

Bioenergetic Analysis of Suspension Cells: Hematopoietic Stem Cells and Lymphocytes

A Real-Time Assay that Quantifies the ATP and Biosynthetic Demands of Immune Cell Proliferation, Differentiation, and Effector Function

Application Brief

Introduction

Normal suspension cells, such as hematopoietic cells and lymphocytes, are responsible for supplying oxygen to the body and protecting the host. Cellular metabolism is markedly dynamic in immune responses during proliferation and immune effector functions^{1,2}. For example, antigen-stimulated T cells display a metabolic switch to aerobic glycolysis accompanied by cell growth and proliferation, similar to the Warburg effect observed in cancer cells. Manipulation of the lymphocyte-specific metabolic control pathways may prove useful in treating diseases characterized by immune hyperactivation, including leukemia and autoimmune disorders.

Immobilization of non-adherent cells in Agilent Seahorse XF Cell Culture Microplates enables bioenergetic analysis of lymphocytes and hematopoietic cells with the Agilent Seahorse XF Analyzer. Immobilization can be achieved by using microplates coated with Cell-Tak Adhesive, a formulation of non-immunogenic, polyphenolic proteins extracted from the marine mussel.

Cappasso, *et al.*³ investigated the role of the voltage-gated proton channel HVCN1 in the cellular metabolism of B lymphocyte activation using B cells immobilized with Cell-Tak. Bioenergetic analyses of the B cells were generated by measuring the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of B cells as indications of mitochondrial respiration and glycolysis, respectively. The authors assessed mitochondrial respiration and glycolysis in B cells activated for 24 hours with F(ab')₂ anti-IgM. The B cell receptor (BCR) activated B cells experienced an increase in both mitochondrial respiration and glycolysis when compared to non-stimulated B cells. (Figure 1).

The authors then showed that genetic deletion of HVCN1 significantly impaired the increase in metabolism of activated B cells, but not that of resting B cells. These impairments result from disrupted signal transduction of the BCR complex and downstream signaling of Akt activation. It was determined that after BCR stimulation, cells lacking HVCN1 displayed lower oxygen consumption and acidification than did wild-type cells (Figure 1), which indicated impaired energy production via mitochondrial oxidative phosphorylation and glycolysis in the absence of HVCN1 in the activated B cells.



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Consistent with a defect in cellular metabolism, HVCN1-deficient B cells showed less proliferation *in vitro* after stimulation with F(ab')₂ anti-IgM, and defective antibody response *in vivo*.

Results and Discussion

Immobilization of suspension cells makes it possible to monitor mitochondrial respiration and glycolysis of leukocytes in the Agilent Seahorse XF Analyzer, and to connect cellular energy metabolism to cellular function, for example, immune responses or dysfunctions such as leukemia.

Liu; *et al.*⁴ demonstrated that polycomb repressor Bmi1 has an important role in maintaining mitochondrial function and redox homeostasis of thymocytes and hematopoietic stem cells. The authors analyzed the mitochondrial function of freshly isolated Bmi1^{-/-} murine thymocytes. They showed that intact Bmi1^{-/-} thymocytes had both reduced basal mitochondrial oxygen consumption and reduced mitochondrial oxidative capacity. Consistent with these results, the authors found significant impairment in the function of isolated Bmi1^{-/-} mitochondria including reduced electron flow, increased NAD(P)H levels, and increased MitoSox staining, supporting their hypothesis that mitochondria are the major source of increased ROS level in Bmi1^{-/-} thymocytes.

Abnormal metabolism is a hallmark of many types of cancers including leukemia. In another study, Wu; *et al.*⁵ investigated the effect of the anticancer drug Imatinib, which targets the Bcr-Abl oncogene, on Bcr-abl-expressing K562 leukemia cells. It was found that K562 cells treated with Imatinib for 48 hours reduced mitochondrial respiration and glycolysis, which was accompanied by a decreased cell proliferation rate. These results suggest that Imatinib's anticancer effect is mediated, at least in part, through altering cellular energy metabolism.

Gurumurthy; *et al.*⁶ demonstrated that Lkb1 tumor suppressor is critical for the maintenance of energy homeostasis in haematopoietic cells, independent of AMP-activated protein kinase (AMPK), and mammalian target of rapamycin (mTOR) signaling. It was found that Lkb1-deficient bone marrow cells exhibit mitochondrial defects with markedly reduced mitochondrial respiratory capacity, among the other metabolic defects. These results define a central role for Lkb1 in broadly maintaining energy homeostasis in hematopoietic cells through a novel metabolic checkpoint.

One concern about assaying immune cells such as B and T lymphocytes immobilized on a culture surface, is the potential for cell activation by the crosslinking of the cells during immobilization. This is unlikely for at least two reasons. Firstly, the immobilized cells are analyzed within a very short time period, typically 2 hours, which precedes the surface expression of early activation marker CD26⁷. Secondly, as shown in Figure 1, it is evident that stimulated and unstimulated B cells showed a clear bioenergetic difference suggesting the naive cells are not activated by the immobilization. Poly-D-lysine (another nonspecific attachment factor) treated plates, have also been used successfully to immobilize lymphocytes for XF bioenergetic assay.

