

The Stromal Microenvironment Modulates Mitochondrial Oxidative Phosphorylation in Chronic Lymphocytic Leukemia Cells^{1,2}



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Abstract

Peripheral blood chronic lymphocytic leukemia (CLL) cells are replicationally quiescent mature B-cells. In short-term cultures, supporting stromal cells provide a survival advantage to CLL cells by inducing transcription and translation without promoting proliferation. We hypothesized that the stromal microenvironment augments malignant B cells' metabolism to enable the cells to cope with their energy demands for transcription and translation. We used extracellular flux analysis to assess the two major energy-generating pathways, mitochondrial oxidative phosphorylation (OxPhos) and glycolysis, in primary CLL cells in the presence of three different stromal cell lines. OxPhos, measured as the basal oxygen consumption rate (OCR) and maximum respiration capacity, was significantly higher in 28 patients' CLL cells cocultured with bone marrow-derived NK.Tert stromal cells than in CLL cells cultured alone ($P = .004$ and $<.0001$, respectively). Similar OCR induction was observed in CLL cells cocultured with M2-10B4 and HS-5 stromal lines. In contrast, heterogeneous changes in the extracellular acidification rate (a measure of glycolysis) were observed in CLL cells cocultured with stromal cells. Ingenuity Pathway Analysis of CLL cells' metabolomics profile indicated stroma-mediated stimulation of nucleotide synthesis. Quantitation of ribonucleotide pools showed a significant two-fold increase in CLL cells cocultured with stromal cells, indicating that the stroma may induce CLL cellular bioenergy and the RNA building blocks necessary for the transcriptional requirement of a prosurvival phenotype. The stroma did not impact the proliferation index (Ki-67 staining) of CLL cells. Collectively, these data suggest that short-term interaction (≤ 24 hours) with stroma increases OxPhos and bioenergy in replicationally quiescent CLL cells.

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Introduction

Chronic lymphocytic leukemia (CLL) is characterized by the accumulation of quiescent mature B cells. CLL is a compartmentalized disease; malignant cells reside in bone marrow, spleen, lymph nodes, and peripheral blood. Therefore, CLL cells inhabit diverse microenvironments, and in general, the CLL cell population is either slowly proliferating or quiescent [1].

Several investigations have focused on the crosstalk between CLL cells and the cellular components of the microenvironment. In one study, CLL cells from the bone marrow, peripheral blood, or lymph nodes of the same patients showed different mRNA signatures depending on the microenvironment with which they were

Abbreviations: BCR, B-cell receptor; CLL, chronic lymphocytic leukemia; ECAR, extracellular acidification rate; ETC, electron transport chain; MM, mitochondrial mass; MOMP, mitochondrial outer membrane potential; MRC, maximum respiration capacity; mtDNA, mitochondrial DNA; mTOR, mechanistic target of rapamycin; NTP, ribonucleoside triphosphate; OCR, oxygen consumption rate; OxPhos, mitochondrial oxidative phosphorylation; qPCR, quantitative polymerase chain reaction; ROS, reactive oxygen species; XF, extracellular flux.

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interacting [2]. The lymph nodes provided the niche for proliferation, and CLL cells from the lymph nodes showed the distinct signature for growth, proliferation, and DNA replication. The lymphoid tissue has been found to harbor nascent CLL cells [3]. In short, at least a portion of CLL cells that reside in the lymph nodes are proliferating; this change in biology is due to signals from the non-CLL cells of this microenvironment.

The microenvironment of the bone marrow has been shown to provide anti-apoptotic signals to resident CLL cells. In vitro investigations clearly demonstrate that stromal cells provide a survival advantage (but no proliferation advantage) to CLL cells by protecting them from apoptosis [4–6]. Mechanistic studies have further elucidated that stromal interaction induces the anti-apoptotic members of the BCL-2 family at the transcript and protein levels; after coculture with stromal cells, the levels of MCL-1, BCL-2A1, and BCL-XL proteins in the CLL cells are increased [6–10].

The stroma-mediated induction of anti-apoptotic proteins in CLL cells also results in the resistance of these primary cells to a variety of chemotherapeutic agents [11–14]. The coculture of CLL cells with stromal cells induces a cascade of events, such as the phosphorylation of Ser2 and Ser5 in the C-terminal domain of RNA polymerase II; the elevation of the global RNA synthesis rate; the amplification of pro-survival transcripts; and the up-regulation of protein synthesis. This expansion in macromolecule synthesis is without an increase in CLL cell replication, which remains inert [8]. Collectively, these findings indicate that stroma-induced apoptosis resistance may be mediated through transcriptional and translational changes of the signaling proteins that regulate the expression of prosurvival proteins.

The B-cell receptor (BCR) and CD19 pathways, both pivotal to the survival and maintenance of CLL B cells, are also activated by microenvironmental factors [8,15,16]. Microenvironmental factors have also been found to stimulate CLL cells, resulting in the activation of the BCR pathway. Stroma-derived factors such as SDF-1 activate BCR and its downstream targets in CLL cells [4]. At the apex of this pathway are transcription factors, such as nuclear factor kappa-light-chain-enhancer of activated B cells, which induce transcription in CLL lymphocytes.

Specific microenvironmental events, such as the stimulation of CpG sites and activation of toll-like receptors, are associated with an increase in the proliferation index of CLL cells. The stimulation of these pathways and the BCR axis increases thymidine incorporation, indicating the replication of CLL cells. Various chemokines and cytokines in this microenvironment also influence CLL cell behavior [17–19].

Collectively, these data suggest that bone marrow stromal cells, through direct contact and signaling, generate a microenvironment that provides survival and anti-apoptotic signals without affecting cellular replication. Despite the absence of DNA synthesis, CLL cells that are exposed to stromal cells have augmented RNA and protein production; these processes require a bioenergy reservoir and consumption.

