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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, we compared gene expression profiles between normal and acute myeloid leukemia cells from various publicly available datasets. We identified CD99, a gene that is upregulated in patients with acute myeloid leukemia. In 186 patients from The Cancer Genome Atlas - acute myeloid leukemia dataset, CD99 was overexpressed in patients with FLT3-ITD and was downregulated in patients with TP53 mutations. CD99 is a transmembrane protein expressed on leukocytes and plays a role in cell adhesion, trans-endothelial migration and T cells differentiation. CD99 gene encodes two isoforms with distinct expression and functional profiles in both normal and malignant tissues. Here, we report that though the CD99 long isoform initially induces an increase in cell proliferation, it also induces higher levels of reactive oxygen species (ROS), DNA damage, apoptosis and 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, cell migration as well as 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 of both ERK and SRC phosphorylation. Altogether, our study provides new insights into the role of CD99 isoforms in acute myeloid leukemia 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\(^1\). To identify target genes that were differentially overexpressed in AML compared with normal hematopoietic cells, we leveraged genomics data and discovered \textit{CD99}.

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

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

Patient Samples

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

Patient Datasets and gene expression analysis

The TCGA dataset was downloaded from oncomine\textsuperscript{19,20}. Patient data from the GSE7186\textsuperscript{21}, GSE13159\textsuperscript{22}, GSE1159\textsuperscript{23}, GSE15434\textsuperscript{24}, GSE3077\textsuperscript{25}, GSE425\textsuperscript{26}, GSE1241\textsuperscript{27} and GSE17855\textsuperscript{28} datasets were downloaded from the GEO database. (Detailed methods in supplementary file).

Cell lines and primary blasts

AML cell lines were grown in RPMI supplemented with 10% fetal bovine serum (FBS) and 100U/mL penicillin. peripheral blood mononuclear cells (PBMCs) and mononuclear cells from healthy BM 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 USC. For THP-1 and MOLM-13 xenograft experiments, 2.5X10\textsuperscript{6} cells were injected via tail-vein (IV) into 4-6 week-old NOD-scid /Il2rg\textsuperscript{-/-} (NSG) mice (Jackson) . For primary blasts
xenograft experiment, 1X10^6 cells were IV engrafted into irradiated mice. (Detailed methods including plasmids, primer sequences, antibodies and in vivo experiments are available in supplementary file).

**Results**

**CD99 is upregulated in AML.**

In an effort to identify target genes that were differentially overexpressed in AML, we compared gene expression profiles between normal and AML cells from various public datasets available. We found that *CD99* was significantly upregulated in AML compared with normal cells in five datasets with available measurements of *CD99* RNA levels in both leukemia and normal cells (median ranking among upregulated 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 samples (GSE7186: 3.5-fold; p=0.0072; Figure.1A). Consistently, *CD99* was significantly overexpressed in blasts of 542 patients with AML compared with PBMCs 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; Figure.S1A). A 2.3-fold increase in *CD99* expression (GSE13164: p<0.001; Figure.S1B) in blasts of 257 patients compared with PBMCs of 58 HD. In the GSE995 dataset, *CD99* expression was 3-fold higher in blasts from nine patients compared with that in cells from six HD (p=0.028; 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) (Figure.1SD). Transcript Ensembl IDs and their description are listed in Table.S1. The transcript expression of the two isoforms were positively correlated (Figure 1SE and F).
Several reports suggested that CD99 is upregulated on leukemia stem cells\textsuperscript{29-31}. 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\(^+\)CD38\(^+\) and CD34\(^+\)CD38\(^-\) subpopulation compared with CD34\(^-\)CD38\(^-\) and CD34\(^-\)CD38\(^+\).

**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-Exact \( P=0.04 \), 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; \( p=0.03 \) Figure.S1G, 1.4-fold; \( p<0.001 \) 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 \( p=0.002 \); Figure.S1I-N). ENST00000611428.5 (CD99-S) was not significantly associated with FLT3-ITD after adjusting for multiple hypotheses testing (\( p=0.049 \)).

CD99 expression was significantly lower (1.9-fold; \( p<0.001 \)) in patients with mutated TP53 (\( N=15 \)) compared with patients with TP53-WT (\( N=171 \)) (Figure S1O; Fisher-Exact \( P=0.0006 \), Table.S1). CD99 expression was not associated with other mutations (Figure.S2 and Table.S2).
**CD99 expression levels according to patients’ clinical characteristics**

We dichotomized patients into high and low 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 in CD99-high and 52.1 CD99-low groups (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 and poor-risk group (1.2-fold; p< 0.01, 2-fold; p<0.0001 respectively; Figure.2A). According to AML French-American-British (FAB) classification data, patients with M5-AML had lower CD99 expression compared with M1, M2, M3 and M4 (Figure.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 (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 ATRA and have a better outcome, thus they were excluded from the survival analyses. When patients were stratified based on cytogenetics 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, Figure.S4A). Yet, CD99 levels were not significantly different between CN-AML and CA-AML (p=0.33, Figure.S4B).
Event-free survival (EFS) between CD99-high and CD99-low patients was not significantly different (median: 15.6 vs 13.3 months; \( p=0.13 \); Figure.S4C,D,E).

In the GSE425 dataset of 71 patients with CA-AML, CD99-high patients survived significantly longer than CD99-low patients (\( p=0.04 \), Figure.S5B). No significant difference in OS was found when we included CN-AML patients or analyzed them separately (Figure.S5A,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 (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 (\( p=0.364 \); Table.S4).

We also assessed the association of CD99 transcript’s upregulation with overall survival 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 (Figure.S6A-G).

**Differential effect of CD99 isoforms 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+ cells and two healthy donors PBMC lysates (Figure.3B and S7A). Similarly, qPCR analysis showed that CD99 transcripts were upregulated in AML blast samples (N=9) and AML cell lines (KG-1a, U937, THP-1, and MOLM-13) compared with HD PBMCs (N=3) and CD34+ cells. Transcript ENST00000482405.7 is higher in CD34+ cells than in AML cell lines (Figure.S7B; qPCR primers are listed in Table.S5).
We then examined the effect of CD99 knock-down on cell viability. Transducing cells with lentiviral-CD99-shRNA significantly reduced primary AML blasts viability (N=4; 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 (Figure.S7D). THP-1 and MV4-11 cells had ~35-50% decrease in cell viability when transiently transfected by elecroporation with CD99-siRNA compared with negative-control-siRNA (p=0.001 and p=0.001 respectively; Figure.S7E,F,G).

