

# Clinical and preclinical characterization of CD99 isoforms in acute myeloid leukemia

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## ABSTRACT

In an effort to identify target genes in acute myeloid leukemia (AML), we compared gene expression profiles between normal and AML cells from various publicly available datasets. We identified *CD99*, a gene that is up-regulated in AML patients. In 186 patients from The Cancer Genome Atlas AML dataset, *CD99* was over-expressed in patients with *FLT3*-ITD and was down-regulated in patients with *TP53* mutations. *CD99* is a trans-membrane protein expressed on leukocytes and plays a role in cell adhesion, trans-endothelial migration, and T-cell differentiation. The *CD99* gene encodes two isoforms with distinct expression and functional profiles in both normal and malignant tissues. Here we report that, although the *CD99* long isoform initially induces an increase in cell proliferation, it also induces higher levels of reactive oxygen species, DNA damage, apoptosis and a subsequent decrease in cell viability. In several leukemia murine models, the *CD99* long isoform delayed disease progression and resulted in lower leukemia engraftment in the bone marrow. Furthermore, the *CD99* monoclonal antibody reduced cell viability, colony formation, and cell migration, and induced cell differentiation and apoptosis in leukemia cell lines and primary blasts. Mechanistically, *CD99* long isoform resulted in transient induction followed by a dramatic decrease in both ERK and SRC phosphorylation. Altogether, our study provides new insights into the role of *CD99* isoforms in AML that could potentially be relevant for the preclinical development of *CD99* targeted therapy.

## Introduction

The outcome of patients with acute myeloid leukemia (AML) remains dismal due to the high relapse rate.<sup>1</sup> To identify target genes that were differentially over-expressed in AML compared with normal hematopoietic cells, we leveraged genomic and transcriptomic data and discovered *CD99*.

*CD99*, a membrane glycoprotein,<sup>2</sup> is normally expressed in cortical thymocytes, mature plasma cells, granulosa cells of the ovary, Sertoli cells of the testis, and pancreatic islet cells.<sup>3</sup> The *CD99* gene encodes two distinct proteins that are produced by alternative splicing of the *CD99* transcript. The alternative spliced short isoform results from a deletion in its intracytoplasmic fragment.<sup>2</sup> *CD99* plays a role in cell migration,<sup>4</sup> adhesion, differentiation of thymocytes and T cells,<sup>2</sup> and regulation of diapedesis.<sup>5</sup> In cancer cells, *CD99* is highly expressed on the cell surface of Ewing's sarcoma (EWS),<sup>6</sup> gliomas<sup>7</sup> and other mesenchymal,<sup>8,9</sup> hematopoietic,<sup>10-12</sup> and epithelial cancers.<sup>13,14</sup> In EWS, engagement with the anti-*CD99* antibody enhanced apoptosis and sensitivity to chemotherapy.<sup>15</sup> High *CD99* correlated with enhanced invasion of glioma cells.<sup>7</sup>

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CD99 immunoreactivity was found in AML but rarely in myeloproliferative disorders, myelodysplastic syndromes, remission, and normal marrow samples.<sup>16</sup> A recent study, however, showed that CD99 is a disease stem cell marker, and CD99 antibody proved beneficial in xenograft mice models of myeloid malignancies.<sup>17</sup> With growing evidence that CD99 plays a role in cancer, and particularly in AML, and that CD99 isoforms are differentially expressed and play different roles in different hematopoietic cells,<sup>18</sup> investigating the roles of the two isoforms is crucial for CD99 preclinical development as a therapeutic target. Here we characterize CD99 upregulation in patients with AML and its association with clinical and molecular characteristics, and determine the function of CD99 long (L) and short (S) isoforms in preclinical leukemia models.

## Methods

### Patients' samples

Diagnostic or relapse blood was obtained from AML patients treated at the Norris Comprehensive Cancer Center at the University of Southern California (USC) after obtaining written informed consent. The use of human materials was approved by the Institutional Review Boards of the USC in accordance with the Declaration of Helsinki.

### Patient datasets and gene expression analysis

The Cancer Genome Atlas (TCGA) AML dataset was downloaded from oncomine.<sup>19,20</sup> Patients data from the GSE7186,<sup>21</sup> GSE13159,<sup>22</sup> GSE1159,<sup>23</sup> GSE15434,<sup>24</sup> GSE3077,<sup>25</sup> GSE425,<sup>26</sup> GSE12417,<sup>27</sup> and GSE17855<sup>28</sup> datasets were downloaded from the GEO database. Details of analysis methods are available in the *Online Supplementary Appendix*.

### Cell lines and primary blasts

Acute myeloid leukemia cell lines were grown in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% fetal bovine serum (FBS) and 100U/mL penicillin. Peripheral blood mononuclear cells (PBMC) from AML patients and from healthy donors were isolated by density gradient centrifugation using Ficoll-Paque. Primary cells were grown in RPMI plus 20% FBS and cytokine cocktails CC100 (Flt3L, SCF, IL-3 and IL-6).

### In vivo studies

Animal protocols were approved by the Institution for Animal Care and Use Committee (IACUC) of the USC. For THP-1 and MOLM-13 xenograft experiments,  $2.5 \times 10^6$  cells were injected *via* tail-vein (IV) into 4-6 week-old NOD-*scid* /*Il2rg*<sup>-/-</sup> (NSG) mice (Jackson). For the primary blasts xenograft experiment,  $1 \times 10^6$  cells were IV engrafted into irradiated mice. Details of the methods used, including plasmids, primer sequences, antibodies and *in vivo* experiments are available in the *Online Supplementary Appendix*.

## Results

### CD99 is up-regulated in acute myeloid leukemia

In an effort to identify target genes that were differentially over-expressed in AML, we compared gene expression profiles between normal and AML cells from various available public datasets. We found that CD99 was significantly up-regulated in AML compared with normal cells in five datasets with available measurements of CD99

RNA levels in both leukemia and normal cells (oncomine median ranking among up-regulated measured genes 155,  $P=0.013$ ; TCGA data did not have CD99 levels in normal cells). CD99 was significantly higher in 23 AML samples compared with six normal bone marrow (BM) samples (GSE7186: 3.5-fold,  $P<0.0001$ ) (Figure 1A). Consistently, CD99 was significantly over-expressed in blasts of 542 patients with AML compared with PBMC from 74 healthy donors (HD) (GSE13159: 2-fold,  $P<0.0001$ ) (Figure 1B). Similarly, CD99 expression was 1.8-fold higher in blasts from 285 patients with AML compared with HD cells (GSE1159:  $P=0.003$ ) (*Online Supplementary Figure S1A*). There was a 2.3-fold increase in CD99 expression (GSE13164:  $P<0.001$ ) (*Online Supplementary Figure S1B*) in blasts of 257 AML patients compared with PBMC of 58 HD. In the GSE995 dataset, CD99 expression was 3-fold higher in blasts from nine AML patients compared with that in cells from six HD ( $P=0.028$ ) (*Online Supplementary Figure S1C*). We also analyzed the expression of CD99 protein coding transcripts in patients with AML (GSE106291;  $n=246$ ). ENST00000381192.10 (CD99-L) and ENST00000611428.5 (CD99-S) were the top expressed transcripts (Figure 1C). The combined expression of ENST00000482405.7 and ENST00000611428.5 (both code for CD99-S) was significantly higher ( $P=0.0073$ ) than the level of ENST00000381192.10 (CD99-L) (*Online Supplementary Figure S1D*). Transcript Ensembl IDs are described in *Online Supplementary Table S1*. The transcript expression of the two isoforms were positively correlated (*Online Supplementary Figure S1E* and *F*).

Several reports suggested that CD99 is up-regulated on leukemia stem cells.<sup>29-31</sup> To validate this, we assessed CD99 expression in the GSE3077 dataset that has gene expression data of leukemia blasts obtained from 23 patients with AML sorted according to their CD34 and CD38 expression levels. We found that CD99 expression was significantly higher (1.34-fold,  $P<0.001$ ) (Figure 1D) in the CD34<sup>+</sup>CD38<sup>+</sup> and CD34<sup>+</sup>CD38<sup>-</sup> combined subpopulation compared with CD34<sup>-</sup>CD38<sup>-</sup> and CD34<sup>-</sup>CD38<sup>+</sup> combined.

### CD99 overexpression is positively associated with FLT3-ITD and reversely with TP53 mutations

We assessed the association between CD99 upregulation and the presence of AML mutations. In the TCGA dataset, CD99 expression was significantly higher ( $P=0.004$ ) in patients with FLT3-ITD ( $n=37$ ) compared with patients with FLT3 point mutations ( $n=16$ ) and FLT3 wild-type ( $n=133$ ) (1.3 fold,  $P=0.002$ ) (Figure 1E). Similarly, there was a significant association between high CD99 (above the median) and the presence of FLT3-ITD mutation (Fisher's exact test,  $P=0.04$ ) (*Online Supplementary Table S2*). A similar association was found in the GSE17855 and GSE15434 datasets with childhood AML and patients with cytogenetically normal karyotype, respectively. CD99 is significantly higher in patients with FLT3-ITD compared with patients with FLT3 wild-type (FLT3-WT) (1.3-fold,  $P=0.03$ , *Online Supplementary Figure S1G*; 1.4-fold,  $P<0.001$ , *Online Supplementary Figure S1H*; respectively). We next examined the association of each CD99 transcript with FLT3-ITD mutation. ENST00000381187.8 and ENST00000624481.4 were significantly higher in FLT3-ITD compared with FLT3-WT patients ( $P=0.001$  and  $P=0.002$ ) (*Online Supplementary Figure S1I-N*). ENST00000611428.5 (CD99-S) was not significantly associated with FLT3-ITD after adjusting for

multiple hypotheses testing (unadjusted  $P=0.049$ ).

