der(18)t(17;18)(q11.2;q21), +mar1 [2]/44, idem, add(X)(q22), -3, add(6)(p23), add(8)(q24), add(12)(q13), -17, der(18)t(17;18)(q11.2;q21), +mar1,-Y [9]	NA
9	M	62	AML with myelodysplasia changes	Normal	WT
10	M	78	Therapy-related AML	Normal	NA
11	F	86	AML with maturation	NA	NA
12	F	44	Acute myelomonocytic leukemia	Normal	WT
13	M	61	AML without maturation	Normal	NA
14	F	36	AML with maturation	47, XX, +21	WT
15	M	50	Acute monocytic leukemia	45, X, -Y,?t(3;11;10)(q29;q23;p12)	WT
16	M	22	Acute monocytic leukemia	46, XY, add(8)(p11.2),?t(10;11)(p12;q23)	WT
17	F	65	AML without maturation	Normal	NA
18	M	45	AML with maturation	44, XY, del(5)(q?), -17, -19, i(21)(q10), -22, -22, +mar1, +mar2 [2]/45, idem, add(11)(q23), add(12)(p11.2), +16, +19, add(20)(p13), +21, -i(21), -mar2 [16]	WT
19	M	71	AML with myelodysplasia changes	46, XY, add(3)(p21), add(5)(q22), der(8)t(8;8)(p?21;q11.2), add(13)(p11.2), add(17)(p11.2), add(17)(q25) [cp5]/46, XY, add(3)(p21), add(5)(q22), der(8)t(8;8)(p?21;q11.2), add(13)(p11.2), add(17)(p11.2), add(17)(q25), add(21)(q22) [5]	NA
20	M	70	Therapy-related AML	47, XY, +X, t(10;20)(p11.2;q13.1) [1]/ 46, XY, t(10;20)(p11.2;q13.1) [19]	WT
21	M	22	Acute monocytic leukemia	46, XY, add(8)(p11.2),?t(10;11)(p12;q23) [7]/ 46, idem, t(1;3)(q21;q29), -2, del(4)(q?), add(22)(q13), +marl [11]	WT
22	M	70	Therapy-related AML	Normal	WT
23	F	76	AML with myelodysplasia changes	46, XX, t(11;19)(q23;p13.1)	WT
24	F	90	AML with maturation	46, XX, t(8;21)(q22;q22) [1]/45, idem, -X [17]	WT
25	F	55	Acute myelomonocytic leukemia	Normal	ITD
26	M	67	Therapy-related AML	Normal	WT
27	F	77	AML with maturation	46, XX, t(11;13)(p13;q12)	NA
28	M	61	AML with maturation	Normal	ITD

Abbreviations: AML, acute myelogenous leukemia; F, female; FLT3, fms-like tyrosine kinase 3; ITD, internal tandem duplication; M, male; Normal, 46, XX, in a female or 46, XY in a male; NA; not assessed; Pt, patient; WHO, World Health Organization (leukemia classification); WT, wild type. Square brackets indicate the number of analyzed cells.

Table 2. PCR primers

Gene	*Accession no.*	*Direction*	*Primer*
BCL2L12 transcript variant 1	NM_138639	Forward	5′-CCCTCGGCCTTGCTCTCT-3′
		Reverse	5′-GGGCCACCAAAGCATAGAAG-3′
CD82	NM_001024844	Forward	5′-GGCGGGATGGGCTCAGCCTG-3′
		Reverse	5′-TCAGTACTTGGGGACCTTGC-3′
18S		Forward	5′-AAACGGCTACCACATCCAAG-3′
		Reverse	5′-CCTCCAATGGATCCTCGTTA-3′

Colony-forming assay

The colony-forming assay was performed with methylcellulose medium H4034 (StemCell Technologies, Vancouver, BC, Canada), as previously described.[24]

Bone marrow transplantation and engraftment

NOD.Cg-*Rag1*^{tm1Mom} *Il2rg*^{tm1Wjl}/SzJ mice (Stock Number, 007799) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA)[24] and bred in a pathogen-free environment in accordance with the guidelines of the Kochi University School of Medicine; 6-week-old female and male animals were utilized for the experiments. Human MOLM13 cells (5×10^6) transfected with control shRNA ($n = 14$) or BCL2L12-specific shRNA1 ($n = 14$) were injected via the tail vein and cell engraftment was analyzed using flow cytometry after staining of peripheral blood monoclonal cells with human CD82 phycoerythrin-conjugated monoclonal antibody (BioLegend).

Statistical analysis

All statistical analyses were performed using the statistical software GraphPad PRISM (GraphPad Software Inc., San Diego, CA, USA). Differences were analyzed by Student's *t*-test and were considered significant at a *P*-value of < 0.05 and highly significant at a *P*-value of < 0.01. Mouse survival was calculated using the Kaplan–Meier method, and survival curves were compared by log-rank test.

