Exosome-driven lipolysis and bone marrow niche remodeling support leukemia expansion

Bijender Kumar,[1,2] Marvin Orellana,[1°] Jamison Brooks,[1] Srideshikan Sargur Madabushi,[1] Paresh Vishwasrao,[1] Liliana E. Parra,[1] James Sanchez,[3] Amandeep Salhotra,[2,3] Anthony Stein,[2,3] Ching-Cheng Chen,[2] Guido Marcucci[2,3] and Susanta Kumar Hui[1]*

[1]Department of Radiation Oncology, Beckman Research Institute,City of Hope National Medical Center, [2]HematologicalMalignancies, Stem Cell Transplantation Institute, City of Hope National Medical Center and [3]Department of Hematology and HCT, City of Hope National Medical Center, Duarte, CA, USA

[°]Current address: Department of Stem Cell Transplantation and Cellular Therapy, MD Anderson Cancer Center, Houston, TX, USA

Correspondence: BIJENDER KUMAR - bkumar1@mdanderson.org

SUSANTA KUMAR HUI - shui@coh.org

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Supplementary Information

Materials and Methods:

Mice

C57BL/J(B6) and NOD-SCID-γc$^{-/-}$ (NSG) mice were maintained by the Animal Resource Center of City of Hope. MLL-AF9 knock-in (KI,*Kmt2a*$^{tm2(MLLT3)Thr}$/KsyJ) mice were obtained from Jackson Laboratory, and BCR-ABL knock-in (KI) mice were provided by Guido Marcucci, City of Hope. Mouse care and experimental procedures were performed in accordance with the guidelines and protocols approved by the Institutional Animal Care and Use Committee (IACUC) at City of Hope.

MLL-AF9 and BCR-ABL transduction transplantation leukemia mice models

Briefly, 5000 HSC cells from C57/Bl6J mice were isolated and transduced with viral particles encoding MLL-AF9-GFP or BCR-ABL-GFP leukemic oncogenes twice in 3 days using spinoculation (8μM polybrene, Santa Cruz Biotechnology, Dallas, TX) in Iscove's Modified Dulbecco's Media (IMDM) media (Gibco, Grand Island, NY,) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Flowery Branch, GA) and low growth factors conditions in hypoxia. The transduced cells were plated in fresh media after 48 hours and allowed to expand for another 7 days in methylcellulose IMDM media (STEMCELL Technologies, Cambridge, MA). The flow sorted GFP+ cells were subsequently injected through the retro-orbital sinus of 8-10-week-old CD45.1 mice. The mice developed leukemia at 4-5 months post transplantation; the BM cells from these leukemic mice were harvested to engineer more aggressive second-generation leukemic mice(1). The advantage of these models lies in their capacity to engraft without any myeloablative radiation or chemotherapy during transplantation leaving the normal HSC niche unperturbed mimicking a spontaneous human leukemia development. For this study, 1x10^6 MLL-AF9-GFP+ or BCR-ABL-GFP+ cells were injected into 8-10-week-old C57/Bl6J mice, and organs were harvested when the mice developed leukemia at 15-20 days post transplantation characterized by severe anemia and leukocytosis in blood. The GFP+ cells percentage was analyzed using flow cytometry in the live BM cells as a measure of leukemic burden in the mice. The age and sex matched controls mice were used for bone marrow niche populations comparison with leukemic mice.

Cell lines and patient samples

Leukemic cell lines (MV411 & NALM6; ATCC) were cultured in IMDM with 10% FBS. Mycoplasma testing by PCR was performed every three months on cell lines in culture. AML patients and control samples (peripheral blood or BM) were obtained under a City of Hope-

Institutional Review Board—approved protocol with informed consent from patients or healthy individuals (see Supplementary Table 1 for more information). BM CD34+ cells were enriched using human CD34+ microbeads kit (Miltenyi Biotec, Auburn, CA).

Quantitative real-time PCR

RNA was extracted from sorted stroma cells using the RNeasy RNA isolation kit (Qiagen, Germantown, MD) and was reverse transcribed into cDNA using the Sensiscript RT kit (Qiagen). The cDNA for specific genes was amplified using TaqMan assays on a ViiA 7 Real-Time PCR System (Applied Biosystems). Data were analyzed using the ViiA 7 software (Applied Biosystems). The TaqMan probes used are listed in the Supplementary Table 2.

ELISA

Plasma was obtained from normal and leukemic mice peripheral blood. For BM analysis, normal and leukemic mice bones (2 tibias and 2 femurs/mice) were crushed and re-suspended in 500 µl PBS, after which was performed sandwich based ELISA for Perilipin and PNPLA2 (ATGL, Lifespan Biosciences Inc.Seattle WA) according to the manufacturer's recommended protocols and analyzed at 450 nm. White adipose tissue (WAT) from normal and leukemic mice was lysed and quantified for PNPLA2 expression.