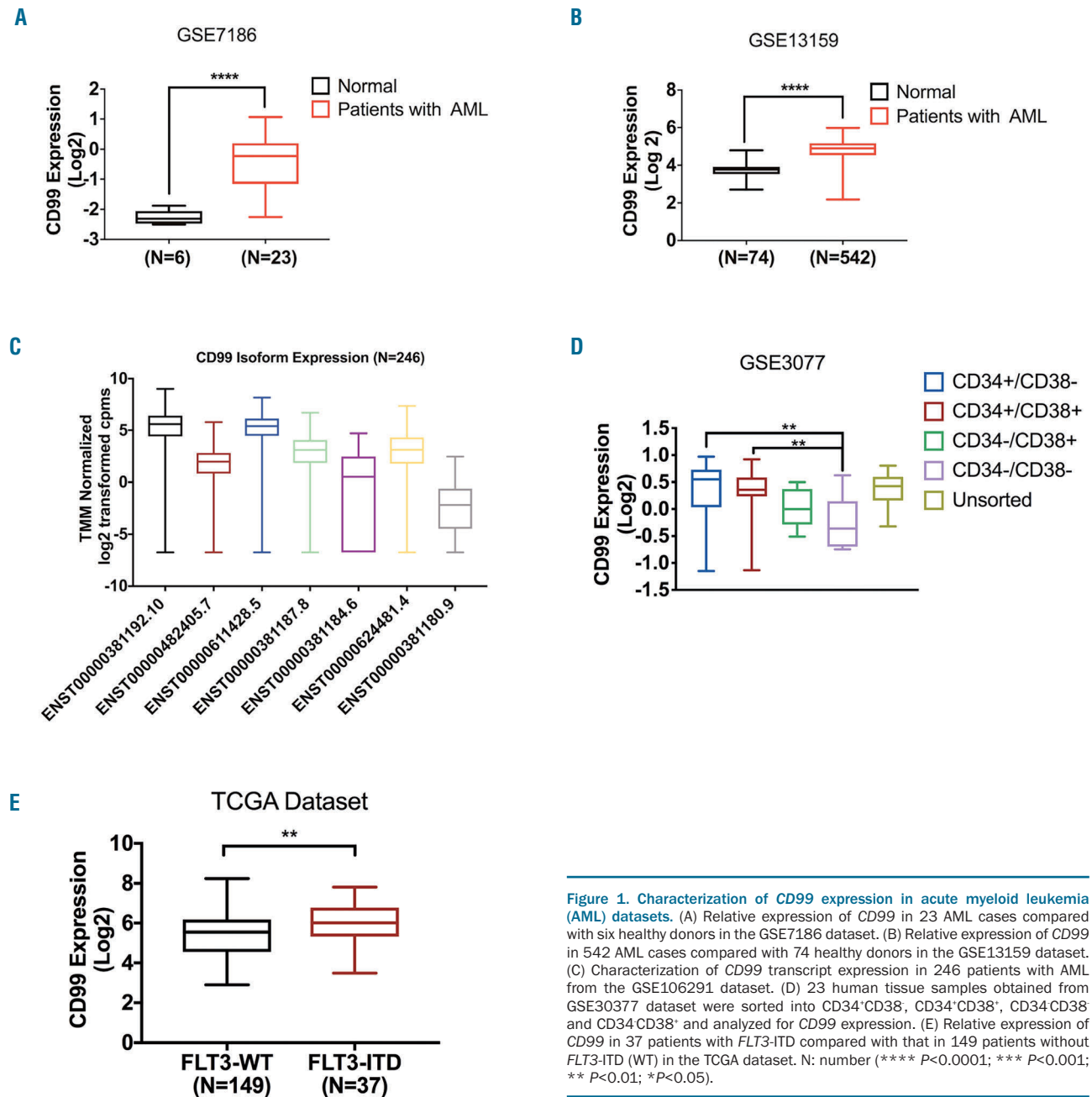
*CD99* expression was significantly lower (1.9-fold,  $P<0.001$ ) in patients with mutated *TP53* ( $n=15$ ) compared with patients with *TP53*-WT ( $n=171$ ) (Online Supplementary Figure S1O; Fisher's Exact test,  $P=0.0006$ , Online Supplementary Table S1). *CD99* expression was not associated with other mutations (Online Supplementary Table S2 and Online Supplementary Figure S2).

**CD99 expression levels according to patients' clinical characteristics**

We dichotomized patients into high and low groups based on *CD99* median expression. Patients with *CD99*-low had a significantly lower percentage of bone marrow (BM) blasts (median, 66.2 vs. 72.5,  $P=0.024$ ), peripheral

blood (PB) blasts (median, 23.7 vs. 50.7,  $P<0.0001$ ), and white blood cell (WBC) count (median, 27.7 vs. 45.8,  $P=0.019$ ). The median age was 57.8 years in *CD99*-low and 52.1 years in *CD99*-high groups (Online Supplementary Table S3).

According to the National Comprehensive Cancer Network (NCCN), AML is classified into favorable, intermediate and poor-risk groups based on patients molecular and cytogenetic characteristics. *CD99* expression was significantly higher in the favorable-risk group than that in the intermediate-risk (1.2-fold,  $P<0.01$ ) and poor-risk (2-fold,  $P<0.0001$ ) groups (Figure 2A). According to the AML French-American-British (FAB) classification data, patients with M5-AML had lower *CD99* expression compared with M1, M2, M3 and M4 (Online Supplementary Figure



**Figure 1. Characterization of *CD99* expression in acute myeloid leukemia (AML) datasets.** (A) Relative expression of *CD99* in 23 AML cases compared with six healthy donors in the GSE7186 dataset. (B) Relative expression of *CD99* in 542 AML cases compared with 74 healthy donors in the GSE13159 dataset. (C) Characterization of *CD99* transcript expression in 246 patients with AML from the GSE106291 dataset. (D) 23 human tissue samples obtained from GSE30377 dataset were sorted into CD34<sup>+</sup>CD38<sup>-</sup>, CD34<sup>+</sup>CD38<sup>+</sup>, CD34<sup>-</sup>CD38<sup>+</sup> and CD34<sup>-</sup>CD38<sup>-</sup> and analyzed for *CD99* expression. (E) Relative expression of *CD99* in 37 patients with *FLT3*-ITD compared with that in 149 patients without *FLT3*-ITD (WT) in the TCGA dataset. N: number (\*\*\*\*  $P<0.0001$ ; \*\*\*  $P<0.001$ ; \*\*  $P<0.01$ ; \* $P<0.05$ ).

S3A). Based on leukemia cytogenetics, no significant differences in *CD99* expression was found in patients with complex karyotype, Inv (16), t (15; 17), t (8,21), del (7q) / 7q- and trisomy 8, compared with normal karyotype (Online Supplementary Figure S3B).

### CD99 overexpression is associated with better clinical outcome

The overall survival (OS) of *CD99*-high patients (based on median cut-off) was significantly longer than that of *CD99*-low patients (median: 27 vs. 11.2 months;  $P=0.0026$ ) (Figure 2B). Patients with t(15;17) are treated with all-trans retinoic acid (ATRA) and have a better outcome, thus they were excluded from the survival analyses. When patients were stratified into cytogenetically normal (CN) and cytogenetically abnormal (CA), we found that in CA-AML, but not in CN-AML, *CD99*-high survived significantly longer than *CD99*-low patients (CA-AML: median OS: 32.3 vs. 11 months,  $P=0.02$ , Figure 2C; CN-AML:  $P=0.24$ , Online Supplementary Figure S4A). Yet, there was no significant difference in *CD99* levels between CN-AML and CA-AML ( $P=0.33$ ) (Online Supplementary Figure S4B). There was no significant difference in event-free survival (EFS) between *CD99*-high and *CD99*-low patients (median: 15.6 vs. 13.3 months;  $P=0.13$ ) (Online Supplementary Figure S4C-E).

In the GSE425 dataset of 71 patients with CA-AML, *CD99*-high patients survived significantly longer than *CD99*-low patients ( $P=0.04$ ) (Online Supplementary Figure S5B). No significant difference in OS was found when we

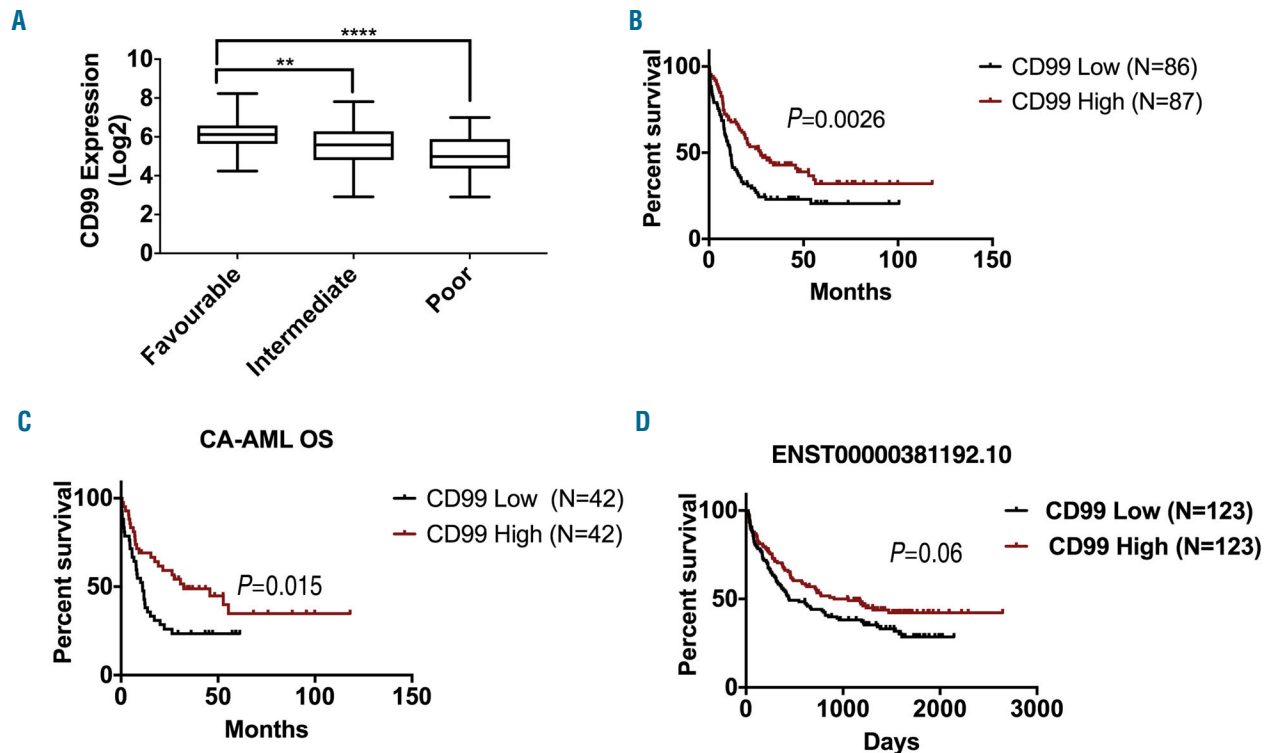
included CN-AML patients or analyzed them separately (Online Supplementary Figure S5A and C). In the GSE12417 dataset, which includes only CN-AML patients, no significant difference between *CD99*-high and *CD99*-low patients ( $n=163$ ) was observed (Online Supplementary Figure S5D).

In a multivariable analysis, high *CD99* was not significantly associated with OS when adjusted by age, cytogenetic risk, transplant status, *DNMT3A* mutation status, and *TP53* mutation status ( $P=0.364$ ) (Online Supplementary Table S4).

We also assessed the association of *CD99* transcript upregulation with OS in 246 patients with AML using median cut-off for each transcript. High ENST00000381192.10 (*CD99*-L) exhibited a trend of association with better OS (GSE106291: median OS: 908 vs. 445 days;  $P=0.06$ ) (Figure 2D). No difference in survival between high and low expression was observed for the other transcripts (Online Supplementary Figure S6A-G).

### Differential effect of CD99 isoform expression on leukemia growth

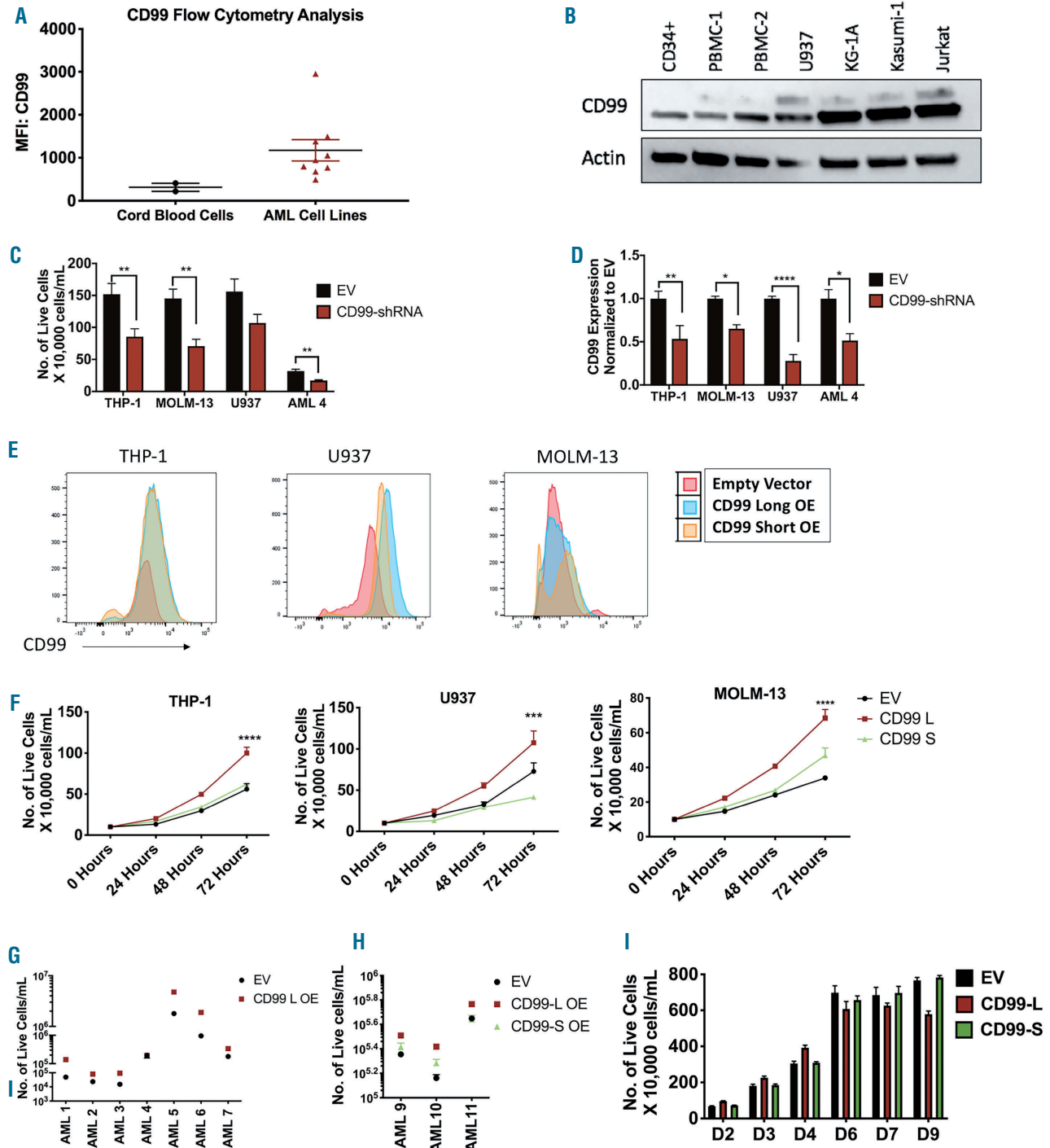
In a panel of AML cell lines (KG-1, KG-1A, MOLM13, MV4-11, Kasumi-1, THP-1, NB4, U937, UOC-M1), *CD99* surface levels are higher than that of healthy cord blood cells (Figure 3A). Western blots revealed 28 and 32kDa bands corresponding to *CD99*-L and -S in U937, KG-1A and Kasumi-1 cells, but only the lower band was recognized in THP-1, MOLM-13, *CD34*<sup>+</sup> cells and two HD BMC lysates (Figure 3B and Online Supplementary Figure



**Figure 2.** Association of *CD99* expression with patients' clinical characteristics in acute myeloid leukemia (AML) datasets. (A) Relative expression of *CD99* according to the National Comprehensive Cancer Network (NCCN) risk status classification in the The Cancer Genome Atlas (TCGA) dataset. (B) Overall survival (OS) of 173 patients grouped based on *CD99* median expression into *CD99* high ( $n=87$ ) and *CD99* low ( $n=86$ ) from the TCGA dataset. (C) OS of cytogenetically abnormal (CA-AML) cases grouped based on *CD99* median expression into *CD99* high ( $n=42$ ) and *CD99* low ( $n=42$ ). (D) OS of 146 patients grouped based on ENST00000381192.10 transcript isoform based on median expression into high ( $n=123$ ) and low ( $n=123$ ) from the GSE106291 dataset. (\*\*\*\* $P<0.0001$ ; \*\* $P<0.01$ ).

S7A). Similarly, quantitative polymerase chain reaction (qPCR) analysis showed that *CD99* transcripts were up-regulated in AML blast samples (n=9) and AML cell lines (KG-1a, U937, THP-1, and MOLM-13) compared with

HD PBMC (n=3) and CD34<sup>+</sup> cells. Transcript ENST00000482405.7 is higher in CD34<sup>+</sup> cells than in AML cell lines (Online Supplementary Figure S7B; for qPCR primers see Online Supplementary Table S5).



**Figure 3. Effect of CD99 isoform expression on acute myeloid leukemia (AML) cell proliferation.** (A) Relative CD99 expression in cord blood cells (n=2) and AML cell lines (n=9) measured by flow cytometry. (B) Western blot analysis of CD99 isoform expression in CD34<sup>+</sup> cells, healthy donor peripheral blood mononuclear cells (PBMC) and AML cell lines. (C) Viability of CD99-shRNA knockdown in THP-1, MOLM-13, U937 and AML-4 cells measured 96 hours (h) after transfection using trypan-blue and alamar-blue. (D) Quantitative polymerase chain reaction (qPCR) analysis using *CD99* TaqMan assay to confirm *CD99* knockdown in THP-1, MOLM-13, U937 and AML-4 cells transfected with CD99-shRNA plasmid (or EV) for 96 h. CD99 expression was normalized to EV cells. (E) CD99 expression in EV, CD99-L OE and CD99-S OE cells measured by flow cytometry in THP-1, U937 and MOLM-13 cells. (F) Proliferation assay of EV, CD99-L and CD99-S measured at 24, 48 and 72 h by trypan-blue (n=4) in THP-1, U937, and MOLM-13 cells. (G) Viability of AML blasts over-expressing CD99-L or EV measured 96 h after lentiviral transduction using trypan-blue (n=7). Data reported as total number of live cells/mL. (H) Viability of AML blasts over-expressing CD99 L, CD99-S or EV measured 96 h after lentiviral transduction using trypan-blue (n=3). Data reported as the number of live cells/mL. (I) Long-term proliferation assay of EV, CD99-L and CD99-S cells measured at days 2, 3, 4, 6, 7 and 9 by trypan-blue assay. (\*\*\*\**P*<0.0001; \*\*\**P*<0.001; \*\**P*<0.01; \**P*<0.05).

We then examined the effect of *CD99* knockdown on cell viability. Transducing cells with lentiviral-*CD99*-shRNA significantly reduced primary AML blast viability ( $n=4$ ) (Figure 3C and *Online Supplementary Figure S7C*) and AML cell lines (THP-1, MOLM-13, and U937) (40-60%;  $P<0.05$ ) (Figure 3C). Knockdown was confirmed by qPCR (Figure 3D) and western blot (*Online Supplementary Figure S7D*). THP-1 and MV4-11 cells had an approximately 35-50% decrease in cell viability when transiently transfected by electroporation with *CD99*-siRNA compared with negative-control-siRNA ( $P=0.001$  and  $P=0.001$ , respectively) (*Online Supplementary Figure S7E-G*).

We also established a gain-of-function approach to study *CD99*-L and *CD99*-S isoform functions. We performed lentiviral transduction to over-express *CD99*-L and *CD99*-S in THP-1, U937 and MOLM-13 cells expressing variable endogenous levels of *CD99* isoforms (Figure 3E and *Online Supplementary Figure S7H and I*). *CD99*-L transduced cells had increased cell proliferation at 72 hours (h) compared with their respective empty vector (EV) controls and *CD99*-S transduced cells, respectively, counted by trypan-blue in THP-1 (1.78-fold,  $P<0.001$ ; 1.61-fold,  $P<0.01$ ), U937 (1.47-fold,  $P<0.001$ ; 2.59-fold,  $P<0.0001$ ), and MOLM-13 cells (2-fold,  $P<0.0001$ ; 1.45-fold,  $P<0.0001$ ) (Figure 3F). This was also confirmed by alamar-blue assay (*Online Supplementary Figure S8A*) and by BrdU staining (1.6-fold,  $P=0.006$ ) (*Online Supplementary Figure S8B*) suggesting enhanced metabolic activity and DNA synthesis in these cells, respectively.

We also ectopically over-expressed *CD99*-L in AML blasts ( $n=7$ ) (*Online Supplementary Table S6*) using lentiviral transduction. Overexpression of *CD99*-L was confirmed using western blot and fluorescence microscopy for green fluorescent protein (GFP) (*Online Supplementary Figure S8C and D*). Higher cell number was observed in lenti-*CD99* blasts compared with lenti-EV transduced blasts 96 h after viral transduction (2.8-fold,  $P<0.0001$ ) (Figure 3G). We also observed a modest increase in the number of colonies on day 14 in 3 of 6 patient samples over-expressing *CD99*-L compared with their respective controls: AML-3 (6 vs. 15,  $P=0.02$ ), AML-4 (6 vs. 14,  $P=0.02$ ), and AML-5 (42 vs. 128,  $P=0.001$ ) (*Online Supplementary Figure S8E*). Furthermore, we ectopically expressed *CD99*-L and *CD99*-S in three additional AML blast samples (*Online Supplementary Figure S8F*). Cell viability was measured 96 h after transduction using trypan-blue and alamar-blue. A higher number of live cells was observed in *CD99*-L transduced blasts compared with lenti-EV (1.5-fold,  $P<0.0001$ ) and *CD99*-S transduced blasts (1.3-fold,  $P<0.0001$ ) (Figure 3H). More colonies were observed in one sample over-expressing *CD99*-L compared with their respective controls; AML-10 (49.5 vs. 101.5,  $P=0.04$ ) (*Online Supplementary Figure S8G*).

However, long-term culture of *CD99*-L transduced cells showed a subsequent drop in cell viability, and cells could not be maintained in culture for more than 4-6 weeks. To validate this, we performed a long-term culture assay for ten days starting approximately two weeks post viral transduction. Proliferation of *CD99*-L cells started to decline by day 5 of initial serum stimulation compared with EV and *CD99*-S cells, even when cell density and nutrients were accounted for (Figure 3I). In  $CD34^+$  cells, we observed no significant change in the number of viable cells between cells transduced with *CD99*-L, -S isoform or EV (*Online Supplementary Figure S8H*) at 96 h post transduction. Transducing cells with lentiviral-*CD99*-shRNA

resulted in a slight decrease in cell viability (*Online Supplementary Figure S8I*).

### Ectopic expression of *CD99* long isoform enhances reactive oxygen species levels, DNA damage, and induces cell apoptosis

Because the initial enhanced proliferation of *CD99*-L cells was serum induced and reversed with further expansion *in vitro*, we speculated that the serum-induced cell growth would stimulate higher production of reactive oxygen species (ROS) in these cells. Indeed, THP-1 cells transduced with *CD99*-L exhibit a 2.5- and 1.6-fold increase in ROS levels compared with *CD99*-S and EV cells, respectively (Figure 4A and B). Because of their higher ROS levels, we asked whether DNA damage is increased in these cells. Western blot analysis showed that *CD99*-L cells exhibit a higher level of the DNA damage marker H2Axy compared with *CD99*-S and EV cells (Figure 4C). Furthermore, apoptosis was also enhanced in *CD99*-L transduced cells measured by annexin-V staining in THP-1 (*CD99*-L vs. EV: 3.48-fold,  $P=0.001$ ; *CD99*-L vs. *CD99*-S: 6.32-fold,  $P=0.0027$ ), U937 (*CD99*-L vs. EV: 3.26-fold,  $P=0.07$ ; *CD99*-L vs. *CD99*-S: 3.67-fold,  $P=0.10$ ), and MOLM-13 cells (*CD99*-L vs. EV: 4.88-fold,  $P=0.0032$ ; *CD99*-L vs. *CD99*-S: 4.2-fold,  $P=0.0042$ ) (Figure 4D and E). The enhanced apoptosis was also confirmed by the increase of cleaved caspase-3 (Figure 4F).

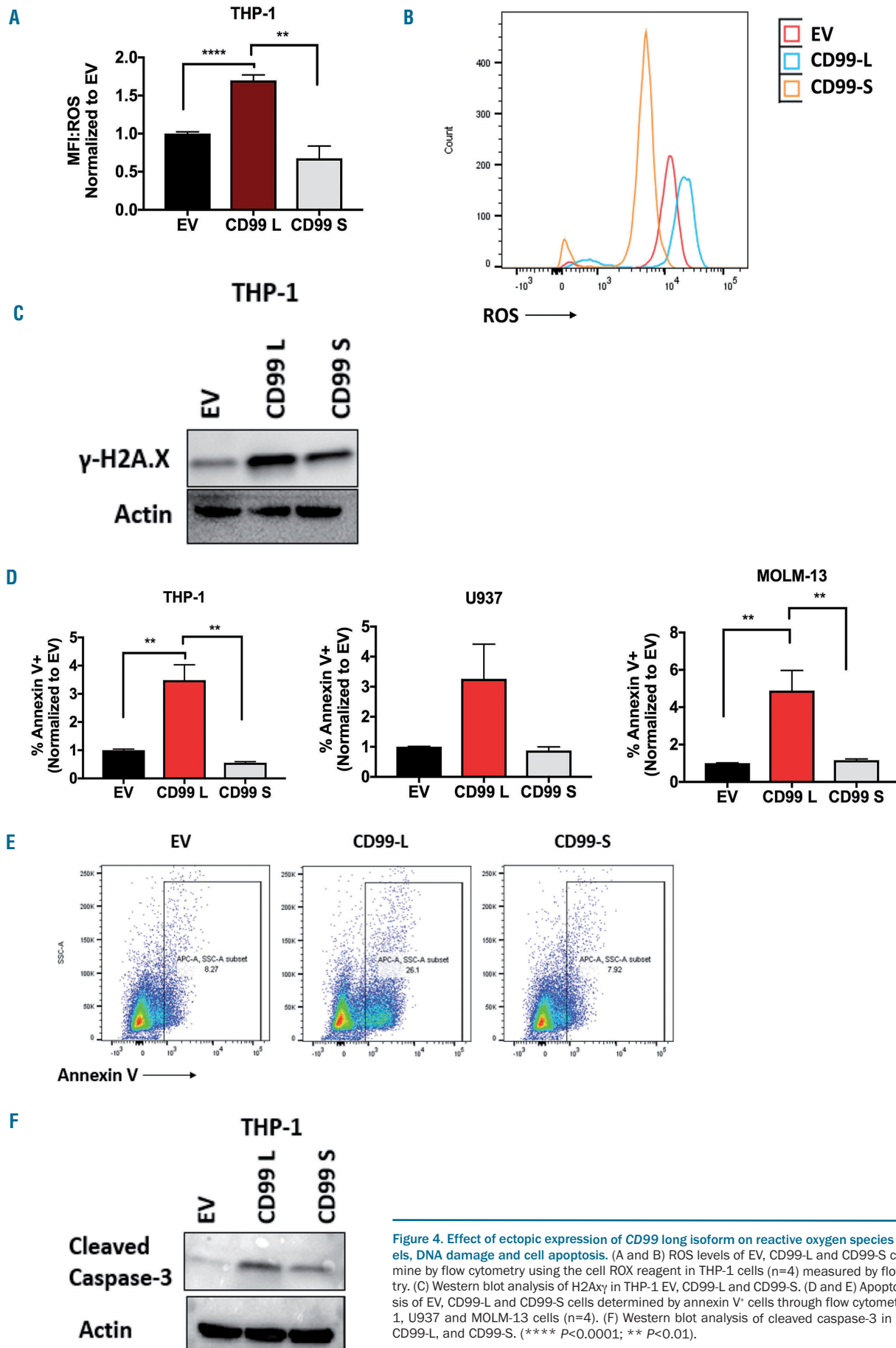
### Ectopic expression of *CD99*-L isoform induces myeloid differentiation and reduces cell migration

Previous studies have demonstrated that *CD99* homotypic interaction in *CD99* expressing cells<sup>32-34</sup> plays a role in monocyte trans-endothelial migration.<sup>4</sup> Thus, we asked which isoform is responsible for cell homotypic interaction, and whether they affect cell migration and myeloid differentiation differently. Cells were seeded at  $1 \times 10^5$  cells/mL per well in a 6-well-plate and images were taken 6 h later; *CD99*-L THP-1 cells displayed higher cell aggregation compared with EV and *CD99*-S cells (Figure 5A). *CD99*-L cells exhibit decreased migration towards SDF-1 $\alpha$  in a transwell chamber compared with EV and *CD99*-S cells; THP-1 (70%,  $P<0.0001$ ; 66%,  $P<0.0001$ ), U937 (80%,  $P<0.0001$ ; 83%,  $P<0.0001$ ), and MOLM-13 (80%,  $P<0.0001$ ;  $P=0.0032$ ) (Figure 5B and C).

*CD99*-L expressing cells showed an increase in *CD11b* surface marker measured by flow cytometry 24 h after cells were seeded in THP-1 (*CD99*-L vs. EV: 2-fold,  $P=0.0027$ ; *CD99*-L vs. *CD99*-S: 1.63-fold,  $P=0.043$ ), U937 (*CD99*-L vs. EV: 1.56-fold,  $P=0.01$ ; *CD99*-L vs. *CD99*-S: 1.29-fold,  $P=0.11$ ), and MOLM-13 (*CD99*-L vs. EV: 1.89-fold,  $P<0.0001$ ; *CD99*-L vs. *CD99*-S: 1.68-fold,  $P<0.079$ ) (Figure 5D).

### Ectopic expression of *CD99* long isoform delayed leukemia engraftment in acute myeloid leukemia murine models

Next, we investigated the effect of ectopic expression of *CD99* isoforms in murine leukemia models. THP-1 cells stably over-expressing *CD99*-L ( $n=6$ ), *CD99*-S ( $n=3$ ), or EV ( $n=6$ ) were injected into NSG mice. Mice were sacrificed on day 30-32 post implantation. Mice engrafted with *CD99*-L cells had smaller spleens compared with EV mice (0.04g vs. 0.07g,  $P=0.010$ ) and *CD99*-S mice (0.04g vs. 0.06g,  $P=0.0003$ ) (Figure 6A and B). hCD45 flow analysis revealed that *CD99*-L mice had significantly less BM engraftment

