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Analysis of human Natural Killer Cell metabolism

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Dear Editor of the *Journal of Visualized Experiments*, Dr. Bajaj,

Please, find enclosed the revised manuscript by Traba et al., entitled "Analysis of Natural Killer cell metabolism", JoVE61466R1, which I am submitting for publication in the *Journal of Visualized Experiments*.

This manuscript describes a method to purify Natural Killer (NK) cells from human blood and measure their metabolic activity. This protocol has proved valid to evaluated changes in glycolysis and mitochondrial respiration NK cells at rest and upon stimulation with soluble IL-15. The manuscript has been revised addressing the Editor comments.

I hope our manuscript is now acceptable for publication in the *Journal of Visualized Experiments*.

Best regards,

A handwritten signature in black ink, appearing to read "Olga M. Anton", enclosed within a large, loopy oval shape.

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KEYWORDS:

Natural Killer cells, metabolism, extracellular flux, glycolysis, mitochondria, respiration, electron transport chain, flow cytometry

SUMMARY:

In this paper, we describe a method to measure glycolysis and mitochondrial respiration in primary human Natural Killer (NK) cells isolated from peripheral blood, at rest or following IL15-induced activation. The protocol described could be easily extended to primary human NK cells activated by other cytokines or soluble stimuli.

ABSTRACT:

Natural Killer (NK) cells mediate mainly innate anti-tumor and anti-viral immune responses and respond to a variety of cytokines and other stimuli to promote survival, cellular proliferation, production of cytokines such as interferon gamma (IFN γ) and/or cytotoxicity programs. NK cell activation by cytokine stimulation requires a substantial remodeling of metabolic pathways to support their bioenergetic and biosynthetic requirements. There is a large body of evidence that suggests that impaired NK cell metabolism is associated with a number of chronic diseases including obesity and cancer, which highlights the clinical importance of the availability of a method to determine NK cell metabolism. Here we describe the use of an extracellular flux analyzer, a platform that allows real-time measurements of glycolysis and mitochondrial oxygen consumption, as a tool to monitor changes in the energy metabolism of human NK cells. The method described here also allows for the monitoring of metabolic changes after stimulation of NK cells with cytokines such as IL-15, a system that is currently being investigated in a wide range of clinical trials.

INTRODUCTION:

Natural Killer (NK) cells are innate lymphocytes that mediate anti-tumor and anti-viral responses.

NK cells comprise 5-15% of all lymphocytes in human peripheral blood, and can be also found in spleen, liver, bone marrow and lymph nodes. NK cells do not express polymorphic clonotypic receptors, such as T-cell receptors (TCR) or B-cell receptors (BCR). In contrast, the activation of their cytolytic functions is prompted by the engagement of receptors that recognize invariable ligands on the surface of a target cell^{1,2}.

Resting human NK cells isolated from peripheral blood can survive for several days in culture medium supplemented with human serum. Activation of NK cells by cytokines such as IL-15 or IL-2 drives the cells to proliferation and to an increase of their killing ability, amongst other effects³⁻⁵. Several studies have shown a direct correlation between NK cell activation and changes in their metabolic activity⁶. These metabolic changes are destined to meet the particular requirements of the cells in terms of energy and biosynthesis.

Aerobic cells and organisms obtain energy through a series of chemical reactions that involve the catabolism and oxidation of carbohydrates, fat and proteins. Through a combination of glycolysis, the tricarboxylic acid (TCA) cycle and oxidative phosphorylation, eukaryotic cells meet the majority of their ATP demand and obtain intermediates required as building blocks for macromolecules essential for cell growth and proliferation. The process of glycolysis (**Figure 1A**) starts with the entry of glucose in the cell. In the cytosol, glucose is phosphorylated and transformed into pyruvate (with a net production of 2 molecules of ATP per glucose molecule), which can be reduced to lactate or transported into the mitochondria to be transformed into Acetyl-CoA and enter the TCA cycle. The TCA cycle continues cycling fueled with more molecules of Acetyl-CoA and produces CO₂ (that eventually will diffuse outside the cell and, by reacting with H₂O in the medium, will generate carbonic acid that will lead to the acidification of the medium) and NADH, the molecule in charge of donating electrons to the electron transport chain (ETC). The electrons travel through different protein complexes and are finally accepted by oxygen. These complexes (I, III and IV) also pump H⁺ from the mitochondrial matrix into the intermembrane space. As a consequence of the electrochemical gradient generated, the H⁺ will enter again to the matrix through the complex V (ATP-synthase), investing the potential energy accumulated into the generation of ATP.

