B-cell Receptor Signaling Regulates Metabolism in Chronic Lymphocytic Leukemia

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Abstract

Peripheral blood chronic lymphocytic leukemia (CLL) cells are quiescent but have active transcription and translation processes, suggesting that these lymphocytes are metabolically active. Based on this premise, the metabolic phenotype of CLL lymphocytes was investigated by evaluating the two intracellular ATP-generating pathways. Metabolic flux was assessed by measuring glycolysis as extracellular acidification rate (ECAR) and mitochondrial oxidative phosphorylation as oxygen consumption rate (OCR) and then correlated with prognostic factors. Further, the impact of B-cell receptor signaling (BCR) on metabolism was determined by genetic ablation and pharmacological inhibitors. Compared with proliferative B-cell lines, metabolic fluxes of oxygen and lactate were low in CLL cells. ECAR was consistently low, but OCR varied considerably in human patient samples (n = 45). Higher OCR was associated with poor prognostic factors such as ZAP 70 positivity, unmutated IGHV, high B2M levels, and higher Rai stage. Consistent with the association of ZAP 70 and IGHV unmutated status with active BCR signaling, genetic ablation of BCR mitigated OCR in malignant B cells. Similarly, knocking out PI3Kδ, a critical component of the BCR pathway, decreased OCR and ECAR. In concert, PI3K pathway inhibitors dramatically reduced OCR and ECAR. In harmony with a decline in metabolic activity, the ribonucleotide pools in CLL cells were reduced with duvelisib treatment. Collectively, these data demonstrate that CLL metabolism, especially OCR, is linked to prognostic factors and is curbed by BCR and PI3K pathway inhibition.

Implications: This study identifies a relationship between oxidative phosphorylation in CLL and prognostic factors providing a rationale to therapeutically target these processes.

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Introduction

Unlike solid tumors, which are highly proliferative, chronic lymphocytic leukemia (CLL) is an indolent malignancy characterized by the relentless accumulation of quiescent, immunologically dysfunctional mature B cells that fail to undergo apoptosis. These cells reside in different compartments such as bone marrow, spleen, lymph nodes, and peripheral blood, which provide diverse microenvironments. Although the population is either slowly proliferating or quiescent (1), these cells are transcriptionally and translationally active, suggesting that CLL cells are not inert and should have an active metabolic profile (2). Furthermore, this metabolome précis should correspond to the aggressiveness of the disease, which is categorized based on prognostic factors such as ZAP 70 positivity (3, 4), unmutated nature of immunoglobulin variable region heavy chain (IGHV) status (5), higher Rai stage (6), and increased abundance of B2M microglobulin (B2M; ref. 7).

Changes in the metabolome, which has been intensively investigated in solid tumors and is initiated and activated by growth factors through growth-factor-receptor nexus, which invariably includes the PI3Kα isoform–specific signal transduction pathway. The maintenance and production of healthy and malignant B cells occur through the B-cell receptor (BCR) axis. A primary node in the BCR pathway is the PI3K/AKT cassette, which affects downstream activation of survival factors in CLL cells. Among the four isoforms of PI3K, malignant CLL and other B cells rely on PI3Kδ/γ, the primary effector of the BCR network. Our recent study suggested that in B-cell malignancies, PI3Kδ mimicked a signaling pathway that was previously identified in solid tumors with PI3Kα (8). Several investigations have established that the PI3Kα-mediated signaling cascade influences the cellular metabolome, including glycolysis and energy production (9). Newer studies further demonstrated a role for AKT in this pathway and AKT-dependent and AKT-independent activation of the cellular metabolome (10, 11). However, the role of PI3Kδ/γ in regulating the metabolome is not yet elucidated.

Based on these reports, we hypothesized that malignant CLL lymphocytes should have an active metabolism and may show differential metabolome précis that are in line with the aggressiveness of the disease based on prognostic markers. BCR and

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PI3K induced changes in the metabolome should enhance the nucleotide biosynthesis needed for increased gene transcription and protein translation. In accordance with these expectations, genetic or pharmacological intervention in this pathway should impact metabolic changes.

To test these hypotheses, we compared the metabolic phenogram in freshly isolated peripheral blood CLL cells from 45 patients. Bioenergetic profiles of CLL samples suggested diverse range in oxidative phosphorylation (OxPhos) rates which were associated with prognostic characterization of the disease. Genetic targeting of the BCR pathway or knocking out PI3K resulted in mitigation of both OxPhos and glycolysis. Corollary to this observation, pharmacological inhibition induced similar results and correspondingly demonstrated a decrease in bioenergy.

**Materials and Methods**

**CLL patient sample collection**

Peripheral blood samples were collected from 77 CLL patients (Supplementary Table S1). All patients had given written informed consent in accordance with the Declaration of Helsinki and under a protocol approved by the Institutional Review Board (IRB) of The University of Texas MD Anderson Cancer Center.

**Normal peripheral blood mononuclear cells, normal and malignant B-cell collection, and isolation**

Peripheral blood (100 mL) was obtained in green top tubes from healthy human donors under a MD Anderson Cancer Center IRB–approved protocol. Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll–Hypaque procedure. Normal B cells were purified by negative selection using the Easy Sep Human B Cell Enrichment Kit (Stemcell Technologies). For optimal comparison, the same numbers of normal PBMCs (generally containing T-lymphocytes), normal B lymphocytes, or malignant CLL B cells were used for all assays.

For CLL cells, cells were isolated from peripheral blood, and PBMCs were separated by Ficoll–Hypaque density centrifugation (Atlanta Biologicals). All experiments were performed using freshly isolated CLL cells, and purity of this cell population was ≥95%. Cells were cultured in RPMI-1640 medium with l-glutamine and 10% human serum. For CLL and other primary cells to remain quiescent during the 24 hours of culture, cells were kept for 24 hours in conditions, cells were kept for 24 hours in culture. These primary cells are replicationally quiescent and remain quiescent during the 24 hours of culture.

**Drugs**

Duvelisib (IPI-145) was obtained from Infinity Pharmaceuticals and used at 1 μmol/L. Idelalisib (GS1101) was obtained from Gilead Sciences and MK-2206 from Selleck Chemicals; 1 μmol/L idelalisib and 2.5 μmol/L MK-2206 were used in the experiments. These concentrations were selected based on reported plasma concentrations of these drugs during clinical trials. All compounds were dissolved in DMSO.

**B-cell line cultures**

Wherever needed, the mantle cell lymphoma (MCL) cell lines (JeKo-1, Sp53, and Mino) were used either as control or as proliferative cell types. These three MCL lines were obtained from Dr. Hesham Amin, MD Anderson Cancer Center and represent BCR-reliant B-cell lines (12). JeKo-1 and Sp53 were grown in RPMI 1640 with 10% FBS, and Mino was grown in RPMI 1640 with 20% FBS and were used within six passages. These cell lines were authenticated by the MD Anderson core facility using short tandem repeat DNA profiling and were routinely tested for *Mycoplasma* infection by the same core facility using the Lonza MycoAlert Kit.

**Cytotoxicity assays**

To determine apoptotic and necrotic cells, CLL lymphocytes were stained with annexin/propidium iodide and counted using flow cytometry as described previously (13).

**Extracellular flux assays**

Extracellular flux assays (Seahorse Bioscience) were used to measure the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of CLL cells. In most cases, CLL cells (5 × 10^5) were plated in RPMI-1640 + 10% human serum in 6-well plates, with or without duvelisib for 24 hours. For each assay, after treatment, cells were counted, and equal numbers of viable cells were used and plated onto XF microplates coated with Cell-Tak (BD Biosciences) and allowed to adhere for 4 to 6 hours. RPMI-1640 medium was replaced with XF base (OCR) or glycolysis base (ECAR) media as recommended by Seahorse Bioscience. Five technical replicates for each condition were plated. Glycolysis and cell mitochondrial stress tests were performed as described previously (Supplementary Fig. S1; ref. 14). Generally for OCR, data were expressed as basal OCR, which is the starting level of OCR (Supplementary Fig. S1). Oligomycin, an ATP coupler, is added, followed by FCCP, which acts as ETC accelerator. Increase in OCR above basal respiration after FCCP addition is spare respiratory capacity while total is maximal respiratory capacity. Addition of rotenone and antimycin A shuts down mitochondrial respiration (Supplementary Fig. S1A). ECAR is measured under basal condition, which increases after addition of glucose that provides glycolytic flux. Addition of oligomycin measures the glycolytic capacity (Supplementary Fig. S1B).

**Mitochondrial reactive oxygen species, membrane potential, and mass**

CLL cells were stained with MitosOX Red, tetramethylrhodamine ethyl ester perchlorate (TMRE), and MitoTracker Deep Red FM (Life Technologies), and analyzed using FACs for mitochondrial reactive oxygen species (ROS), membrane potential and mass, respectively. Geometric means of cytometric data were obtained using FlowJo software (FlowJo).

**PCR for mtDNA copy number**

QiaAMP DNA mini kit (Qiagen) was used to extract DNA from CLL samples according to the manufacturer’s protocol. qPCR SYBR green master mix was used to amplify the mitochondrial DNA from 4 CLL patient samples. All samples were run in triplicate. These experiments were conducted in collaboration with Dr. Benny Kaipparettu’s laboratory, Baylor College of Medicine, Houston.

**Electron transport chain activity analysis**

Frozen cell pellets were lysed and then used for electron transport chain (ETC) enzyme assays according to published procedures (15, 16). Briefly, the enzymes were assayed at 30°C using a temperature-controlled spectrophotometer; Ultraspec 6300 pro, Biochrom Ltd. Each assay was performed in triplicate. The activities of the five enzymes were measured using...
appropriate electron acceptors/donors. These experiments were conducted in collaboration with Dr. Benny Kaipparettu’s laboratory, Baylor College of Medicine, Houston.

