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Adipose tissue attracts and protects acute lymphoblastic leukemia cells from chemotherapy

Rocky Pramanik, PhD^{a,f}, Xia Sheng^a, Brian Ichihara^a, Nora Heisterkamp, PhD^{b,c,d}, and Steven D. Mittelman, MD, PhD^{a,b,c,e,*}

^aCenter for Endocrinology, Diabetes & Metabolism, Children's Hospital Los Angeles, CA, USA

^bThe Saban Research Institute, Children's Hospital Los Angeles, CA, USA

^cDepartment of Pediatrics, Keck School of Medicine, University of Southern California, Los Angeles, CA, USA

^dDepartment of Pathology, Keck School of Medicine, University of Southern California, Los Angeles, CA, USA

^eDepartment of Physiology & Biophysics, Keck School of Medicine, University of Southern California, Los Angeles, CA, USA

^fDepartment of Neurological Surgery, Keck School of Medicine, University of Southern California, Los Angeles, CA, USA

Abstract

Obesity is associated with an increased risk of acute lymphoblastic leukemia (ALL) relapse. Using mouse and cell co-culture models, we investigated whether adipose tissue attracts ALL to a protective microenvironment. Syngeneically implanted ALL cells migrated into adipose tissue within ten days. *In vitro*, murine ALL cells migrated towards adipose tissue explants and 3T3-L1 adipocytes. Human and mouse ALL cells migrated toward adipocyte conditioned media, which was mediated by SDF-1 α . In addition, adipose tissue explants protected ALL cells against daunorubicin and vincristine. Our findings suggest that ALL migration into adipose tissue could contribute to drug resistance and potentially relapse.

Keywords

adipocytes; acute lymphoblastic leukemia; ALL; AMD3100; C-X-C chemokine receptor type 4; CXCR4; microenvironment; SDF-1 α ; stromal derived-factor-1 alpha

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Corresponding author: Steven D. Mittelman, M.D., Ph.D., Center for Endocrinology, Diabetes & Metabolism, Children's Hospital Los Angeles, Los Angeles CA 90027, Mailstop 93. smittelman@chla.usc.edu.

**Author Contributions:

RP and XS contributed equally to this work. RP developed the idea and hypothesis, designed and performed the experiments, analyzed the experimental results, and wrote the manuscript. XS designed and performed experiments and analyzed results. BI performed the migration assay using cells and tissue explants, helped RP in measuring SDF-1 α by ELISA and Western blot. NH helped with the design and analysis of the study, and assisted with the manuscript. SDM directed and oversaw the project and helped with data analysis and preparation of the manuscript.

Conflict of Interest:

The authors have no conflicts of interest to disclose.

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Introduction

Acute lymphoblastic leukemia (ALL) is the most common type of cancer in children. With aggressive combination chemotherapy, the overall cure rate is about 80% in children [1] but only 50% in adults [2]. Leukemia relapse continues to be a problem, and is thought to be due to drug resistance [3]. While many studies of drug resistance have focused on acquired gene mutations in leukemia cells, in some studies the leukemia microenvironment has also been shown to play a major role in *de novo* chemotherapy resistance in ALL [4]. It is thought that leukemia cells home to the bone marrow, which acts as a niche that protects ALL cells from drug-induced death [4, 5]. Since bone marrow is comprised of many different cell types (e.g. mesenchymal stem cells, osteoblasts, endothelial cells, hematopoietic cells, adipocytes), it is not clear which marrow cells are responsible for ALL homing and induction of chemotherapy resistance.

There is growing evidence that adipocytes may interact with cancer cells to promote invasion, proliferation, and/or drug resistance [6–8]. Adipocytes secrete numerous factors which have roles in cancer cell proliferation, migration, and metastasis, such as insulin-like growth factor 1, leptin, platelet-derived growth factor, matrix metalloproteinase 11, interleukin 6 and stromal cell-derived factor 1 (SDF-1 α) [9–12]. Some of these factors may contribute to the strong associations observed between obesity and cancer mortality [13] including studies which show that obesity is associated with increased risk of relapse in ALL [14, 15]. However, the precise mechanisms whereby adipocytes may contribute to ALL relapse remain unknown.

Previously, we observed the presence of transplanted ALL cells in the fat depots of obese mice by fluorescence microscopy after vincristine treatment [8]. However, since these mice had developed a substantial leukemia burden, and we did not look for ALL cells in other organs, it was not known whether the leukemia cells actively and preferentially migrated into the adipose tissue. Furthermore, the mechanism(s) regulating leukemia cell migration into adipose tissue are not known. In the present study, we report that adipocyte secretion of SDF-1 α induces ALL cells to migrate into adipose tissue, further demonstrating the importance of adipocytes in the leukemia cell microenvironment.

Materials and Methods

Materials

AMD3100, daunorubicin, and vincristine were purchased from Sigma Chemicals (St. Louis, MO). Mouse recombinant SDF-1 α , leptin, adiponectin, MCP-1, resistin and RANTES were obtained from Peprotech (Rocky Hill, NJ). All other chemicals and reagents were tissue culture grade.

Mouse Tissue explants

All mouse experiments were approved by the Children's Hospital Los Angeles Institutional Animal Care and Use Committee, and were performed in accordance with the USPHS Policy on Humane Care and Use of Laboratory Animals. C57Bl/6J mice were raised on a 60 kCal% fat diet (Research Diets, New Brunswick, NJ) at the Jackson Laboratory (Bar Harbor, ME). Mice were euthanized at 20 weeks of age by cardiac perfusion with PBS/Heparin under anesthesia. Various tissues were removed rapidly and washed in cold PBS. Small pieces of each tissue (100 mg) were washed twice with RPMI plus 10% FBS, and cultured in the same medium. One day later, the culture medium was changed with fresh medium. Tissue explants were then cultured for an additional two days without media change before use for migration or chemotherapy protection assay.

Cell lines

Murine pre-B 8093-ALL and GFP positive 8093-ALL cells have been previously described [8, 16]. Human leukemia cell lines RS4;11, BV173, SD1 and K562, and Murine fibroblastic 3T3-L1 and OP-9 cells were purchased from ATCC. The human primary leukemia cell strains ICN13, BLQ1, UCSF02, US.7 and TXL-2 [17, 18] were kindly provided by Yong-mi Kim and Markus Müschen. ChubS7 cell line was described before [19]. 3T3-L1, OP-9, and ChubS7 cells were cultured at confluence in a 24-well plate and differentiated as previously described [8, 19]. Pre-adipocyte (FCM) and adipocyte conditioned media (ACM) were collected after 48 hour conditioning of 3T3-L1, OP-9 and ChubS7 pre-adipocytes and adipocytes.

Migration assay

For migration of mouse ALL, FCM and ACM were made by culturing 3T3-L1 cells in RPMI medium containing 10% FBS for 2 days. Migration of mouse ALL toward feeder layers were set up with a confluent monolayer of 3T3-L1 and OP9 pre-adipocytes and adipocytes. RPMI medium containing 10% FBS was incubated with the feeder layers for 48 hours prior to the assays. For migration of human ALL cells, we generated serum-free ACM in Opti-MEM medium (Invitrogen, Carlsbad, CA) with differentiated 3T3-L1 adipocytes. Migration assays were all performed in 24-well tissue culture plates, using TransWell inserts with 5 μ m (for 8093-ALL cells) or 8 μ m (for human cells) pores (Millipore, Billerica, MA). ALL cells in RPMI with 10% FBS were seeded into the top chambers. The bottom chambers contained cultured tissue explants (held down by a 1 mm pore size nylon mesh), pre-adipocyte or adipocyte monolayers, or conditioned media. After 1.5 hours (8093-ALL cells) or 3 hours (human leukemia cells), viable cells in each chamber were quantified by trypan blue exclusion. The number of cells (% migration) that migrated to the bottom chamber was calculated from the total cell count in top and bottom chambers. In some experiments where differentiated adipocytes were used, leukemia cells were collected from the bottom chambers by vigorous pipetting, and counted using trypan blue exclusion.

SDF-1 α concentrations in plasma, obese and control mice tissue explant conditioned media, and 3T3-L1 and OP9 FCM and ACM, were measured in duplicate by ELISA (RayBiotech, Norcross, GA).

Western blot

Total protein was extracted from murine or human leukemia cells with protein isolation buffer [8]. Lysates were sonicated briefly and centrifuged for 10 minutes at 14,000 rpm. The supernatant was used for protein measurement. Twenty micrograms of protein was subjected to SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were blocked in 5% milk and probed simultaneously with CXCR4 and actin antibody (Cell Signaling Technology) because CXCR4 and actin have clearly distinct molecular weights.

In vivo leukemia cell transplantation

To determine whether leukemia cell migration into adipose tissue is an early event *in vivo*, GFP positive 8093-ALL cells were transplanted retro-orbitally into 20-week old syngeneic obese and lean C57Bl/6J mice (10,000 cells per mouse, [8], 6 mice per group). Ten days after engraftment, blood was collected from the submandibular plexus by cheek bleeds, and then mice were sacrificed by cardiac perfusion.

For FACS analysis of leukemia cells, various tissues (brain, lung, liver, muscle, spleen, and kidney), were removed and washed once with cold PBS then digested with Liberase (Roche) as per their instruction manual to prepare stromal vascular fraction (SVF). Blood was processed with BD Pharm Lyse (BD Biosciences) according to the instruction manual. Bone

marrow cells were collected from femurs by flushing with PBS and then pelleted at 300g for 5 minutes. Processed blood, bone marrow and SVF were subjected to FACS analysis in a FACScan (BD Bioscience) machine. DAPI was added to each sample to distinguish live cells. Tissues from a non-transplanted mouse were used as negative controls, respectively, for setting up GFP+ gating.

Statistical analyses

All statistical tests were performed with GraphPad Prism (GraphPad Software, Inc., La Jolla, CA). Two-sided student's t-tests were used to compare control and ACM groups, and tissue explants. Square-root transformation was used when data were not normally distributed. The data are presented as mean $\pm$ SD. A p value equal or less than 0.05 was taken as statistically significant.

Results

ALL cells migrate into adipose tissue

To investigate whether leukemia cells actively migrate into adipose tissue *in vivo*, we transplanted 20 week-old obese C57Bl/6 mice syngeneically with GFP positive 8093 ALL cells. We collected tissues at an early time point, ten days after implantation, to measure homing to different locations. The presence of 8093 cells in these tissues was analyzed by FACS. At this early time point, there were few circulating ALL cells, with all 6 obese mice and 4 of 6 control mice having detectible leukemia, though at <0.1% of events. Half the mice from each group had detectible leukemia in the bone marrow and 2 obese and 3 control mice had detectible leukemia in the spleen, all at <11% of events (Fig. 1A). Thus, the leukemia burden in these mice was similar to one would expect in a patient prior to clinical symptoms.

At this time point, all mice also had detectible leukemia in their visceral fat pads, and most in other fat pads as well. When leukemia cells were present in marrow, spleen and liver, they were present in large numbers (>0.5% of events on average), while the burdens in kidney, lung, and brain were lower (Fig. 1B). Leukemia burden was also high in both visceral and perirenal fat depots. There was a significantly higher burden of leukemia cells in visceral fat from obese compared to control mice. Although diet group had no effect on leukemia burden in any other tissue, obese mice had significantly more fat in all four depots compared to lean mice (Fig. 1C), and therefore the absolute number of leukemia cells in these depots would be expected to be higher in the obese mice.

The leukemia burden in the adipose tissue apparently increased over time; in two additional mice, we found that adipose tissue leukemia burden was substantially higher when mice were sacrificed 21 days after leukemia transplantation (not shown). Therefore, leukemia cells appear to migrate into adipose tissue early after transplantation, when the leukemia burden is still relatively low.

To test whether adipose tissue attracts leukemia cells, we performed *ex vivo* migration assays with mouse adipose tissue explants. Murine preB ALL cells migrated through TransWells toward various fat depots (3–15% within 90 minutes; n=3, Fig. 1D). Consistent with the *in vivo* findings, very few cells migrated toward muscle or control media.

Leukemia cells exhibit chemotaxis towards adipocytes

Adipose tissue is comprised of multiple cell types, including adipocytes, endothelial cells, stromal cells, and immune cells. Since adipocytes occupy the most volume in adipose tissue, we next tested whether the chemotaxis of leukemia cells was specifically toward adipocytes.

Murine preB ALL cells migrated toward differentiated 3T3-L1 and OP9 adipocytes, and to media conditioned by these adipocytes (Fig. 2A). Undifferentiated pre-adipocytes also caused significant migration of leukemia cells; for 3T3-L1 cells, migration toward pre-adipocytes and preadipocyte-conditioned media was less than toward adipocytes or ACM. However, migration toward OP-9 adipocytes and preadipocytes (and respective conditioned media) was similar. Interestingly, when both chambers contained adipocyte conditioned media (ACM, Fig. 2B), an intermediate number of 8093 cells migrated through the TransWell, implying that ACM increases both directional and non-directional leukemia cell motility. Migration toward ACM was relatively rapid, with about half of the total migration taking place within the first 60 minutes of incubation (Fig. 2C).

SDF-1 is the adipocyte-derived chemoattractant responsible for leukemia cell migration

Several adipocyte-derived molecules function as chemoattractants to immune cells. To identify which molecule(s) were responsible for the adipocyte-mediated chemoattraction, we tested various adipocyte-secreted molecules for their ability to stimulate 8093-ALL cell migration. In a screening of 6 different recombinant molecules (SDF-1 α , leptin, adiponectin, resistin, MCP-1 and RANTES), we found only SDF-1 α to significantly stimulate migration of 8093-ALL cells in our experimental conditions (data not shown; doses tested 10–180 ng/mL). Murine recombinant SDF-1 α induced migration of all 3 human ALL cell lines we tested, to a similar degree as ACM (Fig. 3A). In addition, three out of five primary human leukemia cell strains showed a significantly increased migration toward SDF-1 α (TXL2, ICN13, US.7, BLQ1 and UCSF02, Fig. 3B).

We next tested whether adipocytes secrete SDF-1 α . Media conditioned for 48 hours by 3T3-L1 and OP9 preadipocytes and adipocytes were measured using an SDF-1 α ELISA kit. In both cell lines, adipocytes secreted significantly more SDF-1 α than pre-adipocytes (Fig. 3C). We also measured SDF-1 α secretion by tissue explants from control and obese mice. There was no significant effect of diet group on SDF-1 α secretion in any of the tissues (Supplementary Fig. 1).

To confirm that adipocyte-derived SDF-1 α was responsible for the ALL chemotaxis, we used AMD3100, a specific inhibitor of CXCR4. AMD3100 inhibited 8093 leukemia cell migration toward SDF1 α and 3T3-L1 ACM (Fig. 3D) in a dose dependent manner. At the highest dose tested (0.8 μ g/mL), AMD3100 completely blocked ALL migration toward 3T3-L1 ACM. Interestingly, AMD3100 also blocked migration of ALL toward OP-9 conditioned media, though at higher doses, possibly due to the higher concentration of SDF-1 α . These results demonstrate that SDF-1 α is the functional chemoattractant for ALL cells in ACM.

We also verified that the ALL cells which migrated toward ACM express the SDF-1 α receptor, CXCR4. While the pre-B ALL cell lines which migrated toward ACM expressed CXCR4, the myelogenous leukemia cell line K562 did not migrate toward ACM (data not shown) and had lower CXCR4 levels (Fig. 3E).

Adipose tissue protects leukemia cells from chemotherapy in vitro

Since we previously found that adipocytes in vitro protect ALL cells against various chemotherapeutic agents, we tested the effects of weight-matched tissue explants on drug resistance of 8093-ALL cells. As shown in Fig. 4, subcutaneous and visceral fat pads from obese and control mice both protected 8093-ALL cells from daunorubicin. Both fat pads also protected ALL from vincristine, though this did not reach statistical significance for the obese mice. Muscle protected ALL cells from in some conditions, while lung and kidney showed no significant protection for either drug.

Discussion

The tumor microenvironment plays an important role in cancer cell survival and growth. Primary leukemia cells do not survive and proliferate well *in vitro* unless they are cultured with stromal cells [20, 21]. It is thought that the bone marrow is the most important microenvironment for pre-B ALL cells *in vivo*. However, since a primary role of lymphocytes is immune surveillance, it would not be surprising to find that leukemia cells migrate throughout the body and would be found in a variety of tissues. Recent studies have elaborated a complex trafficking of immune cells in adipose tissue, especially in the obese state [22, 23]. In accordance, we previously found transplanted leukemia cells in mouse adipose tissue after chemotherapy [8]. In the present study, we have demonstrated that adipose tissue is actively infiltrated by ALL cells, even when the leukemia burden is similar to that seen in patients with minimal residual disease [0.01% in blood, [24]]. Our current results show that leukemia cells migrate into adipose tissue, probably under the regulation of SDF-1 α (stromal cell-derived factor-1 α) secreted by adipocytes.

Immune cell migration is regulated by chemokines [25, 26], including SDF-1 α . SDF-1 α , also known as CXCL12, is a chemoattractant for cells of lymphoid origin, including hematopoietic cells, mature lymphocytes, and leukemia cells [27–29]. It acts by binding to the surface receptor CXCR4, causing multiple intracellular changes including actin cytoskeletal reorganization and activation of integrins and adhesion molecules [30]. While it was first identified in bone marrow stromal cells, SDF-1 α has since been found to be expressed in other cell types, such as fibroblasts, adipocytes, and endothelial cells [31–33]. Many of these cells reside in adipose tissue, and indeed we found adipose tissue to be a significant producer of SDF-1 α *ex vivo*.

Numerous immune cells are found in normal adipose tissue, including T and B lymphocytes [34, 35]. Although obesity clearly increases the number of T cells found in adipose tissue in mice [22, 36, 37], its effect on B cells is less clear [36, 37]. These lymphocytes are believed to interact with adipose tissue macrophages, and may play a role in obesity-induced insulin resistance and diabetes [22, 38]. Whether adipocytes attract non-malignant lymphocytes via SDF-1 α secretion has not been determined.

Although adipocytes express and secrete SDF-1 α , serum levels are actually lower in obese humans [39, 40]. Since SDF-1 α is produced in multiple tissues of the body, it is not clear whether decreased serum levels reflect a decrease in production from adipose tissue or other tissues, or perhaps even an increase in SDF-1 α clearance from the blood. In any case, as SDF-1 α is a chemokine, its activity depends on local gradients in capillary beds of tissue. Since obese patients can have several fold higher amounts of adipose tissue than lean patients, circulating leukemia cells in obese patients are likely exposed to more adipose tissue capillary beds, where they can encounter a local SDF-1 α gradient and have the opportunity to migrate into adipose tissue. Indeed, this hypothesis is consistent with our finding that adipose tissue from control and obese leukemic mice have a similar ALL burden per milligram of adipose tissue in most depots (Fig 1B), while the increased amount of adipose tissue (Fig 1C) likely reflects a higher overall number of leukemia cells in adipose tissue of obese mice.

Interestingly, adipocyte expression of SDF-1 α decreases during differentiation [22], though our data show that secretion is higher from both 3T3-L1 and OP-9 adipocytes than from preadipocytes (Fig 3C). However, ALL migration was similar toward the conditioned media of differentiated and undifferentiated OP-9 cells. Migration of ALL toward tissue explants was also not directly proportional to SDF-1 α concentrations measured in their respective conditioned media. Since AMD3100 completely abolished ALL migration toward towards

OP9 conditioned media, it is likely that this migration is dependent upon SDF-1 α . However, there may be other signals which modulate this SDF-1 α dependent migration.

While it has been shown that SDF-1 α regulates bone marrow trafficking of leukemia cells [41], this is the first study to demonstrate that pre-B acute lymphoblastic leukemia cells migrate toward adipose tissue. Although this occurred in mice, it is not known whether adipose infiltration by leukemia cells is common in humans. There is some evidence from the literature that this can occur with lymphoid malignancies. For example, Maitra et al. reported on 9 patients with precursor B-cell lymphoblastic leukemia with no evidence of marrow or blood involvement, and found that at least one of those patients had blast cells within subcutaneous fat [42]. In addition, malignant lymphocytes have been described as “rimming” adipocytes in subcutaneous tissue [43]. However, to our knowledge there has been no systematic study examining the frequency of leukemia cells in adipose tissue, the true prevalence of this phenomenon is not known.

Since we have previously demonstrated that adipocytes protect leukemia cells from chemotherapy [8] and absorb chemotherapeutic agents [44], it is possible that leukemia cells which migrate into adipose tissue gain a survival advantage due to this microenvironment. Leukemia cells in this environment could remain in a dormant state, or could receive survival signals which allow them to resist chemotherapy, which could contribute to the increased relapse rate observed in children and adults who are obese at the time they are diagnosed with leukemia [14, 45]. In fact, it is possible that adipocyte secretion of SDF-1 α could contribute to the links between obesity and poor outcome from other cancers which express CXCR4, such as colon [46], breast [47], and prostate [48]. Strategies which block leukemia cell migration into adipose tissue may improve outcomes in both lean and obese patients. Indeed, AMD3100 is currently being investigated as an adjunct to chemotherapy for ALL, based on the theory that this could mobilize leukemia stem cells from the bone marrow niche to the bloodstream, where they are more susceptible to chemotherapy [49–51]. Our findings suggest that this and other CXCR4 antagonists might also block leukemia cells infiltration to adipose tissue, which could be an additional benefit of treatment, particularly in patients with excess adipose tissue.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Migration of ALL cells toward adipose tissue. Summary of flow cytometry analysis of ALL cells (GFP positive) in blood, bone marrow, various tissues and SVF of adipose tissue. Six obese (HF) and six control (LC) mice were transplanted. (A) The number of mice that had detectable amount of ALL cells is shown. The detection limit is set for each tissue using samples from a non-transplanted mouse. (B) Quantification of ALL cells in the sites detected in (A) analyzed by flow cytometry. Percentages are calculated by dividing the amount of GFP+ cells by the total number of live cells in mice with detectable amounts of ALL cells. No ALL cells were detected in muscle (N.D.). (* $p < 0.05$, HF visceral vs. LC visceral) (C) Weight of fat depots from obese and lean mice. (***) $p < 0.001$ (D) Tissue

explants from obese mice were cultured for 2 days and 8093 ALL cells were plated in transwells above the explants and incubated for 90 minutes. % migration is calculated as number of cells in bottom chamber divided by total number of cells counted in both chambers. (* $p < 0.05$ and ** $p < 0.01$ vs. no tissue, $n=3$)

Figure 2.

Migration of ALL cells to adipocytes and ACM. (A) Migration of 8093 murine ALL cells toward pre-adipocyte conditioned medium (FCM), ACM, pre-adipocyte feeder layer (Fibro), and adipocyte feeder layer (Adipo) of 3T3-L1 cells and OP9 cells. Percentage of migrated cells is calculated as before. Control condition was performed with RPMI medium in both chambers. (* $p < 0.05$ and ** $p < 0.01$ vs. control, $n = 3$) (B) ACM causes both directional and non-directional movement of 8093 ALL cells. Top and bottom chambers are filled with either RPMI (C) or ACM (A). (** $p < 0.01$ and * $p < 0.05$ vs. medium, $n = 4$) (C) Time course of 8093 ALL migration toward ACM ($n = 2$).

Figure 3.

Migration of ALL cells toward SDF-1α and blocking by AMD3100. (A) Human ALL cell lines were incubated in control medium, and migration toward control, ACM, or SDF-1α (60 ng/mL) was quantified. (B) Migration of primary human ALL cell strains migrate toward 60 ng/mL SDF1α. (*p<0.05, **p<0.01, and ***p<0.001 vs. control, n=3) (C) ELISA quantification of SDF-1α secretion by 3T3-L1 and OP9 pre-adipocytes and adipocytes, measured in media conditioned for 48 hours. (**p<0.01, n=3) (D) Inhibition of migration of 8093 cells towards SDF-1α (n=3) or ACM (n=4) after pre-incubation of cells with the indicated amounts of AMD3100. (*p<0.05, **p<0.01, and ***p<0.001 vs. zero dose of AMD3100) (E) Migration of 8093 cells towards OP9 FCM and ACM (n=3) after pre-incubation of cells with the indicated amounts of AMD3100 (**p<0.01, and ***p<0.001 vs. zero dose of AMD3100). (F) Western blot for CXCR4 in leukemia cell lines. β-actin is included as loading control.

Figure 4.

Protection of ALL cells by fat explants against daunorubicin and vincristine. Weight-matched tissue explants (subcutaneous fat, visceral fat, muscle, lungs, and kidneys) from control and obese mice (n=6 each) were excised after cardiac perfusion. The doses of daunorubicin (19nM) and vincristine (3nM) were EC₉₉ doses in the control conditions without tissue explants. ALL cells were quantified using trypan blue exclusion after 48 hours of drug treatment (*p<0.05, **p<0.01, and ***p<0.001 vs. no tissue control).