Both glycolysis and mitochondrial respiration can be blocked at different points by using inhibitors. The knowledge and usage of these inhibitors was the basis for the development of the extracellular flux assay. By measuring two simple parameters in real time such as pH and oxygen, the extracellular flux analyzer infers the rate of glycolysis and mitochondrial respiration in a 96-well plate. The glycolysis stress test is performed in a basal medium without glucose (**Figure 1B**)⁷. The first measurements of the extracellular acidification rate (ECAR) are indicative of glycolysis-independent acidification. It is referred to as non-glycolytic acidification and correlates with CO₂ produced by the TCA that, as explained before, combines with H₂O in the medium to generate H⁺ (TCA-linked ECAR). The first injection is glucose to induce glucose utilization and boost glycolysis. The second injection combines both rotenone, a Complex I inhibitor, and antimycin A, a Complex III inhibitor together, to block the ETC. Cells respond to this dramatic decrease in mitochondrial ATP production by activating glycolysis to maintain cellular ATP levels, and this represents the amount of glycolysis that is not used by cell in the basal state but could be

potentially recruited in response to increases in ATP demand (compensatory glycolysis). The third injection is the glucose analog 2-DG, which competes with glucose as a substrate for the enzyme hexokinase. The product of this phosphorylation, 2-deoxyglucose-6-phosphate cannot be transformed into pyruvate, and therefore glycolysis is blocked, which lowers the ECAR to its minimum. The ECAR measured at this point includes other sources of extracellular acidification that are not attributed to glycolysis or respiratory activity as well as any residual glycolysis not fully inhibited by 2-DG (post 2-DG-acidification).

The mitochondrial stress test is performed in a medium with glucose (**Figure 1C**)⁸. The first measurements of the oxygen consumption rate (OCR) correspond to the base line of mitochondrial respiration (basal respiration). The first injection is oligomycin, which inhibits the return of protons through the ATP synthase (complex V), blocking ATP synthesis and thus rapidly hyperpolarize the mitochondrial membrane, which prevents further proton pumping through respiratory complexes, and leads to a decrease in OCR. The comparison between the baseline respiration and the value given by addition of oligomycin represents the ATP-linked respiration. The remaining oligomycin-insensitive rate of oxygen consumption is called proton leak, which represents the flow of protons through the lipid bilayer or proteins in the inner mitochondrial membrane such as the adenine nucleotide translocase⁹. The second injection is the uncoupler 2,4-dinitrophenol (DNP), an ionophore that induces a massive entry of H⁺ into the mitochondrial matrix, which leads to depolarization of the mitochondrial membrane and disruption of mitochondrial ATP synthesis. Cells respond to the dissipation of the proton-motive force by increasing the rate of electron transport and oxygen consumption to maximum levels in a futile attempt to recover membrane potential (maximal respiratory capacity). The difference between the maximal respiratory capacity and the basal respiration is the spare respiratory capacity of cell, which represents the amount of respiration that is not used by the cell to generate ATP in the basal state but could be potentially recruited in response to increases in ATP demand or under conditions of stress⁸. The third injection is a combination of rotenone and antimycin A. This injection completely stops the ETC and OCR decreases to its lowest level, with the remaining oxygen consumption being non-mitochondrial (caused by NADPH-oxidases, etc.).

Changes in metabolic pathways could somehow predict the functioning of NK cells, as it has been suggested that continuous activation of NK cells with cytokines in vitro could lead to NK cell exhaustion by the study of different metabolic pathways^{10,11}. The correlation between NK cell metabolic status and function is very important from the point of view of cancer immunotherapy. In this field, activation of NK cells with infusion of IL-15, alone or in combination with monoclonal therapeutic antibodies have been tested in order to improve tumor cell killing¹²⁻¹⁴. The knowledge of the metabolic status of the NK cells in response to these treatment strategies would provide a valuable predictor of NK cell activation status and killing function.

The study of metabolic pathways in other myeloid and lymphoid cells such as monocytes, T and B cells has been described¹⁵ and optimized methods have been published¹⁶. In this protocol we provide a method that combines both an NK isolation protocol that yields high numbers of pure and viable NK cells and an optimized protocol to measure metabolic activity using an extracellular flux analyzer. Here we show that this is a valid method for the study of metabolic changes in

resting and IL-15 activated human NK cells. For the extracellular flux assay, parameters such as cell number and drug concentrations have been tested and optimized. Compared with other respirometric methods, the extracellular flux analyzer is fully automated and able to test in real time, with very low quantities of cells, up to 92 samples simultaneously, and thus allows high throughput screenings (with multiple samples and replicates) in a relatively quick manner¹⁷.

This method can be used by researchers interested in assessing NK cell function by studying NK cell metabolism. It could be applied as well to cells activated by other cytokines, antibodies or soluble stimuli.

PROTOCOL:

All the experiments were performed in accordance with the Declaration of Helsinki's ethical principles of medical research. Peripheral blood samples from donors were obtained from the NIH Department of Transfusion Medicine under the 99-CC-0168 IRB approved protocol, with patient written informed consent.

1. Reagent preparation

1.1. Reagents for the isolation of NK cells

NOTES: Prepare these reagents in a cell culture hood.