We hypothesized that stromal cells have an active role in modulating the energy metabolism of malignant B cells that enables them to cope with their energy demands. We assessed the two major ATP-generating pathways, mitochondrial oxidative phosphorylation (OxPhos) and glycolysis, in primary CLL cells in the presence of three different adherent stromal cell lines. Our data demonstrate that stromal cell interaction augmented CLL cells' biosynthesis of not only

ATP but also other nucleotides. Furthermore, stromal cells mediated a modulation of the OxPhos pathway without changing the glycolysis route of ATP generation in CLL cells.

Patients, Materials, and Methods

Patient Sample Collection

Peripheral blood samples from 58 CLL patients (Supplemental Table 1) were collected in green-top tubes containing sodium heparin. All patients had given written informed consent in accordance with the Declaration of Helsinki and under a protocol approved by the Institutional Review Board of The University of Texas MD Anderson Cancer Center.

Cell Isolation and Coculture

Peripheral blood mononuclear cells were separated by Ficoll-Hypaque (Atlanta Biologicals, Flowery Branch, GA) density gradient centrifugation. CLL cells were cultured in RPMI-1640 medium with L-glutamine plus 10% human serum. All experiments were performed using freshly isolated CLL cells; the purity of this cell population was $\geq 95\%$. Depending on the experiment, CLL cells were cocultured with stromal cells (NK.Tert, M2-10B4, or HS-5) for 2, 24, or 48 hours as indicated in the figure legends. CLL and stroma cells were plated at a ratio of 100:1 as described previously [14]. The culture conditions for the stromal cell lines are given in Supplemental Table 2.

Cytotoxicity Assays

For cytotoxicity assays, CLL cells (1×10^6) were washed with $1 \times$ phosphate-buffered saline (PBS) and resuspended in $100 \mu\text{L}$ of $1 \times$ Annexin V binding buffer with $5 \mu\text{L}$ of Annexin V–fluorescein isothiocyanate as described previously [20]. Samples were incubated in the dark for 15 minutes at room temperature, and then $400 \mu\text{L}$ of $1 \times$ propidium iodide buffer (Annexin V binding buffer, $0.625 \mu\text{g}/\text{mL}$ propidium iodide) was added to the reaction. Cells were analyzed with a BD Accuri flow cytometer (BD Biosciences, Franklin Lanes, NJ). Ten thousand events were recorded per sample.

Metabolite Mass Spectrometry

CLL cells were cocultured with NK.Tert cells for 24 hours, washed twice with ice-cold $1 \times$ PBS, counted with a Coulter counter, and then saved as pellets at -80°C . Cellular metabolites extracted from 5×10^6 cells using 80% methanol were assessed at the mass spectrometry facility at Beth Israel Deaconess Medical Center for levels of polar metabolites (e.g., nucleobases, nucleotides, sugars, amino acids, polyamines) [21]. The changes in CLL cell metabolites after coculture were expressed as the ratios of values obtained for each metabolite after coculture versus suspension culture. The metabolites were then grouped into pathways using Kyoto Encyclopedia of Genes and Genomes identifiers. Pathway analysis was performed using Ingenuity Pathway Analysis (Qiagen Bioinformatics, Redwood, CA).

Extracellular Flux Assays

Extracellular flux (XF) assays (Seahorse Bioscience, Chicopee, MA) were used to measure the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of CLL cells in suspension culture or after co-culture with stromal cells for 2 or 24 hours as indicated in the figure legends. For OCR both the basal OCR and maximum respiration capacity were assessed. For ECAR, both the glycolytic flux and glycolytic capacity were evaluated. Stromal cells

(5×10^3) were first plated on XF microplates coated with Cell-Tak (BD Biosciences, San Jose, CA) and allowed to adhere for 4–6 hours. CLL cells (5×10^5) were then plated onto the stromal cells on the XF microplates. RPMI-1640 medium was replaced with XF base media (for OCR assessment) or glycolysis base media (for ECAR assessment) as recommended by Seahorse Bioscience. Five technical replicates for each condition were plated as described previously [22]. The median OCRs and standard deviations of the technical replicates were calculated using Prism 6 software (GraphPad Software, San Diego, CA).

Ribonucleotide Pools

After 24 hours of culture in suspension or co-culture with stromal cells, CLL cells were carefully removed, transferred to 15-mL conical tubes, and centrifuged at 1500 rpm at 4°C for 5 minutes. The cells were then washed with 10 mL of ice-cold PBS and pelleted by centrifugation. Perchloric acid was used to extract nucleotide pools from CLL cells. All four ribonucleoside triphosphates (NTPs) were separated on an ion-exchange column using high-performance liquid chromatography. Standard curves were generated to quantitate nucleotide concentrations as described previously [22].

Immunoblotting

CLL cells cultured in suspension or on stroma were washed, and protein extracts were probed using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE) as described previously [22]. Primary antibodies included an OxPhos antibody cocktail (Abcam, Cambridge, UK) and a GAPDH antibody (Cell Signaling Technology, Danvers, MA).

Glucose/Glutamine Uptake Assays

For the substrate uptake assays, CLL cells cocultured with stromal cells for 24 hours were gently removed without perturbing the stromal cells and then washed twice with ice-cold 1X PBS. Equal numbers of CLL cells from the original suspension cultures or the stromal cocultures were suspended in glucose- or glutamine-free media (DMEM D5030 medium supplemented with NaCl), to which either 0.5 μ L of [3 H]2-deoxy-d-glucose (specific activity, 28 Ci/mmol) or 1 μ L of [3 H]l-glutamine (specific activity, 50.3 Ci/mmol) was added (both reagents from Perkin Elmer, Waltham, MA). The cells were incubated for the indicated times. The radioactivity was quantified using a scintillation counter.