RESULTS

Gene expression profiles in CD34$^+$/CD38$^-$ AML cells expressing CD82-specific shRNA

To explore the function of CD82 in CD34$^+$/CD38$^-$ AML cells, CD34$^+$/CD38$^-$ cells isolated from AML patients ($n = 3$, cases 1–3) were transduced with CD82-specific shRNA and their gene expression profiles were compared with the cells transduced with control shRNA by microarray analysis. The threefold differences in more than two AML cases were considered as significant. Between CD82-depleted and control CD34$^+$/CD38$^-$ AML cells, 995 genes were found differentially expressed in at least two AML samples (NCBI; GSE64527). Among them, anti-apoptotic BCL2 family genes *BCL2L12* (NM_138639) and *BCL2L2* (NM_004050) were the only genes downregulated in CD82-depleted CD34$^+$/CD38$^-$ AML samples isolated from all three AML patients (Supplementary Table S1). We previously showed that CD34$^+$/CD38$^-$ AML cells aberrantly expressed CD82.[24] These observations suggested the possibility that BCL212 and BCL2L2 may be overexpressed by CD82 in leukemia cells. In addition, other researchers performed the comparison of mRNA expression between AML cells such as KG-1, U937 and THP-1 cells and normal monocytes isolated from healthy volunteers using microarray. They found that the expression of BCL2L12 but not BCL2L2 was significantly upregulated in AML cells (Table 3, GDS2251, http://www.ncbi.nlm.nih.gov/geoprofiles/29050864).[28] Given that BCL2L12 overexpression was associated with poor outcome in AML,[19] *BCL2L12* may be an important gene involved in maintenance of survival of AML cells. We therefore investigated the relationship between CD82 and BCL2L12 in AML cells in this study.

Table 3. The level of BCL2L12 in myeloid leukemia cells

Cells	*Value*	*Rank*
Human blood monocytes	58.8	69
KG-1	153.1	81
THP-1	169.9	83
U937	231.4	86

Publicly available microarray databases showed BCL2L12 was highly expressed in myeloid leukemia cells compared with normal blood monocytes.

CD82 regulates BCL2L12 expression in leukemia cells

To validate the results of microarray (Supplementary Table S1), we performed real-time RT-PCR that showed that levels of BCL2L12 decreased by 0.3-fold in CD34$^+$/CD38$^-$ AML cells after depletion of CD82 by half by shRNA ($n = 5$, cases 4–7 and 19; Figure 1a and figure not shown),[29] whereas forced expression of CD82 in CD34$^+$/CD38$^+$ AML cells by 10-fold (figure not shown)[29] demonstrated a twofold increase in BCL2L12 ($n = 6$, cases 1–3, 17, 19 and 20; Figure 1b). We also compared BCL2L12 mRNA levels in CD34$^+$/CD38$^-$/CD82$^+$ and CD34$^+$/CD38$^-$/CD82$^-$ AML cells isolated from the patients ($n = 10$, cases 1, 2, 6–9, 19, 22, 27 and 28). As expected, BCL2L12 expression was 10-fold higher in CD34$^+$/CD38$^-$/CD82$^+$AML cells than their CD82-negative counterparts (Figure 1c). To explore the relationship between BCL2L12 and CD82, we used CD82-overexpressing MOLM13 and Kasumi-1 cells. Similarly, in CD82$^+$ MOLM13 and Kasumi-1 cells, there was a twofold increase in BCL2L12 expression compared with CD82$^-$ cells (Figure 1d). These results suggested that CD82 might regulate BCL2L12 expression in leukemia cells.

CD82 and BCL2L12 expression in AML cells

We have previously shown that CD82 expression is upregulated in CD34$^+$/CD38$^-$ AML cells ($68 \pm 27\%$) compared with their CD34$^+$/CD38$^+$ counterparts ($30 \pm 19\%$).[24] Here, we found that BCL2L12 levels in CD34$^+$/CD38$^-$ cells were higher than in CD34$^+$/CD38$^+$ cells ($n = 7$, cases 1, 3, 8 and 23–26; Supplementary Figure S1A). A trend of positive correlation between CD82 and BCL2L12 mRNA levels was shown in CD34$^+$/CD38$^-$ AML cells isolated from patients ($r = 0.73$, Supplementary Figure S1B). Moreover, we compared CD82 and BCL2L12 expression in the cells isolated from AML patients ($n = 10$, cases 10–19) and bone marrow mononuclear cells (BMMNCs) from healthy volunteers ($n = 4$) by real-time RT-PCR. The levels of CD82 and BCL2L12 were 7757- and 13-fold higher, respectively, in AML cells than in BMMNCs (Figure 2a). The analysis of 10 AML patients showed a trend ($r = 0.36$) of positive correlation between CD82 and BCL2L12 mRNA expression (Figure 2b). We next examined the protein levels of BCL2L12 in AML cells isolated from the patients ($n = 10$, cases 10–19), BMMNCs isolated from a healthy volunteer ($n = 1$) and AML cell lines by western blotting (Figure 2c). Consistent with mRNA data, BCL2L12 protein expression was higher in AML cells than in BMMNCs, although the levels varied among cell lines (Figure 2c). Similarly, flow cytometry analysis indicated that CD82 surface expression in AML