Adipose differentiation of MSCs

Bone marrow sorted $Sca1^+$ MSCs from C57BL/Ka (B6) mice were expanded and cultured in adipocyte differentiation media (R&D systems, Minneapolis, MN) for 2 weeks and co-cultured with leukemic cells, exosomes, and exogenous cytokines with and without ATGL and HSL inhibitors. To study adipocytes differentiation experiments using adipo-primed MSCs, $CD45^-$ $CD31^-$ $Ter119^-$ $CD166^-$ $CD146^-$ $Sca1^+$ $CD24^-$ adipocyte-primed progenitor cells from control and AML/ALL mice bones were cultured in the MEM-alpha medium (Gibco, Waltham, MA) containing 15% FBS at 37 °C with 5% CO_2 in a 96-well plate for 3 days followed by in adipocyte induction media culture for 14 days.

Adipose co-culture

The fully differentiated adipocytes were used for leukemia, exosomes or leukemia conditioned media culture experiments. Briefly, $5x10^5$ adipocytes were cultured in lower transwell chamber of corning transwell (0.4 µm pores, Sigma, St. Louis MO) and $5x10^5$ leukemic cells were plated in upper chamber for 24h., allowing only the cells secreted cytokines or exosomes to pass through the pores into respectively chambers. For adipocytes proinflammatory genes

expression profiling experiment, $2x10^6$ adipocytes were cultured alone or with 10 µg normal or leukemic exosomes for 24h. The adipocytes were harvested and RNA extraction and RQ-PCR analysis was performed.

Pharmacological Inhibitors

GW1929 (PPARγ agonist) and Zoledronic acid (Sigma Aldrich, St. Louis, MO, 100 µg/kg in-vivo dose), CAY10499 (Caymanchem, Ann Arbor, MI) and Atglistatin (Selleckchem, Houston, TX) were purchased and used in the study. GW1929 (15 mg/kg) daily through oral gavage route for 3 weeks was used to enhance BM adipogenesis in the recipient mice.

BODIPY transfer

Adipocyte-specific lipids and oils were stained with 5 µM solution of BODIPY (493/505nm, Thermo Fisher Scientific, Waltham, MA) at 37°C for 20 mins, and excessive dye was removed by washing with PBS twice. The BODIPY stained cells were visualized on confocal microscope using 488 nm (FITC) channel. The BODIPY stained adipocytes were co-cultured with leukemia cells and analyzed for uptake by leukemic cells using flow cytometry.

Free fatty acid uptake assay

For lipid visualization and transfer, adipocytes were incubated with dodecanoic acid fluorescent fatty acid (FA) analog, DAA (Molecular Devices, San Jose, CA) for 3 hours and washed 3 times in PBS. MV4-11 and BCR-ABL+ cells were then cultured alone, with labeled adipocytes with and without ATGL/HSL inhibitors for 24 hours. AML blasts were then removed, and fluorescence was subsequently measured by flow cytometry to indicate transfer of fluorescent-labeled FAs from adipocytes to AML.

Western immunoblotting

Abdominal white adipose tissue was harvested from normal and leukemic mice, and Western analyses using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on proteins from adipose tissue lysates and adipocyte monolayer from co-cultures were extracted using RIPA assay buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4, 1 µg/ml leupeptin, and a cocktail of phosphatase and protease inhibitors (Sigma-Aldrich, St. Louis, MO). Immunoblotting for ATGL (1:1000), pHSL (S563, 1:1000), β-Actin (1:2000) was performed with 20 µg of protein lysate using cell signaling technology (Danvers, MA) antibodies as per the manufacturer's instructions.

Exosome isolation and characterization from plasma and cell cultures

Leukemia-derived exosomes were isolated and characterized as described previously (1). Culture media or FBS was centrifuged at 100,000 g for 10 h to remove bovine-derived exosome contamination from FBS. Centrifuged media was filtered using a 0.2 μm filter to remove the FBS exosomes pellet, then collected in a flask and used for culturing the cells.

For exosome isolation, the AML/ALL cells were cultured directly with the centrifuged media in hypoxia. The primary AML and normal control cells were cultured in low cytokine (SCF, TPO, FLT3-L, all 5 ng/ml) conditions. Supernatants were collected 48 h later, centrifuged at 300 g and 800 g for 10 min at 4 °C to remove whole cells, centrifuged again at 10000 g for 10 min at 4 °C to remove cellular debris, and filtered through a 40-μm membrane, prior to exosome preparation by centrifugation at 100000 g for 1 h. The exosome pellet was washed twice in a large volume of PBS and underwent centrifugation at 100 000 g for 1 h and used for co-culture experiments. The exosomes pellet was dissolved in RIPA buffer and quantified using BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL). Exosome-specific protein expression was confirmed using Western Blot for anti-TSG101 (1:1000) antibody. The other exosomes specific CD9 & CD63 markers expression was confirmed using flow cytometry analysis.