Mitochondrial Reactive Oxygen Species, Membrane Potential, Mass, and DNA Copy Number

CLL cells (1×10^6) from the suspension cultures and corresponding 24-h co-cultures were washed twice with ice-cold 1x PBS. The cells were then stained with MitoSOX Red, tetramethylrhodamine ethyl ester perchlorate, and MitoTracker Deep Red FM (Life Technologies, Carlsbad, CA); incubated at 37°C for 10 minutes; and analyzed using flow cytometry for mitochondrial reactive oxygen species, mitochondrial outer membrane potential, and mass, respectively. The geometric means of cytometric data were obtained using FlowJo software (FlowJo, Ashland, OR). For copy number analysis, DNA was extracted from CLL samples using the QiaAMP DNA mini kit (Qiagen, Venlo, Netherlands). Quantitative polymerase chain reaction (qPCR) with SYBR green master mix was used to amplify the mitochondrial DNA (mtDNA) from four CLL patient samples [23]. All samples were run in triplicate.

Statistical Analysis

To assess differences between CLL cells on stroma versus CLL cells in suspension, we performed two-tailed Student *t*-tests and ANOVA using Prism 6 software (GraphPad Software, San Diego, CA) with

$P > .05$ as the level of significance. To verify the statistical analysis, we used Microsoft Excel software and a two-sample *t* test assuming unequal variances or a paired two-sample *t* test for means where required.

Results

Stromal Cell Interaction Up-Regulates Mitochondrial OxPhos in CLL Cells

We first assessed the effect that stromal cells have on mitochondrial OxPhos in CLL cells. The OCR profiles of NK.Tert cells cultured alone (blue line) and 1 patient's CLL cells cultured alone in suspension (red line) or cocultured with NK.Tert cells (green line) are shown in Figure 1A. The basal OCR (before the addition of oligomycin) of CLL cells cultured alone was 94 ± 5 pmol/min. The OCR of CLL cells decreased after the addition of the ATP synthase activity inhibitor oligomycin but OCR quickly increased (297 ± 49 pmol/min) after the addition of the mitochondrial membrane permeabilizer FCCP. The cells' oxygen consumption response to FCCP determines their maximum respiration capacity (MRC). The OCR decreased sharply after the addition of a combination of antimycin A and rotenone. Compared with CLL cells cultured alone, CLL cells cocultured with NK.Tert cells had a significantly higher basal OCR (162 ± 12 pmol/min) and MRC (379 ± 12 pmol/min). In contrast to CLL cells, NK.Tert cells had a very low basal OCR (13 ± 5 pmol/min), which did not change much. This value was subtracted from the CLL-NK.Tert coculture values. This was confirmed by 15 biological replicates of NK.Tert OCR profiling (data not shown).

This experiment was repeated using CLL lymphocyte samples from several patients. To ensure proper comparison, we used the same number of CLL cells for both culture conditions. The basal OCR of 28 patients' CLL cells cocultured with NK.Tert cells (99 ± 64 pmol/min) was significantly higher than that of the patients' CLL cells cultured alone (78 ± 53 pmol/min; $P = .004$; Figure 1B). Similarly, the MRC of these CLL cells cocultured with NK.Tert cells (155 ± 84 pmol/min) was significantly higher than that of the CLL cells cultured alone (110 ± 80 pmol/min; $P = .0001$; Figure 1C). The stromal cell-mediated increase in CLL cell OCR was an early event; of 12 CLL samples plated on stromal cells for only 2 hours, 11 showed a statistically significant increase in basal OCR ($P = .0001$; Figure 1D). As mentioned before, the OCR value of NK.Tert cells was low. We tested if the interaction with CLL cells modulates stromal cell OCR values. For this, NK.Tert cells were cultured alone or with three different patients' CLL cells. In contrast to that of CLL cells, the OCR of stromal cells cocultured with CLL cells was not affected (Supplemental Figure 1).

To determine whether other stromal cell lines also result in the augmentation of OxPhos activity in CLL cells, we examined the effects of M2-10B4, a murine fibroblast cell line, and HS-5, a human bone marrow-derived cell line, on CLL cell OCR. The basal OCR and MRC augmentation was also observed with these two stroma cell lines (Figure 2, A–D). Compared with CLL cells cultured alone, CLL cells cocultured with M2-10B4 had a higher basal OCR (91 pmol/min) and MRC (40 pmol/min; green line, Figure 2A). The OCR profile of M2-10B4 stromal cells cultured alone (blue line) was lower than that of CLL cells cultured alone (red line, Figure 2A). Among eight patients' CLL samples, the basal OCR of CLL cells cocultured with M2-10B4 cells (171 ± 107 pmol/min) was significantly higher than that of CLL cells cultured alone (114 ± 81 pmol/min; $P = .04$; Figure 2B). CLL cells cocultured with HS-5 stromal cells had a basal OCR profile and