We also established a gain-of-function approach to study CD99-L and CD99-S isoforms functions. We performed lentiviral transduction to overexpress CD99-L and CD99-S in THP-1, U937 and MOLM-13 cells expressing variable endogenous levels of CD99 isoforms (Figure.3E and Figure.S7H, I). CD99-L transduced cells had increased cell proliferation at 72-hrs compared with their respective 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 (Figure.S8A) and by BrdU staining (1.6 –fold; p=0.006; Figure.S8B) suggesting enhanced metabolic activity and DNA synthesis in these cells, respectively.

We also ectopically overexpressed CD99-L in AML blasts (n=7, Table.S6) using lentiviral transduction. Overexpression of CD99-L was confirmed using western-blot and fluorescence microscopy for GFP (Figure.S8C,D). Higher cell number was observed in lenti-CD99 blasts compared with lenti-EV transduced blasts 96-hrs 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 overexpressing 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) (Figure.S8E). Furthermore, we ectopically expressed CD99-L and CD99-S in three additional AML blasts samples (Figure.S8F). Cell viability was measured 96-hrs 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 overexpressing CD99-L compared with their respective controls; AML-10 (49.5 vs 101.5 , p=0.04; Figure.S8G).

However, long-term culture of CD99-L transduced cells showed subsequent drop in cell viability, and cells could not be maintained in culture for more than 4-6 weeks. To validate this, we performed long term culture assay for 10 days starting roughly 2 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 (Figure.S8H) at 96-hrs post-transduction. Transducing cells with lentiviral-CD99-shRNA resulted in slight decrease in cell viability (Figure.S8I).

**Ectopic expression of CD99 long isoform enhances ROS 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 ROS in these cells. Indeed, THP-1 cells transduced with CD99-L exhibit 2.5 and 1.6-fold increase in ROS levels compared with CD99-S and EV cells, respectively (Figure.4A, 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 higher level of the DNA damage marker H2Ax 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
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 play a role in monocytes trans-endothelial migration. 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 1X10^5 cells/mL per well in a 6-well-plate and images were taken 6-hrs 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-1a 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, C).

CD99-L expressing cells showed an increase in CD11b surface marker measured by flow cytometry 24-hrs 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 AML murine models

Next, we investigated the effect of ectopic expression of CD99 isoforms in murine leukemia models. THP-1 cells stably overexpressing 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 (0.04 vs 0.07g; p=0.010) and CD99-S mice (0.04 vs 0.06g; p=0.0003) (Figure.6A, B). hCD45 flow analysis revealed that CD99-L mice had significantly less bone marrow engraftment 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, S10). No difference in engraftment was seen in the liver (Figure.9A) and spleen (Figure.9B) 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 (0.048 vs 0.11g; p=0.004) and CD99-S mice (0.048 vs 0.10g; p=0.026). (Figure.6E, 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; Figure.9C, S11). No difference in engraftment was seen in spleen (Figure.9D). 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 (day 29 and 44). We observed PB engraftment in both mice and in the BM for one mouse (Figure.9E). H&E 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 (Figure.9F). 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 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 primary blasts 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 sacrificed four-month post-engraftment (Figure.6N-O). CD99-L mice had less BM engraftment, though it was not significant
compared with the EV mice (1.52 vs 4.76%, p=0.01; Figure.6P), and a significant decrease in hCD45\(^+\) cells in PB (7.5 vs 26.9 %, p=0.005; Figure.6Q and S9G, S12).

**CD99 monoclonal antibody treatment exhibits antileukemia activity in AML cells**

AML patient blasts (n=7) incubated with monoclonal CD99 antibody (CD99mAB: 20µg/mL) showed a significant decrease in cell viability (48hrs: 0.5±0.02-fold; p<0.0001, Figure.7A). CD99mAb caused a decrease in colony formation in 3/6 patient samples: AML-2 (0.5-fold, p=0.02), AML-6 (0.65-fold, p=0.03) and AML-7 (0.36-fold, p=0.01) (Figure.7B). CD99mAB (5 µg/ml) caused a 50% decrease in cell viability at 48-hrs in THP-1 and MOLM-13 cells but not in healthy donor PBMCs (p<0.0001, 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 CD99mAb (5µg/mL) we found ~70% decrease in cell migration in both THP-1 and MOLM-13 cells (p=0.01 and p=0.004 respectively; Figure.7F, G). CD99mAB treatment of THP-1 cells triggered an increase in CD11b\(^+\) population, indicative of myeloid differentiation (2.79-fold, p=0.04; Figure.7H). Consistently, wright-giemsa stain revealed that CD99mAb (2.5 µg/ml) induced differentiation with morphology resembling more mature cell fates (Figure.7I).

**CD99 modulates ERK and SRC signaling pathways in AML 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\(^{35,36}\). 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\(^{17,37}\). CD99-L induces transient upregulation of P-ERK, P-AKT and P-SRC compared with CD99-S and EV
expressing cells (Figure.8A and S12) measured 72-hrs 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\textsuperscript{35,36}. 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). Also, MDM2 is reduced in cells treated with CD99mAb 2-9hrs post-treatment (Figure.8D), yet P-ERK was not changed (Figure.8E and 8J). Because MDM2 is known to ubiquitinate IGF-1R, we speculated that CD99-induced reduction in MDM2 may upregulate IGF-1R downstream target genes such as Cyclin D1\textsuperscript{38}. \textit{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 \textit{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 1-2hrs followed by a decrease in P-SRC observed 3-9hrs post-treatment (Figure.8I,J).
Discussion

$CD99$ upregulation has been implicated in several malignancies and is mostly known for its role in Ewing sarcoma$^{6,39}$. Chung et al have recently shown that $CD99^+$ cells expressed an antigenic profile that enriches LSCs in the majority of human AML. They also demonstrated antileukemia activity of the $CD99$-antibody both in cellular and murine models$^{17}$. 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+ cell fraction predicts $FLT3$-ITD mutations$^{40}$. Patients with $FLT3$-ITD have dismal clinical outcome. Whether targeting $CD99$ in this patient’s 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 multivariable 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$^{41}$.