Ribonucleotide pools
Perchloric acid was used to extract nucleotide pools, and pools were separated using HPLC as described previously (14). Standard NTPs were used to quantify nucleotide pools, and the concentration was determined based on mean cell volume.

Immunoblotting
Cells were washed and protein extracts were probed using an Odyssey Infrared Imaging System (LI-COR Biosciences) as described previously (14). p-AKT Thr308, AKT, p-MAPK (44/42), MAPK, GAPDH (Cell Signaling Technology) and OxPhos cocktail (Abcam) primary antibodies were used.

Glycolysis/glutamine uptake assays
CLL cells were cultured in glucose free or glutamine-free media and either [3H] 2-deoxy-D-glucose (0.5 μM, specific activity 28 Ci/mmol) or [3H]glutamine (1 μL specific activity 50.3 Ci/mmol) was added (both reagents from PerkinElmer). The radioactivity was quantified using the scintillation counter.

BCR knockout
CRISPR-Cas9 px330 plasmids with BCR target DNA sequence constructs were donated by Dr. Davis. JeKo-1 was used for genetic knock out experiments. This cell line expresses the characteristic MCL proteins including cyclin D1, cyclin E, retinoblastoma (Rb), c-Myc, p21waf1, p27kip-1, Mcl-1, Bcl-2, Bax, and Bcl-xL. JeKo-1 overexpresses cyclin D3 and p16INK4a; however, it lacks p53 (12). JeKo-1 cells were transfected (10 μg of plasmid DNA/million cells) by electroporation (Neon transfection system) according to manufacturer’s protocol. Seventy-two hours after transfection, the cells were stained and analyzed by flow cytometry for BCR heavy chain (IgM) and light chain (κ) expression. The knockout (KO) cells were stained and sorted by FACs on day 6 (Supplementary Fig. S2). Three KO clones were generated, where the Cas9 construct harbored DNA sequences complimentary to different regions of CH2 domain of the IGHM gene, which encodes a vital component of the BCR (TS4: CCCCGCGAATTCGAAAGCTGATC; TS5: CAGGTGGTCCGTCGTCCGGAGGG; TS6: ACTTGGCGGC-GTGGATCACAGGGG). The general approach was to knock in (KI) the GFP-STOP-polyA cassette right after translation initiation site of the PIK3CD leading to PIK3CD promoter driven expression of GFP instead of PIK3CD. The px330 plasmid (Addgene plasmid #42230) coding for Cas9 and gRNA targeting PIK3CD (target site sequence: 5’-CATCTGGAAATGAAAGCAGC-3’) was cloned according to the published protocol (17). The pS-C-B-ampl (Agilent Technologies) was used to carry the KI template sequence that consisted of (from 5’ to 3’): left homology arm (400 bp of PIK3CD DNA sequence upstream of translation initiation site), kozak sequence (GCCACC), GFP with stop codon, bGH PolyA signal sequence, and right homology arm (300 bp of PIK398 sequence downstream of translation initiation site). The PIK3CD KO GFP-positive cells were stained and sorted by FACS on day 6 (Supplementary Fig. S3). After sorting, the cells were placed in DMEM media supplemented with 20% FBS +1 U penicillin–streptomycin solution. As a control for KI specificity, px330 plasmids with nonspecific target site were electroporated together with template plasmid. No GFP-positive cells were detected in the specificity control.

Statistical analysis
Student t tests (paired or unpaired, one-tailed) and ANOVA were performed using Prism 6 software (GraphPad Software) with P > 0.05 as level of significance. For Figs. 1 and 2, data were from 45 CLL subjects. Because they were from different patients, a range was observed between two variables for each parameter such as Rai stage, ZAP 70 status, etc. (Figs. 1B–G and 2A–F). To rigorously analyze these data, we compared each patient data (to incorporate broad range) rather than medians or averages. We also verified the statistical analysis using Microsoft Excel software and two sample t test assuming unequal variances or paired two sample t test for means where required. Although absolute P values were slightly different in Prism versus Excel analyses, significant or nonsignificant likelihood did not change between two analyses.