1.1.1. Prepare NK separation buffer: Supplement PBS (pH 7.4) with 1 mM EDTA and 2% Fetal Calf Serum (FCS) that has previously been heat-inactivated (at 56 °C for 30 min).

1.1.2. Prepare NK culture medium: Supplement Iscove's Modified Dulbecco's Media (IMDM) with 10% Human Serum (HS). Sterile filter and store at 2-8 °C. Warm the medium to 37 °C before adding to the cells.

1.1.3. Resuspend Human IL-15 in PBS at 200 µg/mL.

1.2. Reagents for extracellular flux assay

1.2.1. Prepare assay media: For mitochondrial stress test medium, add 1 mM sodium pyruvate, 2 mM L-glutamine and 10 mM glucose to the base medium; for glycolysis stress test medium, add 2 mM L-glutamine to the base medium.

NOTE: The concentrations of glucose, pyruvate and glutamine are provided as recommended by the extracellular flux analyzer manufacturer. However, media composition can be altered by researchers to perfectly match that of the culture medium, if desired.

1.2.2. Adjust the pH of both media to 7.4 with 0.1 N NaOH using a benchtop pH meter, sterile filter through a 0.2 µm pore size and store at 2-8 °C. Warm media to 37 °C, check pH and readjust to 7.4 before use if required.

NOTE: Only ambient CO₂ is contained in the atmosphere of the extracellular flux analyzer, the use of media without bicarbonate is critical.

1.2.3. Prepare stock solutions for reagents: Oligomycin (ATP synthase inhibitor), 10 mM stock solution in DMSO; 2,4-dinitrophenol (DNP, uncoupler), 1 M stock solution in DMSO; antimycin A (complex III inhibitor), 10 mM stock solution in DMSO; rotenone (complex I inhibitor), 10 mM stock solution in DMSO. Make 30 µL aliquots of all reagents and store at -20 °C.

NOTE: These guidelines are intended for reagents purchased individually and prepared by the researcher. If reagents are purchased from the analyzer manufacturer instead, follow their guidelines for reagent preparation.

2. NK cells isolation from peripheral blood

2.1. Peripheral blood mononuclear cells (PBMCs) preparation from human blood

NOTE: Perform these steps in a cell culture hood. Decontaminate all residues and material in contact with blood with bleach and discard them into the appropriate container to be incinerated.

2.1.1 Pipette 20 mL of Lymphocyte Separation Medium (LSM) into a 50 mL conical tube.

2.1.2 Carefully, while keeping the tube at a 30° angle, pipette 20 mL of blood over the LSM, very gently and touching the wall of the tube. Avoid the mixing of blood with the LSM and create a visible and well-defined interphase between the two fluids.

NOTE: Peripheral blood or enriched leukapheresis products can be used.

2.1.3 Centrifuge the tubes for 25 min, 1000 x g at room temperature. Do not use brake, as it could make both phases in the tube (LSM and blood) mix.

2.1.4 Carefully take out the tubes from the centrifuge and place them in a rack. Check for the presence of a conspicuous layer of cells (mononuclear cells) that will form at the interphase between LSM (clear) and plasma (yellow), while red blood cells pellet at the bottom of the tube.

2.1.5 Gently aspirate the mononuclear cell layer with a 10 mL plastic pipette (around 5-8 mL) and place it in a new 50 mL conical tube. The lymphocyte interphase of up to 2 different tubes can be pooled together.

2.1.6 Wash the mononuclear cells 2x by resuspending in 45 mL PBS and centrifuging at 800 x g for 5 min, at room temperature.

NOTE: After this step, a pellet of peripheral blood mononuclear cells (PBMCs) is obtained and the

researcher can proceed to the NK isolation step.

2.2. NK isolation from PBMCs

2.2.1. Count the cells from 2.1.6 and resuspend them in NK Separation Buffer (1×10^8 PBMCs/mL).

2.2.2. Take 10 mL of the cell resuspension (10^9 PBMCs) and place them into a 50 mL tube.

2.2.3. Add 500 μ L (50 μ L/mL of buffer) of NK cell isolation antibody mix and 10 μ L (1 μ L/ mL of buffer) of anti-CD3 positive isolation antibody mix to the PBMCs and incubate at room temperature for 10 min.

2.2.4. Vortex the magnetic beads and add 1 mL to the mix of PBMCs with antibodies (100 μ L beads/ml PBMCs). Incubate for 10 min at room temperature with occasionally stirring.

2.2.5. Add 35 mL of NK isolation buffer (3.5 ml buffer/ml PBMCs), mix and place on the magnet for 15 min (2.2×10^7 PBMCs/mL). After that time, the beads and cells positively selected (all but NK cells) will have adhered to the walls of the tube.

2.2.6. Carefully collect the supernatant (containing NK cells) with a 50 mL plastic pipette without touching the sides or the bottom of the tube.

2.2.7. