

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

Since the bone marrow (BM) microenvironment and adipose tissue provide an attractive sanctuary for cancer progression and the acquisition of drug resistance phenotypes,¹⁻⁵ interactions between BM niche components and leukemia have recently attracted interest. However, the mechanisms underlying these complex interactions are not well understood. We previously reported that leukemic cells, through their secreted exosomes, induce microenvironment reprogramming to inhibit normal hematopoiesis and create a self-enforcing microenvironment for their own expansion.⁶ Here, using syngeneic acute myeloid leukemia (AML)/acute lymphoblastic leukemia (ALL) transplantation and knock-in leukemic mice models, we aimed to understand how leukemia remodels the BM niche. We describe a novel mechanism through which leukemic cells, via their exosomes,

remodel BM niche subpopulations by enhancing the expression of adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL) enzymes in adipocytes, resulting in increased lipolysis. Furthermore, pharmacological inhibition of ATGL and HSL rescued lipolysis, preserved adipocyte morphology and reduced fatty acid oxidation in leukemic cells. Finally, we show that leukemia progression can be delayed by increasing osteoblasts and adipocytes *in vivo*.

As mesenchymal stem cells, osteoblasts and adipocytes together with other bone stromal cells influence hematopoietic stem cell homeostasis, regeneration and leukemic progression,⁷⁻¹¹ we investigated these populations during leukemogenesis. As found previously,⁶ histological analysis of normal and leukemic mice bones revealed a reduction in mature osteoblasts and adipocyte numbers in both AML and ALL models (Figure 1A, *Online Supplementary Figure S1A*). Significant, progressive declines of osteocalcin (*Ocn*) and peroxisome proliferator-

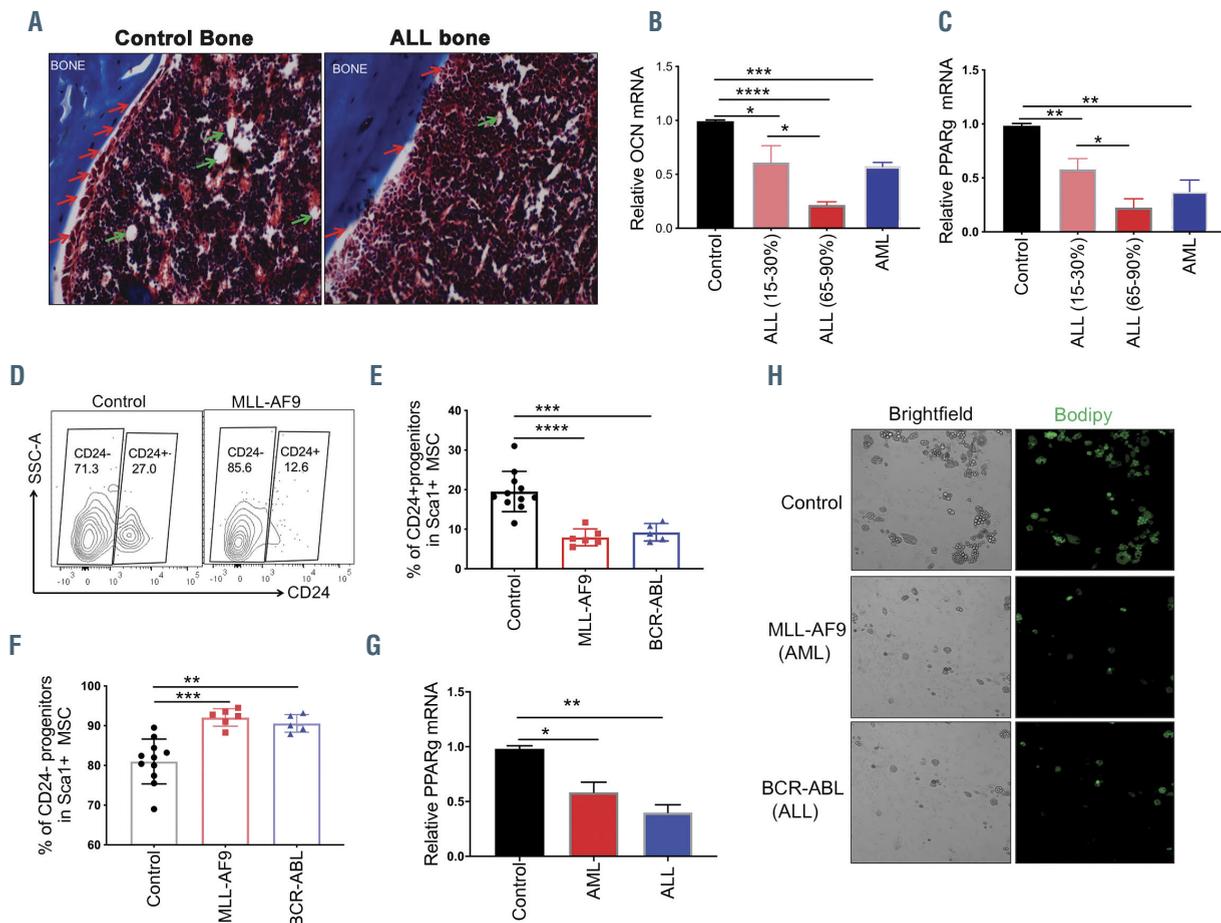


Figure 1. Leukemia leads to exhaustion of mature osteoblasts and adipocytes and expands bone marrow preadipocytes that do not undergo terminal differentiation. (A) 5X Trichrome-stained images of bones from normal and acute lymphoblastic leukemia (ALL) mice, showing reduced osteoblasts and adipocytes in the leukemic mice. Red arrows mark osteoblasts, and green arrows mark adipocytes. (B, C) Quantitative real-time polymerase chain reaction analysis of relative gene expression of osteoblast-specific osteocalcin (*Ocn*) (B) and mature adipocyte-associated *Pparg* (C) mRNA expression between controls and leukemia bone marrow-derived sorted stroma (GFP-CD45⁺ Ter119⁺ CD31⁻ cells). (D) Representative flow-cytometric analysis of CD24 expression within mesenchymal stromal cells (MSC) in bones of 8- to 10-week-old B6/C57 control and MLL-AF9-transplanted leukemic mice. (E) Frequency of GFP-CD45⁺ Ter119⁺ CD31⁻ CD166⁻ Sca1⁺CD24⁺ multipotent bone marrow (BM) MSC in normal mice and syngeneic mice transplanted with acute myeloid leukemia (AML) or ALL (n>6 mice per group in at least three independent experiments). (F) Frequency of GFP-CD45⁺ Ter119⁺ CD31⁻ CD166⁻ Sca1⁺CD24⁻ cells (preadipocytes) in the BM of normal and leukemic mice. (G) *Pparg* mRNA expression by Sca1⁺CD24⁻ cells (preadipocytes) derived from normal and leukemic mice primed for adipose induction for 2 weeks (P=0.0085 and P=0.0284, in ALL and AML, respectively). (H) BODIPY staining images of BM-derived Sca1⁺CD24⁻ cells, after 2 weeks *in vitro* differentiation, from control mice and mice with a high leukemia burden (AML or ALL). The images suggest that the adipocyte differentiation capacity of leukemic preadipocytes is reduced compared to that of normal, control preadipocytes. All data are means ± standard error of mean. Statistical significance was determined by unpaired *t*-tests; *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

activated bone marrow-derived receptor gamma (*Pparg*) mRNA levels were observed in stromal cells (GFP-CD45⁻Ter119⁻CD31⁻) in a leukemia burden-dependent manner in ALL and AML mice (Figure 1B and C) confirming that leukemia progression exhausts mature osteoblasts and adipocytes. Besides reductions in mature niche populations, the frequency of bone stromal cells was also significantly diminished in ALL and AML mice compared to the frequency in control animals ($P < 0.0001$ and $P < 0.0001$, respectively) (Online Supplementary Figure S1B and C) suggesting a progressive inhibition of all BM constituents that support functional hematopoiesis with leukemia development.

Considering the loss of BM stroma, osteoblasts and adipocytes, we next investigated the progenitor populations responsible for replenishing these cells. Sca1⁺CD24⁺ cells from BM have been shown to differentiate into

osteochondrogenic and adipogenic lineages, unlike adipocyte lineage-restricted Sca1⁺CD24⁻ progenitors (preadipocytes).¹² Flow cytometry analysis of these distinct entities in bone stromal cells revealed that the percentage of Sca1⁺CD24⁺ multipotent progenitors (CD45⁻Ter119⁻CD31⁻CD166⁻Sca1⁺CD24⁺) within mesenchymal stem cells in MLL-AF9⁻ and BCR-ABL⁺ leukemic mice was significantly reduced compared to that in healthy controls both in transplantation and knock-in leukemia models ($P < 0.0001$ and $P = 0.0004$, respectively (Figure 1D and E; Online Supplementary Figure S1D)). Although, the frequency of preadipocytes in Sca1⁺ mesenchymal stem cells was significantly higher in leukemia mice (Figure 1F), the *in vitro* differentiation potential of preadipocytes from mice with high BM disease burden (70-85%) was severely compromised, as suggested by reduced *Pparg* mRNA levels (Figure 1G) and BODIPY staining (Figure 1H).

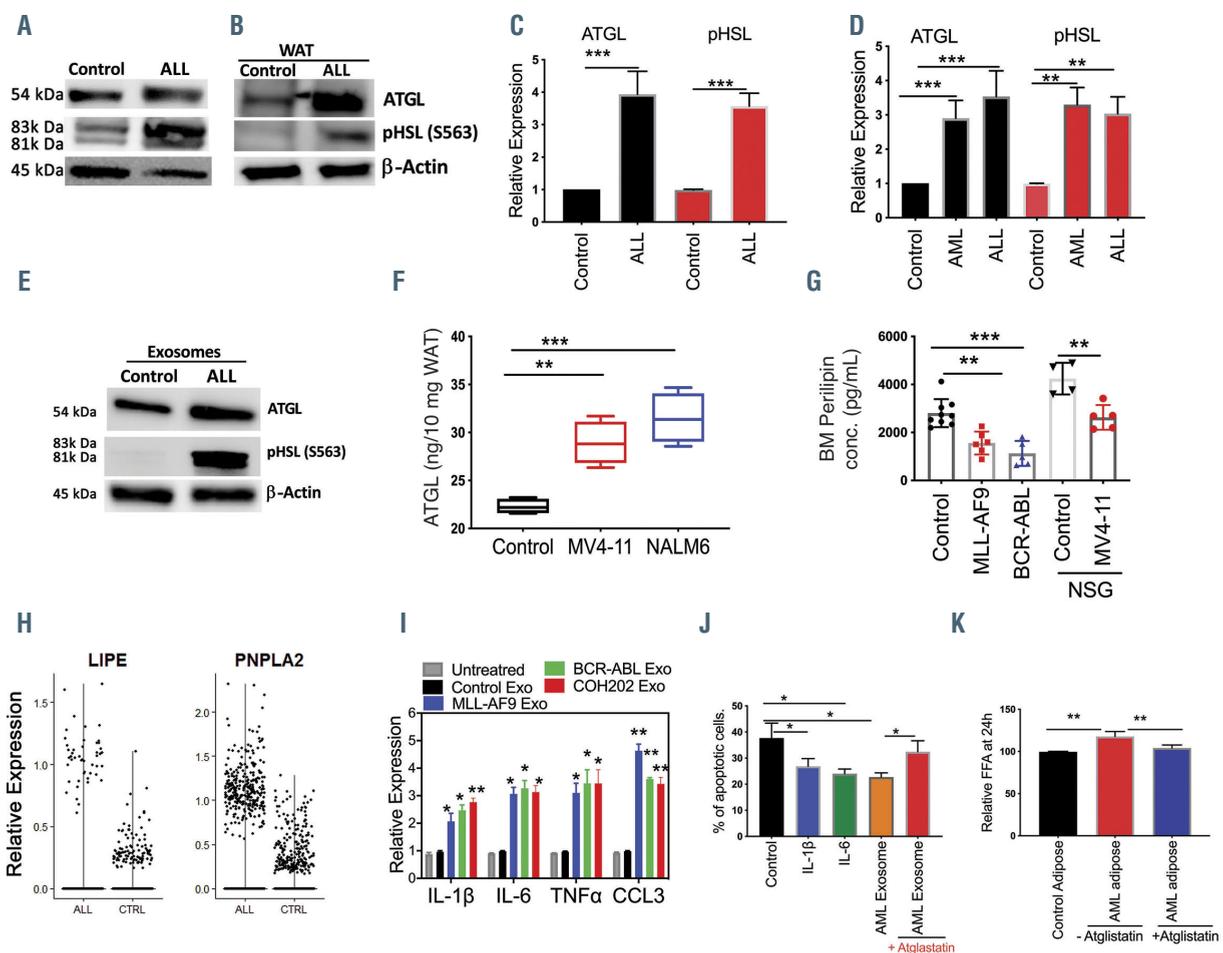


Figure 2. Leukemic cells enhance the expression of lipolytic enzymes through their secreted exosomes, creating an inflammatory microenvironment that promotes the cells' survival. (A) Representative western blot image showing upregulation of the expression of adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL) phosphorylated at serine 563 (pHSL) (S563) in adipocytes co-cultured with acute lymphoblastic leukemia (ALL) cells through transwell insert plates for 24 h. (B) ATGL and pHSL (S563) immunoblot of white adipose tissue (WAT) lysates from normal and ALL mice. (C) Combined normalized expression analysis of ATGL and pHSL (S563) between control and leukemic adipocytes *in vitro*. (D) Combined normalized ATGL and pHSL (S563) expression in WAT from age- and sex-matched normal mice and mice with ALL or acute myeloid leukemia (AML) from three independent experiments. (E) ATGL and pHSL (S563) immunoblot of adipocytes co-cultured with 10 μ g normal cells or AML-derived exosomes for 24 h. (F) Enzyme-linked immunosorbent assay (ELISA) of ATGL protein expression levels from control, MV4-11 and NALM6 leukemic mice WAT at day 15 after transplantation. (G) Perilipin protein levels, determined by ELISA, were significantly lower in leukemic mice BM supernatants than in those from control BM; ** $P < 0.01$, *** $P < 0.001$. (H) Single-cell sequencing revealing relative LIPE (HSL) and PNPLA2 (ATGL) mRNA expression in leukemia and healthy control BM-sorted stroma cells. (I) Relative mRNA expression analysis of interleukin-1 β (IL-1 β), interleukin-6 (IL-6), tumor necrosis factor- α (TNF α), and CCL3 of adipocytes after co-culture with normal and leukemic exosomes for 24 h; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (J) Percentage of MV4-11 apoptotic (annexin V⁺DAPI⁺) cells at 48 h after co-culturing with control, AML exosomes, and exogenous cytokines (IL-1 β and IL-6, 100 ng/mL) \pm 50 μ M atglustatin. (K) Relative levels of free fatty acids (FFA) in culture supernatants at 24 h after culturing adipocytes alone, with AML cells, or with AML in presence of atglustatin. All data are means \pm standard error of mean. Statistical significance was determined by unpaired t-tests; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Additionally, the *bona fide* mesenchymal stem cell marker, PDGFR α ⁺Sca1⁺(PaS), and adipocyte primed leptin receptor (LepR)¹⁵ were also analyzed. The frequency of these markers in leukemia bone stromal cells was significantly increased in a disease-burden-dependent manner (Online Supplementary Figure S2A and B). These data suggest that the leukemia-induced exhaustion of stroma and terminally differentiated adipocytes and osteoblasts pushes the mesenchymal stem cells or preadipocytes to expand to meet the renewed demands to sustain normal hematopoiesis or to support leukemia propagation, but that they fail to differentiate fully. Collectively, these data suggest that cancer-induced microenvironmental reprogramming could be a common feature of acute leukemias despite the genetic heterogeneity of these malignancies.

To gain insights into the molecular mechanisms underlying the reduction of adipocytes in leukemia, we analyzed the expression of genes involved in the pathway of triglyceride hydrolysis, ATGL (also known as PNPLA2) and hormone-sensitive lipase (HSL or LIPE, Ser563 phosphorylation site) in leukemic conditions.¹⁴ The adipocytes co-cultured with leukemic cells through a transwell for 24 h (*in vitro*) and white adipose tissue from leukemic mice (*in vivo*) exhibited upregulation of ATGL and HSL Ser563 protein expression compared to the respective controls (Figure 2A-D).

Next, to identify the driver behind this leukemia-mediated upregulation of ATGL and HSL, we analyzed leukemia exosomes. The exosomes used in the study were validated using standard markers (Online

Supplementary Figure S2C-E). When co-cultured with adipocytes, the leukemia exosomes enhanced adipocyte-specific ATGL and phosphorylated HSL (Ser563) protein levels unlike their normal exosome counterparts (Figure 2E), initiating the lipolysis cascade, suggesting cargo-specific differences between leukemic and normal BM exosomes. Next, we observed a significant upregulation of ATGL in white adipose tissue from leukemic mice (Figure 2F) and significantly reduced levels of a lipid droplet-associated protein, perilipin, in BM plasma, suggesting an inverse correlation between perilipin and ATGL expression (Figure 2G). Single-cell transcriptomics analysis of human BM stroma from ALL patients and healthy controls corroborated the leukemia models and *in vitro* findings and showed an upregulation of LIPE and PNPLA2 gene expression in leukemia stromal cells (Figure 2H). Furthermore, evaluating the clinical significance of these genes related to triglyceride catabolism, we analyzed the RNA sequencing data from the published TARGET phase-II ALL study datasets¹⁵ and observed that overexpression of *LIPE* and *PNPL2* genes is associated with inferior overall survival in ALL patients (Online Supplementary Figure S2F), highlighting the importance of these genes in leukemia-induced microenvironmental dysregulation.

Extending the working hypothesis that exosome-mediated adipose dysfunction favors leukemia growth, we cultured adipocytes with 10 μ g of exosomes derived from healthy subjects, leukemia cell lines and an AML patient (COH202) for 24 h. The adipocytes cultured with leukemia exosomes exhibited enhanced levels of inter-

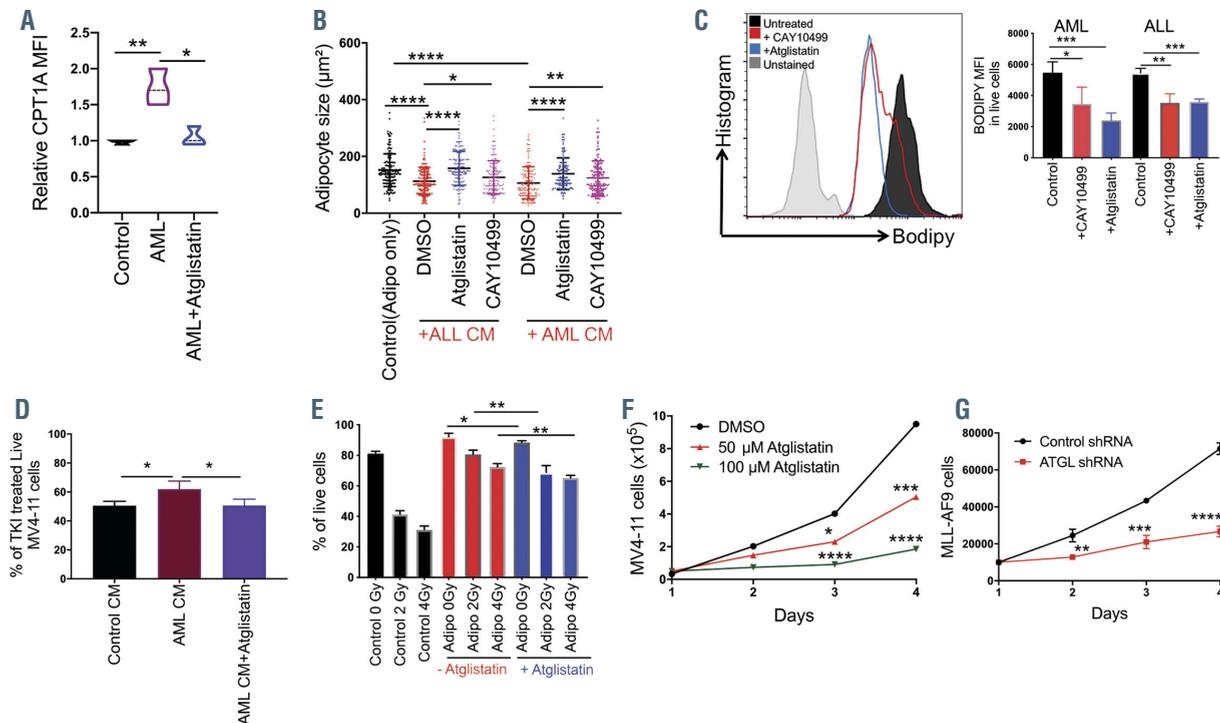


Figure 3. Exosome-induced lipolysis increases fatty acid oxidation in leukemic cells and genetic or pharmacological targeting alone or in combination with chemo-radiotherapy augments leukemia cell apoptosis. (A) Relative intracellular carnitine palmitoyltransferase 1A (CPT1A) mean fluorescence intensity (MFI) expression analysis at 24 h in MV4-11 cells after co-culturing with conditioned media (CM) derived from control adipocytes, acute myeloid leukemia (AML) adipocytes, and AML adipocytes with 50 μ M atglistatin; * P <0.05, ** P <0.005. (B) Quantification of adipocyte size after culturing adipocytes alone, with leukemic cell exosome-enriched CM, and with CM + 50 μ M atglistatin or 200 nM CAY104988 for 48 h; * P <0.05, ** P <0.005, **** P <0.0001. (C) BODIPY (493/505) MFI expression in leukemic cells after culturing with prelabeled adipocytes \pm atglistatin or CAY104988; * P <0.05, ** P <0.005, *** P <0.001. (D) Percentage apoptosis of MV4-11 cells after culture with AML/adipocyte-CM with or without atglistatin, followed by culture in the presence of 5 nM of quizartinib, a tyrosine kinase inhibitor (TKI) for 72 h; * P <0.05. (E) Percentage of live MV411 cells at 48 h (annexin V/DAPI⁺) after radiation and co-culture with non-primed adipocytes, AML-primed adipocytes, and AML-primed adipocytes in the presence of 50 μ M atglistatin. (F) MV4-11 cell numbers on different days after culturing alone and in the presence of 50 μ M and 100 μ M atglistatin. (G) MLL-AF9 cells were transduced with non-targeting scrambled shRNA (control cells) and ATGL shRNA and treated with 1 μ M doxycycline. Cell numbers were determined on days 1, 2, 3, and 4; ** P <0.005, *** P <0.001, **** P <0.0001.

leukin-1 β , interleukin-6, tumor necrosis factor- α and CCL3 mRNA transcripts compared to controls (Figure 2I), suggesting that some of the effects of exosomes on adipocyte differentiation might be attributed indirectly to these pro-inflammatory cytokines (Online Supplementary Figure S3A-C) and perhaps may also be responsible for the differentiation block. This is the first study establishing a role for leukemic exosomes in the induction of adipocyte atrophy by upregulation of ATGL/HSL lipolytic enzyme activity, directly or through inflammatory cytokines.

As a proof of principle, we first cultured the adipocytes with cytokines, AML exosomes alone or in the presence of atglistatin (an ATGL inhibitor) in nutrient-deprived conditions for 48 h. AML cells cultured in these exosome/cytokine conditioned media (CM) had lower apoptosis, possibly by providing lipid substrates from adipocytes to drive mitochondrial β -oxidation (an alternate energy pathway) in leukemic cells during nutrient deficiency. However, atglistatin treatment increased apoptosis by blocking ATGL-mediated free-fatty acid (FFA) release (Figure 2J and K). The inhibition of FFA resulted in reduced leukemic fatty acid oxidation, evidenced by reduced mitochondrial *Cpt1a* gene expression (Figure 3A; Online Supplementary Figure S3D).

Atglistatin or HSL inhibitor (CAY10499) treatment also reversed the leukemia CM-induced reductions in adipocyte size (Figure 3B), possibly by inhibiting exosome-induced triglyceride breakdown. Since leukemia-induced adipocyte breakdown releases FFA into the surrounding tumor environment to drive the leukemic cells' energy processes, we hypothesized that atglistatin or CAY10499 could inhibit FFA release and hence lower the uptake by leukemia cells. To test this hypothesis, we used adipocytes with fluorescently labeled FFA analogs and observed that atglistatin or CAY10499 treatment could significantly reduce BODIPY transfer in leukemic cells (Figure 3C).

Next, we evaluated the benefit of ATGL inhibition in the context of the resistance to chemo/radiotherapy induced by adipocytes and leukemic exosomes. MV4-11 cells were cultured in control or AML adipocyte CM together with or without atglistatin and 5 nM quizartinib (a tyrosine kinase inhibitor) in nutrient-deprived conditions. Compared to treatment with quizartinib alone, the atglistatin and quizartinib combination significantly reduced the survival advantage mediated by leukemia exosome-induced lipolysis, suggesting that atglistatin could be used as combination therapy with quizartinib to enhance its efficacy on AML cells by limiting nutrients released from adipocytes (Figure 3D).

Similarly, atglistatin also increased radiation-induced leukemia killing to overcome the adipocyte-induced resistance of leukemia to radiotherapy (Figure 3E). We further proved that leukemia-specific ATGL is essential for leukemia cell proliferation and showed that the proliferation could be inhibited pharmacologically by atglistatin in a dose-dependent manner ($P=0.001$ and $P<0.0001$ at day 4 at a doses of 50 μ M and 100 μ M atglistatin, respectively) (Figure 3F) or by shRNA-mediated genetic knockdown of the ATGL gene ($P<0.0001$ at day 4) (Figure 3G, Online Supplementary Figure S3E).

Since losses of osteoblasts and adipocytes are key features during leukemia progression, we hypothesized that increasing osteoblast or adipocyte content pharmacologically with either zoledronic acid (osteoblasts) or GW1929 (adipocytes) might delay disease development. The treated mice stroma exhibited increased levels of *Ocn* and *Pparg* genes in BM stroma providing a functional val-

idation of the increases in osteoblast and adipocyte numbers (Online Supplementary Figure S4A and B). Furthermore, zoledronic acid or GW1929 treatment in vivo prolonged the survival of AML and ALL mice (Online Supplementary Figure S4C-E), confirming the earlier belief of a role of osteoblasts and adipocytes in assisting normal hematopoiesis to delay leukemia progression.

In conclusion, we discovered that leukemia induces defective preadipocyte expansion and describe a novel mechanism of leukemic exosome-induced adipocyte loss through the activation of lipolytic genes. Finally, leukemia- or exosome-induced niche reprogramming could be rescued by GW1929 and zoledronic acid to limit the progression of leukemia. This study further strengthens our earlier observations of leukemia exosome-induced BM microenvironmental dysregulation to favor leukemia propagation. Taken together, these findings provide a strong rationale for targeting the defective niche with leukemia exosome inhibition and normalizing BM niche components with pharmacological agents in conjunction with conventional therapies.

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Contributions: BK, MO, JB, SSM, PV and LEP performed experiment; BK and SKH conceived the idea; BK designed experiments, interpreted data and wrote the manuscript; AS, AS, GM and SKH provided the leukemia patients' samples; SSM, JS, GM and SKH edited the manuscript. All authors read and approved the paper.

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References

1. Ye H, Adane B, Khan N, et al. Leukemic stem cells evade chemotherapy by metabolic adaptation to an adipose tissue niche. *Cell Stem Cell*. 2016;19(1):23-37. *Cell Stem Cell*. 2016;19(1):23-37.
2. Tabe Y, Yamamoto S, Saitoh K, et al. Bone marrow adipocytes facilitate fatty acid oxidation activating AMPK and a transcriptional network supporting survival of acute monocytic leukemia cells. *Cancer Res*. 2017;77(6):1453-1464.
3. Mendez-Ferrer S, Bonnet D, Steensma DP, et al. Bone marrow niches in haematological malignancies. *Nat Rev Cancer*. 2020;20(5):285-298.
4. Sheng X, Tucci J, Parmentier JH, et al. Adipocytes cause leukemia cell resistance to daunorubicin via oxidative stress response. *Oncotarget*. 2016;7(45):73147-73159.
5. Schepers K, Campbell TB, Passegue E. Normal and leukemic stem cell niches: insights and therapeutic opportunities. *Cell Stem Cell*. 2015;16(3):254-267.
6. Kumar B, Garcia M, Weng L, et al. Acute myeloid leukemia transforms the bone marrow niche into a leukemia-permissive microenvironment through exosome secretion. *Leukemia*. 2018;32(3):575-587.
7. Frisch BJ, Ashton JM, Xing L, Becker MW, Jordan CT, Calvi LM. Functional inhibition of osteoblastic cells in an in vivo mouse model of myeloid leukemia. *Blood*. 2012;119(2):540-550.
8. Boyd AL, Reid JC, Salci KR, et al. Acute myeloid leukaemia disrupts endogenous myelo-erythropoiesis by compromising the adipocyte bone marrow niche. *Nat Cell Biol*. 2017;19(11):1336-1347.
9. Zhou BO, Yu H, Yue R, et al. Bone marrow adipocytes promote the regeneration of stem cells and haematopoiesis by secreting SCF. *Nat Cell Biol*. 2017;19(8):891-903.
10. Mendez-Ferrer S, Michurina TV, Ferraro F, et al. Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. *Nature*. 2010;466(7308):829-834.
11. Mattiucci D, Maurizi G, Izzi V, et al. Bone marrow adipocytes support hematopoietic stem cell survival. *J Cell Physiol*. 2018;233(2):1500-1511.
12. Ambrosi TH, Scialdone A, Graja A, et al. Adipocyte accumulation in the bone marrow during obesity and aging impairs stem cell-based hematopoietic and bone regeneration. *Cell Stem Cell*. 2017;20(6):771-784.e6.
13. Yue R, Zhou BO, Shimada IS, Zhao Z, Morrison SJ. Leptin receptor promotes adipogenesis and reduces osteogenesis by regulating mesenchymal stromal cells in adult bone marrow. *Cell Stem Cell*. 2016;18(6):782-796.
14. Lass A, Zimmermann R, Oberer M, Zechner R. Lipolysis - a highly regulated multi-enzyme complex mediates the catabolism of cellular fat stores. *Prog Lipid Res*. 2011;50(1):14-27.