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Metabolic reprogramming of bone marrow stromal cells by leukemic extracellular vesicles in acute lymphoblastic leukemia

Suzanne M Johnson¹, Clare Dempsey¹, Amy Chadwick², Stephanie Harrison¹, Jizhong Liu¹, Yujun Di¹, Owen J McGinn¹, Marco Fiorillo², Federica Sotgia², Michael Lisanti², Mayur Parihar³, Shekhar Krishnan⁴, Vaskar Saha¹.⁴.

From: ¹Children’s Cancer Group, Institute of Cancer Sciences, Manchester Academic Health Sciences Centre, University of Manchester, United Kingdom; ²Manchester Centre for Cellular Metabolism, Institute of Cancer Sciences, Manchester Academic Health Sciences Centre, University of Manchester, United Kingdom; ³Tata Medical Center, Kolkata, India, ⁴Tata Translational Cancer Research Centre, Kolkata, India.

Correspondence to:
Vaskar Saha, Children’s Cancer Group, Institute of Cancer Sciences, Manchester Academic Health Sciences Centre, Paterson Building, University of Manchester, United Kingdom M20 4BX
E mail: vaskar.saha@manchester.ac.uk
Cancer cells produce unique heterogeneous vesicles\textsuperscript{1} capable of transferring oncogenic material\textsuperscript{2,3} to other cells\textsuperscript{4,5}, with the potential of modulating a tumour-supportive environment\textsuperscript{6-8}. We have previously reported the presence of lipid-enriched, membrane bound sub-cellular vesicles at the periphery of acute lymphoblastic leukemia (ALL) cell lines\textsuperscript{9,10}. We now extend these findings to describe heterogeneous anucleate vesicles released into extracellular fluids \textit{in vitro} and \textit{in vivo} by primary B-cell precursor (BCP) ALL blasts and cell lines. Leukemic extracellular vesicles (LEVs) were internalized by stromal cells, and induced a metabolic switch.

Extracellular vesicles (EV’s) are enclosed in lipid bilayers originating from the cell of origin, \textcolor{blue}{released by both normal and cancer cells}\textsuperscript{11}. Here, the BCP cell specific membrane protein CD19 present within membrane lipid rafts\textsuperscript{11} was used to identify the cell of origin of EV’s in clinical samples. \textcolor{blue}{We directly compared plasma samples from CD19+ primary BCP-ALL bone marrow aspirates at diagnosis containing >95% malignant blasts with matched remission samples obtained after 28 days of therapy} (Figure 1A, left panel). The diagnostic sample, which predominantly contained BCP ALL cells was significantly enriched in CD19\textsuperscript{+} vesicles, suggesting that the CD19\textsuperscript{+} LEVs identified were of leukemic origin. By contrast, CD61\textsuperscript{+} EV’s were increased in remission marrow samples as expected in a regenerating marrow (Figure 1A, right panel).\textcolor{blue}{NOD.Cg-Prkdc\textsuperscript{scid} ll2rg\textsuperscript{tm1Wjl}/SzJ (NSG) mice are extensively used as a patient derived xenograft model of BCP-ALL, by ourselves\textsuperscript{10,18} and others}. PKH26\textsuperscript{12} labeled BCP cell line SD1 and LEVs derived from SD1 cells were separately transplanted intrafemorally into NSG mice. Bone marrow flushes taken 17 days later showed transplanted LEVs within a proportion of murine bone marrow stromal cells
(BMSC) (Figure 1B, upper). When labeled SD1 cells were introduced (1B, lower), cells and LEVs were detected within the extracellular space. Confocal microscopy and 3-dimensional modeling confirmed LEV internalization of pre-labelled SD1 LEVs by BMSCs after 24 hours \textit{in vitro} co-incubation (Figure 1C). All transplanted mice showed evidence of PKH26\(^+\), human CD19\(^+\) or dual\(^+\) LEVs in peripheral blood plasma (Figure 1D) and femoral flushes (day 14) showed engrafted PKH26\(^+\) ALL cells and murine stromal cells with internalized PKH26\(^+\) LEVs (Figure 1E).

The effect of LEV internalisation by BMSC’s was investigated in the human mesenchymal stem cell (MSC) line HS5\(^{13}\) exposed to LEVs released by the BCP-ALL cell lines SD1 and NALM6. Proliferation and viability assays revealed no significant differences from control (Figure 2A). Despite a sustained increase in AKT phosphorylation over 24 hours (Figure 2B), non-significant reductions in ATP concentrations were observed (Figure 2C). Next, the two major energy producing pathways of the cell and parameters of metabolism were assessed. At 24 hours, HS5+LEVs showed a reduced oxygen consumption rate (OCR) compared to control; were less sensitive to the inhibition of ATP by oligomycin and did not change OCR when electron transport from ATP generation in the mitochondria was uncoupled (Figure 2D). Disrupting the electron transport chain (Rotenone/Antimycin A) reduced OCR to a comparable level in all cells, suggesting that the rate of oxygen consumption due to non-mitochondrial sources was comparable. HS5+LEV have a significantly reduced spare respiratory capacity, an indicator of a decreased ability to respond to stress or metabolic challenge (Figure 2D). Overall these results suggest that uptake of LEVs significantly reduced mitochondrial respiration in recipient stromal cells.
In the absence of glucose, HS5 and HS5+LEVs had comparable extracellular acidification rates (ECAR) (Figure 2E). In the presence of glucose, HS5+LEVs initiated a sharp increase in ECAR compared to control (~5 fold), suggesting a higher glycolytic rate. Inhibiting ATP synthase increased ECAR in both HS5+LEVs and controls, but more sharply in the latter. Following the addition of 2-Deoxy-D-glucose, a competitive inhibitor of glycolysis, ECAR returned to base levels in both control and LEV exposed cells. Thus in the presence of glucose, LEV exposed HS5 showed an increase in ECAR which is suggestive of glycolysis. This was corroborated by the demonstration of significantly increased extracellular lactate production, the end product of aerobic glycolysis, by HS5+LEV (Figure 2F). To investigate this further, OCR and ECAR were evaluated in the same cultures simultaneously. A shift towards a glycolytic phenotype was observed in HS5 cells treated with either NALM6 or SD1 LEVs (Figure 2G). Overall, these results suggest that uptake of LEVs significantly reduced mitochondrial respiration in recipient stromal cells and their ability to respond oxidatively to stress or metabolic challenge. As ATP levels and proliferation rates were similar in HS5 and HS5+LEV, LEVs induced a metabolic switch from oxidative phosphorylation (OXPHOS) to aerobic glycolysis in stromal cells to meet energy requirements.

Our observations show that both in vitro and in vivo, BCP ALL cells release a variety of LEVs into the extracellular fluid and circulation which are taken up by BMSC. LEV exposed BMSC undergo a metabolic switch. A similar reprogramming of stromal cells to glycolytic cancer associated fibroblasts \cite{14,15} has been attributed to exosomes released by chronic lymphocytic leukemia cells \cite{4}. The altered tumour microenvironment promotes leukemic cell survival \cite{16,17} and protects against cytotoxic
effects of chemotherapy. A mechanism of this protective effect of the microenvironment appears to be mediated via an adaptation to oxidative stress with decreased mitochondrial electron transfer \(^{18}\) and a switch to glycolysis \(^{19}\). In this study, we show that the reprogrammed stromal cells generate an excess of lactate which is released into the extracellular fluid. We speculate that this lactate is utilised preferentially by tumour cells as a source of energy, a process we have previously termed the reverse Warburg effect \(^{20}\). Targeting glycolysis \(^{21}\) as well as the redox adaptation \(^{18}\), has been shown to overcome drug resistance in ALL. Modulating the tumour-stromal metabolic interactions offers the development of novel therapeutic strategies to enhance the therapeutic response in ALL and other cancers.
**Cells and cell lines:** HS5 were obtained from ATCC and SD1 and NALM6 from DSMZ.

**Primary human leukemic xenograft:** Animal procedures were approved by CRUK, Manchester Institute's Animal Welfare and Ethical Review Body and performed under a Project License issued by the United Kingdom Home Office. 6-12 week old NSG mice were transplanted intrafemorally with either $1 \times 10^6$ PKH26-labelled ALL cells, SD1 cells or vesicles from $1 \times 10^7$ PKH26-stained ALL cells. Bone marrow flushes from transplanted mice were seeded onto fibronectin, and either imaged live or fixed with 3.7% paraformaldehyde and counter stained with Cell Mask green (Life technologies) and DAPI.

**Assessment of bone marrow stromal cell metabolism:** HS5 cells were seeded at $1 \times 10^4$ cells/ well, whilst SD1 or NALM6 were cultured in serum-free RPMI. DMEM diluted 1:1 with either serum-free RPMI or LEV-containing conditioned media was added and incubated for 24 hours. HS5+/-LEVs were washed and re-plated into XF®96 FluxPaks before equilibration in basal media. The response to both glycolytic and mitochondrial stress were analysed on using a Seahorse XF®96 extracellular flux analyser (Seahorse Bioscience, Massachusetts, United States).

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References


FIGURE 1: LEVs are produced *in vivo* and internalised by stromal cells.

(A) Diagnostic bone marrow plasma is enriched with CD19+ LEVs. Imaging flow cytometry identified CD19 PE_Cy7 (LEV) and CD61 FITC (platelets) positive vesicles in matched diagnostic and remission (Day 28 post-chemotherapy) ALL patient bone marrow plasma samples (1x10^6 events acquired). The number of CD19+ vesicles/ml (LEV: Left panel) were significantly higher at diagnosis and number of CD61+ extracellular vesicles (Platelets: Right panel) significantly higher in remission marrow plasma (** p>0.05; *** p>0.005).

(B) SD1 cells produce LEVs within the bone marrow microenvironment which are internalised by mouse BMSCs. LEVs from 1x10^7 SD1 cells or 1x10^6 SD1 cells labelled with the fluorescent membrane label PKH26 were introduced into the left femur of 5 x NOD-scid IL2rγnull (NSG) mice. Bone marrow flushes taken 17 days post-transplantation were seeded onto fibronectin coated glass coverslips and BMSCs allowed to adhere overnight. Live cell images were taken using bright field and UV illumination with Red Sedat filter at x 40 magnification. Scale bar is 20µm. *Left image* represents an overlay of the images and shows murine BMSCs with internalised PKH26+ LEVs as indicated by intracellular red fluorescence (yellow arrow). Figure shows variable, punctate peri-nuclear red fluorescence within the outer membrane of murine BMSCs, and not around the periphery of the recipient cell. *Right image* shows a cluster of PKH26+SD1 cells (black arrow) and free PKH26+ LEVs (yellow arrow) in the extracellular space, produced within the murine bone marrow. Scale bar is 20µm.
(C) **Confirmation of LEV internalisation by human bone marrow cells *in vitro.*** Isolated SD1 LEV’s were labelled using a lipophilic tracer (Dio C<sub>18</sub>; Green). Labelled LEV’s were added to cultures of bone marrow cells in glass plates for 24 hours. Cells were fixed using paraformaldehyde and counterstained using Cell Mask (red). Serial images were captured at 0.1µm intervals in Z using a Spinning disk confocal system based around an Olympus IX71 microscope. Illumination achieved by white light LED and a 300W Xenon light source for fluorescence and Sedat filters. Composite 3D image from 81 0.1µm Z stacks was achieved using IMARIS software (BITPLANE, Oxford Instruments) which revealed the LEV’s to be fully internalised by the recipient cells as represented by green fluorescence along the mid-line (indicated by a blue star). Scale bar represents 10µm.

(D) **Human CD19<sup>+</sup> LEVs are detected in peripheral blood plasma in mice engrafted with primary ALL cells.** Plasma samples isolated from tail vein bleeds 9 or 20 days after intrafemoral injection of PKH26 labelled human ALL cells were screened for PKH26 and human CD19<sup>+</sup> vesicles. *Top panel:* Synchronus Image stream acquisition of bright field (left), PKH26 (centre) and CD19 PE_Cy7 (right) fluorescence identified vesicles, which originated from the labelled human ALL cells, 9 days post-transplantation. All transplanted mice (n=5) showed evidence of PKH26<sup>+</sup>, or human CD19<sup>+</sup> vesicles in the peripheral blood plasma. As PKH26+ LEVs were generated *in vivo* by transplanted PKH26+ ALL cells, staining is less uniform compared with the human CD19+ antibody labeling post-isolation. Figure shows dual labelled LEVs from 10,000 acquired events. Scale bar is 7µm. *Lower panel:* All transplanted mice showed evidence of PKH26<sup>+</sup> or human CD19<sup>+</sup> (0.73-5.67% at day 9; 2.23-6.40% at day 20) LEVs in the peripheral blood plasma.
Engrafted primary human ALL cells introduced into the mouse bone marrow produce LEVs which are internalised by surrounding mBMSCs. Primary ALL cells were labelled with PKH26 (red) and introduced into the femur of 5 x NOD-scid IL2γnull (NSG) mice. Bone marrow flushes 14 days after injection were seeded onto fibronectin and fixed with paraformaldehyde. Cells were counterstained with cell mask green and DAPI. Panel shows PKH26+ LEVs, visualised as punctate red fluorescent staining within the cell membrane of adherent mBMSCs, were internalised by mBMSCs in vivo (x160 magnification). Captured using a black and white camera, the figure shows composite image and individual filters. Scale bar is 10µm.

**FIGURE 2: LEV uptake by BMSCs alters cell metabolism.**

**(A) LEV uptake by BMSCs did not affect the survival or proliferation of BMSCs.**

HS5 cells were plated into 96 well plates at 4x10⁴ cells per well. Unselected LEVs were isolated from serum-free cultures of SD1 and NALM6 cells. LEV-containing conditioned media (24h) was diluted 1:1 with DMEM/10% FBS and added to the HS5 cells. MTS assay was used to assess proliferation and viability of HS5 cells; HS5 + NALM6 LEVs and HS5 + SD1 LEVs over 96 hours. At least 5 wells were assessed at each time-point and the experiment repeated 3 times. No significant differences were apparent.

**(B) Co-incubation of BMSCs with ALL LEVs results in activation of AKT.**

HS5 cells were seeded into large 10cm cell culture plates at 1.5x10⁵ cells and allowed to adhere. LEVs were isolated from 24h serum free RPMI cultures of SD1 cells. HS5 media was replaced with LEV containing conditioned media diluted 1:1
with DMEM/10% FBS and incubated at 37°C 5% CO₂. Cells were washed and lysed on ice at 1, 3, 5, and 24 hours. 30µg protein was resolved by gradient SDS-PAGE and transferred to PVDF membrane. Blots were probed for pAKT, and then re-probed for AKT and β-Actin. Figure show increased pAKT in HS5+LEV compared to control across all time points. Figure is representative of 3 independent experiments.

(C) ATP production by BMSC + LEV is reduced compared to control BMSCs. ATP production was measured using the Cell Titer Glo assay (Promega) in HS5 cells exposed to LEVs from either NALM6 or SD1 cells for 24 hours. Data are mean and SEM of 4 independent experiments.

(D) BMSC + LEVs have lower resting energetics and are less able to respond to metabolic challenge. HS5 cells were seeded into Seahorse XFe96 cell plates at 1 x 10^4 cells per well and allowed to attach. LEV-containing conditioned media was diluted 1:1 with DMEM/10% FBS and added to the HS5 cells for 24 hours. Cells were washed and equilibrated in basal media. XF cell mitochondrial stress test was performed and oxygen consumption rate (OCR) monitored following the sequential addition of oligomycin an inhibitor of ATP synthase, FCCP (carbonyl cyanide p-trifluoromethoxyphenylhydrazone), which uncouples electron transport from ATP generation in the mitochondria and rotenone/antimycin A which disrupts the electron transport chain using the XFe96 extracellular flux analyser. Individual wells were normalised using sulforhodamine B (SRB). LEV uptake resulted in a lower basal respiration rate and diminished ability to respond to energetic crisis in the BMSCs. ATP-linked respiration, maximal respiration and spare respiratory capacity (difference in the basal respiratory rate and the maximal respiration) were all
significantly diminished in LEV exposed BMSCs, indicating oxidative stress. Figures are representative of 3 independent experiments with 6 replicates per plate; error bars represent SEM of a representative experiment.

ATP-linked respiration: basal respiration – proton leak reveals ATP production

Maximal respiration: The uncoupler FCCP stimulates the respiratory chain to operate at its maximum speed and mimics a physiological “energetic crisis” and is measured after oligomycin through to prior to rotenone and antimycin A injection.

Spare respiratory capacity refers to the difference between the maximal FCCP-stimulated rate of respiration – basal respiration and reflects the cells ability to deal with an energetic crisis.

(E) BMSC + LEVs utilise glucose more readily and have a greater glycolytic capacity. HS5 cells, HS5 + NALM6 LEVs and HS5 + SD1 LEVs cells were washed and equilibrated in assay media prior to basal readings. Glycolysis stress test was performed and extra cellular acidification rate (ECAR) monitored following the sequential addition of glucose, oligomycin and 2-Deoxy-D-glucose (2DG) a competitive inhibitor of glycolysis using an XFe96 extracellular flux analyser). Individual wells were normalised using sulforhodamine B (SRB). Glycolysis and glycolytic capacity were significantly increased in the LEV exposed HS5 cells, whilst the glycolytic reserve was comparable to control cells. Figures are representative of 3 independent experiments with 6 replicates per plate; error bars represent SEM of a representative experiment. Glycolysis as indicated by the first measurement of ECAR (attributed to the breakdown of glucose to pyruvate) taken after glucose addition minus the ECAR prior to glucose addition.
Glycolytic capacity refers to increased rate of glycolysis to meet the metabolic demands of the cell following the addition of oligomycin which blocks mitochondrial ATP synthase minus ECAR prior up glucose.

Glycolytic reserve indicates the increase in glycolysis needed to meet cellular energetic requirements without mitochondrial ATP production as calculated by the glycolytic capacity minus glycolysis measurements.

(F) **BMSC + LEVs produce more lactate compared to control.** HS5 cells were seeded as described and the media harvested after 24 hours. The media from BMSC +/- LEV from either SD1 cells or NALM6 cells was screened for lactate 24 hours later and compared to control. An increase in lactate was evident from BMSCs+LEVs from either leukemic cell line. Data is expressed as concentration (nmoles/cell) as determined from a standard curve and normalised to individual cell counts. Figures are representative of 3 independent experiments with multiple replicates per plate; error bars represent SEM of a representative experiment.

(G) **LEV exposed BMSCs switch to a glycolytic phenotype.** HS5 cells were exposed to LEVs from either NALM6 or SD1 cells for 24 hours, then washed and equilibrated in mitochondrial stress test media. Extracellular acidification and oxygen consumption were measured in the same well. While control HS5 cells maintain aerobic respiration, following exposure to LEVs for 24 hours, they switch to a more glycolytic phenotype and become less dependent on mitochondrial respiration. Figure is representative of 3 independent experiments with 6 replicates per plate; error bars represent SEM of a representative experiment.

* = p <0.05; ** = p <0.01; *** = p <0.005
FIGURE 1

A

LEVs

Platelets

CD19+ LEV's/ml

CD61+ particles/ml

0 1 2 3 4

Patient 1

Patient 2

Patient 3

**

***

***

0 1 2 3 4

CD61+ particles/ml

0 1 2 3 4

CD19+ LEV's/ml

C

PKH26+ SD1 LEVs

PKH26+ SD1 cells

8.1µm

10µm

D

Brightfield

PKH26

CD19 PE_Cy7

MOUSE

CD19+ PKH26+ DUAL+

Day 9

1 1.47% 0.16% 0.48

2 0.73% 0.34% 0.14%

3 0.93% 0.52% 0

4 5.67% 0.87% 0.12%

5 1.78% 0.05% 0

Day 20

1 x x x

2 x x x

3 6.40% 0.60% 0.40%

4 x x x

5 2.23% 0.13% 0.20%

E

Composite

PKH26

Cell Mask Green

DAPI