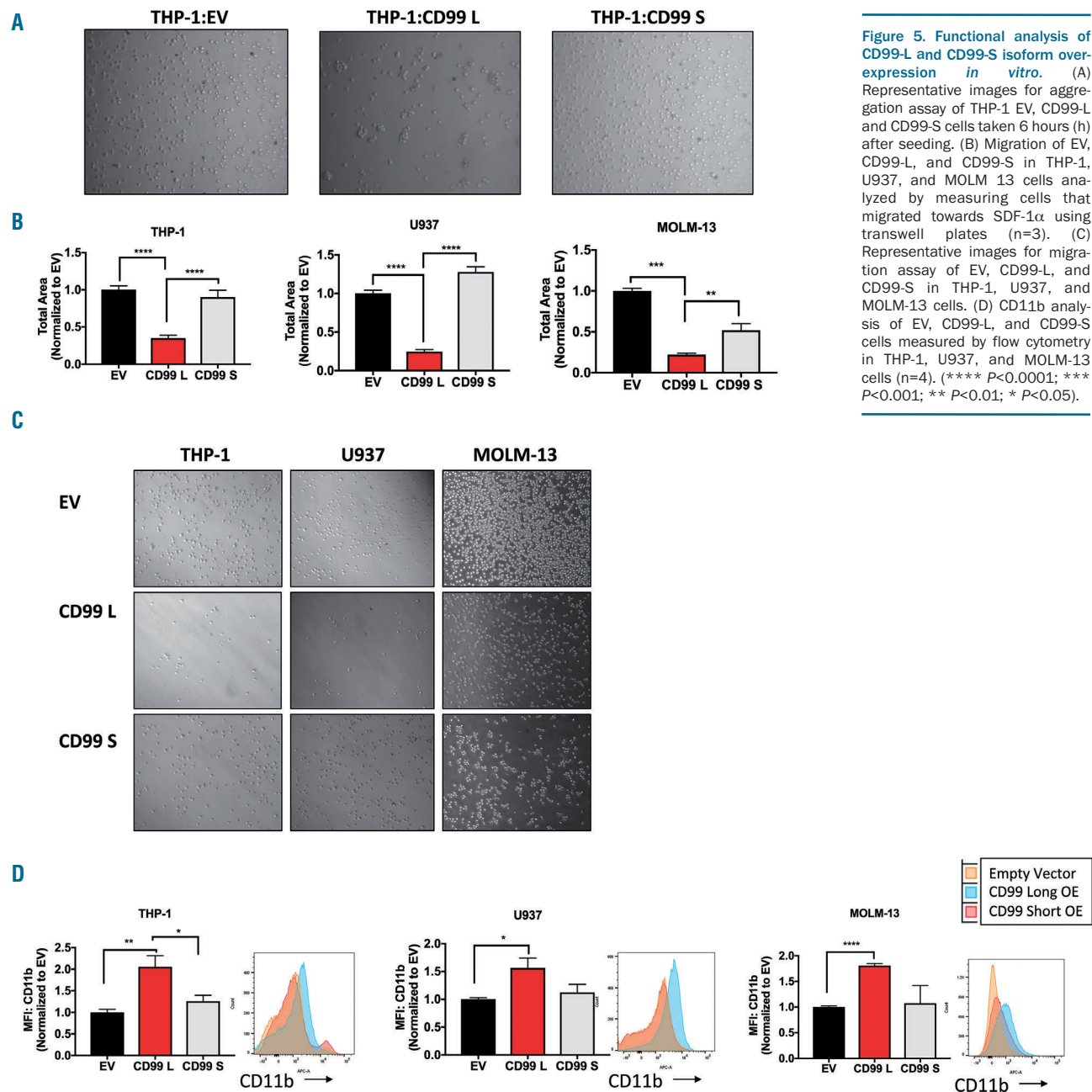


**Figure 4.** Effect of ectopic expression of CD99 long isoform on reactive oxygen species (ROS) levels, DNA damage and cell apoptosis. (A and B) ROS levels of EV, CD99-L and CD99-S cells determined by flow cytometry using the cell ROX reagent in THP-1 cells (n=4) measured by flow cytometry. (C) Western blot analysis of H2A $\gamma$  in THP-1 EV, CD99-L and CD99-S. (D and E) Apoptosis analysis of EV, CD99-L and CD99-S cells determined by annexin V<sup>+</sup> cells through flow cytometry in THP-1, U937 and MOLM-13 cells (n=4). (F) Western blot analysis of cleaved caspase-3 in THP-1 EV, CD99-L, and CD99-S. (\*\*\*\*  $P < 0.0001$ ; \*\*  $P < 0.01$ ).

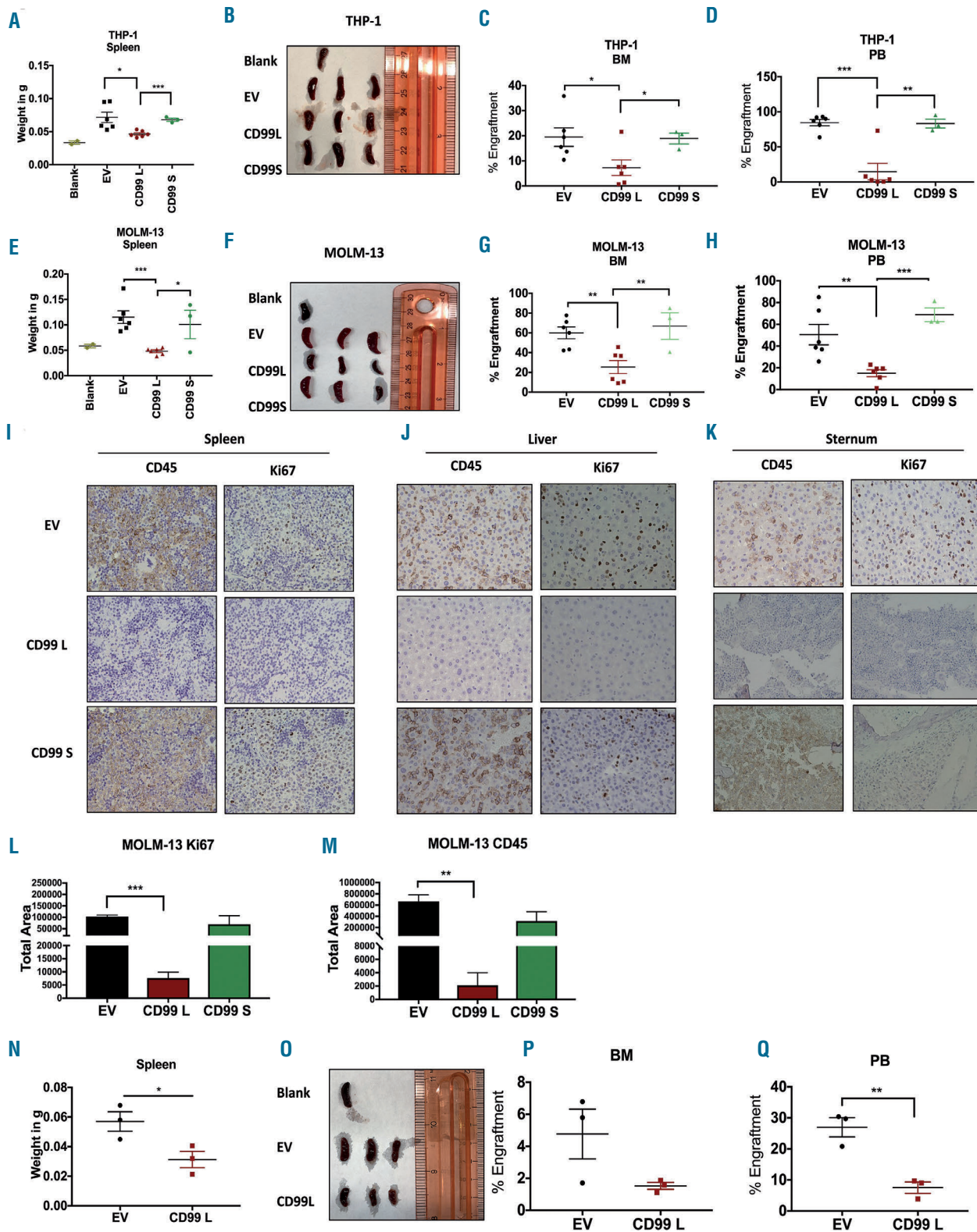
compared with EV (7.29% vs. 19.47%;  $P=0.02$ ) and CD99-S mice (7.29% vs. 18.9%;  $P=0.04$ ) (Figure 6C). CD99-L mice had significantly less PB engraftment compared with EV (14.73% vs. 84.52%;  $P=0.0002$ ) and CD99-S mice (14.73% vs. 83.3%;  $P=0.006$ ) (Figure 6D and *Online Supplementary Figure S9*). No difference in engraftment was seen in the liver (*Online Supplementary Figure S10A*) or spleen (*Online Supplementary Figure S10B*) between groups.

In the MOLM-13 murine model, mice were engrafted with CD99-L, CD99-S and EV mice ( $n=6$  for EV and CD99-L, and  $n=3$  for CD99-S) and were sacrificed on day 19 post transplantation. Mice engrafted with CD99-L cells had smaller spleens than EV mice (0.048g vs. 0.11g,  $P=0.004$ ) and CD99-S mice (0.048g vs. 0.10g,  $P=0.026$ ) (Figure 6E and F). CD99-L mice had significantly less engraftment than the EV mice in the BM (25.38% vs. 59.57%,  $P=0.003$ ) (Figure

6G), PB (15.03% vs. 50.5%,  $P=0.0051$ ) (Figure 6H), and liver (21.6% vs. 44.03%,  $P=0.011$ ) (*Online Supplementary Figures S10C* and *S11*). No difference in engraftment was seen in spleen (*Online Supplementary Figure S10D*). Additionally, to determine whether mice engrafted with CD99-L cells developed deadly leukemia, two CD99-L engrafted mice were sacrificed only when mice appeared sick (days 29 and 44). We observed PB engraftment in both mice and in the BM of one mouse (*Online Supplementary Figure S10*). Hematoxylin & Eosin staining of the liver, spleen and sternum showed that EV and CD99-S tissues presented a higher percentage of infiltrated blasts compared with the CD99-L mice tissues (*Online Supplementary Figure S10*). We also found higher CD45 immunostaining (CD99-L vs. EV,  $P=0.004$ ; CD99-L vs. CD99-S,  $P=0.13$ ) and Ki67 staining (CD99-L vs. EV,  $P=0.0002$ ; CD99-L vs. CD99-S,  $P=0.17$ ) in