Results
CLL B-cell metabolic flux is comparatively lower than that of proliferating B-cell lines and is associated with patient prognostic factors
Most cancer cells have high metabolic requirements owing to a high proliferation index. Due to the indolent nature of CLL lymphocytes and nonadherent culture conditions, not many studies have been conducted to decipher the bioenergetic profile of CLL B cells. We performed a comprehensive study, encompassing the metabolic phenotype of CLL cells, its association with prognostic features, and role of the BCR pathway. Glycolysis (measured as ECAR) and oxidative phosphorylation (OxPhos; measured as OCR) profiles were analyzed in normal
PBMCs (n = 10), normal B-lymphocytes (n = 11) and malignant CLL B-lymphocytes (N = 45) as well as malignant B cell lines (three cell lines). In each case, for appropriate comparison, the number of cells was kept constant and for each data point, five technical replicate values were obtained. We used normal PBMC and normal B-lymphocytes to compare them with malignant B-lymphocytes (i.e., CLL cells) as all of these cell types are replicationally quiescent. We used cell lines to compare replicationally quiescent CLL B-lymphocytes with proliferating MCL cell lines. OCR and ECAR were lower in malignant CLL lymphocytes (n = 45), normal B lymphocytes (n = 11), and PBMC (n = 10) compared with that in all three B-lymphoma cell lines. The latter were highly metabolic both aerobically and anaerobically (Fig. 1A) and had two- to thirty-fold greater values for ECAR and OCR than that in the quiescent normal or neoplastic lymphocytes.

Among the patients’ malignant lymphocyte samples, there was very little glycolytic disparity, whereas the mitochondrial respiration varied. This was not due to heterogeneity of the cell population as all samples have ≥95% CLL cells. The ECAR values ranged from 1 to 15 mPoles/min for 5 × 10^5 (median 5.5; n = 45) CLL cells. In contrast, for the same number of CLL lymphocytes, the median for OCR was 19 pMoles/min (range, 5–190, n = 45; Fig. 1A). To understand the basis for this diversity in the OCR, we compared basal OCR with prognostic markers of the disease. Unpaired Student t test was used for statistical analyses and P values are listed on the graph. Rai and Binet are staging systems used for standard classification and prognosis in CLL (6, 18). Compared with lower Rai stage (n = 34), increased mitochondrial respiration (OCR) was observed at higher Rai stage (n = 20; Fig. 1B). In contrast to low B2M (n = 38), the presence of high levels of B2M (>3.5 µg/mL; n = 15), another poor prognostic feature (19, 20), also showed increased OCR (Fig. 1C). Both ZAP 70-positive (n = 29) and IGHV unmutated samples (n = 27) had higher OCR compared with their counterparts (Fig. 1D and E). In contrast to these prognostic characteristics, LDH, gender, age, and CD38 expression did not significantly associate with OCR (Fig. 1F and G; Supplementary Fig. S4A and S4B). Though cytogenetic lesions such as loss of ATM (11q deletion) and p53 (17p deletion) are associated with adverse prognosis, the OCR values were not significantly different among diverse cytogenetic cohorts (Fig. 1H).

While OCR accounts for mitochondrial metabolism through the OxPhos pathway, the ECAR represents glycolysis. Unpaired Student t test was used for statistical analyses and P values are listed on the graph. In comparison with OCR, prognostic factors, such as ZAP 70 and IGHV status, increased B2M and higher Rai stage did not correlate with ECAR (Fig. 2A–D). Level of LDH also did not affect ECAR (Fig. 2E). On the other hand, the ECAR readings indicated a higher rate in male patients (n = 14) compared with female cohorts (n = 12), which needs to be further explored (Fig. 2F). In conclusion, CLL lymphocyte metabolome preciso suggested diversity in OCR, which was higher in samples from patients with greater Rai stage, increased B2M, unmutated IGHV, and higher level of ZAP 70.

Genetic deletion of BCR receptor affects mitochondrial respiration in JeKo-1, an MCL cell line

CLL subgroups defined by B2M overexpression, ZAP 70 positivity, and unmutated IGHV locus have been associated with increased B-cell receptor signaling (21), denoting that BCR pathway manipulation may affect metabolism. We generated a knock-out (KO) of BCR in a malignant B-cell line, JeKo-1, to determine if abrogating B-cell signaling impacts OxPhos and ECAR. CRISPR-Cas9 technique was used to generate three clones (MC4, MC5, and MC6) of BCR KO by targeting the CH2 region of the gene encoding the heavy chain of the IgM. All three clones demonstrated significantly reduced OCR levels when compared with WT cells both for basal OCR; 15% decrease (Fig. 3A), and spare respiratory capacity; ~50% decline (Fig. 3B), demonstrating that BCR signaling plays an important role in regulating B-cell metabolism. The BCR KO cell lines were further validated for their lack of ability to respond to IgM stimulation. BCR WT cells displayed an increase in p-ATK Thr308 and p-MAPK Thr202/Tyr204, whereas in the KO clones, these residues were minimally phosphorylated, compared with WT cells (Fig. 3C) confirming abrogation of the BCR pathway. The decrease in OCR was not due to change in growth, as both WT and BCR KO cells had a similar population-doubling time (Fig. 3D). Similar to OCR, ECAR was also reduced in BCR KO clones (not shown).

PI3K p110δ knock out in MCL cell line (JeKo-1) affects glycolysis and OxPhos

A pivotal node in the BCR pathway is PI3Kδ. Hence, we further tested if genetic ablation (KO) of PI3KCD (coding for p110δ) would also impact OxPhos in malignant B cells (JeKo-1). JeKo-1 PIK3CD KO cells were analyzed for glycolysis and OxPhos, along with unmodified cells (WT) from a parallel culture. The PIK3CD KO cells displayed 20% reduction in both glycolytic flux (Fig. 4A and B) and glycolytic capacity (Fig. 4A and C) as compared with the WT cells. A more profound decrease in respiration levels, i.e., 25% decline in basal OCR (Fig. 4D) and 40% reduction in spare respiratory capacity (Fig. 4F) as compared with the PI3Kδ WT cells, was observed. Real-time RT-PCR assays in these cells confirmed for reduced transcript levels of PI3KCD (not shown). Similar to BCR KO cells, compared with PI3Kδ WT cells, the PIK3CD KO cells did not differ in proliferation ability (Fig. 4G) or cell size (not shown).

Pharmacological inhibition of the PI3K/AKT pathway diminishes CLL mitochondrial OxPhos and intracellular NTP pools

Because PI3Kδ knockout isoform demonstrated lower OxPhos and glycolysis in malignant B cells, we evaluated if a similar result is obtained in CLL lymphocytes by employing PI3K δ-specific pharmacological inhibitors. Unpaired Student t test was used for statistical analyses and P values are listed on the graph (Fig. 5). The PI3Kδγ inhibitor, duvelisib (IPI-145), is currently in phase III clinical trials for CLL (Duo trial, NCT02004522; NCT02049515; www.clinicaltrials.gov). We tested ability of duvelisib to alter the OxPhos pathway in CLL cells. Duvelisib treatment of CLL cells mitigated both glycolysis and aerobic respiration (Fig. 5A–D). Both glycolytic flux and capacity were mitigated in 4 of 5 samples tested (Fig. 5A and B). Although there was heterogeneity in reduction of basal OCR (7/9), maximum respiratory capacity was significantly reduced upon duvelisib treatment in all samples tested (Fig. 5C and D). Again, the most profound effect was on maximum respiratory capacity. Similar to basal OCR and maximum respiratory capacity, spare respiratory capacity was also reduced after treatment with duvelisib (Supplementary Fig. S5). Decline in ECAR and OCR was not due to duvelisib-
induced modest cell death (data not shown) as equal number of untreated and duvelisib-treated CLL cells were plated for the assay. Because glycolysis is hampered by inhibition of AKT, whose role in glucose uptake and utilization is well established, we hypothesized that duvelisib treatment would inhibit glucose uptake. Surprisingly, an assessment of the uptake of [3H]2-deoxy-D-glucose demonstrated that glucose uptake was not significantly impacted by duvelisib treatment (Supplementary Figure 1).

The CLL bioenergetics profile and relationship of CLL OCR with disease prognostic markers. Bioenergetics was measured for 5 x 10^5 freshly isolated CLL cells after 24 hours of culture. Five technical replicates were used for ECAR and OCR assays. A, Bioenergetic profile of CLL cells from 45 patients (red circles) compared with PBMCs (n = 10, blue triangles), B cells from healthy donors (n = 11, inverted green triangles), and B-lymphoma cell lines (Jeko-1 (maroon), Mino (purple) and Sp53 (orange, squares). B, OCR correlation with patient Rai stage. Basal OCR of CLL cells was compared with disease Rai stage: low grade (stages 0, 1, n = 34), high grade (stages 2–4, n = 20). B, OCR correlation with patient β2 microglobulin (β2M) levels. Basal OCR from CLL cells was compared according to β2M cutoff levels determined by CLL International Prognostic Index (≤3.5 µg/mL (n = 38), >3.5 µg/mL (n = 15). D, OCR correlation with ZAP 70 status. Basal OCR of CLL cells obtained from ZAP 70-negative (neg; n = 20) and positive (pos; n = 29) patients was compared. E, OCR correlation with IGHV mutational status. Basal OCR of CLL cells was compared based on the mutational status of IGHV: mutated (M; n = 19) or unmutated (U; n = 27). F, OCR correlation with patient lactate dehydrogenase (LDH) levels. Basal OCR from CLL cells was compared for patients with different LDH levels: low (L; 300–500 µg/mL, n = 8), medium (M; 501–700 µg/mL, n = 26), high (H; >700 µg/mL, n = 17). G, OCR correlation with patient gender. Basal OCR of CLL cells was compared based on the gender of the patient, male (n = 27) or female (n = 27). Statistical significance was calculated using unpaired Student t test for B–G, H. Basal OCR of CLL cells were compared for patients with various chromosomal abnormalities by extracellular flux analysis: 1q deletion (n = 8), 13q deletion (n = 9), trisomy 12 (+12; n = 8), 17p deletion (n = 5). C.K. (n = 7) indicates complex karyotype with 2 or more of these deletions, O (n = 16) indicates other chromosomal abnormalities and U (n = 21) indicates unknown karyotype. Statistical significance was calculated using ANOVA P = 0.4.
Fig. S6A). Furthermore, glutamine uptake (Supplementary Fig. S6A), as well as mitochondrial ROS, mitochondrial membrane potential, mitochondrial mass (Supplementary Fig. S6B), and mitochondrial DNA copy number (Supplementary Fig. S6C), were not affected. No significant changes in the protein levels or the enzyme activity of respiratory chain complexes (I–V) were observed after duvelisib treatment (Supplementary Fig. S6D and S6E). However, PI3Kδ/γ inhibition resulted in 25% to 50% decreases in intracellular ribonucleotide triphosphate pools measured in 7 patient samples (Fig. 5E). Because duvelisib inhibits both PI3Kδ and γ, we tested if a PI3K δ isoform inhibition or downstream AKT is responsible for the decrease in overall energy metabolism. We, therefore, examined the effects of a PI3Kδ inhibitor, idelalisib, and an AKT inhibitor, MK-2206. Each of these two inhibitors were used as single agent. Idelalisib mitigated glycolysis in all samples (5/5) tested (Fig. 5F and G) and lowered basal OCR in 5 of 6 samples and maximum respiration in all samples (Fig. 5H and I); similarly, MK2206 alone curbed glycolysis (5/6; Supplementary Fig. S7A and S7B) and OCR (basal OCR was reduced in 4/6 samples and maximum respiration capacity in 5/6 samples; Supplementary Fig. S7C and S7D), suggesting that inhibition of the AKT pathway at least in part underlies the mitigation of glycolysis and OxPhos pathways.