Flow cytometry

After removing muscles and connective tissue, femurs and tibias were crushed with mortar and pestle in PBS 0.1% BSA buffer, and extra PBS buffer was added to separate BM from bone fragments. The bone marrow and bone cells were incubated with ACK lysis buffer to remove red blood cells (RBCs) followed by PBS washing. The bone fragments were incubated at 37 °C with collagenase I (3 mg/ml; Sigma, St Louis, MO) in DMEM media and gently agitated for 45 min. The digested bones fragments were then filtered with a 40 μm strainer (BD Biosciences, San Jose, CA), pelleted at 200 *g* at 4°C, and resuspended in PBS. The bone cells were blocked with anti-CD16/32 antibody and stained with CD45, Ter119, CD31, Sca1, CD146, CD166, CD140a, CD24, Leptin receptor and AmCyan 405 (viability dye) for 30-45 min at 4°C in the dark (Supplementary Table 3). The unbound antibodies were removed by centrifugation at 300 *g* at 4 °C, and cells were re-suspended in PBS 0.1% BSA buffer. The stained cells were then sorted using a FACSAriaIII sorter (BD Biosciences, San Jose, CA).

For different mesenchymal stromal progenitors and mature populations analysis, at least 3000-5000 events per sample were collected within the $CD45^{-}Ter119^{-}CD31^{-}$ gate and analyzed using FlowJo software (FlowJo, Ashland, OR).

Apoptosis analysis in leukemic cells after different inhibitor treatments was performed using Annexin V and PI/DAPI staining for 45 min and analyzed using FACSAria III sorter (BD Biosciences). Mitochondrial CPT1A intracellular staining was performed on the fixed & permeabilized MV4-11 cells followed by analysis using FACSAriaIII sorter (BD Biosciences, San Jose, CA).

Histology

Isolated bones were cleaned from surrounding tissue, fixed/decalcified in 4% paraformaldehyde fixative (Thermo Fisher Scientific, Waltham, MA), and incubated with 10% EDTA for 1 week to soften the bones and with 20% sucrose solution incubation for 1 hour followed by paraffin embedding. 10-15-micron-thick sections were used for H&E and trichrome staining.

shRNA transduction and leukemia proliferation:

The MLL-AF9 cells were transduced with SMARTvector inducible mouse pnpla2 (ATGL) and non-targeting control shRNAs (Dharmacon, Lafayette, CO). The stably transduced and puromycin-selected leukemic cells were treated with 1 μM doxycycline for 24h to induce conditional knockdown of the target genes along with turbo RFP expression and analyzed for leukemia cell proliferation. More than 95% of leukemic cells in the culture exhibited RFP expression after doxycycline induction, and further knockdown efficiency was conformed using RQ-PCR.

Single-cell RNA-seq library preparation, sequencing and Seurat analysis

Single cell sequencing of flow sorted human stroma (live $CD33^{-}CD123^{-}lineage^{-}CD45^{-}$ $CD235a^{-}$ $CD41^{-}$ $CD31^{-}$ cells) from a healthy control and ALL patient was performed using 10X Genomics (Qiagen, Germantown, MD) Chromium platform kit by following the manufacturer's protocol to capture, barcode and generate Gel Beads-in-Emulsion of the single cells. Briefly, along with the reverse transcription master mix, single cell suspensions were loaded onto 10x Genomics Single Cell 3′ Chips with gel beads coated with oligonucleotides to capture mRNA inside the droplets by 30 bp oligo-dT after cell lysis and provide barcodes to index cells (16 bp) as well as transcripts (10 bp UMI). Following reverse transcription, cDNAs with both barcodes were amplified, and a library was constructed using the Single Cell 3′ Reagent Kit (v2 chemistry) for each sample. The resulting libraries were sequenced on an Illumina Hiseq2500 in a 26+8+101 bp paired-end mode. The data was further aligned and analyzed using R toolkit Seurat software(2).

Lipolysis genes expression and patient's survival correlation

To study the impact of LIPE and PNPLA2 gene expression on Acute lymphoblastic leukemia (ALL) patients survival, the TCGA cBioPortal for Cancer Genomics from Memorial Sloan Kettering Cancer Center, New York was used to analyze from the earlier published RNA sequencing datasets from the TARGET pediatric ALL Phase-II study(3).

Statistics: Statistical analysis was performed using the 2-tailed unpaired *t* test, with the exception that for mice survival analysis experiments, the log-rank (Mantel-Cox) test was used for comparison of differences between multiple groups. The difference was considered significant when the p value was <0.05. Kaplan-Meier survival curves and all graphs and statistical analyses were generated using GraphPad Prism software. ns=not significant, *=p<0.05, **=p<0.01, ***=p<0.0005, ****=p<0.00005.

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Supplementary figures legends

Supplementary Figure S1: (A) 10X H&E stained images of normal and AML mice diaphysis and trabecular bone regions, Red arrows mark osteoblasts and green arrows mark adipocytes. (B) Gating strategy for characterization and sorting of bone-derived $Sca1^{+}CD24^{+}$ progenitors in the 10-12-week old leukemic mice. The preadipocytes and multipotent precursors were denoted as live $GFP^{-}CD45^{-}Ter119^{-}CD31^{-}CD166^{-}Sca1^{+}CD24^{-/+}$ cells populations. (C) Bone stroma ($GFP^{-}CD45^{-}Ter119^{-}CD31^{-}$) cells percentages in normal and leukemia mice. (D) The percentages of multipotent $GFP^{-}CD45^{-}Ter119^{-}CD31^{-}CD166^{-}Sca1^{+}CD24^{+}$ progenitors in Sca1+ MSCs in age/sex matched normal, MLL-AF9-KI and BCR-ABL-KI bone stroma.

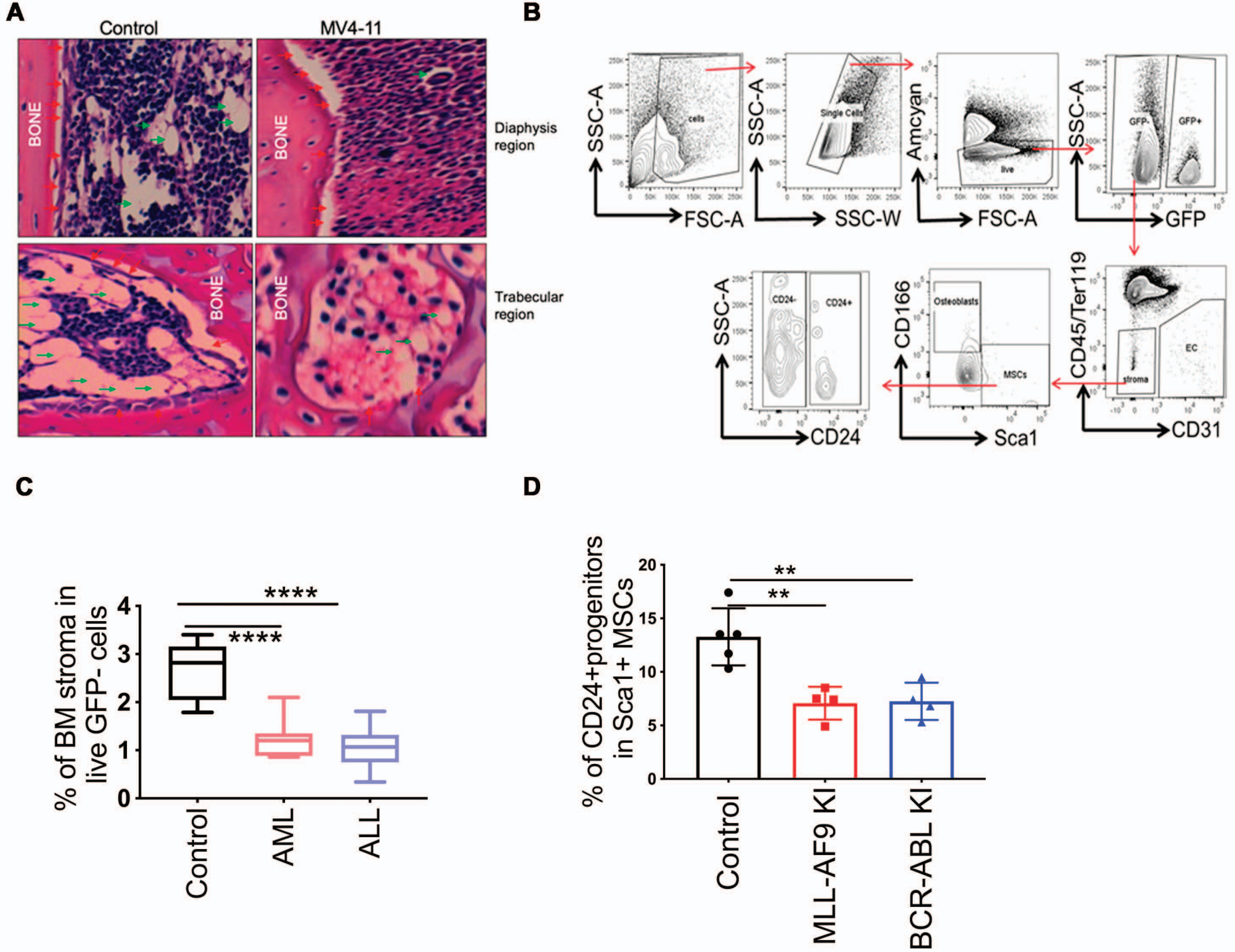
Supplementary Figure S2: (A) Gating strategy and the percentage of Leptin receptor (LEPR) expressing cells in normal and leukemic (ALL) mice bone stroma ($GFP^{-}CD45^{-}Ter119^{-}CD31^{-}$ cells). (B) Gating strategy of PaS ($PDGFRa^{+}\,Sca1^{+}$ cells) MSC population in mice bone stroma cells and leukemia burden dependently increased frequency in the ALL mice compared to normal stroma. (C) TSG101, exosome marker Immunoblot analysis from 10 µg protein extract from MV-411 cell line and a primary AML patient (COH202)- derived exosomes. (D) NanoSight size distribution histogram plot of purified leukemia exosomes confirming the purity of exosome preparation (30-150nM median size range). (E) Flow cytometry histogram plots of leukemia exosomes showing CD9 and CD63 expression. (F) Overall survival in months data of LIPE and PNPL2 overexpressing and normal pediatric ALL patients. All values mean ± SEM means, *** =p<0.0005, ****=p<0.0001.

Supplementary Figure S3: (A) Oil Red staining as analyzed by absorbance at 518 nm was significantly reduced in AML and ALL exosome co-cultured adipocytes (p=0.04 and p=0.03, respectively), and Atglistatin and CAY10948 treatment partially rescued leukemia-induced lipolysis and increased adipocyte-specific Oil Red absorbance (p=0.032 and p=0.02, respectively). (B) Representative Oil Red staining of adipocytes treated with AML conditioned media, IL-1β (200 ng/ml), IL-6(200 ng/ml), TNFα (100 ng/ml) and Atglistatin for 48h. (C) Relative Oil Red absorbance at 518 nm of adipocytes treated with the conditions listed in (B), *=p<0.05, ** =p<0.005.(D) Representative FACS plot of MV4-11 cells to monitor intracellular CPT1A expression after culture with normal and AML-conditioned adipocytes. (E) Relative *Pnpla2* (ATGL) mRNA expression in MLL-

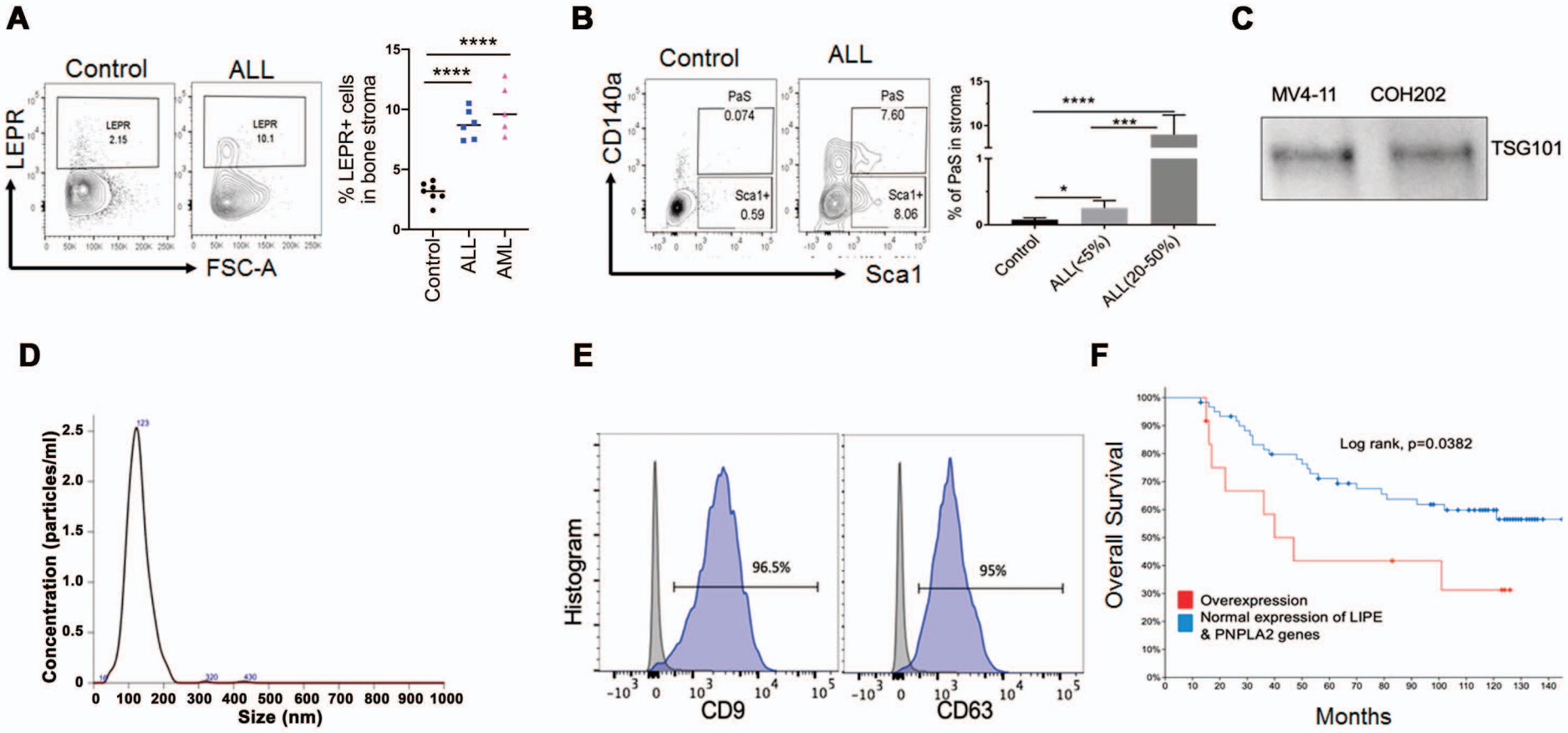
AF9 cells after 24h atglistatin treatment and conditional shRNA knockdown at 48h after doxycycline induction, *=P<0.05.

Supplementary Figure S4: (A) Relative *Ocn* mRNA expression from mice bone marrow stroma cells pretreated with zolendronic acid (ZA) or DMSO (1 Vs 1.60, p=0.0215. (B) Relative *PPAR*γ mRNA expression from bone marrow stroma cells pretreated with ZW1929(15 mg/kg for 3 weeks) or DMSO (1 Vs 1.76, p=0.0159). (C) Log rank survival analysis of B6 mice injected with 1 x 10^6 MLL-AF9-GFP+ cells followed by ZA or DMSO treatment (28.5 vs. 21 days, long rank test p=0.0010). (D) Log rank survival analysis of B6 mice injected with 1 x 10^6 BCR-ABL-GFP+ cells followed by ZA or control treatment from days 4-8. (24.5 vs. 16.5 days, p=0.0016). (E) Log rank survival analysis of control and GW1929-pretreated mice for 3 weeks followed by injection of 1 x 10^6 BCR-ABL-GFP cells (16 Vs 23 days, p=0.0009).

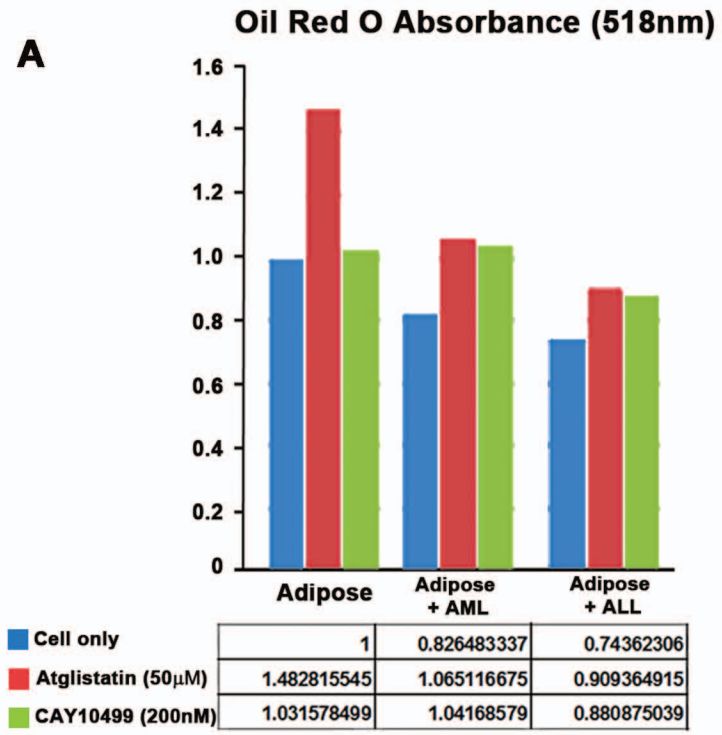
Supplementary Figure S1

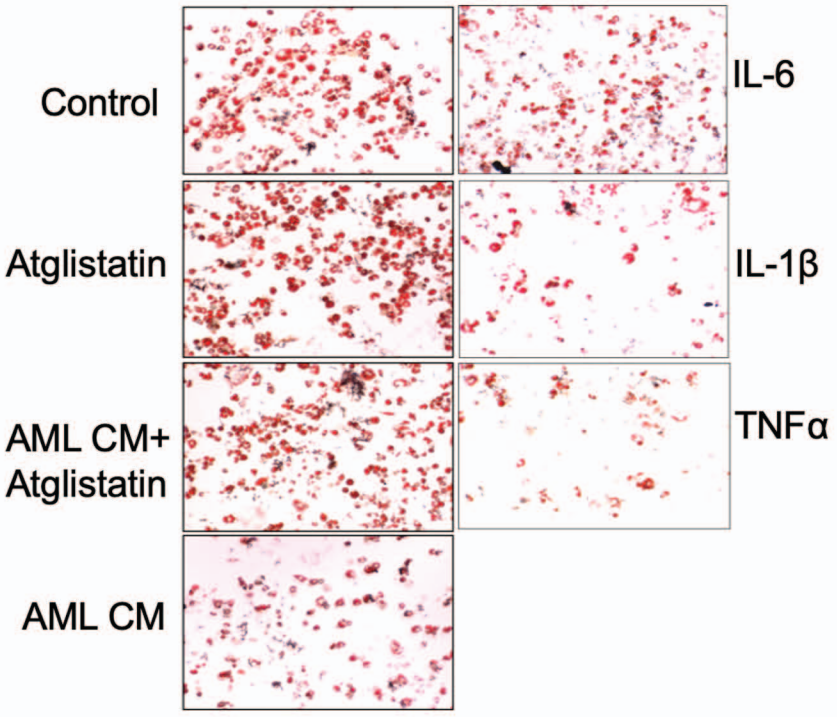


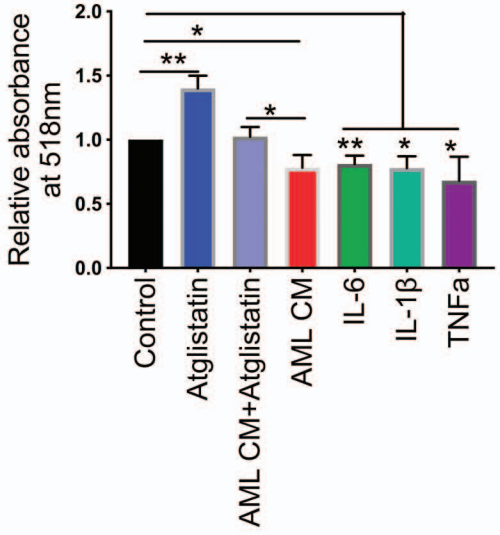
Supplementary Figure S2

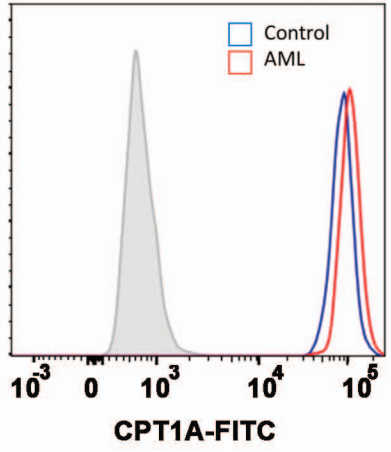


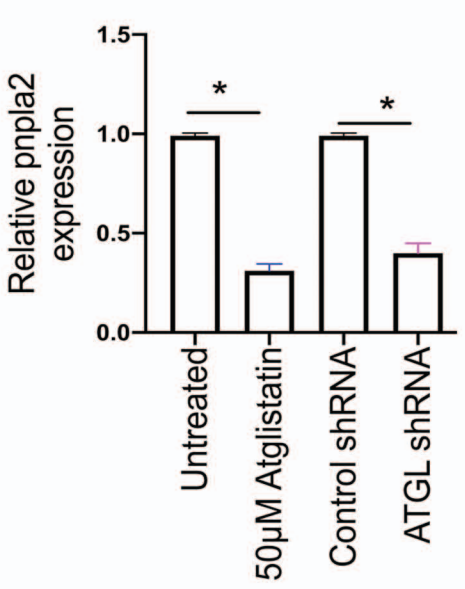
Supplementary Figure S3



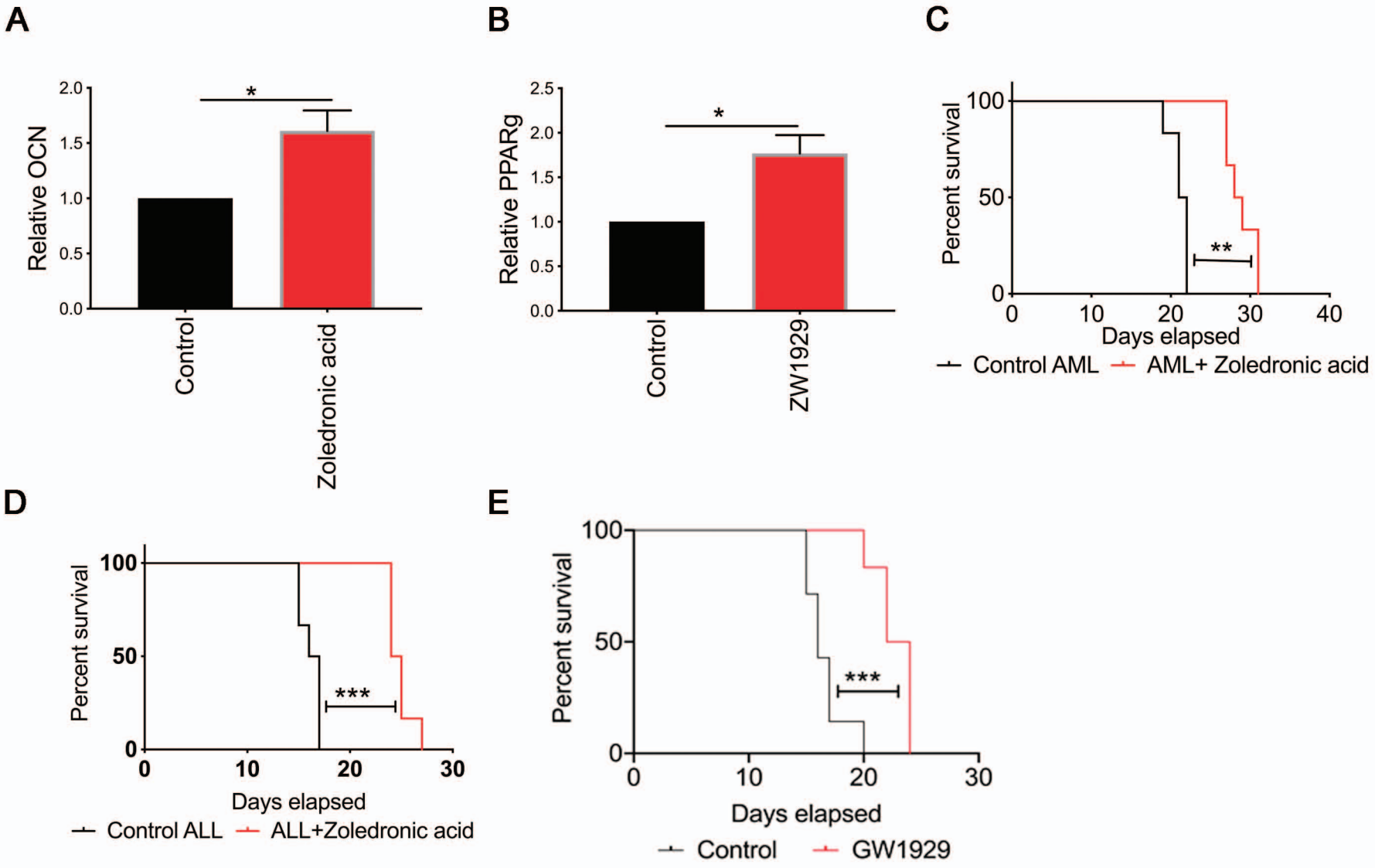








Supplementary figure S4



Supplementary Table S1: Hematological and genetic features of the leukemia patients used in the study.

ID	Cytogenetics	Disease	Risk Status	Gene(s) Mutated	BM blast %
COH200	47XX,+1	AML	high	FLT-TKD, TET2, WT1	20
COH201	46XY	ALL	high	NA	30
COH202	t(2;17), der(8)t(8;8)	AML	high	FLT-3 ITD	85

Supplementary Table S2: TaqMan probes used in the current study

Gene	TaqMan Assay ID
GAPDH	Mm99999915_g1
IL-6	Mm00446190_m1
BGLAP (OCN)	Mm03413826_mH
PPARγ	Mm00440940_m1
IL-1β	Mm00434228_m1
CCL3	Mm00441259_g1
TNFa	Mm00443258_m1
Pnpla2(ATGL)	Mm00503040_m1

Supplementary Table 3: Antibodies and chemicals used in the current study.

Antibody/Compound	Dilution	Cat. No.	Source
BV605 anti-mouse Sca1 Antibody	1:100	108134	BioLegend
PE/Cyanine5 anti-mouse CD45 Antibody	1:100	103110	BioLegend
PB anti-mouse TER-119/Erythroid cell Antibody	1:100	116232	BioLegend
Mouse Leptin R Biotinylated Antibody	1:25	BAF497	R&D systems
APC antihuman CD140 (PDGFRα) antibody	1:100	323512	BioLegend
Purified Anti-mouse CD16/32	1:200	101302	BioLegend
PerCP/Cy5.5 Anti-mouse CD146 Antibody	1:50	134710	BioLegend
PE Anti-mouse CD166 Antibody	1:50	FAB1172P	R&D systems
PE/Cy7 Anti-mouse CD31 Antibody	1:100	102418	BioLegend
A700 Anti-mouse CD24 Antibody	1:100	101835	BioLegend
Anti-Human Annexin V Antibody	1:100	640936	BioLegend
A488 Anti-Human Anti-CPT1A antibody	1:100	ab171449	abcam
LIVE/DEAD™ Fixable Violet Dead Cell Stain	1µg/ml	L34955	Thermo Fischer Scientific
DAPI (4',6-Diamidino-2-Phenylindole, Dilactate)	1µg/ml	422801	BioLegend
Propidium Iodide	1µg/ml	P3566	Thermo Fischer Scientific
Phospho-HSL (Ser563) Antibody	1:1000	4139	Cell Signaling Technology
Perilipin-1 (D1D8) XP® Rabbit mAb	1:1000	9349S	Cell Signaling Technology
ATGL (30A4) Rabbit mAb	1:1000	2439S	Cell Signaling Technology
β-Actin (D6A8) Rabbit mAb (HRP Conjugate)	1:1000	12620S	Cell Signaling Technology
TSG101 antibody	1:1000	SAB2702167	Sigma Aldrich
PE antihumanCD9 antibody	1:100	312106	BioLegend
APC/Cyanine7 antihuman CD63 antibody	1:100	353045	
BODIPY™ 493/503	5µM	D3922	Thermo Fischer Scientific
APC/Cyanine7 Streptavidin	1:200	405208	BioLegend
Atglistatin		S7364	Selleckchem
CAY10499		359714-55-9	Caymanchem
Polybrene		sc-134220	Santacruz biotechnology
Zoledronic acid monohydrate		SML0223	Sigma Aldrich
GW1929		G5668	Sigma Aldrich
Quizartinib (AC220)		S1526	Selleckchem

Cytosine β-D-arabinofuranoside		C1768-500MG	Sigma Aldrich
Permeabilization Wash Buffer(10X)		421002	BioLegend
Annexin V Binding Buffer		422201	BioLegend
SMARTvector Inducible Mouse Pnpla2 shRNA		V3SM11256 08EG66853	Dharmacon
SMARTvector Inducible Non-targeting Control shRNA		VSC6571 50	Dharmacon
Free Fatty acid quantification kit		MAK044-1KT	Sigma Aldrich