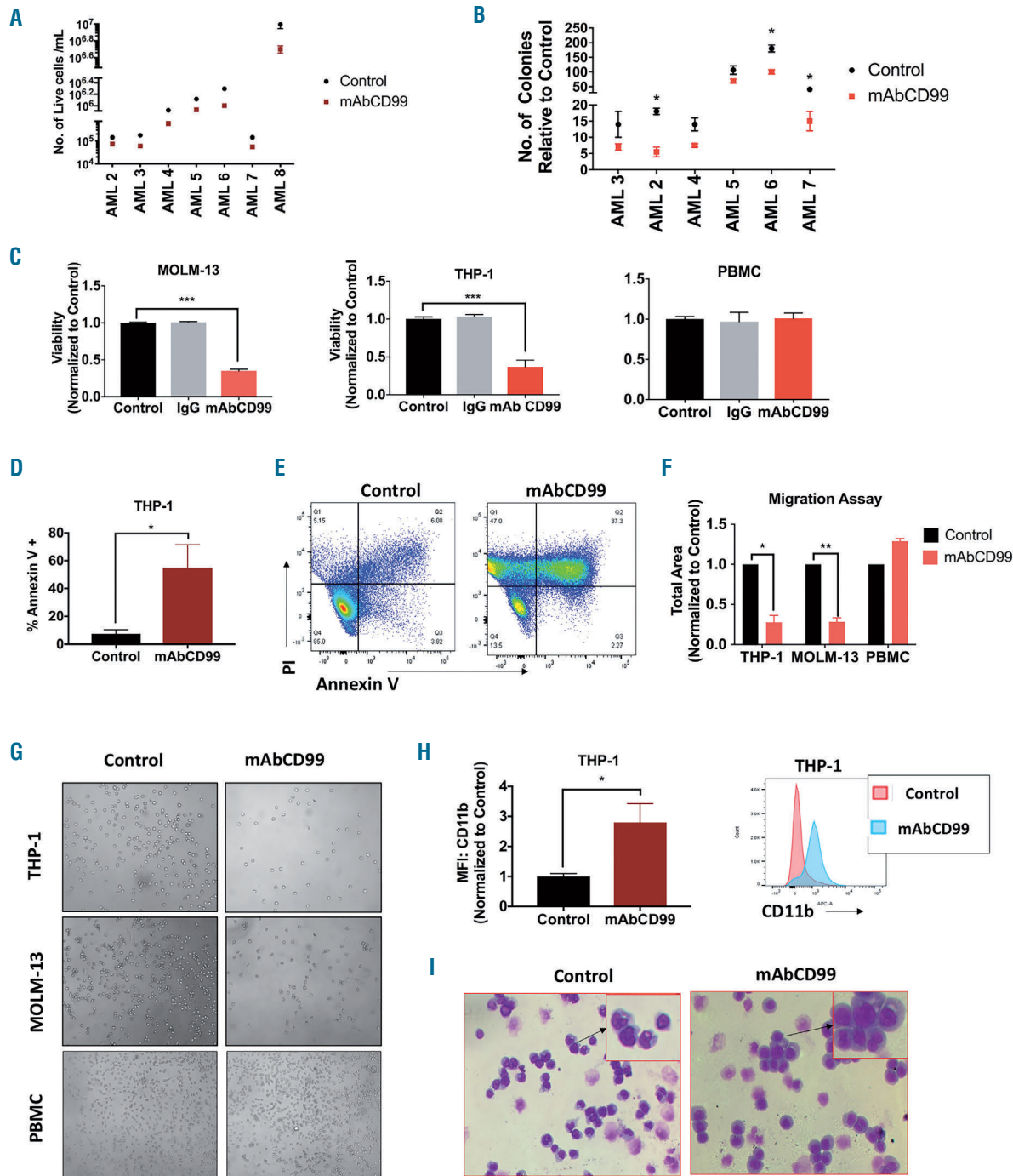


**Figure 6. Effect of CD99-L and CD99-S isoform overexpression in acute myeloid leukemia (AML) murine models.**  $2.5 \times 10^6$  THP-1 EV (n=6), CD99-L (n=6), or CD99-S (n=3) cells were implanted in mice and sacrificed when sick (days 30-32 post implantation). (A and B) Spleen weight and images of blank, EV, CD99-L, and CD99-S mice at time of sacrifice. (C and D) Bone marrow (BM) and peripheral blood (PB) engraftment of THP-1 cells in EV, CD99-L and CD99-S mice determined by quantitative analysis of CD45<sup>+</sup> cells through flow cytometry.  $2.5 \times 10^6$  MOLM-13 EV (n=6), CD99-L (n=6), or CD99-S (n=3) cells were implanted in mice and sacrificed when mice were sick. (E and F) Spleen weight and images of blank, EV, CD99-L, and CD99-S mice at time of sacrifice. (G and H) BM and PB engraftment of MOLM-13 cells in EV, CD99-L, and CD99-S mice determined by quantitative analysis of CD45<sup>+</sup> cells through flow cytometry. (I-K) Representative images of immunostaining for CD45 and Ki67 on collected tissues of (I) spleen, (J) liver, and (K) sternum. (L and M) Quantitative analysis of Ki67 and CD45 cells in the liver tissues of mice engrafted with MOLM-13 EV, CD99-L, and CD99-S cells quantified using ImageJ.  $1 \times 10^6$  primary AML cells over-expressing EV (n=3) or CD99-L (n=3) cells were engrafted in mice and sacrificed when mice were sick (day 19 post-cell transplantation). (N and O) Spleen weight and images of EV and CD99-L mice at the time of sacrifice (4-months post transplantation). (P and Q) BM and PB engraftment of primary AML cells in EV and CD99-L mice determined by quantitative analysis of CD45<sup>+</sup> cells by flow cytometry. (\*\*\*\* $P < 0.0001$ ; \*\*\* $P < 0.001$ ; \*\* $P < 0.01$ ; \* $P < 0.05$ ).

EV and CD99-S tissues compared with CD99-L tissues, confirming a decrease in leukemia engraftment in mice with CD99-L cells (Figure 6I-M).

In a primary blast murine model, we engrafted primary AML samples (AML-4) transduced with CD99-L (n=3) or EV (n=3) in sub-lethally irradiated mice. Mice were sac-

rificed four months post engraftment (Figure 6N-O). CD99-L mice had less BM engraftment, though this was not significant compared with the EV mice (1.52 vs. 4.76%,  $P=0.1$ ) (Figure 6P), and a significant decrease in hCD45<sup>+</sup> cells in PB (7.5% vs. 26.9%,  $P=0.005$ ) (Figure 6Q and *Online Supplementary Figures S10 and S12*).

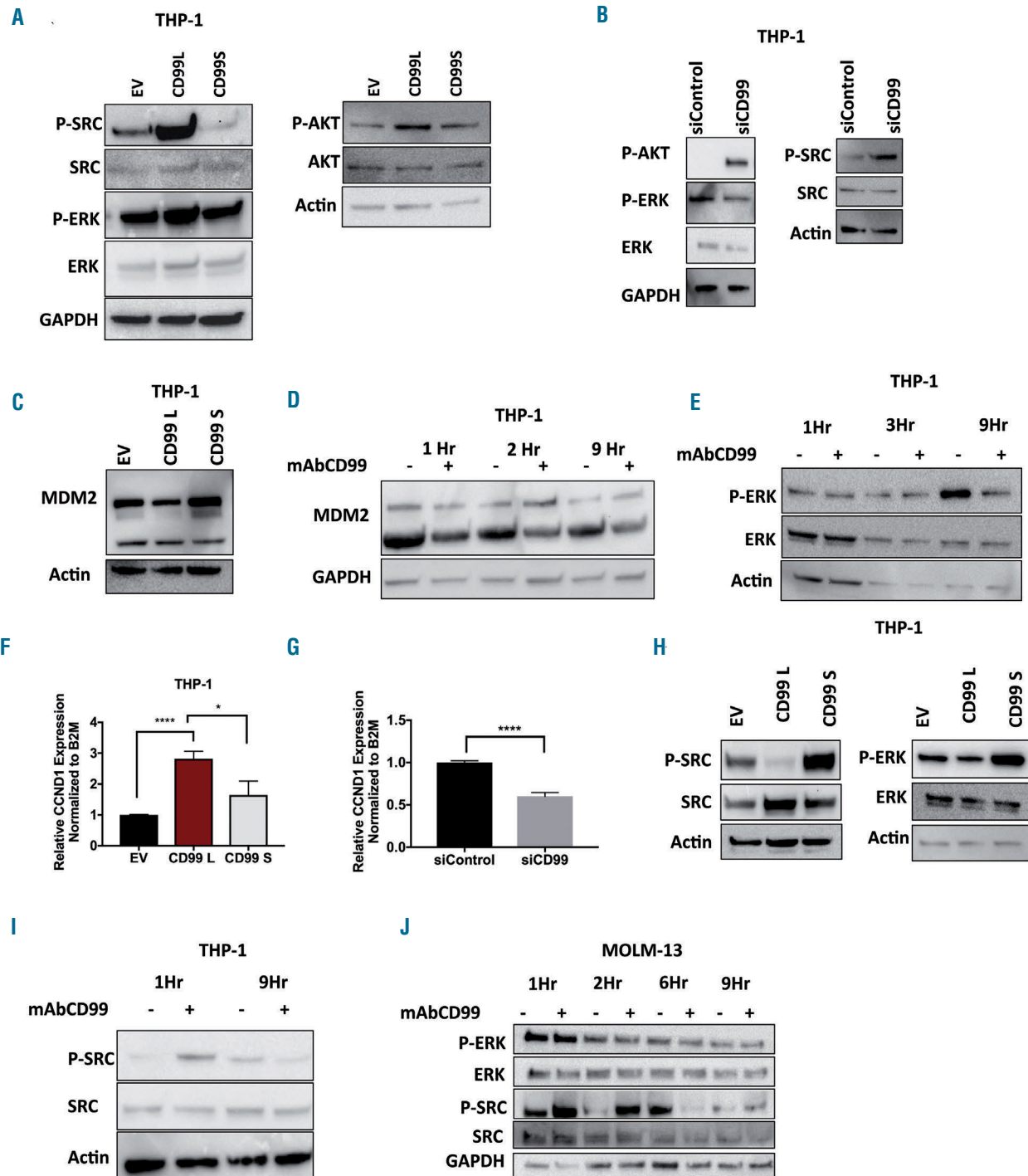


**Figure 7. Effect of CD99 mAb on acute myeloid leukemia (AML) cells.** (A) Cell viability of AML blasts treated with 20µg/mL of mAbCD99 for 48 hours (h) and measured using trypan-blue (n=7). (B) Total number of colonies comparison between AML blasts treated with mAb CD99 and control blasts (n=6). (C) Viability of THP-1, MOLM-13 and healthy donor peripheral blood mononuclear cells (PBMC) treated with 5µg/mL of mAbCD99 for 48 h and measured using alamar blue. (D and E) Apoptosis measured in THP-1 and MOLM-13 cells treated with mAbCD99 for 72 h and stained with Annexin V for flow cytometry analysis. (F and G) Quantitative analysis and representative images of migration of THP-1, MOLM-13 and PBMC treated with mAb CD99 towards SDF-1α performed in a transwell plate. (H) CD11b measured by flow cytometry in THP-1 cells 72 h post treatment with 2.5 µg/mL of mAb CD99. (I) Representative images for Wright-Giemsa staining of THP-1 cells treated with 2.5 µg/mL of mAb CD99 for 72 h. (\*\* $P<0.001$ ; \*\* $P<0.01$ ; \* $P<0.05$ ).