**Discussion**

Most cancer cells have high metabolic requirements owing to a high proliferation index. Due to the indolent nature of CLL lymphocytes and nonadherent culture conditions, not many studies have been conducted to decipher the bioenergetic profile of CLL B cells. Transcriptome characterization has found that genes involved in metabolic pathways were upregulated in CLL cells compared with normal lymphocytes (22).
activity in noncycling (quiescent) CLL cells was recently demonstrated (23). Additionally, free oxygen radicals in cells are heterogeneous among patients with CLL but significantly higher in CLL cells from patients with prior chemotherapy (24). CLL bioenergetic rewiring is also associated with kinase sensitivity; for example, CLL was classified into subsets based on the dasatinib-induced differential dependency on mitochondrial respiration to cope with cellular stress and meet the ATP demands (25).

Prevalence of oxidative stress resulting in increased ROS production in CLL cells (24) and targeting this by natural compounds (26) has been reported before. These observations were further confirmed recently by Jitschin and colleagues (27). They further identified that altered mitochondrial metabolism in CLL contributes to oxidative stress. Focusing on normal versus malignant B-cell biology, they compared ATP levels \( (n = 5) \), as well as ECAR and OCR values \( (n = 4) \), in healthy subjects and CLL patients. Their data suggested that increased oxidative phosphorylation, rather than aerobic glycolysis, was coupled to bioenergy synthesis in CLL cells.

Our current investigation evaluated the metabolic characteristic to comprehensively determine ECAR and OCR profiling of freshly isolated malignant lymphocytes from peripheral blood of 45 CLL patients. We further focused to identify if prognostic factors and cytogenetics are associated with this broad range of OCR values. We extended the work to establish the role of the BCR pathway in CLL mitochondrial metabolism. The bioenergetics phenogram showed a relatively low metabolic profile for CLL cells compared with proliferating B-lymphoma cells (Fig. 1A). Moreover, the oxidative profile measured as OCR was significantly higher. For OCR, our data demonstrate a range of 5 to 200 pmol/min/5 \( \times 10^5 \) cells. This range is similar to what was reported in 4 CLL subjects (27). However, in normal B cells, both OCR and ECAR values were lower in their investigations compared with what was observed in the present study. This could be due to highly stressed B cells as reflected in the abnormally low endogenous ATP pool, which is less than 10% of the malignant CLL cells.

Higher number of CLL samples allowed us to compare OCR and ECAR values based on prognostic factors. Rai and Binet are staging systems used for standard classification and prognosis in CLL (6, 18). In our study, increased mitochondrial respiration (OCR) was observed at higher Rai stage (Fig. 1B). Higher metabolic state was also reported with increased Binet stage using an NMR-based metabolic monitoring assay (23). The presence of...
high levels of B2M, another poor prognostic feature (19, 20), also showed a trend for increased OCR (Fig. 1C). In contrast, the cytogenetic abnormalities did not affect OCR values (Fig. 1H). While we do not know why cytogenetic lesions did not associate with increased OCR, we can provide some explanations. For example, the gene p53 or ATM may be fully functional in the second allele. Also, 

in vivo in human body, these features are associated with aggressive/more proliferative tumor. During 

in vitro culture of blood CLL cells, this characteristic is lost, as more than 98% of the cells are in G0–G1 phase and do not replicate. Among patients with CLL, there are two major cohorts, one with an indolent course that does not require treatment and with patients surviving for more than two decades and another with aggressive disease that requires immediate therapeutic intervention. Several prognostic factors and risk stratification have been proposed for therapeutic intervention (19). While genetic lesions such as loss of ATM (11q deletion) and p53 (17p deletion) are associated with adverse prognosis, these anomalies are not common in previously untreated patient populations (28). Recent genome analyses have revealed limited additional markers of poor prognosis that were identified in whole exome and genome sequencing (22, 28, 29).

Figure 4.
Impact of PIK3CD knockout in MCL cell line on glycolysis and OxPhos. A, Graphical representation of glycolysis stress profile of WT and PIK3CD KO JeKo-1 cells. WT (blue curve) and PIK3CD KO (red curve) cells were analyzed for B, glycolytic flux and C, glycolytic capacity. D, Mitochondrial OxPhos in wild-type and PIK3CD KO JeKo-1 cells. Graphical representation of WT (blue curve) and PIK3CD KO (red curve) JeKo cell mitochondrial stress profile. WT and PIK3CD KO cells were analyzed for E, basal OCR and F, spare respiratory capacity. Student paired two-tailed t test was used in the statistical analysis for B, C, E, F, G, WT and PIK3CD KO JeKo-1 clones were plated at a concentration of (2 x 10^5)/mL in RPMI 1640 media supplemented with 10% FBS and 1% penicillin-streptomycin solution on day 1, and the cell numbers were measured on days 2, 3, 4, and 5.
Among the molecular markers that consistently surface in risk stratification analyses is the mutational status of immunoglobulin heavy chain (IGHV; ref. 19). Earlier studies established that unmutated IGHV was associated with more aggressive disease (5), high CD38 expression (30) and correlated with ZAP 70 expression (3, 31). Patients with unmutated IGHV genes had far inferior median survival than patients with mutated IGHV genes (32). Gene expression profiling investigations identified a multitude of genes differentially expressed in these two subsets of CLL (33). Both ZAP 70-positive and IGHV unmutated samples had higher OCR compared with their normal counterparts (Fig. 1D and E). IGHV unmutated samples showed significantly higher OCR, which may reflect on the aggressiveness of this subtype of CLL. Serum metabolome analyses from patients with CLL also suggested differences between these two molecular subgroups (34). ZAP 70 is typically present in normal T cells and is involved with T-cell signaling (35). However, its presence in malignant B cells as well as its prognostic importance has been established for CLL (3). ZAP 70 expression in CLL cells is associated with the BCR signaling pathway (21) and IGHV unmutated status (33). IGHV-unmutated status responded differently to during B-cell activation with a higher preponderance and increase in nucleotide metabolism genes. These markers (ZAP 70 positivity and IGHV unmutated status) correlate with progression-free survival (36). Akin to their role in aggressiveness of the disease, CLL cells from these patients had relatively higher OCR. Because we studied these cells in culture and they were obtained from peripheral blood, this difference was not due to proliferation index, as all cells were quiescent. In summary, OCR values showed wide variability in CLL subjects, and this heterogeneous range correlated with prognostic markers such as Rai stage, β2M expression level, and status of IGHV mutation and ZAP 70.