$CD99$ 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 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$^{18}$. RNAseq 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 healthy donor PBMCs and AML cell lines. However, western blot ability 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\textsuperscript{42}. Overexpression of CD99-S isoform, however, resulted in decreased cell aggregation and increased cell migration in osteosarcoma and prostate cancer cells\textsuperscript{42}. In Ewing’s sarcoma, CD99-S inhibited cell differentiation and contributed to the maintenance of stemness\textsuperscript{43}. Downregulation of CD99-L transformed B-lymphocytes to Hodgkin and Reed–Sternberg phenotype\textsuperscript{44}. 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\textsuperscript{45}, only a slight increase in P16 was observed in CD99-L cells (data not shown).

Consistently, CD99-L delayed disease progression in AML murine models. Whether CD99 interaction with the murine microenvironment inhibits cell homing to the bone marrow is unclear. Yet, we observed no change in CXCR4 and CD49d (VLA-4a) on the surface of CD99-L cells (data are 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\textsuperscript{37}; they also found that ERK is essential for CD99 homotypic aggregation\textsuperscript{46}. CD99 homotypic interactions driving a negative feedback loop or via its subsequent binding with caveolin may explain the later inhibition of ERK and SRC\textsuperscript{42}. CD99 was found to drives terminal differentiation of osteosarcoma cells via activation of membrane-bound/cytoplasmic ERK rather than affecting its nuclear localization\textsuperscript{47}. Yet in EWS, CD99 knockdown led to prolonged nuclear
phosphorylation of ERK1/2 and neural differentiation\textsuperscript{48}. Also, \textit{CD99} mAb was shown to act as an agonist and reported similar phenotypes to those observed in our study\textsuperscript{46,49}. Both CD99-L ectopic expression and CD99 mAb induced similar cellular effects and likewise modulated P-SRC. Yet, further biochemical studies are required to establish whether CD99 mAb acts as an agonist for CD99 long isoform.

In summary, our results present unique insights into the clinical, functional and mechanistic role of CD99 isoforms in AML. The mechanisms by which \textit{CD99} is upregulated and those that govern its function in leukemia initiation remain to be investigated.
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Figure Legends

Figure 1: Characterization of CD99 expression in AML datasets. A) Relative expression of CD99 in 23 AML cases compared with 6 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 isoform expression in 246 patients with AML from the GSE106291 dataset. D) 23 human tissue samples obtained from GSE30377 dataset were sorted into CD34+CD38−, CD34+CD38+, CD34-CD38- and CD34-CD38+ and analyzed for CD99 expression. E) Relative expression of CD99 in 37 patients with FLT3-ITD compared with that in 133 patients with FLT3-WT in the TCGA dataset.

Figure 2. Association of CD99 expression with patient clinical characteristics in AML datasets. A) Relative expression of CD99 according to the NCCN risk status classification in the 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) Overall survival (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.

Figure 3. Effect of CD99 on 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+ cells, healthy donor PBMC and AML cells lines. C) Viability of CD99-shRNA knockdown in THP-1, MOLM-13, U937 and AML 4 cells measured 96 hours after transfection using trypan-blue and alamar-blue. D) 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 hours. 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 hours by trypan-blue (n=4) in THP-1, U937, and MOLM-13 cells. Data reported as number of live cells. G) Viability of AML blasts overexpressing CD99-L or EV measured 96 hours after lentiviral transduction using trypan-blue (n=7). Data reported as total number of live cells/mL. H) Viability of AML blasts overexpressing CD99-L, CD99-S or EV measured 96 hours 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 day 2, 3, 4, 6, 7 and 9 by trypan-blue assay.

**Figure 4. Effect of ectopic expression of CD99 long isoform on ROS levels, DNA damage and cell apoptosis.** A-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 H2Ax in THP-1 EV, CD99-L and CD99-S. D-E) Apoptosis analysis of EV, CD99-L and CD99-S cells determined by annexin V+ 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.

**Figure 5. Functional analysis of CD99-L and CD99-S isoform overexpression in vitro.** A) Representative images for aggregation assay of THP-1 EV, CD99-L and CD99-S cells taken 6 hours after seeding. B) Migration of EV, CD99-L and CD99-S in THP-1, U937 and MOLM 13 cells analyzed by measuring cells that migrated towards SDF1-α using trans well plates (n=3). C) Representative images for migration assay of EV, CD99-L and CD99-S in THP-1, U937 and MOLM-13 cells. D) CD11b analysis of EV, CD99-L and CD99-S cells measured by flow cytometry in THP-1, U937 and MOLM-13 cells (n=4).
Figure 6. Effect of CD99-L and CD99-S isoform overexpression in AML murine models. 2.5X10^6 THP-1 EV (n=6), CD99-L (n=6) or CD99-S (n=3) cells were implanted in mice and sacrificed when sick (day 30-32 post-implantation). A-B) Spleen weight and images of blank, EV, CD99-L and CD99-S mice at the time of sacrifice. C-D) Bone marrow and peripheral blood engraftment of THP-1 cells in EV, CD99-L and CD99-S mice determined by quantitative analysis of CD45+ cells through flow cytometry. 2.5X10^6 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-F) Spleen weight and images of blank, EV, CD99-L and CD99-S mice at the time of sacrifice. G-H) Bone marrow and peripheral blood engraftment of MOLM-13 cells in EV, CD99-L and CD99-S mice determined by quantitative analysis of CD45+ 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-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. 1X10^6 primary AML cells overexpressing EV (n=3) or CD99-L (n=3) cells were implanted in mice and sacrificed when mice were sick (day 19 post cell transplantation). 1X10^6 primary AML cells overexpressing EV (N=3) or CD99-L (N=3) were engrafted in mice. N-O) Spleen weight and images of EV and CD99-L mice at the time of sacrifice (four-month post-transplantation). P-Q) Bone marrow and peripheral blood engraftment of primary AML cells in EV and CD99-L mice determined by quantitative analysis of CD45+ cells by flow cytometry.