### CD99 monoclonal antibody treatment exhibits antileukemia activity in acute myeloid leukemia cells

Acute myeloid leukemia patient blasts ( $n=7$ ) incubated with monoclonal CD99 antibody (mAbCD99: 20  $\mu\text{g}/\text{mL}$ )

showed a significant decrease in cell viability (48 h:  $0.5 \pm 0.02$ -fold,  $P < 0.0001$ ) (Figure 7A). Treatment with mAbCD99 caused a decrease in colony formation in 3 of 6 patient samples: AML-2 (0.5-fold,  $P=0.02$ ), AML-6 (0.65-



**Figure 8.** Effect of CD99 on ERK and SRC signaling pathways in acute myeloid leukemia (AML) cells. (A) Western blot analysis of SRC and ERK signaling pathways in THP-1 cells transduced with EV, CD99-L, and CD99-S lenti-virus for 72 hours (h). (B) Western blot analysis of SRC and ERK signaling pathways in THP-1 cells transfected with siCD99 for 48 h. (C) MDM2 western blot analysis in THP-1 cells transduced with EV, CD99-L, and CD99-S lenti-virus. (D) MDM2 analysis performed by western blot in THP-1 cells treated with mAbCD99 for 1, 2, and 9 h. (E) ERK signaling pathway analyzed in THP-1 cells treated with mAbCD99 for 1, 3, and 9 h. (F) Cyclin D1 mRNA expression measured by taqman assay in THP-1 cells transduced with EV, CD99-L and <CD99-S lenti-virus. (G) CCND1 mRNA expression measured by taqman assay in THP-1 cells transfected with siCD99. (H) Western blot analysis of SRC and ERK signaling pathways in THP-1 stable cells transduced with EV, CD99-L and CD99-S lenti-virus. (I) SRC signaling pathway analyzed in THP-1 cells treated with mAbCD99 for 1 and 9 h. (J) ERK and SRC signaling pathway analyzed in MOLM-13 cells treated with mAbCD99 for 1, 2, 6, and 9 h. (\*\*\*\* $P < 0.0001$ ; \* $P < 0.05$ ).

fold,  $P=0.03$ ), and AML-7 (0.36-fold,  $P=0.01$ ) (Figure 7B). Treatment with mAbCD99 (5  $\mu\text{g}/\text{mL}$ ) caused a 50% decrease in cell viability at 48 h in THP-1 and MOLM-13 cells but not in healthy donor PBMC ( $P<0.0001$  and  $P<0.001$ , respectively) (Figure 7C). The decrease in viability was accompanied by an increase in Annexin-V apoptosis stain in THP-1 (7.35-fold,  $P=0.04$ ) (Figure 7D and E). Four hours after treatment with mAbCD99 (5  $\mu\text{g}/\text{mL}$ ) we found an approximately 70% decrease in cell migration in both THP-1 and MOLM-13 cells ( $P=0.01$  and  $P=0.004$ , respectively) (Figure 7F and G). Treatment of THP-1 cells with mAbCD99 triggered an increase in CD11b<sup>+</sup> population, indicative of myeloid differentiation (2.79-fold,  $P=0.04$ ) (Figure 7H). Consistently, Wright-Giemsa stain revealed that mAbCD99 (2.5  $\mu\text{g}/\text{mL}$ ) induced differentiation with morphology resembling more mature cell fates (Figure 7I).

### CD99 modulates ERK and SRC signaling pathways in acute myeloid leukemia cells

Because the initial enhanced proliferation of CD99-L expressing cells was serum stimulated and transient, we speculated that growth factor-induced signaling pathways are affected by CD99. In EWS and osteosarcoma, CD99 was found to modulate ERK pathways.<sup>35,36</sup> Thus, we examined the effect of ectopic expression of CD99 isoforms on AKT and ERK kinase activity. We also assessed changes in SRC signaling, previously shown to be affected by CD99.<sup>17,37</sup> CD99-L induces transient upregulation of P-ERK, P-AKT and P-SRC compared with CD99-S and EV expressing cells (Figure 8A and *Online Supplementary Figure S13*) measured 72 h post viral transduction. CD99 knockdown (against both S and L) decreased P-ERK but increased P-AKT and P-SRC (THP-1 cells express mainly CD99-S isoform) (Figure 8B). In EWS, treatment with CD99-antibody induced cell death *via* rapid decrease of MDM2 and activation of IGF-1R and ERK signaling.<sup>35,36</sup> Thus, we asked whether MDM2 is affected by CD99 expression. CD99-L cells exhibit lower MDM2 protein levels compared with CD99-S and EV cells (Figure 8C). In addition, MDM2 is reduced in cells treated with CD99mAb 2-9 h post treatment (Figure 8D), yet P-ERK was not changed (Figure 8E and J). Because MDM2 is known to ubiquitinate IGF-1R, we speculated that CD99-induced reduction in MDM2 may up-regulate IGF-1R downstream target genes such as Cyclin D1.<sup>38</sup> *CCND1* mRNA levels in CD99-L cells were higher than that in CD99-S (1.75-fold,  $P=0.02$ ) or EV (2.8-fold,  $P<0.0001$ ) (Figure 8F). CD99 knockdown also decreased *CCND1* mRNA ( $P<0.0001$ ) (Figure 8G). We also examined P-ERK and P-SRC in stable cells maintained in culture for >2 weeks (Figure 8H). Contrary to the early effect, we found a dramatic decrease in P-SRC in CD99-L cells. Similarly, cells treated with anti-CD99mAb showed a rapid increase in P-SRC observed within 1-2 h followed by a decrease in P-SRC observed 3-9 h post treatment (Figure 8I and J).

### Discussion

CD99 upregulation has been implicated in several malignancies and is mostly known for its role in EWS.<sup>6,39</sup> Chung *et al.* have recently shown that CD99<sup>+</sup> cells expressed an antigenic profile that enriches leukemia stem cells in the majority of human AML. They also demon-

strated anti-leukemia activity of the CD99-antibody both in cellular and murine models.<sup>17</sup> Because AML is a heterogeneous disease, it is essential to identify patients with high CD99 expression that may benefit from treatments aimed to target this gene. Our analysis revealed an association between CD99 overexpression and the presence of *FLT3*-ITD. A previous study has shown that CD123/CD99/CD25(+) cells in a CD34<sup>+</sup> cell fraction predict *FLT3*-ITD mutations.<sup>40</sup> Patients with *FLT3*-ITD have a dismal clinical outcome. Whether targeting CD99 in this patient population provides a therapeutic advantage remains to be investigated. Despite its upregulation in AML, high CD99 was associated with better outcome. Yet this association was only observed in CA-AML and was not significant in multivariate survival analysis. The inverse correlation between high CD99 and *P53* mutations is likely driving its association with better clinical outcome as *P53* mutations are most common in complex karyotype patients and associated with inferior outcome.<sup>41</sup>

CD99 is expressed as short and long isoforms with tissue-specific differential expression. However, the different roles of CD99 isoforms in normal and malignant tissues is only supported by limited research. Considering the increased interest in CD99 as a therapeutic target in AML, investigating the roles of its isoforms in preclinical studies is essential.<sup>18</sup> RNA sequencing data of patients' samples revealed that transcripts coding for CD99-S and L isoforms are both expressed in AML. We further observed varying levels of CD99 isoform protein expression in HD PBMC and AML cell lines. However, the ability of western blot to compare isoform levels is limited by the antibodies that are generated against different epitopes. Furthermore, CD99 is highly glycosylated, which also affects the size of the protein band. Due to the limited number of cells in our experiments, we were unable to distinguish CD99 isoform protein expression in primary AML blasts.

The two isoforms have distinct, and maybe opposite, functions in malignancies. Studies of the ectopic expression of CD99-L isoform supported an oncosuppressor function in osteosarcoma.<sup>42</sup> Overexpression of CD99-S isoform, however, resulted in decreased cell aggregation and increased cell migration in osteosarcoma and prostate cancer cells.<sup>42</sup> In EWS, CD99-S inhibited cell differentiation and contributed to the maintenance of stemness.<sup>43</sup> Downregulation of CD99-L transformed B lymphocytes to Hodgkin and Reed-Sternberg phenotype.<sup>44</sup> Our data suggest that CD99-L cells are more responsive to serum stimuli with increased DNA synthesis and cell growth; however, over time, these cells accumulate higher ROS and DNA damage, resulting in apoptosis. We speculate that CD99 homotypic interaction is likely driving the later reduction of cell viability in CD99-L cells. While this phenomenon resembles oncogene-induced senescence,<sup>45</sup> only a slight increase in P16 was observed in CD99-L cells (*data not shown*).

CD99-L consistently delayed disease progression in AML murine models. Whether CD99 interaction with the murine microenvironment inhibits cell homing to the BM is unclear. Yet we observed no change in CXCR4 and CD49d (VLA-4a) on the surface of CD99-L cells (*data not shown*). Mechanistically, CD99-L induced P-SRC and P-ERK is likely driving the initial increase in cell growth. A CD99-derived agonist peptide that specifically interacts with conserved motifs in the CD99 extracellular domain was previously found to inhibit fibronectin-mediated  $\beta$ 1

integrin activation through the SHP2/ERK/PTPN12/FAK signaling pathway;<sup>37</sup> they also found that ERK is essential for CD99 homotypic aggregation.<sup>46</sup> CD99 homotypic interactions driving a negative feedback loop or *via* its subsequent binding with caveolin may explain the later inhibition of ERK and SRC.<sup>42</sup> CD99 was found to drive terminal differentiation of osteosarcoma cells *via* activation of membrane-bound/cytoplasmic ERK rather than affecting its nuclear localization.<sup>47</sup> Yet, in EWS, CD99 knockdown led to prolonged nuclear phosphorylation of ERK1/2 and neural differentiation.<sup>48</sup> In addition, CD99mAb was shown to act as an agonist, and reported similar phenotypes to those observed in our study.<sup>46,49</sup> Both CD99-L ectopic expression and CD99mAb induced similar cellular effects, and, likewise, modulated P-SRC. However, further biochemical studies are required to establish whether CD99mAb acts as an agonist for CD99 long isoform.

In summary, our results present unique insights into the

clinical, functional, and mechanistic roles of CD99 isoforms in AML. The mechanisms by which CD99 is up-regulated and those that govern its function in leukemia initiation remain to be investigated.

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### References

- Lowenberg B. Acute myeloid leukemia: the challenge of capturing disease variety. *Hematology Am Soc Hematol Educ Program*. 2008;1-11.
- Gelin C, Aubrit F, Phalipon A, et al. The E2 antigen, a 32 kd glycoprotein involved in T-cell adhesion processes, is the MIC2 gene product. *Embo J*. 1989;8(11):3253-3259.
- Dworzak MN, Fritsch G, Buchinger P, et al. Flow cytometric assessment of human MIC2 expression in bone marrow, thymus, and peripheral blood. *Blood*. 1994;83(2):415-425.
- Schenkel AR, Mamdouh Z, Chen X, et al. CD99 plays a major role in the migration of monocytes through endothelial junctions. *Nat Immunol*. 2002;3(2):143-150.
- Sullivan DP, Watson RL, Muller WA. 4D intravital microscopy uncovers critical strain differences for the roles of PECAM and CD99 in leukocyte diapedesis. *Am J Physiol Heart Circ Physiol*. 2016;311(3):H621-H632.
- Ambros IM, Ambros PE, Strehl S, et al. MIC2 is a specific marker for Ewing's sarcoma and peripheral primitive neuroectodermal tumors. Evidence for a common histogenesis of Ewing's sarcoma and peripheral primitive neuroectodermal tumors from MIC2 expression and specific chromosome aberration. *Cancer*. 1991;67(7):1886-1893.
- Seol HJ, Chang JH, Yamamoto J, et al. Overexpression of CD99 Increases the Migration and Invasiveness of Human Malignant Glioma Cells. *Genes Cancer*. 2012;3(9-10):535-549.
- Pelmus M, Guillou L, Hostein I, et al. Monophasic fibrous and poorly differentiated synovial sarcoma: immunohistochemical reassessment of 60 t(X;18)(SYT-SSX)-positive cases. *Am J Surg Pathol*. 2002;26(11):1434-1440.
- Hartel PH, Jackson J, Ducatman BS, Zhang P. CD99 immunoreactivity in atypical fibroxanthoma and pleomorphic malignant fibrous histiocytoma: a useful diagnostic marker. *J Cutan Pathol*. 2006;33 Suppl 2:24-28.
- Dworzak MN, Fritsch G, Fleischer C, et al. CD99 (MIC2) expression in paediatric B-lineage leukaemia/lymphoma reflects maturational disease status. *Leukemia*. 2007;21(6):1198-1203.
- Haferlach T, Kohlmann A, Wicczorek L, et al. Clinical utility of microarray-based gene expression profiling in the diagnosis and subclassification of leukemia: report from the International Microarray Innovations in Leukemia Study Group. *J Clin Oncol*. 2010;28(15):2529-2537.
- Valk PJ, Verhaak RG, Beijnen MA, et al. Prognostically useful gene-expression profiles in acute myeloid leukemia. *N Engl J Med*. 2004;350(16):1617-1628.
- Klein HU, Ruckert C, Kohlmann A, et al. Quantitative comparison of microarray experiments with published leukemia related gene expression signatures. *BMC Bioinformatics*. 2009;10:422.
- Barnes M, Freudenberg J, Thompson S, Aronow B, Pavlidis P. Experimental comparison and cross-validation of the Affymetrix and Illumina gene expression analysis platforms. *Nucleic Acids Res*. 2005;33(18):5914-5923.
- Bullinger L, Dohner K, Bair E, et al. Use of gene-expression profiling to identify prognostic subclasses in adult acute myeloid leukemia. *N Engl J Med*. 2004;350(16):1605-1616.
- Metzeler KH, Hummel M, Bloomfield CD, et al. An 86-probe-set gene-expression signature predicts survival in cytogenetically normal acute myeloid leukemia. *Blood*. 2008;112(10):4193-4201.
- Balgobind BV, Van den Heuvel-Eibrink MM, De Menezes RX, et al. Evaluation of gene expression signatures predictive of cytogenetic and molecular subtypes of pediatric acute myeloid leukemia. *Haematologica*. 2011;96(2):221-230.
- Han L, Qiu P, Zeng Z, et al. Single-cell mass cytometry reveals intracellular survival/proliferative signaling in FLT3-ITD-mutated AML stem/progenitor cells. *Cytometry A*. 2015;87(4):346-356.
- Bonardi F, Fusetti F, Deelen P, van Gosliga D, Vellenga E, Schuringa JJ. A proteomics and transcriptomics approach to identify leukemic stem cell (LSC) markers. *Mol Cell Proteomics*. 2013;12(3):626-637.
- Kikushige Y, Shima T, Takayanagi S, et al. TIM-3 is a promising target to selectively kill
- Sung CO, Ko YH, Park S, Kim K, Kim W. Immunoreactivity of CD99 in non-Hodgkin's lymphoma: unexpected frequent expression in ALK-positive anaplastic large cell lymphoma. *J Korean Med Sci*. 2005;20(6):952-956.
- Buxton D, Bacchi CE, Gualco G, et al. Frequent expression of CD99 in anaplastic large cell lymphoma: a clinicopathologic and immunohistochemical study of 160 cases. *Am J Clin Pathol*. 2009;131(4):574-579.
- Jung KC, Park WS, Bae YM, et al. Immunoreactivity of CD99 in stomach cancer. *J Korean Med Sci*. 2002;483-489.
- Yoo SH, Han J, Kim TJ, Chung DH. Expression of CD99 in Pleomorphic Carcinomas of the Lung. *J Korean Med Sci*. 2005;20(1):50-55.
- Ventura S, Aryee DNT, Felicetti F, et al. CD99 regulates neural differentiation of Ewing sarcoma cells through miR-34a-Notch-mediated control of NF- $\kappa$ B signaling. *Oncogene*. 2015;35(30):3944-3954.
- Zhang PJ, Barcos M, Stewart CC, et al. Immunoreactivity of MIC2 (CD99) in acute myelogenous leukemia and related diseases. *Mod Pathol*. 2000(4):452-458.
- Chung SS, Eng WS, Hu W, et al. CD99 is a therapeutic target on disease stem cells in myeloid malignancies. *Sci Transl Med*. 2017;9(374).
- Pasello M, Manara MC, Scotlandi K. CD99 at the crossroads of physiology and pathology. *J Cell Commun Signal*. 2018;12(1):55-68.
- Gao J, Aksoy BA, Dogrusoz U, et al. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. *Sci Signal*. 2013;6(269):p11.
- Ley TJ, Miller C, Ding L, et al. Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia. *N Engl J Med*. 2013;368(22):2059-2074.
- Andersson A, Ritz C, Lindgren D, et al. Microarray-based classification of a consecutive series of 121 childhood acute leukemias: prediction of leukemic and genetic subtype as well as of minimal resid-

- acute myeloid leukemia stem cells. *Cell Stem Cell*. 2010;7(6):708-717.
32. Bernard G, Zoccola D, Deckert M, et al. The E2 molecule (CD99) specifically triggers homotypic aggregation of CD4+ CD8+ thymocytes. *J Immunol*. 1995;154(1):26-32.
  33. Cerisano V, Aalto Y, Perdichizzi S, et al. Molecular mechanisms of CD99-induced caspase-independent cell death and cell-cell adhesion in Ewing's sarcoma cells: actin and zyxin as key intracellular mediators. *Oncogene*. 2004;23(33):5664-5674.
  34. Byun HJ, Hong IK, Kim E, et al. A splice variant of CD99 increases motility and MMP-9 expression of human breast cancer cells through the AKT-, ERK-, and JNK-dependent AP-1 activation signaling pathways. *J Biol Chem*. 2006;281(46):34833-34847.
  35. Manara MC, Terracciano M, Mancarella C, et al. CD99 triggering induces methuosis of Ewing sarcoma cells through IGF-1R/RAS/Rac1 signaling. *Oncotarget*. 2016;7(48):79925-79942.
  36. Guerzoni C, Fiori V, Terracciano M, et al. CD99 triggering in Ewing sarcoma delivers a lethal signal through p53 pathway reactivation and cooperates with doxorubicin. *Clin Cancer Res*. 2015;21(1):146-156.
  37. Lee KJ, Kim Y, Yoo YH, et al. CD99-Derived Agonist Ligands Inhibit Fibronectin-Induced Activation of beta1 Integrin through the Protein Kinase A/SHP2/Extracellular Signal-Regulated Kinase/PTPN12/Focal Adhesion Kinase Signaling Pathway. *Mol Cell Biol*. 2017;37(14).
  38. Warsito D, Sjostrom S, Andersson S, Larsson O, Sehat B. Nuclear IGF1R is a transcriptional co-activator of LEF1/TCF. *EMBO Rep*. 2012;13(3):244-250.
  39. Kavalar R, Pohar Marinsek Z, Jereb B, Cagran B, Golouh R. Prognostic value of immunohistochemistry in the Ewing's sarcoma family of tumors. *Med Sci Monit*. 2009;15(8):CR442-452.
  40. Angelini DF, Ottone T, Guerrero G, et al. A Leukemia-Associated CD34/CD123/CD25/CD99+ Immunophenotype Identifies FLT3-Mutated Clones in Acute Myeloid Leukemia. *Clin Cancer Res*. 2015;21(17):3977-3985.
  41. Kadia TM, Jain P, Ravandi F, et al. TP53 mutations in newly diagnosed Acute Myeloid Leukemia—Clinico-molecular characteristics, response to therapy, and outcomes. *Cancer*. 2016;122(22):3484-3491.
  42. Scotlandi K, Zuntini M, Manara MC, et al. CD99 isoforms dictate opposite functions in tumour malignancy and metastases by activating or repressing c-Src kinase activity. *Oncogene*. 2007;26(46):6604-6618.
  43. Lee EJ, Lee HG, Park SH, Choi EY. CD99 type II is a determining factor for the differentiation of primitive neuroectodermal cells. *Exp Mol Med*. 2003;35(5):438-447.
  44. Kim SH, Shin YK, Lee IS, et al. Viral latent membrane protein 1 (LMP-1)-induced CD99 down-regulation in B cells leads to the generation of cells with Hodgkin's and Reed-Sternberg phenotype. *Blood*. 2000;95(1):294-300.
  45. Michaloglou C, Vredeveld LC, Soengas MS, et al. BRAFE600-associated senescence-like cell cycle arrest of human naevi. *Nature*. 2005;436(7051):720-724.
  46. Hahn MJ, Yoon SS, Sohn HW, et al. Differential activation of MAP kinase family members triggered by CD99 engagement. *FEBS Lett*. 2000;470(3):350-354.
  47. Sciandra M, Marino MT, Manara MC, et al. CD99 drives terminal differentiation of osteosarcoma cells by acting as a spatial regulator of ERK 1/2. *J Bone Miner Res*. 2014;29(5):1295-1309.
  48. Rocchi A, Manara MC, Sciandra M, et al. CD99 inhibits neural differentiation of human Ewing sarcoma cells and thereby contributes to oncogenesis. *J Clin Invest*. 2010;120(3):668-680.
  49. Lee HJ, Kim E, Jee B, et al. Functional involvement of src and focal adhesion kinase in a CD99 splice variant-induced motility of human breast cancer cells. *Exp Mol Med*. 2002;34(3):177-183.