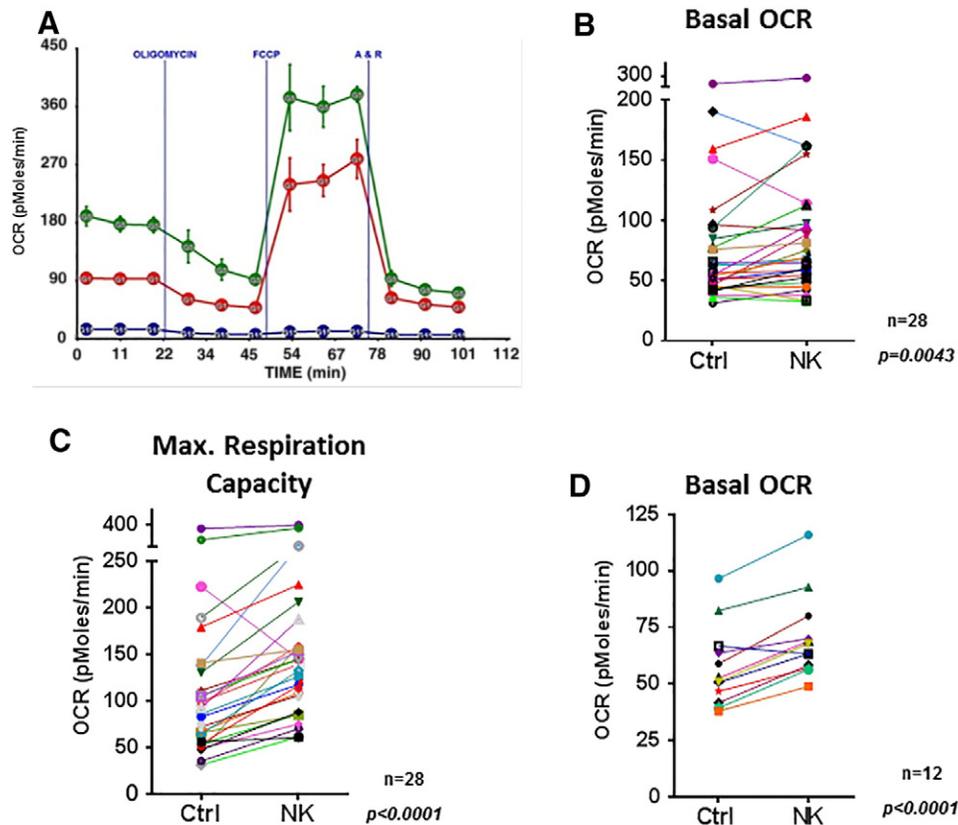


Figure 1. Stromal cells up-regulate mitochondrial OxPhos in CLL cells. The OxPhos of CLL cells (5×10^5) cultured in suspension or cocultured with NK Tert stromal cells (5×10^3) was measured. For each CLL coculture, the OCR values of the stromal cells (blue line) were subtracted from the CLL co-culture OCR values (green line). The same number of CLL cells was used for all suspension and coculture conditions. Five technical replicates were used for the OCR assays. (A) Mitochondrial stress test profile of 1 CLL patient sample (#30). CLL cells cultured alone (red line), CLL cells cocultured with NK Tert stromal cells (green line), and NK Tert cells cultured alone (blue line) were assayed. (B) The OCRs of CLL cells cultured in suspension or cocultured with stromal cells for 24 hours were compared ($P = .0043$, paired t-test; $n = 28$). (C) Similarly, the maximum respiration capacities of CLL cells cultured in suspension or cocultured with NK Tert cells for 24 hours were compared ($P < .0001$, paired t-test; $n = 28$). (D) The basal OCRs of CLL cells cultured in suspension or cocultured with stromal cells for 2 hours were compared ($n = 12$; $P < .0001$, paired t-test; $n = 12$).

MRC profile similar to those of CLL cells cocultured with NK Tert or M2-10B4 cells (green line, Figure 2C). Among three patients' samples, the basal OCR of CLL cells cocultured with HS-5 cells (89 ± 30 pmol/min) was higher than that of CLL cells cultured alone (74 ± 36 pmol/min; Figure 2D). Compared with NK Tert cells cultured alone, both M2-10B4 and HS-5 cells cultured alone had higher OCRs. As before, these values were subtracted from the CLL OCR readouts. Although stroma protected CLL cells from spontaneous apoptosis (Supplemental Figure 2), the stroma-mediated increase in CLL OCR was not due to an increase in cell number, as equal numbers of CLL cells were used for OCR readings. Similarly, the OxPhos augmentation in CLL cells cocultured with stromal cells was not due to an increase in the proliferation index, as both CLL cells cultured alone and those cocultured with stromal cells stained negative for Ki67 (not shown).

For the experiments mentioned above, stroma contact was imperative, as conditioned NK Tert or M2-10B4 media (data not shown) or exogenous SDF-1 and/or BAFF were not sufficient to augment CLL OCR (Supplemental Figure 3, A and B).

Stromal Cell Interaction Induces a Heterogeneous Response in CLL Glycolysis

To assess stromal cells' effect on CLL cell glycolysis, we measured ECAR in 29 patients' CLL cells cultured alone or cocultured with

NK Tert stromal cells. The ECAR profiles of NK Tert cells cultured alone and 1 patient's CLL cells cultured alone or cocultured with NK Tert cells are shown in Figure 3A. After the addition of glucose, the glycolytic flux of CLL cells cultured alone (9 ± 3 mpH/min) or cocultured with NK Tert cells (11 ± 2 mpH/min) did not differ significantly (Figure 3A). However, the flux from the NK Tert cells needs to be deducted (7 ± 3 mpH/min) from the readout in the stroma coculture condition.

Glycolytic capacity determines the ability of cells to utilize glycolysis for ATP generation upon OxPhos inhibition. Glycolysis was measured in CLL cells by oligomycin (a mitochondrial ATP synthase inhibitor) injection. The glycolytic capacity of CLL cells cultured alone was 30 ± 3 mpH/min. CLL cells cocultured with NK Tert cells had slightly higher glycolytic capacity (3 mpH/min higher), but this difference was not significant. The glycolytic capacity of NK Tert stromal cells cultured alone was 16 ± 3 mpH/min. As expected, the ECAR of CLL cells cultured alone decreased sharply after the addition of the glycolytic pathway inhibitor 2-deoxy-D-glucose; the ECAR profile of NK Tert cells was similar to that of CLL cells (Figure 3A). The glycolytic flux and capacity of CLL cells cultured alone were variable and in general smaller than those of CLL cells cocultured with NK Tert cells (Figure 3, B and C).

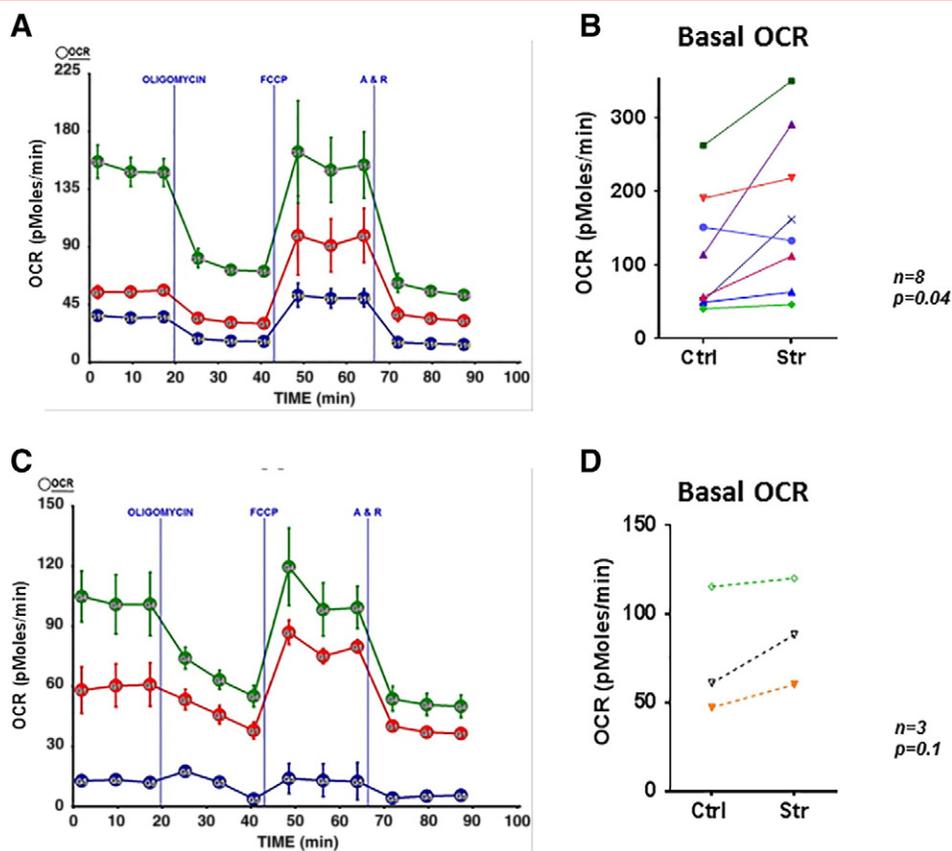


Figure 2. Effect of other stromal cell lines on mitochondrial OxPhos in CLL cells. CLL cells were cocultured with 2 other stromal cell lines (M2-10B4 and HS-5), and the ratio of CLL cells to stromal cells was maintained at 100:1. (A) Cell mitochondrial stress test profile of 1 patient sample (#45). CLL cells cultured alone (red line), CLL cells cocultured with M2-10B4 stromal cells (green line), and M2-10B4 cells cultured alone (blue line) were analyzed. (B) The basal OCRs of CLL cells cultured in suspension versus those of CLL cells cocultured with M2-10B4 cells were analyzed and plotted after the OCR values of the stromal cells alone were subtracted from the CLL co-culture values ($P = .04$, paired t-test; $n = 8$). (C) Cell mitochondrial stress test profile of 1 patient sample (#54). CLL cells cultured alone (red line), CLL cells cocultured with HS-5 cells (green line), and HS-5 cells cultured alone (blue line) were analyzed. (D) The basal OCRs of CLL cells cultured in suspension versus those of CLL cells cocultured with HS-5 cells were analyzed as in (B) ($P = .1$, paired t-test; $n = 3$).

The glycolytic flux of 24 patients' CLL cells cultured alone (mean 11 ± 4 mpH) was slightly smaller than that of CLL cells cocultured with NK.Tert cells (12 ± 10 mpH; Figure 3B). Similarly, the glycolytic capacities of these patients' CLL cells cultured alone or cocultured with NK.Tert cells were 25 ± 17 mpH and 29 ± 7 mpH, respectively (Figure 3C). The ECAR profiles of five patients' CLL cells cultured alone or cocultured with NK.Tert cells for 2 hours were not significantly different (dotted lines, Figure 3, B and C).

Stromal Cell Interaction Does Not Significantly Affect Substrate Uptake and Mitochondrial Functionalities in CLL Cells

Because stromal cells mediated OxPhos augmentation in CLL cells, we investigated whether stromal cells also induce CLL cells to switch their carbon source. We measured substrate uptake in seven patients' CLL cells cultured alone or cocultured with NK.Tert cells (Figure 4A). Compared with CLL cells cultured alone, CLL cells cocultured with stromal cells generally had lower glucose uptake, which indicated these cells' utilization of an alternative carbon source.

Previous studies have shown that many malignant cell types increasingly catabolize glutamine to supplement their increasing metabolic needs [24]; hence, we measured glutamine uptake in CLL

cells cultured alone or cocultured with stromal cells. Compared with CLL cells cultured alone, CLL cells cocultured with stromal cells had a heterogeneous increase in glutamine uptake, although this increase was not statistically significant (Figure 4A).

We assessed the effect of stromal cells on other CLL mitochondrial functionalities, such as reactive oxygen species, mitochondrial outer membrane potential, and mitochondrial mass. Flow cytometry revealed no significant differences in these parameters in CLL mitochondria (Figure 4B).

Because OCR assays revealed that stromal cells up-regulate the mitochondrial electron transport chain activity in CLL cells, we sought to determine whether stromal cells also affect mtDNA copy number and the expression levels of OxPhos complex proteins. The OxPhos protein expression levels ($n = 6$; Figure 4C) and the mtDNA copy numbers ($n = 4$; Figure 4D) of CLL cells cultured alone or cocultured with NK.Tert cells did not differ significantly.

Stromal Cell Interaction Up-Regulates Metabolic Pathways and Intracellular Ribonucleotide Pools in CLL Cells

To determine how stromal cells affect the overall metabolomics of CLL cells, we used mass spectrometry to analyze five patients' CLL