Figure 7. Effect of CD99 mAB on AML cells. A) Cell viability of AML blasts treated with 20µg/ml of mAbCD99 for 48 hours and measured using trypan-blue (n=7). B) Total number of colonies between AML blasts treated with CD99 mAB and control blasts (n=6). C) Viability of THP-1, MOLM-13 and healthy donor PBMCs treated with 5µg/mL of mAbCD99 for 48 hours and measured using alamar blue. D-E) Apoptosis measured in THP-1 and MOLM-13 cells treated with mAbCD99 for 72 hours and stained with Annexin V for flow cytometry analysis. F-G)
Quantitative analysis and representative images of migration of THP-1, MOLM-13 and PBMCs treated with CD99 mAB towards SDF-1 α performed in a transwell plate. H) CD11b measured by flow cytometry in THP-1 cells 72 hours post-treatment with 2.5 µg/mL of CD99 mAB. I) Representative images for Wright-Giemsa staining of THP-1 cells treated with 2.5 µg/mL of CD99 mAB for 72 hours.

**Figure 8. Effect of CD99 on ERK and SRC signaling pathways in 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. B) Western blot analysis of SRC and ERK signaling pathways in THP-1 cells transfected with siCD99 for 48 hours. C) MDM2 western blot analysis in THP-1 cells transduced with EV, CD99-L and CD99-S lentivirus. D) MDM2 analysis performed by western blot in THP-1 cells treated with mAbCD99 for 1, 2 and 9 hours. E) ERK signaling pathway analyzed in THP-1 cells treated with mAbCD99 for 1, 3 and 9 Hours. F) Cyclin D1 mRNA expression measured by taqman assay in THP-1 cells transduced with EV, CD99-L and CD99-S lentivirus. G) Cyclin D1 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 lentivirus. I) SRC signaling pathway analyzed in THP-1 cells treated with mAbCD99 for 1 and 9 Hours. J) ERK and SRC signaling pathway analyzed in MOLM-13 cells treated with mAbCD99 for 1, 2, 6 and 9 Hours.
Figure 5

A) THP-1:EV, THP-1:CD99L, THP-1:CD99S

B) THP-1, U937, MOLM-13

C) THP-1, U937, MOLM-13

D) THP-1, U937, MOLM-13
Clinical and preclinical characterization of CD99 isoforms in acute myeloid leukemia

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Supplementary materials:

The supplemental materials include supplemental methods, tables and figures.
Supplementary Methods:

Patient Datasets and statistical analysis

For the TCGA dataset, 186 patients with previously untreated AML were studied, all of whom had been diagnosed and received treatment according to the National Comprehensive Cancer Network (NCCN) guidelines between November 2001 and March 2010 was downloaded from oncomine. The GSE3077 dataset had microarray data for CD34 and CD38 population in 23 patients with AML. CD99 isoform analysis was performed using the GSE106291 dataset that consisted of 246 all of whom received intensive induction treatment. The cohort consists of an unselected patient population (mixed cytogenetics) and additional refractory patients (most with poor cytogenetics). The median age for the patients was 58 years old (range 18-74). M3 patients were excluded from this analysis. CD99 transcript counts were extracted from GSE106291 annotated with GRCh38 release-96 build using Salmon (v0.9.1)^1. Counts were normalized (TMM) using edgeR ^2 and log2 transformed counts per million were extracted.

For gene expression analysis, patients were divided into two quartiles based on CD99 median expression. To investigate the associations between CD99 expression levels and the clinical and molecular characteristics, Student’s t-test and univariate analysis were used in the hypothesis testing for categorical and continuous variables, respectively. The Mantel-Cox log-rank test was used to estimate the association between CD99 expression and EFS and OS of the patients. Univariate and multivariable analysis were performed using STATA3. The statistical cutoff value was adjusted to p-value ≤0.05. All other analyses were performed using the GraphPad Prism software packages.

Cell lines and primary blasts
THP-1, MV4-11, KG-1, and Kasumi-1 were purchased from ATCC. MOLM 13, U937, KG-1A and NB4 cells were kindly provided by Dr. Wendy Stocks's lab. All AML cell lines were authenticated at the USC Cell Authentication Core.

**Plasmids**

The PLVX-CD99 L-AcGFP-C1 and PLVX-CD99 S- AcGFP -C1 was constructed by cloning the CD99 cDNA from U937 into the Apa1 and Xho1 sites of the PLVX-Zsgreen-C1 (Clontech, CA, USA). Primers used to generate PLVX-CD99 L-Zsgreen-C1 are as follows: Forward: CGCTCTGGGCGCACC, Reverse: AACAAATTGAAGGGC. Primers used to generate PLVX-CD99 S-Zsgreen-C1 are as follows: Forward: CGCTCTGGGCGCACC, Reverse: TCAGCCATCATTTC. For the PLVX- AcGFP -C1, the GFP is fused with CD99 on the N-Terminal. For CD99 shRNA plasmid PLKO.1 backbone was used. The PLKO.1 CD99 sh RNA plasmid was generated by using the following target sequence: CCATCTCTAGCTTCATTGCTT. Primers used to generate this are as follows: Forward:CCGGCCATCTCTAGCTTCATTGCTTCTCGAGAAGCAATGAAGCTAGAGATGGTTTG, Reverse: AATTCAAAAAACCATCTCTAGCTTCATTGCTTCTCGAGAAGCAATGAAGCTAGAGATGG.

**Transient transfection and viral induction**

Transient knockdown was performed using 10 nmol of siRNA per reaction using 200 ul of electroporation buffer in an electroporator. Lentivirus infection was performed by transfecting 293T cells with PLVX plasmids together with psPAX and MD2.G packing plasmids using Calcium Phosphate Transfection Kits (Clontech, CA, USA). Virus was collected 72 hours after transfection, filtered and concentrated using PEG reagent at 1:4 dilution, pelleted 24 hours later and resuspended in RPMI supplemented with 10% FBS and 1% Antibiotics. 1X10^5 suspension cells were infected with the virus and expanded after puromycin selection. Two
separate batches of lentiviral infection were performed for THP-1, U937, and MOLM-13 cells. For the CD99-shRNA experiments, CD99-shRNA plasmid was added to cells for 96 hours and cells were used for various assays.

**Migration Assay**

Migration assay was performed using the modified Boyden chambers (cat.no:3436, Corning, New York, USA) that consisted of Transwell-coated matrigel membrane filter inserts with 8 µm pores in 24-well tissue culture plates. The assay was performed as previously described \(^3\). Briefly, 1X10^5 cells in duplicates of THP-1, MOLM-13 and isolated healthy PBMCs were re-suspended in 100ul RPMI media containing 10% FBS and treated with 5µg/mL of mAbCD99 for 30mins. The cells were then seeded in the insert wells and 600 ul of RPMI media containing 10% FBS and SDF-1α were added to the lower chamber and incubated for 4 hours at 37°C. The experiment was performed twice. Following this, cells migrated towards SDF-1α were analyzed by capturing multiple images of the lower chamber for each well and analyzed using ImageJ software. Similarly, for the overexpression experiment, duplicate experiments from two separate batches of infection for THP-1, MOLM-13 and U937 cells stably overexpressing CD99 L, CD99 S or EV cells were seeded and analyzed.

**Immunoblotting**

For immunoblotting, cells were lysed in Pierce-Protease lysis buffer (cat.no:8788, Thermo Fisher, MA, USA), supplemented with a protease inhibitor mix (cat.no: A32959, Thermo Fisher, MA, USA). Protein concentrations were determined using the BCA protein assay reagent (Pierce). 10 µg of total cellular lysates was added to each lane of SDS-PAGE gels. Trans-blot (BioRad) was used to semi-dry transfer. Then lysates were resolved by SDS-PAGE and transferred to a PVDF membrane. Membranes were blocked with 5% non-fat milk or BSA and probed with indicated
antibodies. Horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology) were used for detection. Anti-CD99 antibody used was 013 (Thermofisher, MA5-12287), Anti- H2Axγ (Santacruz, sc-517348), MDM2 (SantaCruz, sc-965), SRC (Cell Signaling, cat.no. 2123), P-SRC (Cell Signaling, cat.no.6943), ERK (Cell Signaling, cat.no.9102), P-ERK (Cell Signaling, cat.no.9101), Actin (Cell Signaling, cat.no.3700). Western blot band density was evaluated using ImageJ analysis. Immunodetection was achieved with the ECL super signal reagent and detected by a Bio-Rad ECL machine.

RNA extraction and RNA expression quantification

Total RNA was extracted using TriZOL reagent (Invitrogen, CA, USA) as previously described. cDNA was synthesized using SuperScript III reagents (Invitrogen, CA, USA) according to the manufacturer’s instructions. Quantitative real-time RT-PCR (qRT-PCR) was performed using commercially available TaqMan Gene Expression Assay primers and probes. The expression levels were normalized to B2M for microRNA expression. qPCR analysis for isoform analysis was performed using SYBR green assay. For CD99 isoform analysis, the following primers were used forward (CD99 L and CD99 S): GTGATCCCCGGGATTGTG; CD99 L reverse: CTATTTCTCTAAAAGAGTACG; CD99 S reverse: CCTAGGTCTTCAGCCATC.

Wright-geimsa assay

THP-1 cells treated were treated with 2.5µg/mL of mAbCD99 for 3 days following which cells were washed and fixed onto a glass slide using a cytospin. Cells were then stained with quick stain wright-geimsa stain for 1 min and imaged.

BrdU staining assay

For the BrdU staining assay, the APC BrdU Flow kit was used (act no. 552598, BD Biosciences, San Jose, USA). Cells were synchronized by starvation. Following this BrdU was added to the cells for 12 hours and the assay was performed as per the manufacturer’s protocol.
Flow analysis

Cell surface expression of CD99 (cat.no:12-0997-42, eBioscience, CA, USA), CD11b (cat.no:A18613), CD45(cat.no:25-0459-41, eBioscience, CA, USA) and Apoptosis detection kit APC (cat.no:88-8007-74, eBioscience, CA, USA) were analyzed using the LSRII flow cytometer. Cells were stained with PE-conjugated anti-CD99 or anti-annexin V, APC conjugated CD11b or PE-Cy7-A conjugated anti-CD45 for 15 mins analyzed. The data was processed using FloJo software.

Viability and clonogenic assays

Cells were treated with mAbCD99 anti-CD99 mAb clone H036-1.1 (Thermofisher), cells were seeded at 1X10^5 cells/well in a 96-well plate with 3 replicates. 48-hours later, alamar blue assay was performed per the manufacturer’s protocol (Invitrogen). For CD99 overexpression viability assay, stable cells expressing CD99 L, CD99-S or EV cells from two separate viral transductions. The experiment was performed in duplicates for each set of transduced cells. The number of live cells was counted at 24, 48 and 72-hours using trypan blue. For primary blasts overexpression, viability was determined 96 hours after infection with CD99 L, CD99-S or EV lentiviral particles using trypan blue. Methylcellulose clonogenic assays were carried out by plating 5×10^4 primary blasts in MethoCult (StemCell Technologies) as previously described and counted 14 days later.

Aggregation assay

Cells overexpressing CD99-L or CD99-S (or EV) were seeded at a concentration of 1X10^5 cells/mL for 6 hours and images were taken using a fluorescent microscope.

ROS assay
ROS assay was performed using Cell ROX deep red reagent (Invitrogen, Cat no.C10422) according to the manufacturer’s protocol and measured using flow cytometry.

**In vivo studies**

For the xenograft experiments, 4- to 6-week-old NOD-scid /Il2rg^-/- (NSG) mice were used. For the THP-1 xenograft model, 2.5X10^6 THP-1 cells were injected via tail vein injection. 2 separate experiments were conducted and results were summarized together. For the first experiment mice were implanted with empty vector (n=3) and CD99 L (n=3). For the second experiment mice were implanted with empty vector (n=3), CD99 L (n=3) and CD99 S (n=3). For both the experiments, mice were sacrificed once the control mice were sick. For the MOLM-13 experiment, mice were implanted with 2.5X10^6 cells of empty vector (n=3), CD99 L (n=3) or CD99 S (n=3) and were sacrificed only when sick. For the primary cell experiment, cells were transduced with CD99 L (or EV) for 96 hours. Prior to engraftment 4- to 6-week-old NOD-scid /Il2rg^-/- (NSG) mice were irradiated using the X-ray irradiator at a dose of 250 cGy and 24 hours later, 1X10^6 cells were engrafted via tail vein in empty vector (n=3) and CD99 L(n=3) mice. Mice were sacrificed four months after engraftment. For all the *in vivo* experiments, bone marrow, peripheral blood, liver and spleen tissues were stained for CD45 and analyzed using Flow cytometry.
Supplemental tables:

**Tables S1: CD99 isoform Transcript ID and description**

| Name     | Transcript ID | bp    | Protein | Biotype            | Isoform     | CCD5     | Uniprot |
|----------|---------------|-------|---------|--------------------|-------------|----------|---------|
| CD99-205 | ENST00000381192.10 | 1129  | 185aa   | Protein coding     | Long isoform | CD514119 | P14209  |
| CD99-210 | ENST00000611428.5 | 1243  | 160aa   | Protein coding     | Short isoform| CD575947 | P14209  |
| CD99-208 | ENST00000482405.7 | 842   | 160aa   | Protein coding     | Short isoform| CD575947 | P14209  |
| CD99-212 | ENST00000624481.4 | 1089  | 184aa   | Protein coding     |             | CD583452 | A0A096LP69 |
| CD99-204 | ENST00000381187.8 | 892   | 169aa   | Protein coding     |             | CD548071 | P14209  |
| CD99-203 | ENST00000381184.6 | 918   | 177aa   | Protein coding     | -           | A8MQT7  |         |
| CD99-202 | ENST00000381180.9 | 533   | 76aa    | Protein coding     | -           | A6NJ7   |         |
| CD99-211 | ENST00000623253.4 | 573   | 160aa   | Nonsense mediated decay |             | CD575947 | P14209  |
| CD99-201 | ENST00000381177.7 | 756   | 22aa    | Nonsense mediated decay | -           | A6NGF6  |         |
| CD99-206 | ENST00000449611.6 | 604   | No protein | Processed transcript | -           | -       |         |
| CD99-207 | ENST00000482293.6 | 466   | No protein | Processed transcript | -           | -       |         |
| CD99-214 | ENST00000646103.1 | 1278  | No protein | Retained intron     | -           | -       |         |
| CD99-209 | ENST00000497752.7 | 815   | No protein | Retained intron     | -           | -       |         |
| CD99-215 | ENST00000647297.1 | 583   | No protein | Retained intron     | -           | -       |         |
| CD99-213 | ENST00000645950.1 | 497   | No protein | Retained intron     | -           | -       |         |
Table S2: Mutational characteristics of patients with AML in the TCGA dataset according to CD99 expression

|                  | CD99 Low | CD99 High | p value (WT vs Mutated) | Fischer Exact Test |
|------------------|----------|-----------|-------------------------|--------------------|
| FLT3-ITD, no. (%)| Present  | 37 (30)   | 12 (25.2)               | 24 (47.5)          |
|                  | Absent   | 149 (70)  | 81 (74.7)               | 69 (70.3)          |
| p=0.002          | P=0.0402 |
| IDH1, no. (%)    | Mutated  | 17 (9.0)  | 8 (8.4)                 | 9 (9.6)            |
|                  | Wild type| 169 (91.0)| 85 (91.5)               | 84 (90.3)          |
| p=0.08           | P=1      |
| IDH2, no. (%)    | Mutated  | 17 (9.5)  | 6 (6.3)                 | 11 (12.7)          |
|                  | Wild type| 169 (91.5)| 87 (93.6)               | 82 (87.2)          |
| p=0.11           | P=0.3    |
| RUNX1, no. (%)   | Mutated  | 17 (8.5)  | 8 (8.4)                 | 9 (10.4)           |
|                  | Wild type| 169 (91.5)| 85 (91.5)               | 84 (89.5)          |
| p=0.50           | P=1      |
| TET2, no. (%)    | Mutated  | 17 (9.0)  | 10 (10.6)               | 7 (7.4)            |
|                  | Wild type| 169 (91)  | 83 (89.4)               | 86 (92.5)          |
| p=0.98           | P=0.6    |
| NRAS, no. (%)    | Mutated  | 14 (7.9)  | 8 (8.4)                 | 6 (6.3)            |
|                  | Wild type| 172 (91.4)| 85 (91.5)               | 87 (93.6)          |
| p=0.2            | P=0.7    |
| CEBPA, no. (%)   |                  |           |                        |                    |
| p=0.1            | P=0.08    |
|          | Mutated | Wild type | WT1, no. (%) | p=0.92  | P=0.5  |
|----------|---------|-----------|--------------|---------|--------|
| Mutated  | 13 (9.0)| 173 (91)  | 11 (9.0)     |         |        |
| Wild type| 3 (10.6)| 90 (89.4) | 4 (10.6)     |         |        |
|          | 10 (7.4)| 83 (92.5) | 7 (7.4)      |         |        |

|          | DNMT3A, no. (%) | p=0.9  | P=0.31  |
|----------|------------------|--------|---------|
| Mutated  | 49 (26.0)        | 28 (29.7)| 21 (22.3)|
| Wild type| 137 (73.9)       | 65 (70.2)| 72 (77.65)|

|          | NMP1, no. (%)    | p=0.24 | P=0.4  |
|----------|------------------|--------|--------|
| Mutated  | 50 (26.0)        | 28 (29.7)| 22 (22.3)|
| Wild type| 136 (73.9)       | 65 (70.2)| 71 (77.65)|

|          | TP53         | p=0.001 | P=0.0006 |
|----------|--------------|---------|-----------|
| Mutated  | 15           | 14 (93)| 1 (6.6)   |
| Wildtype | 171          | 79 (46.2)| 92 (53.8) |
Table S3: Clinical characteristics of the acute myeloid leukemia (AML) cohort in the TCGA dataset according to CD99 median expression.

|                          | Total | CD99 Low | CD99 High | p value (CD99 Low vs High) | Fischer Exact |
|--------------------------|-------|----------|-----------|---------------------------|---------------|
| Sex, no. (%)             |       |          |           |                           | P=0.55        |
| Female                   | 85 (45.7) | 45 (53)  | 40 (47)   |                           |               |
| Male                     | 101 (55.3) | 48 (47.5) | 53 (52.5) |                           |               |
| Age, years (range)       |       |          |           | p=0.017                   |               |
| Median                   | 57.5  | 57.82    | 52.1      |                           |               |
| Mean                     | 55    | 57.82 ± 1.555 | 52.17 ± 1.764 |                   |               |
| WBC count                |       |          |           | p=0.019                   |               |
| Median                   | 16.55 | 27.34946 | 45.84516  |                           |               |
| Mean                     | 36.59731 | 27.35 ± 3.885 | 45.85 ± 5.912 |                   |               |
| PB blasts                |       |          |           | p<0.0001                  |               |
| Median                   | 34    | 23.76667 | 50.76923  |                           |               |
| Mean                     | 37.34254 | 23.77 ± 2.858 | 50.77 ± 3.042 |                   |               |
| BM blasts                |       |          |           | p=0.024                   |               |
| Median                   | 73    | 66.21505 | 72.51613  |                           |               |
|                | Mean   | 69.36559 | 66.22 ± 2.155 | 72.52 ± 1.749 |                  |
|----------------|--------|----------|---------------|---------------|------------------|
| NCCN subtype,no| Vs Favorable |
| Favorable      | 34     | 8        | 26            |               |                  |
| Intermediate   | 112    | 60       | 52            | P=0.0029      |                  |
| Poor           | 40     | 25       | 15            | P=0.001       |                  |
| FAB subtype,no |         |          |               |               |                  |
| M0             | 16     | 5        | 11            |               |                  |
| M1             | 43     | 14       | 29            |               |                  |
| M2             | 41     | 19       | 22            |               |                  |
| M3             | 18     | 6        | 12            |               |                  |
| M4             | 40     | 27       | 13            |               |                  |
| M5             | 21     | 17       | 4             |               |                  |
| M6             | 2      | 1        | 1             |               |                  |
| M7             | 3      | 3        |               |               |                  |
Table S4: Multivariate analysis of overall survival of patients with AML.

| Multivariant Analysis | OS (categorical CD99) | Hazard Ratio (95% Conf. Interval) |
|-----------------------|-----------------------|----------------------------------|
| Age                   | 0.033                 | 1.016617 (1.001304, 1.032165)    |
| Intermediate Risk     | 0.001                 | 3.135137 (1.601362, 6.137954)    |
| Poor Risk             | 0.000                 | 4.704015 (2.109915, 10.48751)    |
| Transplant status     | 0.000                 | 0.4344322 (.2781283, .6785766)   |
| DNMT3A                | 0.108                 | 1.396372 (.9291901, 2.098445)    |
| TP53                  | 0.05                  | 1.95511 (.9791412, 3.903891)     |
| CD99                  | 0.36                  | 0.839078 (.546331, 1.22522)      |
### Tables S5: Primer match list for CD99 primer set 1 and set 2 for all CD99 transcripts

| Transcript       | ID  | AA  | Primer Set-1 Forward | Primer Set-1 Reverse | Primer Set-2 Forward | Primer Set-2 Reverse |
|------------------|-----|-----|----------------------|----------------------|----------------------|----------------------|
| ENST00000381192.10 | 205 | 185 | Y                    | Y                    | N                    | N                    |
| ENST00000381187.8 | 204 | 169 | Y                    | Y                    | N                    | N                    |
| ENST00000381184.6 | 203 | 177 | Y                    | N                    | N                    | N                    |
| ENST00000482405.7 | 208 | 160 | Y                    | N                    | Y                    | Y                    |
| ENST00000611428.5 | 210 | 160 | Y                    | Y                    | N                    | N                    |
| ENST00000624481.4 | 212 | 184 | Y                    | Y                    | N                    | N                    |
| ENST00000381180.9 | 202 |  76 | N                    |                   | N                    | N                    |
| Patient ID | AML Status sample | FLT3 Mutation |
|------------|-------------------|---------------|
| AML 1      | Diagnosis         | NA            |
| AML 2      | Diagnosis         | NA            |
| AML 3      | Diagnosis         | NA            |
| AML 4      | Diagnosis         | ITD           |
| AML 5      | Relapse           | ITD           |
| AML 6      | Diagnosis         | WT            |
| AML 7      | Diagnosis         | WT            |
| AML 8      | Relapse           | ITD           |
| AML 9      | Diagnosis         | NA            |
| AML 10     | Diagnosis         | NA            |
| AML 11     | Diagnosis         | NA            |
Figure S1: A) Relative expression of CD99 in 285 AML cases as compared with 5 healthy donors in the GSE71159 dataset. B) Relative expression of CD99 in 257 AML cases as compared with
58 healthy donors in the GSE13164 dataset. C) Relative expression of CD99 in 9 AML cases as compared with 6 healthy donors in the GSE995 dataset. E) Correlation between ENST00000482405.7 and ENST00000381192.10 CD99 transcript. F) Correlation between ENST00000611428.5 and ENST00000381192.10 CD99 transcript. G) Relative expression of CD99 in 48 patients with FLT3 ITD as compared with 189 patients with FLT3 WT in the GSE 17855 dataset. H) Relative expression of CD99 in 90 patients with FLT3 ITD as compared with 161 patients with FLT3 wildtype. I-N) Relative expression of various CD99 transcripts in 49 patients with FLT3 ITD as compared with 197 patients with FLT3 wild type in the GSE106291 dataset. O) Relative expression of CD99 in 15 patients with TP53 mutation as compared with 171 patients with TP53 wild type in the TCGA dataset. Differences among groups were analyzed using Student’s T test. (* p< 0.05, ** <0.005, ***p<0.001, ****p<0.0001)).
Supplementary Figure 2:

Figure S2: Relative expression of CD99 in patients with WT1, CEBPA, IDH1, IDH2, RUNX1, RUNX2, NRAS and DNMT3A mutation compared with the wildtype.