Figure 5.
Effect of PI3K inhibitors on cellular metabolism in primary CLL lymphocytes. CLL cells were assayed for cell survival 24 hours after drug treatment and equal numbers of untreated or duvelisib-treated CLL cells were plated for the assay. Five technical replicates were used for ECAR and OCR assays. A, Duvelisib-induced changes in glycolytic flux. CLL cells were treated with 1 μmol/L duvelisib for 24 hours (n = 5 patient samples) and assayed for glycolytic flux along with untreated controls. B, Duvelisib-treated patient samples in A were analyzed for glycolytic capacity (n = 5 patient samples). C and D, Duvelisib induced alterations in mitochondrial respiration. C, Basal OCR and D, maximum respiration capacity were compared in untreated CLL cells and cells treated for 24 hours with 1 μmol/L duvelisib (n = 9 patient samples). E, Influence of duvelisib on the intracellular nucleotide pools in CLL lymphocytes. NTP pools were extracted from CLL cells either untreated or treated for 24 hours with duvelisib (n = 7 patient samples) and analyzed using HPLC. F and G, Effect of PI3K inhibitor on CLL glycolysis. Cells either untreated or treated for 24 hours with PI3K inhibitor 1 μmol/L idelalisib (n = 5 patient samples) and were compared for changes in F, glycolytic flux and G, glycolytic capacity. H and I, Effect of PI3K inhibition on CLL OxPhos. CLL cells either untreated or treated for 24 hours with PI3K inhibitor, 1 μmol/L idelalisib, (n = 6 patient samples) were compared for changes in H, basal OCR and I, maximum respiratory capacity. Ctrl, control untreated; IPI, IPI-145 (duvelisib); GS, GS-1101 (idelalisib).
The presence of ZAP 70 and unmutated IGHV is known to enhance cell signaling through the BCR pathway that is pivotal to the maintenance of B cells (33). Abrogating the BCR pathway (via BCR knockout) affected OxPhos, demonstrating that BCR signaling plays an important role in regulating CLL cell metabolism (Fig. 3A and B). Two major players in the BCR pathway are PI3K and BTK. Increased active BTK (phosphorylated BTK) has been reported for unmutated IGHV harboring CLL cohort (37). The PI3K axis is constitutively active in CLL cells that are influenced by the microenvironment. Inhibiting PI3K signaling by PIK3CD KO mitigated both glycolysis and OxPhos of malignant B cells (Fig. 4A–F). KO of both the BCR and PI3K pathway without much effect on cell growth (Figs. 3 and 4) in Jeko cells may be due to presence of cMyC in this cell line. Furthermore, additional cell lines such as LY19 and Mino did not proliferate after genetic KO of PIK3CD, and such manipulations would be challenging in CLL lines such as LY19 and Mino. However, we are extending data from MCL cell lines to the CLL cells; we understand that the biology would be different in these two B-cell malignancies. We are extrapolating MCL cell line data to CLL given the similarity between the two for dependence on the BCR signaling. To further evaluate our results, we used three pharmacological inhibitors in CLL cells. Pharmacological inhibition of PI3Kα by idelisib has shown efficacy in the clinic for patients with B-cell malignancies (38, 39). Duvelisib binds to the ATP-binding site of the kinases, thereby preventing activation of the PI3Kα/β isoforms (40). This antagonist mitigated glycolysis as well as mitochondrial OxPhos (Fig. 5A–D). PI3Kα signals to downstream AKT, which plays a major role in glycolysis by directly regulating glucose uptake (41), which is in line with duvelisib-mediated diminished ECAR in CLL cells. However, glucose uptake in CLL malignant lymphocytes was not significantly affected by the drug (Supplementary Fig. S5A). It can be speculated that glucose utilization by CLL cells is being affected because uptake of glucose did not decrease, and yet glycolysis and OxPhos were significantly downregulated.

Decline in the intracellular ATP pool is consistent with mitigation of OxPhos by the drug (42, 43). Decrease in other NTP pools suggest that overall energy metabolism is affected by inhibiting the PI3K pathway (Fig. 5E). We have not evaluated mechanisms for changes in other intracellular NTP levels. CTP and UTP pools may be affected as pyrimidine nucleotide biosynthesis is linked to mitochondrial electron transport chain by the enzyme dihydroorotate dehydrogenase (DHODH) that resides in the inner mitochondrial membrane, the only enzyme in that pathway that is located in the mitochondria. AKT, downstream effector of PI3K, also regulates purine nucleotide biosynthesis. It reduces ubiquinone to ubiquitol and converts dihydroorotate to orotate (44). Additionally, AKT activity promotes mTOR pathway signaling, a known regulator of pyrimidine and purine synthesis (45).

Not much is known about the PI3K/AKT pathway and its role in mitochondrial respiratory function in CLL cells; however, hyperactivation of AKT leading to increased mitochondrial respiration and thereby ATP synthesis by the E4-BP1–mediated protein translation pathway has been reported (46). Inhibition of this mitochondrial respiration by the above-mentioned target-specific drugs further supports the notion that mitochondrial respiration is regulated by AKT. Collectively, our PI3K inhibition data in malignant CLL lymphocytes and its role in metabolomics are in concert with prior reports with healthy mature B lymphocytes. Survival of B lymphocytes was mitigated by BCR knockout but rescued exclusively by PI3K signaling (47). Suppression of this pathway was pivotal for anergy (48) and anergic or activated B cells required a change in metabolic status (49).

The fact that CLL with poor prognostic factors such as IGHV unmutated status, presence of ZAP 70, and higher Rai stage etc. were associated with increased OCR suggests that for this subgroup, inhibition of oxidative phosphorylation should be tested as a strategy. Inhibitors of OxPhos have been designed and tested in preclinical setting (50) and one is already in the clinic (NCT02882321; ClinicalTrials.Gov).

In conclusion, the key features of our current investigation are as follows. First, metabolic phenograms of quiescent CLL cells suggest an active OxPhos pathway, and this was strongly correlated with the aggressiveness of the disease and adverse prognostic factors such as ZAP 70 positivity, IGHV mutation status, Rai stage, and β2M levels. Finally, OxPhos, glycolysis, and nucleotide biosynthesis were abrogated by PI3K/AKT pathway inhibition.

Disclosure of Potential Conflicts of Interest

W. Wierda reports receiving a commercial research grant from Gilead. V. Gandhi reports receiving a commercial research grant from Infinity Pharmaceuticals and Gilead Sciences. No conflicts of interest were disclosed by the other authors.

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