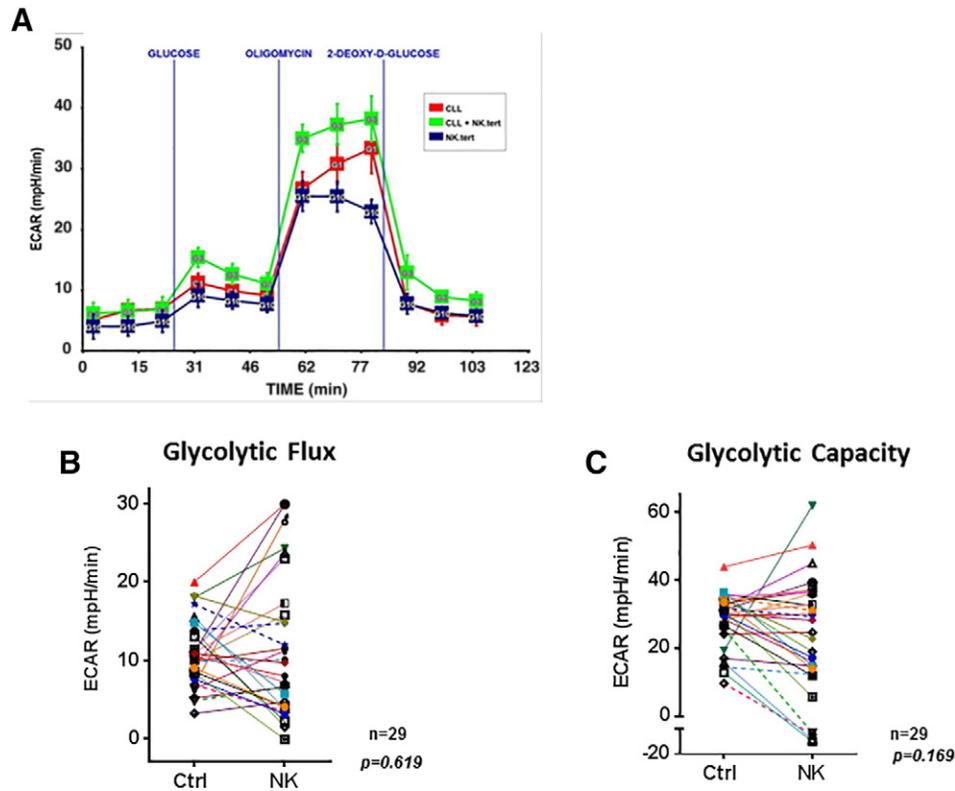


Figure 3. Stromal cells induce heterogeneous glycolysis in CLL cells. The glycolysis of CLL cells (5×10^5) cultured in suspension or cocultured with NK. Tert stromal cells (5×10^3) was measured. OCR values from the stroma OCR profile (blue line) were subtracted for each CLL coculture (green line). (A) Glycolysis stress test profile of 1 CLL patient sample (#9). CLL cells cultured alone (red line), CLL cells cocultured with NK. Tert cells (green line), and NK. Tert cells cultured alone (blue line) were analyzed. (B) CLL cells cultured in suspension or cocultured with NK. Tert cells ($n = 29$) for either 2 hours ($n = 9$, dashed lines) or 24 hours ($n = 20$, solid lines) were assayed as in (A) and analyzed for glycolytic flux ($P = .619$, paired t-test). (C) The glycolytic capacity of the CLL cells mentioned in (B) was analyzed after the addition of oligomycin ($P = .169$, paired t-test; $n = 29$).

cells cultured alone or cocultured with NK. Tert cells (BIDMC mass spectrometry core). Ingenuity Pathway Analysis software was used to categorize 210 metabolites into different pathways. Among the top 10 pathways significantly up-regulated in CLL cells cocultured with stromal cells were the tricarboxylic acid cycle, gluconeogenesis, and nucleotide de novo biosynthesis pathways (Figure 5A).

Because OxPhos and energy production feed into nucleotide biosynthesis, we measured intracellular NTP pools. Compared with endogenous NTP levels, ATP, GTP, and CTP concentrations showed a 40% increase, and the UTP intracellular level showed a 150% increase, after interacting with stromal cells for 24 hours ($n = 12$; $P < .05$). The increase in the NTP pool was also observed at 48 hours ($n = 6$; $P < .05$) (Figure 5B). Endogenous concentrations were normalized as 100%.

Discussion

In the present study, we demonstrate that while short-term (24- and 48-h) cocultures with stromal cell lines do not induce glycolysis in CLL cells, they do up-regulate mitochondrial OxPhos, as evidenced by the augmentation of CLL cells' basal levels of oxygen consumption and maximum respiratory capacity. Bone marrow stromal cell line interactions also augmented ATP and other nucleotide pools in CLL lymphocytes. We selected short-term co-cultures because 24- and 48-h co-cultures have been shown to offer protection against spontaneous apoptosis by increasing anti-apoptotic protein levels

[6–10]. Additionally, 24 or 48 hours of stroma-CLL interaction does not result in CLL cell proliferation, which is known to modulate metabolism [8]. The metabolomics of long-term (6 days) co-cultures have been reported previously [25], but whether metabolomics were modulated by proliferation cues is unclear.

Peripheral blood CLL cells are replicationally quiescent, but this condition can be reversed when the cells reach the proper microenvironment, such as that of the lymph nodes. In bone marrow, CLL cells appear to remain dormant. One transcriptome characterization study showed that, compared with normal lymphocytes, CLL cells have up-regulated expression of genes involved in metabolic pathways [26]. Recent studies have also demonstrated that the microenvironment can modulate the energy metabolism of CLL cells [25,27]. In a recent report, we demonstrated that although freshly obtained blood CLL cells are non-dividing, they are metabolically active [28]. Furthermore, these cells rely on OxPhos for their metabolic needs, and this process has been associated with poor prognostic features such as IGHV unmutated disease, ZAP70 positivity, increased Rai stage, and higher $\beta 2$ -M [28–31]. In addition, levels of free oxygen radicals in CLL cells are generally heterogeneous among CLL patients but are significantly higher in CLL cells from patients who have received chemotherapy [32].

Similar to CLL B-cells, peripheral blood T lymphocytes are also replicationally quiescent and rely on OxPhos to manufacture their ATP [33,34]. When activated, however, the pathway to energy

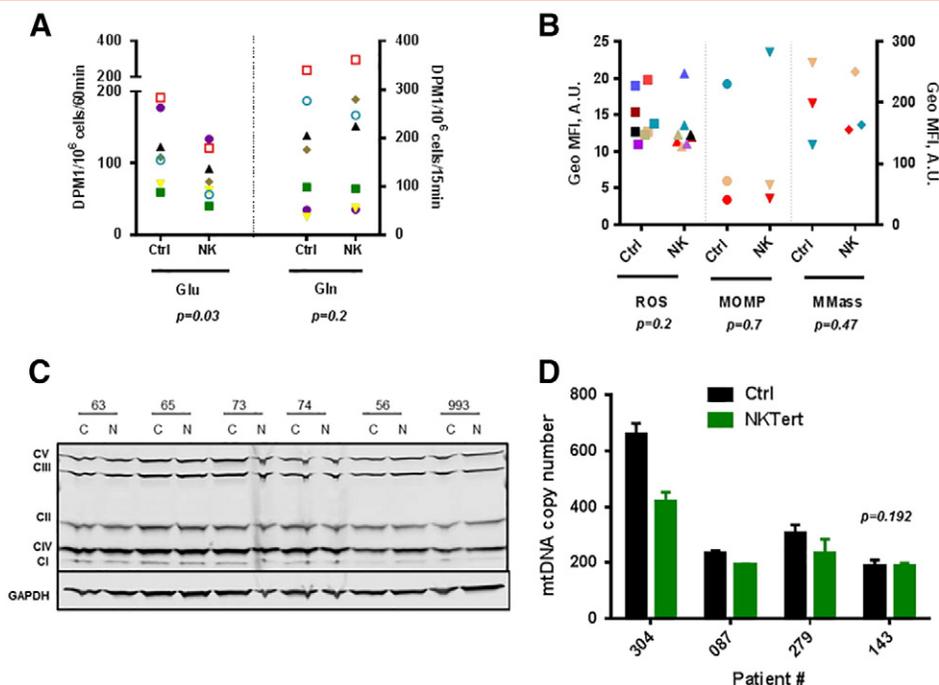


Figure 4. Stromal cells do not significantly affect substrate uptake and mitochondrial functionalities in CLL cells. (A) Effect of stroma on glucose (Glu) and glutamine (Gln) uptake in CLL cells. [³H]-Deoxy-D-glucose was used to determine the cellular uptake of the substrate in CLL patient samples in suspension versus CLL patient samples in cocultures with NK. Tert cells ($P = .03$; paired t-test; $n = 7$). Three technical replicates were used for each suspension and cocultured condition. Disintegrations per minute (DPM)/60 minutes were normalized to 10⁶ cells. [³H]-glutamine was used similarly, and its cellular uptake was measured in CLL patient samples before and after NK.Tert coculture; DPM/15 minutes were normalized to 10⁶ cells ($P = .2$; paired t-test; $n = 7$). (B) Mitochondrial functional assays in CLL cells. The geometric means (determined from flow cytometry data) of patients' CLL cells were analyzed to compare mitochondrial reactive oxygen species (ROS) (on the left y-axis) before and after NK. Tert coculture ($P = .2$). Similarly, three patient samples were analyzed for mitochondrial outer membrane potential (MOMP) and mitochondrial mass (on the right y-axis) before and after coculture ($P = .47$). (C) Immunoblot analysis of whole-cell extracts of 6 patients' CLL cells cultured alone (control; C) and cocultured with NK. Tert cells (N). Proteins were extracted and analyzed using antibodies against all 5 mitochondrial respiratory chain complexes (I, II, III, IV, and V). (D) Effect of stromal cells on CLL mtDNA copy number. DNA was extracted from four patients' CLL cells cultured alone or cocultured with NK.Tert cells. qPCR analysis for mtDNA in CLL cells cultured alone (black bars) and CLL cells cocultured with stromal cells (green bars) was performed in triplicate ($P = .192$).

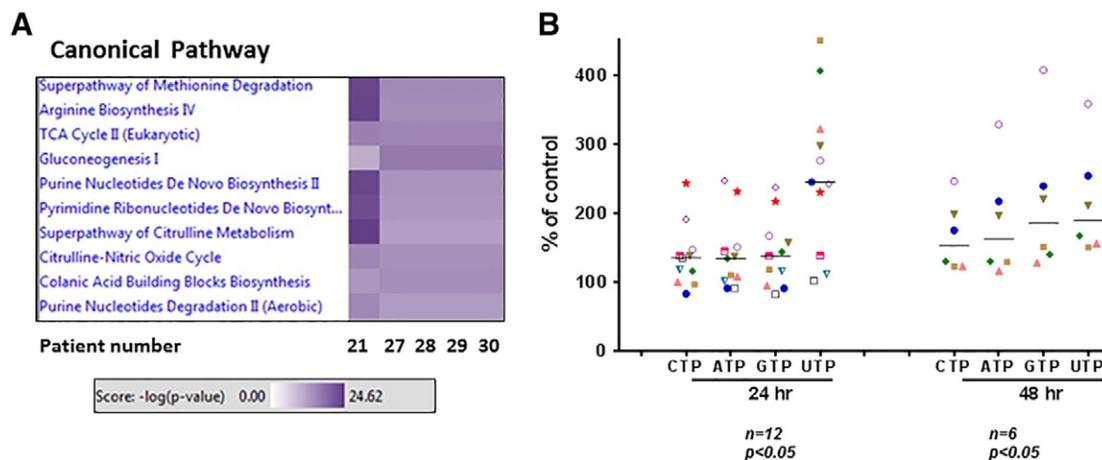


Figure 5. Stromal cells influence CLL metabolic pathways and cellular bioenergy. (A) CLL cells from 5 patient samples were cultured alone or cocultured with NK. Tert cells for 24 hours. Metabolites extracted with 80% methanol were assessed by mass spectrometry and analyzed using Ingenuity Pathway Analysis. Changes in metabolite concentrations were expressed as the ratios of metabolite concentrations in CLL cells cultured alone to metabolite concentrations in CLL cells cocultured with NK. Tert cells. (B) Influence of NK. Tert stromal cells on the intracellular NTP pools of CLL cells. NTP pools were extracted from CLL cells after 24 hours ($n = 11$) or 48 ($n = 6$) h of culture and analyzed with high-performance liquid chromatography.