Supplementary Figure 3

A)

Figure S3: A) Relative expression of CD99 in the different FAB subtypes in the TCGA dataset.
B) Relative expression of CD99 based on different cytogenetics of patients in the TCGA dataset.
Figure S4: A) OS of cytogenetically normal (CN-AML) cases grouped based on CD99 median expression into CD99 high (N=45) and CD99 low (N=44) in the TCGA dataset. B) Relative CD99 expression in CN-AML vs CA-AML groups in the TCGA dataset. Differences among groups were analyzed using Student’s T test. C) Event free survival (ES) of 171 patients grouped based on CD99 median expression into CD99 high (N=86) and CD99 low (N=85). D) ES of cytogenetically abnormal (CA-AML) cases grouped based on CD99 median expression into CD99 high (N=41) and CD99 low (N=41). E) ES of cytogenetically normal (CN-AML) cases grouped based on CD99 median expression into CD99 high (N=45) and CD99 low (N=44). Red line: CD99 high group, black line: CD99 low group.
Figure S5: A) Overall survival (OS) of 115 patients grouped based on CD99 median expression into CD99 high (N=58) and CD99 low (N=57) from the GSE425 dataset. (B) OS of cytogenetically abnormal (CA-AML) cases grouped based on CD99 median expression into CD99 high (N=35) and CD99 low (N=36). C) OS of cytogenetically normal (CN-AML) cases grouped based on CD99 median expression into CD99 high (N=22) and CD99 low (N=22). D) OS of 163 cytogenetically normal (CN-AML) patients from the GSE12417 dataset grouped based on CD99 median expression into CD99 high (N=82) and CD99 low (N=81). Red line: CD99 high group, black line: CD99 low group.
Supplementary Figure 6

Figure S6: A-G) Overall survival (OS) of 246 patients grouped based on median CD99 expression for various transcripts of CD99 into high (N=123) and low (N=123) from the GSE106291 dataset.
Supplementary Figure 7

A) Western blot analysis for CD99 expression in THP-1, U937 and MOLM-13 AML cell lines. B) CD99 isoform analysis using two different primer sets for CD34+ cells, healthy donor.

Figure S7: A) Western blot analysis for CD99 expression in THP-1, U937 and MOLM-13 AML cell lines. B) CD99 isoform analysis using two different primer sets for CD34+ cells, healthy donor.
PBMCs, primary patient blasts and AML cell lines. C) Viability assay for primary AML blast (N=3) transfected with shCD99 plasmid (or EV) for 96 hours measured by trypan blue assay. Number of live cells were normalized to EV cells. D) Western blot analysis of CD99 expression in shD99 knockdown cells of THP-1 and U937 cells. E) Expression of CD99 in siCD99 knockdown cells of THP-1 and MV4-11 normalized to siControl cells using Taqman assay (n=3). F) Western blot analysis of CD99 expression in siCD99 knockdown cells of THP-1 and MV4-11 cells. G) Viability assay for siCD99 knockdown cells of THP-1 and MV4-11 performed by alamar blue at 48 hours; Viability is normalized to control cells (n=3). H) Western blot analysis of CD99 overexpression in THP-1 and U937 cells transduced with EV, CD99-L and CD99-S virus. I) Western blot analysis of CD99 overexpression in 293T cells transfected with EV, CD99-L and CD99-S isoform used to generate virus.
**Figure S8:** A) Relative Viability of THP-1, U937 and MOLM-13 cells stably expressing CD99 L or CD99 S isoform measured using alamar blue at 72 hours. Viability is normalized to EV cells (N=4). B) Proliferation assay using BrdU assay in THP-1 cells stably overexpressing CD99 L or
EV measured 24 hours after adding BrdU using flow cytometry. Data normalized to EV cells. C) Western blot analysis for CD99 expression of 3 primary patient blasts infected with CD99-L (or Empty vector) lenti virus for 5 days. D) Representative GFP images for CD99 expression (or Empty vector) in primary patient blasts infected with CD99 (or Empty vector) lenti virus for 5 days. E) Relative number of colonies between AML blasts overexpressing CD99 L and EV blasts (n=6) measure on day 14. F) Representative GFP images for CD99 L, CD99 S (or Empty vector) in primary patient blasts infected with CD99 (or Empty vector) lenti virus for 5 days. G) Relative number of colonies between AML blasts overexpressing CD99 L, CD99 S or EV blasts (n=3) measure on day 14. H) Flow analysis for CD99 overexpression and proliferation assay measured using trypan blue 96 hours after infecting CD34+ healthy donor cells with EV, CD99-L and CD99-S lenti virus. H) Flow analysis for CD99 knockdown at day 6 and proliferation assay measured using trypan blue after infecting CD34+ healthy donor cells with EV or shCD99 lenti virus.
Figure S9: 2.5×10^6 THP-1 EV (n=6), CD99 L (n=6) or CD99 S (n=3) cells were implanted in mice and sacrificed when mice were sick. A-B) Liver and Spleen engraftment of THP-1 cells in EV, CD99 L and CD99 S mice determined by quantitative analysis of CD45+ cells through flow
cytometry. 2.5X10^6 MOLM-13 EV (n=3), CD99 L (n=3) or CD99 S (n=3) cells were implanted in mice and sacrificed when mice were sick. C-D) Liver and Spleen engraftment of MOLM-13 cells in EV, CD99 L and CD99 S mice determined by quantitative analysis of CD45+ cells through flow cytometry. E) CD45 flow cytometry staining of peripheral blood and bone marrow in mice engrafted with MOLM-13 CD99-L cells and sacrificed on day 29 and 44. F) Representative H&E staining images of Liver, spleen and sternum of mice engrafted with MOLM-13 EV, CD99 L and CD99 S cells. (* p< 0.05, ** <0.005))
**Figure S10.** Fac plot data for CD45 staining in the bone marrow and peripheral blood of mice engrafted with THP-1 EV, CD99-L or CD99-S for two separate experiments.
**Figure S11.** Fac plot data for CD45 staining in the bone marrow and peripheral blood of mice engrafted with MOLM-13 EV, CD99-L or CD99-S for two separate experiments.
**Supplementary Figure 12:**

Fac plot data for CD45 staining in the bone marrow, peripheral blood and spleen of mice engrafted with primary AML blasts infected with EV or CD99-L lenti virus. Blank mice stained with CD45 used as a control.

**Figure S12.** Fac plot data for CD45 staining in the bone marrow, peripheral blood and spleen of mice engrafted with primary AML blasts infected with EV or CD99-L lenti virus. Blank mice stained with CD45 used as a control.
Figure S13. Western blot analysis of P-AKT and P-ERK in U937 cells infected with EV of CD99-L lenti virus.
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