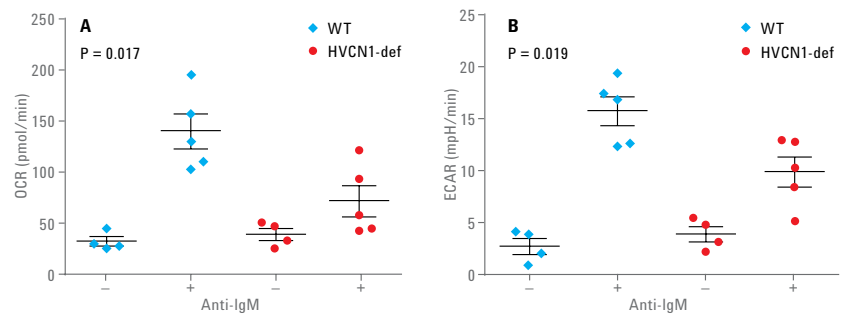


Figure 1. HVCN1 ion channel deficiency in B cells results in impaired cellular metabolism. Metabolic rates in wild-type and HVCN1-deficient B cells before (-) and after (+) stimulation for 24 hours with F(ab')₂ anti-IgM (20 µg/mL), presented as the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR), measures of mitochondrial respiration and glycolysis, respectively. Each symbol represents an individual mouse; longer horizontal lines indicate the mean, and small horizontal lines indicate the standard error. P values, Student's T-test. Data are representative of three experiments with four mice (time 0) or five mice (24 hours).

Materials and Methods

Cells and compounds

Agilent Seahorse XF V7 Cell Culture Microplates were coated with Cell-Tak (BD Bioscience) prior to plating B cells, as described in the Agilent Seahorse protocol.

B cells were purified from the spleens of 8 to 12-week-old mice either by negative selection with anti-CD43 magnetic beads, with a purity of ~95 %, or by positive selection with anti-B220 and anti-CD19 magnetic beads (Miltenyi Biotech), with a purity of ~98 %. Cells were cultured in RPMI complete medium containing 10 % (v/v) FCS, penicillin, and streptomycin, l-Glutamax, and 50 μ M 2-mercaptoethanol. Splenic B cells were stimulated for various times at 37 °C with goat anti-mouse IgM F(ab')₂ fragment (20 μ g/mL). They were stimulated for 24 hours with F(ab')₂

anti-IgM (20 μ g/mL). Stimulated or control B cells were resuspended in bicarbonate-free and low buffered DMEM (pH 7.4) containing 11 mM glucose, 2 mM glutamine and 1 mM pyruvate.

XF Bioenergetic analysis

Bioenergetic analyses of lymphocytes were performed in the Agilent Seahorse XF Analyzer. The Agilent Seahorse XF Analyzer creates a transient micro-chamber of only a few microliters in specialized cell culture microplates. This enables OCR and ECAR to be monitored in real time^{7,8}.

Wild-type and HVCN1-deficient B cells stimulated for 24 hours were plated in a Cell-Tak-coated 24-well Agilent Seahorse XF V7 Cell Culture Microplate at 1×10^6 cells per well in 100 μ L of assay medium. The cells were allowed to become attached for 30 minutes in a 37 °C non-CO₂ incubator. A 500 μ L

amount of assay medium was added to each well after the cells were stably attached to the bottom of the wells, and incubated for one additional hour prior to the XF bioenergetic assay. Low buffered bicarbonate-free DMEM assay medium contains 11 mM glucose, 2 mM glutamine, and 1 mM pyruvate. Four basal OCR and ECAR of the control and stimulated cells were measured, and the average of two basal rates of each sample were plotted (Figure 1).

Immobilization of suspension cells makes it possible to monitor mitochondrial respiration and glycolysis of leukocytes in the Agilent Seahorse XF Analyzer, and to connect cellular energy metabolism to cellular function, for example, immune responses or dysfunction such as leukemia.

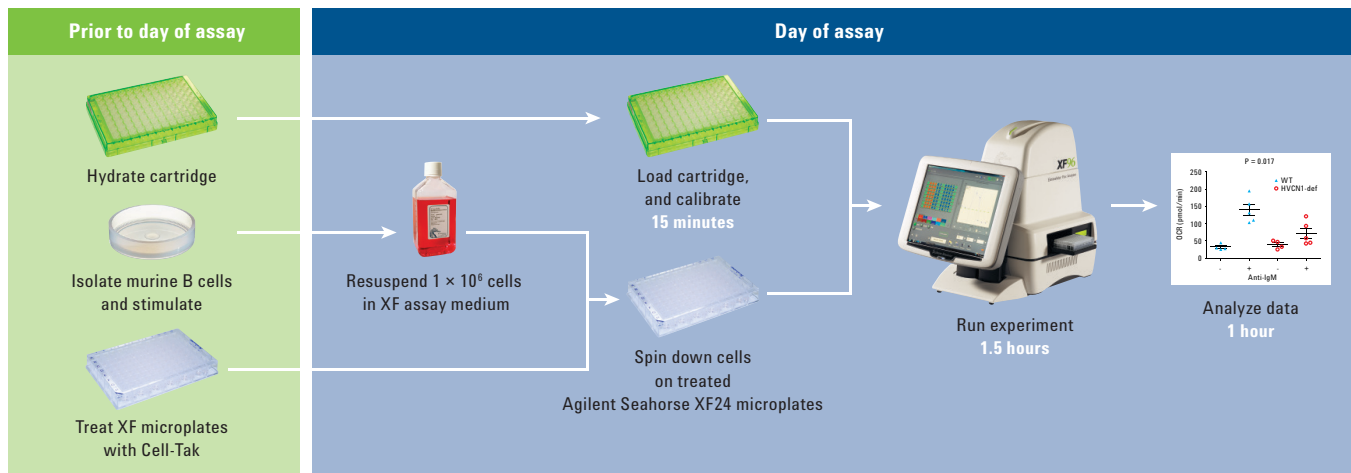


Figure 2. Flow chart of the XF assay.

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