production changes to glycolysis [35,36] and replicationally dormant cells start proliferating. In a previous study, the majority of a population of dividing fibroblasts that were brought to a state of quiescence by contact inhibition or serum starvation were in non-cycling G0 and G1 phase. The cells remained metabolically active, but had decreased their glycolytic capacity [37]. In contrast to our finding of a decrease in glucose uptake in CLL cells cocultured with stroma for 24 hours, in another study, malignant B lymphocytes such as CLL cells showed a rapid amplification of glucose uptake and glycolysis after being cocultured with stromal cells for 6 days. This process was associated with a modulation of the gene expression of the enzymes involved in the glycolytic pathway in a Notch-MYC-dependent manner [25]. Such long-term cultures result in CLL cell proliferation owing to the CLL cells' interaction with nurse-like cells, and this proliferation changes the dynamics of CLL-stroma interaction [4]. In another study, [38] nuclear magnetic resonance monitoring revealed marked metabolic activity in non-cycling CLL cells, and glycolysis was rapidly up-regulated in response to hypoxia. In general, a large portion of CLL cells is a resting population, and a small intracell fraction is proliferating [39]. Many of the early stromal signals do not induce B-lymphocyte proliferation but activate several processes that require steady-state energy production. In the present study, we investigated the energy production route in CLL lymphocytes after stromal interaction without proliferation.

Several approaches in the current study demonstrated that the stromal microenvironment up-regulates mitochondrial respiration in CLL cells. First, CLL cells from all patient samples tested had higher OCRs after being cocultured with NK.Tert stromal cells for 24 hours. Second, this increase in OCR was also observed in 11 samples after 2 hours of coculture. Third, this modulation in metabolomics was not unique to NK.Tert stromal cells; it was also observed with 2 additional stromal cell lines. Fourth, this increase was not due to the stromal cell OCR, as our data plotted the difference between the OCR of CLL cells on stroma and the OCR of stroma alone. Collectively, these stringent and rigorous assays established that stromal cell lines mediate the up-regulation of OxPhos in CLL cells without changing the division status of the CLL lymphocytes.

The CLL microenvironment is diverse, and in this environment, malignant B cells interact with other cell types, including bone marrow stromal cells, macrophages, monocytes, nurse-like cells, and T cells. Similarly, the oxygen tension in the microenvironment is variable even in hypoxic niches in highly vascularized bone marrow [40]. The current work focused on stromal influence on CLL cells in normoxic conditions. Previous reports have indicated a role of hypoxia inducible factor in stroma and CLL cell interactions, in CLL cell survival and on CLL cell plasticity when residing in hypoxic niches [41–43]. However, impact of hypoxia on CLL metabolomics needs to be interrogated.

Multiple lines of evidence have suggested that the bone marrow stroma microenvironment positively impacts CLL survival [29]. In previous studies, we and others have shown that stroma induces the survival of CLL cells [8,14] and the phosphorylation of AKT and S6, indicating the activation of the PI3K/AKT/mTOR pathway [20]. However, the downstream effectors of these pathways impacting mitochondrial metabolism remain elusive. The stroma-mediated uptake of cysteine by CLL cells for glutathione synthesis promotes cell survival and overcomes drug resistance [27]. This activation cascade has also been observed when the BCR pathway in CLL cells is directly stimulated by IgM ligation [20]. A consequence of this

activation may be an increase in the transcript and protein levels of early response genes, such as MCL-1 [8].

In the present study, because stromal cells augmented the OCR of CLL cells, we expected that stromal cells would also increase the cellular ATP pool in CLL lymphocytes. Our data demonstrated that stromal cell lines amplified not only ATP but also other pools, including pyrimidine triphosphates (UTP and CTP). In our experiments, the CLL cells and stromal cells were plated at a ratio of 100:1, and the CLL cells were separated from the stromal cells before nucleotide extraction. The concentration of the stromal cell NTP pool was similar to that of CLL cells, and the OCR values of stromal cells did not change after co-cultures. The endogenous NTP pool of the entire population of stromal cells (at a ratio of 100 CLL cells to 1 stromal cell) was too small to increase CLL NTP pool size. Hence, neither stromal cell contamination nor nucleotide exchange between CLL cells and stromal cells would explain the increase in the NTP pools of CLL cells.

Preliminary data from a metabolite analysis of CLL cells cocultured with stromal cells revealed that NAD⁺ levels were augmented ($P = .036$), suggesting an increase in the mitochondrial complex I activity of the electron transport chain. Although the exact molecular mechanism of the rapid increase in stroma-mediated oxygen consumption in CLL is unknown, the increase of substrate availability to the ETC complexes may have amplified OxPhos. We also found that orotate levels were marginally elevated in CLL cells cocultured with NK.Tert stromal cells (Supplemental Figure 4). Orotate is produced from dihydroorotate by dihydroorotate dehydrogenase (DHODH), an enzyme that resides in mitochondria and is critical to the de novo biosynthesis of uridine monophosphate [44], which is phosphorylated to UTP [45]. Furthermore, UTP is converted to CTP directly by CTP synthase [45]. Hence, it is plausible that stromal cell interaction induces DHODH, thereby increasing orotate levels, UTP levels, and consequently CTP pools. Therefore, the effects of stromal cells on the OxPhos pathway and electron transport chain may influence pyrimidine biosynthesis. Among purines, the consequence of OxPhos is ATP production, which directly feeds into GTP synthesis [46]. Carbon tracing experiments will more accurately delineate the substrate flow in the metabolic pathways, which were not evaluated in the present study. Integrin-mediated signaling pathways can be examined in this context as well.

Specific antagonists of OxPhos are being investigated in leukemia patients in a clinical trial (NCT02882321; ClinicalTrials.gov). Such inhibitors need to be tested to evaluate the attenuation of OxPhos in CLL cells that are interacting with stroma. We are conducting such experiments using IACS-10759, an OxPhos inhibitor, in CLL cells cultured alone or cocultured with stroma. Our postulate is that OxPhos will be mitigated by IACS-10759 in CLL cells growing in suspension as well as on stromal cells.

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