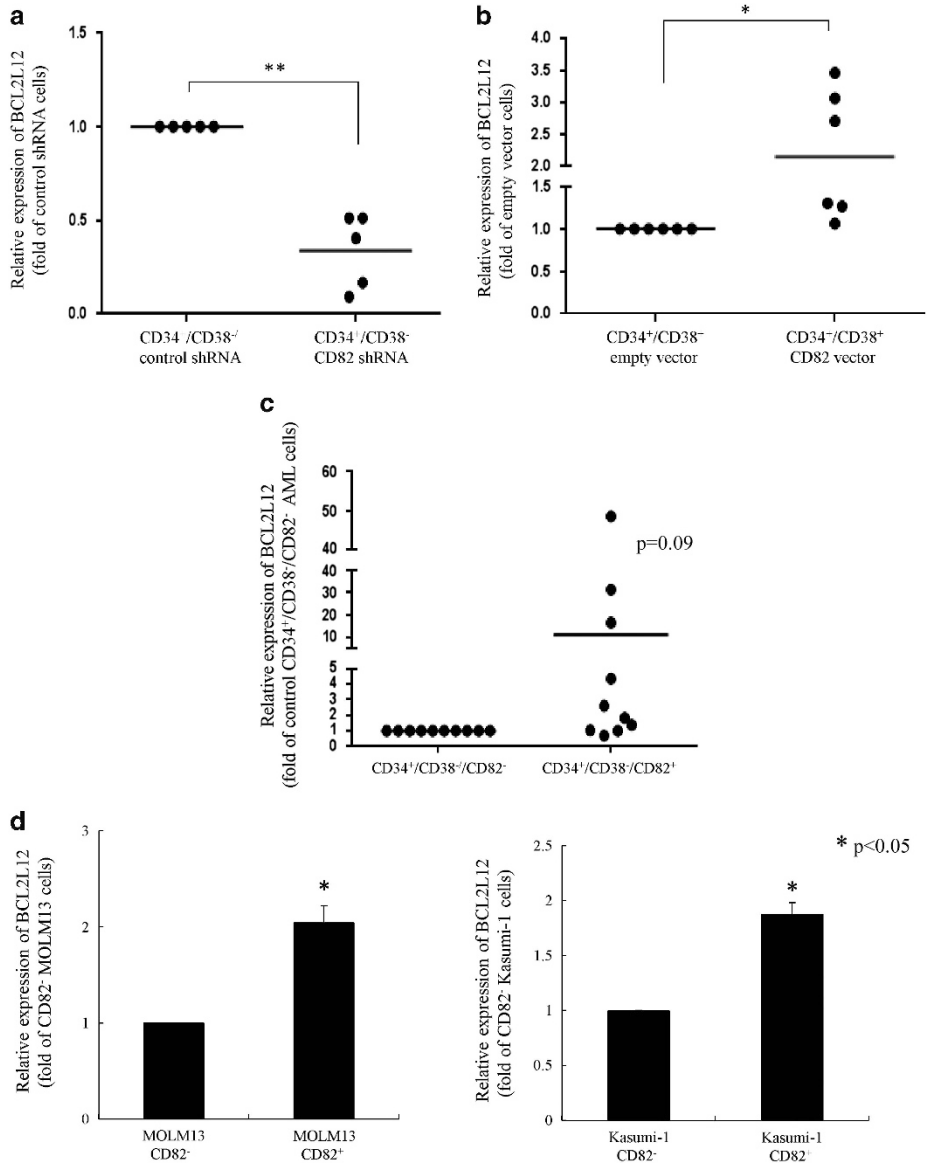

Figure 1. The effect of CD82 on BCL2L12 expression in $CD34^+/CD38^-$ AML cells. (**a**) $CD34^+/CD38^-$ AML cells ($n = 5$ patients) and (**b**) $CD34^+/CD38^+$ AML cells ($n = 6$ patients) transduced with CD82 shRNA or CD82-expressing lentiviral particles were collected and analyzed for BCL2L12 mRNA expression by real-time RT-PCR. Each dot represents BCL2L12 level in each patient and the mean is indicated by the line. $^*P < 0.05$, $^{**}P < 0.01$. (**c**) $CD34^+/CD38^-/CD82^+$ and $CD34^+/CD38^-/CD82^-$AML cells ($n = 10$ patients) were isolated by cell sorting and analyzed for BCL2L12 mRNA expression by real-time RT-PCR. (**d**) $CD82^+$ and $CD82^-$ MOLM13 and Kasumi-1 cells were sorted and analyzed for BCL2L12 mRNA expression by real-time RT-PCR. $^*P < 0.05$.

cells was higher than in BMMNCs (Figure 2d). A trend of positive correlation between CD82 and BCL2L12 protein levels were shown in AML cells isolated from patients ($r = 0.3$, Supplementary Figure S1C).

Molecular mechanisms underlying CD82 regulation of BCL2L12 expression in leukemia cells

We previously showed that CD82 regulated activation of signal transducer and activator of transcription 5 (STAT5) and AKT in AML cells.[24] Thus, we examined whether CD82 modulated the expression of BCL2L12 via STAT5 or AKT signal pathway. The shRNA-mediated downregulation of CD82 in MOLM13 and Kasumi-1 cells attenuated BCL2L12 expression at both mRNA and protein levels and dephosphorylated both STAT5 and AKT as compared with control shRNA-transduced cells (Figures 3a and b). Moreover, the blockade of CD82 in these cells by a CD82-specific antibody decreased BCL2L12 expression at both the mRNA and protein levels and increased the expression of BAX and cleaved caspase-3 and phosphorylation of AKT and STAT5 (Figures 3c and d). Notably, forced expression of AKT or STAT5A increased BCL2L12 levels in MOLM13 cells and recovered BCL2L12 expression in the cells treated with CD82-specific shRNA (Figure 3e), confirming that CD82 regulated BCL2L12 levels via the AKT and STAT5 pathways. We next examined whether BCL2L12 expression would respond to pharmacological inhibition of AKT and STAT5 in leukemia cells and found that PI3K inhibitor LY294002 decreased both AKT phosphorylation and BCL2L12 expression (Figure 3f), whereas Janus-associated kinase 2 inhibitor AZ960 decreased STAT5 phosphorylation as well as BCL2L12 levels (Figure 3g). These results provide further support to our hypothesis that AKT and STAT5 signaling positively regulates BCL2L12 levels in leukemia cells.

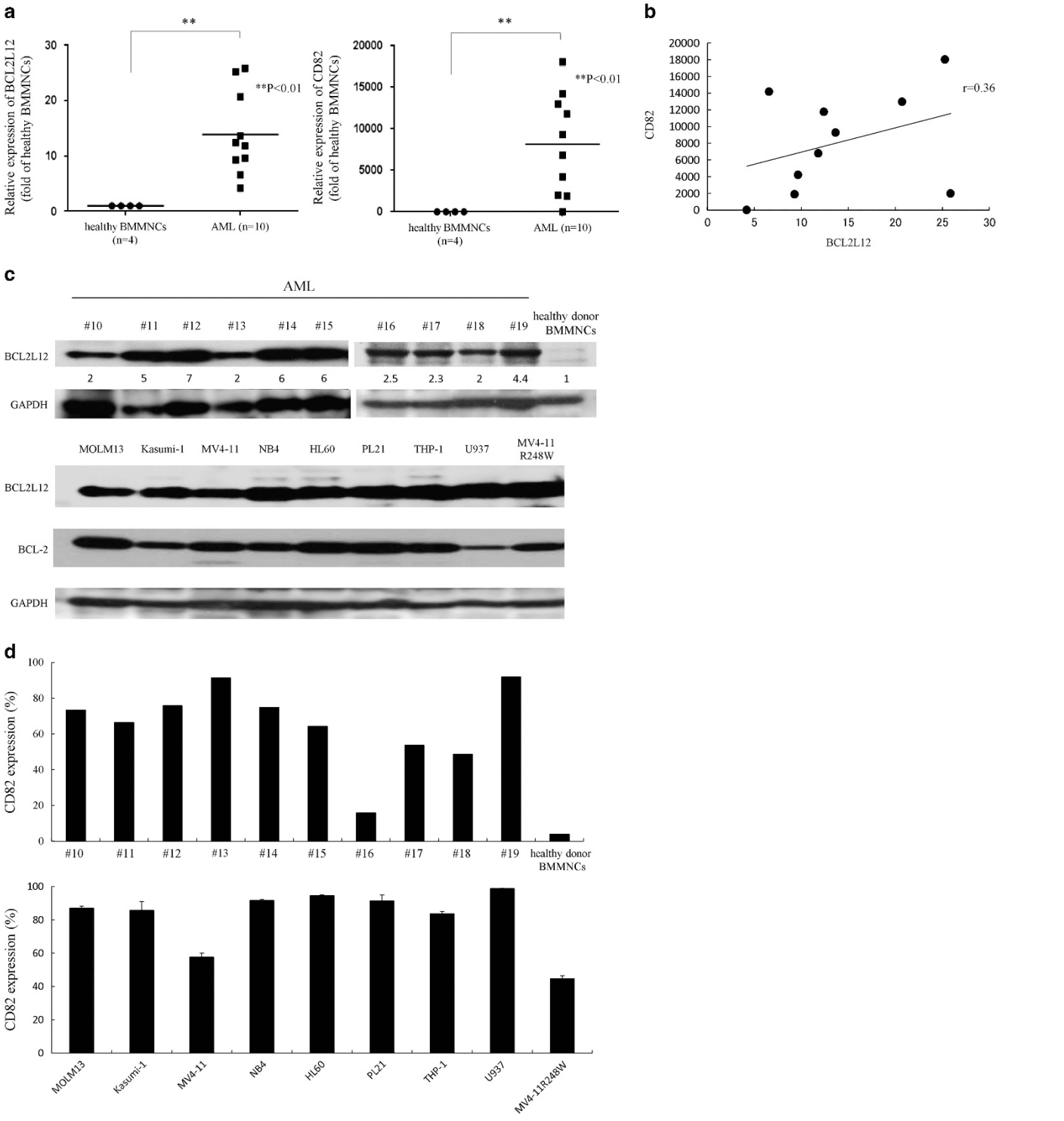

Figure 2. CD82 and BCL2L12 expression in AML cells. (**a**) CD82 and BCL2L12 mRNA levels in AML cells isolated from patients ($n = 10$ patients) and BMMNCs isolated from healthy volunteers ($n = 4$ patients). $^{**}P < 0.01$. (**b**) A scatter plot represents the correlation between CD82 and BCL2L12 mRNA expression in patients' AML cells. (**c**, **d**) BMMNCs isolated from healthy volunteer ($n = 1$), AML cells ($n = 10$ patients), MOLM13, Kasumi-1, MV4-11, NB4, HL60, PL21, THP-1, U937 and MV4-11R248W cells were harvested and analyzed for BCL2L12 and BCL2 expression by western blotting and flow cytometry. Band intensities were quantified with ImageJ software (Wayne Rasband, NIH).

The effect of BCL2L12 on apoptosis in leukemia cells

In Kasumi-1 and MOLM13 cells, BCL2L12-specific shRNAs decreased the expression of BCL2L12 and increased that of BAX and cleaved caspase-3 in parallel with the inhibition of cell survival, as evidenced by Trypan blue staining (Figures 4a–d). On the other hand, downregulation of BCL2L12 did not affect p53 expression and cytochrome *c* translocation from mitochondria into cytosol (Figures 4a–d).

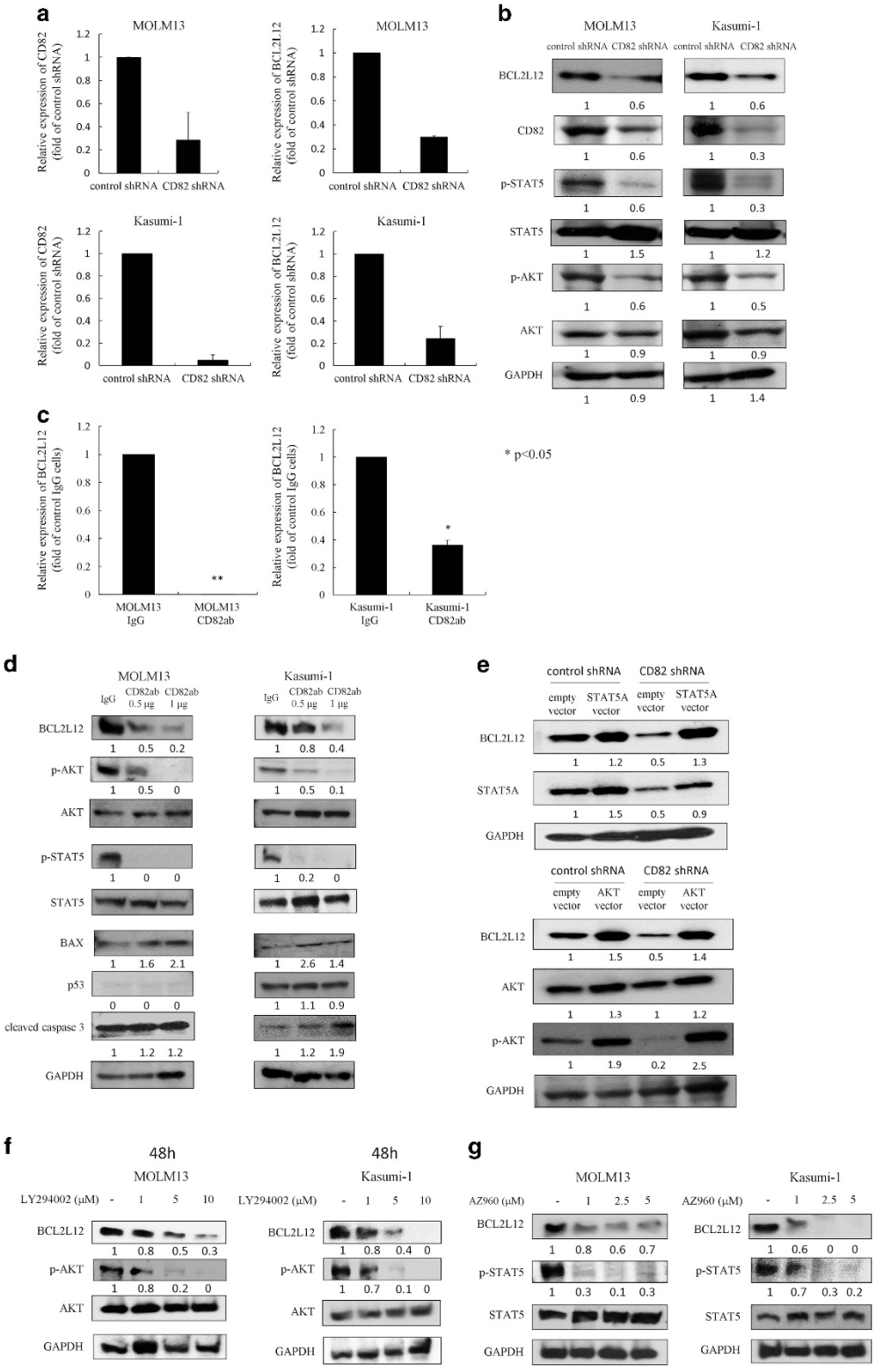

Figure 3. The effect of CD82 on the expression of apoptotic proteins and phosphorylation of STAT5 and AKT in leukemia cell lines. (**a**, **b**) MOLM13 and Kasumi-1 cells transduced with CD82 shRNA were analyzed for BCL2L12 mRNA expression by real-time RT-PCR; (**a**) and for the expression of indicated proteins by western blotting (**b**). Band intensities were quantified using ImageJ software (Wayne Rasband, NIH). The number indicates the relative values of band intensity in CD82 shRNA-transduced cells compared with control shRNA-transduced cells. (**c**, **d**) MOLM13 and Kasumi-1 cells were treated with control IgG or CD82 antibody (0.5 and 1.0 μg/ml) for 96 h and analyzed for BCL2L12, BAX and cleaved caspase-3 mRNA levels by real time RT-PCR; $*P < 0.05$, $**P < 0.01$ (**c**) and for the expression of indicated proteins by western blotting (**d**). (**e**) MOLM13 cells expressing CD82-specific shRNA were transfected with AKT, STAT5A or vehicle for 72 h, harvested and analyzed for the expression of indicated proteins by western blotting. Each lane was loaded with 30 μg of total protein or nuclear protein. (**f**, **g**) MOLM13 and Kasumi-1 cells were treated with the inhibitor of PI3K/AKT signaling LY294002 or the inhibitor of Janus-associated kinase 2 (JAK2) kinase AZ960 for 48 h. The cells were harvested and analyzed for the expression of indicated proteins by western blotting.

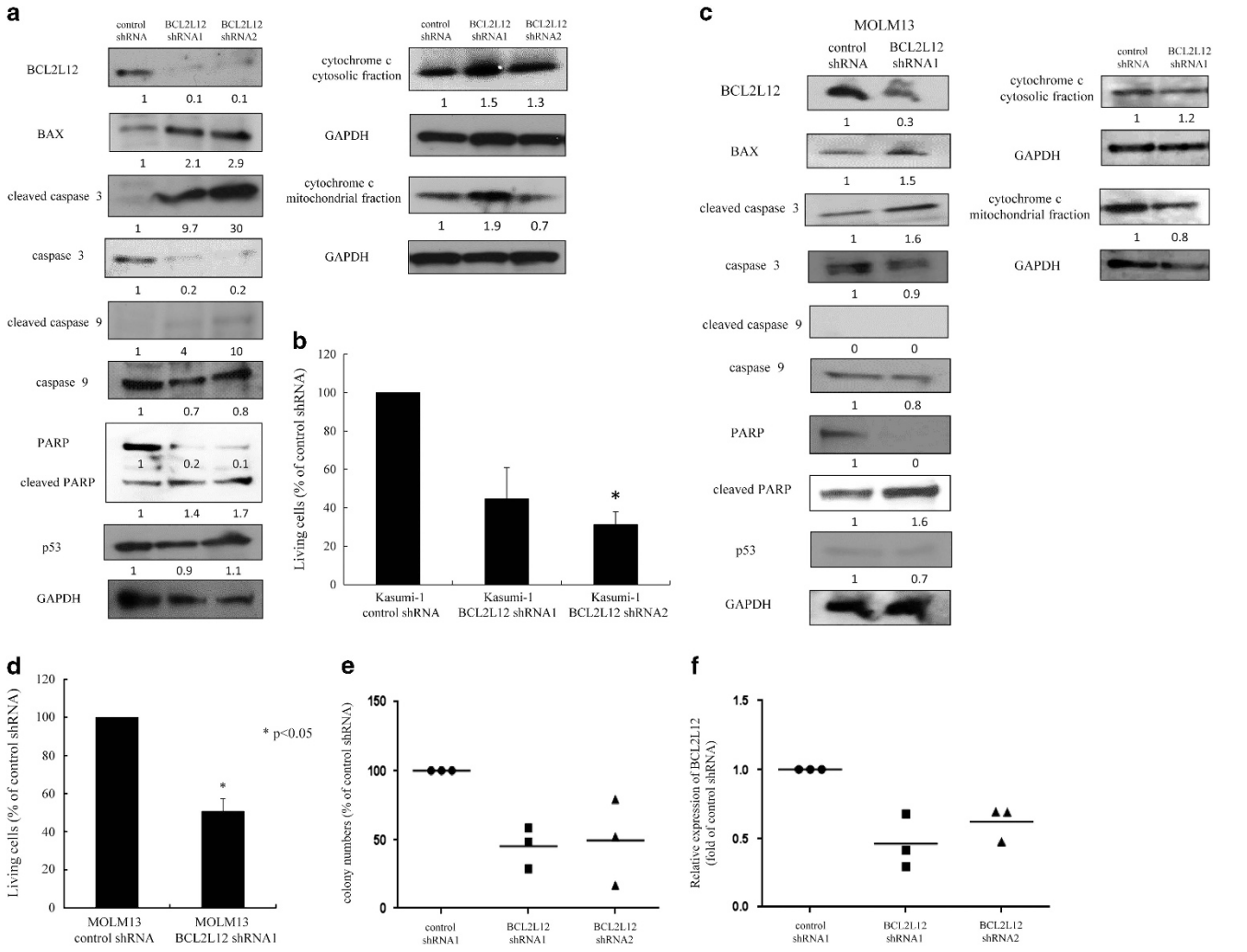

Figure 4. BCL2L12 function in leukemia cells. (**a**) Kasumi-1 cells were transfected with control shRNA or BCL2L12-specific shRNA1 and shRNA2 and analyzed for the expression of indicated proteins by western blotting. (**b**) Kasumi-1 cells transfected with BCL2L12-specific shRNA1 and shRNA2 for 96 h were stained with Trypan blue, and the number of viable cells was counted under a light microscope. $*P < 0.05$. (**c**) MOLM13 cells were transfected with control shRNA or BCL2L12-specific shRNA1 and analyzed for the expression of indicated proteins by western blotting. (**d**) MOLM13 cells transfected with control shRNA or BCL2L12-specific shRNA1 for 96 h were stained with Trypan blue, and the number of viable cells was counted under a light microscope. $*P < 0.05$. (**e**) Colony-forming assay. $CD34^+/CD38^-$ AML cells ($n = 3$ patients) transduced with BCL2L12 shRNAs were cultured in methylcellulose medium for 16 days, and the colonies were counted. (**f**) $CD34^+/CD38^+$ AML cells ($n = 3$ patients) transduced with BCL2L12 shRNA were collected and analyzed for BCL2L12 mRNA expression by real-time RT-PCR. Each dot represents BCL2L12 level in each patient and the mean is indicated by the line.

Then, we examined whether BCL2L12 regulated the survival of $CD34^+/CD38^-$ AML cells. The results show that the shRNA-mediated downregulation of BCL2L12 in $CD34^+/CD38^-$ AML cells decreased colony-forming ability of these cells by ~50% compared with the control cells ($n = 3$, cases 3, 19 and 21; Figures 4e and f).

BCL2L12 effects *in vivo*

We next explored BCL2L12 effects on the engraftment of AML cells in severely immunocompromised mice. MOLM13 cells (5×10^6) transduced with control shRNA or BCL2L12-specific shRNA1 were intravenously transplanted into NOD.Cg-*Rag1*^{tm1Mom} *Il2rg*^{tm1Wjl}/SzJ mice and their engraftment and animal survival were examined (Figure 5). At 3 weeks after transplantation, the expression of human CD82 in mouse peripheral blood monoclonal cells indicating the engraftment of MOLM13 cells was analyzed. The engraftment of MOLM13 cells with decreased BCL2L12 expression was significantly impaired compared with the control cells (BCL2L12 shRNA1, 38 ± 12%; control shRNA, 59 ± 16%; Figure 5a). In addition, Kaplan–Meier survival curves demonstrated that immunodeficient mice transplanted with BCL2L12-depleted MOLM13 cells survived longer than those transplanted with the control cells ($P < 0.01$, Figure 5b).

DISCUSSION

Microarray analysis and RT-PCR found that BCL2L12 was downregulated in CD82-depleted $CD34^+/CD38^-$ AML samples isolated from AML patients (Supplementary Table S1 and Figure 1a). A trend of positive correlation between CD82 and BCL2L12 expression at mRNA and protein levels was noted in AML cells isolated from patients (Figure 2b and Supplementary Figure S1C), suggesting the possibility that CD82 might regulate expression of BCL2L12 in AML cells. In fact, downregulation of CD82 by specific shRNA or antibody attenuated BCL2L12 expression and reduced activation of STAT5 and AKT in AML cells, whereas overexpression of AKT or STAT5 restored BCL2L12 levels in

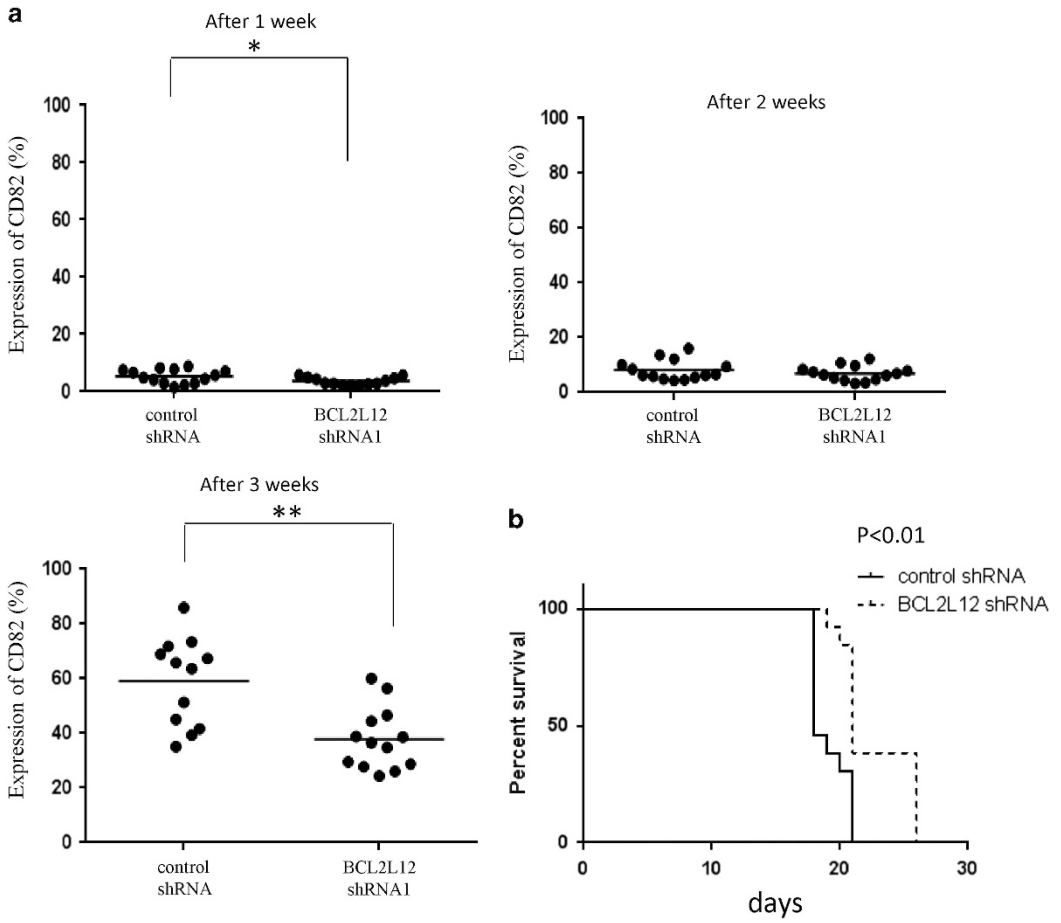

Figure 5. The effect of BCL2L12 on the engraftment of leukemia cells in immunodeficient mice and mouse survival. MOLM13 cells transduced with control shRNA or BCL2L12-specific shRNA1 were transplanted into NOD.Cg-*Rag1*^{tm1Mom} *Il2rg*^{tm1Wjl}/SzJ mice via the tail vein (each group). (**a**) In 1–3 weeks after transplantation, peripheral blood was collected, incubated with CD82 antibodies and analyzed by flow cytometry. *$P < 0.05$, **$P < 0.01$. (**b**) Kaplan–Meier analysis of mouse survival ($P < 0.01$).

CD82-deficient cells (Figure 3), suggesting that in leukemia cells, CD82 positively regulated BCL2L12 expression via AKT and STAT5 signaling (Figure 6).

Amounts of BCL2L12 mRNA and proteins were greater in AML cells than BMMNCs isolated from healthy volunteers (Figure 2). However, we were not able to compare levels of BCL2L12 between CD34$^+$/CD38$^-$ AML cells and CD34$^+$/CD38$^-$ BMMNCs isolated from healthy donors because of the paucity of the latter cells. Therefore, we could not reach the conclusion that levels of BCL2L12 were upregulated in AML cells compared with normal hematopoietic stem/progenitor cells.

We previously showed that CD82 activated AKT and STAT5 in leukemia cells.[30] Long-term maintenance and expansion were impaired in STAT5-deficient leukemia stem cells and hematopoietic stem cells.[31,32] On the other hand, overexpression of STAT5 enhances the self-renewal potential of CD34$^+$ cells.[33] Similarly, activation of AKT by loss of PTEN (phosphatase and tensin homolog deleted on chromosome 10) induces self-renewal and expansion of hematopoietic stem cell via upregulation of anti-apoptotic Mcl-1.[34] These observations suggested that activation of STAT5 and AKT is required for the self-renewal and expansion of primitive hematopoietic stem/progenitor as well as leukemia stem cells.

Downregulation of CD82 in CD34$^+$/CD38$^-$ AML cells by an shRNA significantly impaired engraftment of these cells in severely immunocompromised mice.[24] We have recently shown

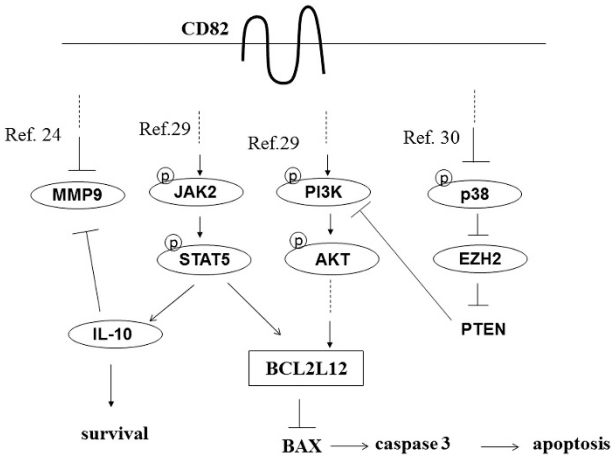

Figure 6. The signal transduction pathways governed by CD82. MMP9, matrix metalloproteinase 9; Ref, reference.

that CD82 negatively regulated matrix metalloproteinase 9 in AML cells, rendering these cells adherent to bone marrow microenvironment (Figure 6).[24] In addition, CD82 activated STAT5 in association with an increase in production of IL-10 that decreased in the level of matrix metalloproteinase 9 in AML cells (Figure 6).[24,30]

CD82 also stimulated expression of polycomb family protein EZH2 (enhancer of zeste homolog 2) via p38 mitogen-activated protein kinase pathway in AML cells, resulting in downregulation of PTEN in association with methylation of its promoter region (Figure 6).[29] As upregulation of PTEN inactivates PI3K/AKT, CD82 may activate PI3K/AKT via downregulation of PTEN. Moreover, the use of CD82 monoclonal antibody in combination with cytarabine (AraC) significantly prolonged survival of immunodeficient mice bearing human AML cells.[35] These results suggested that CD82 pathway may be biologically important target to treat patients with AML.

We found that the levels of CD82 mRNA and protein did not correlate in AML cells ($n = 10$, $r = 0.2$; Supplementary Figure S1d). One possible explanation might be related to the half-life of CD82 protein. Further studies are clearly required to verify the mechanisms by which expression of CD82 is regulated at the mRNA and protein levels.

Blockade of CD82 by the CD82 antibody did not increase p53 levels in leukemia cells (including FLT3/ITD-expressing AML cell line MV4-11 TP53 R248W (data not shown) that harbor a missense mutation CGG → TGG; R248W in the *TP53* gene),[36] suggesting that CD82 may induce apoptosis via a p53-independent pathway. Similarly, the downregulation of BCL2L12 did not affect p53 levels in MOLM13 cells, although it inhibited cell proliferation and increased the levels of pro-apoptotic factors such as cleaved caspase-3 and BAX. Although BCL2L12 inhibited apoptosis via the p53-dependent pathway in glioblastoma multiforme,[16] our results demonstrate that in leukemia cells, BCL2L12 might exert pro-survival effects independently of p53 signaling. CD82/AKT and CD82/STAT5 signaling may inhibit apoptosis in association with the increase in BCL2L12 and decrease in BAX and cleaved caspase-3 levels, thus presenting a mechanism for chemotherapy resistance in AML.

Immunodeficient mice transplanted with BCL2L12-depleted MOLM13 cells demonstrated increased survival compared with those transplanted with control cells; however, they eventually died of leukemia, suggesting that BCL2L12 may partially mediate the survival of leukemia cells. We assume that not only BCL2L12 but also other BCL2 family members may play a role in supporting the survival of AML cells via the CD82 pathway.

BCL2L12 overexpression was associated with poor outcome in AML,[19] although this study cohort was extremely small ($n = 28$) and heterogeneous; five acute promyelocytic leukemia patients whose prognosis is favorable due to the treatment with all-*trans* retinoic acid[37] were included in the study and all of them were negative for BCL2L12 expression.[19] Intriguingly, high expression of BCL2L12 correlated with favorable outcome in gastric and colon cancer.[13,20] Further studies are clearly required to examine the association of BCL2L12 expression and prognosis of AML.

CD82 overexpression in AML cells enhances adhesion and regulate maintenance of AML cell within the bone marrow niche,[24] but suppresses tumor invasion and metastasis and is associated with favorable prognosis in solid cancers such as lung, gastric, colon and cervix cancers.[38–42] These findings suggest that high expression of CD82 and BCL2L12 in colon and gastric cancer may correlate with favorable prognosis, but in hematologic malignancies may be associated with poor outcome, indicating cancer type-specific biological functions of these molecules. Therefore, the manipulation of CD82 and BCL2L12 expression may constitute a therapeutic approach to AML treatment.

In conclusion, our results demonstrate that in leukemia cells, CD82 regulates BCL2L12 expression via STAT5 and AKT signaling, suggesting that BCL2L12 may be a promising therapeutic target to improve survival of AML patients.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

Takayuki Ikezoe contributed to the concept and design, interpreted and analyzed the data and wrote the article. Chie Nishioka performed all experiments and wrote the article. Atsuya Nobumoto and Masayuki Tsuda provided technical support. Akihito Yokoyama provided critical revision and intellectual content.

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Supplementary Information accompanies this paper on the Leukemia website (http://www.nature.com/leu)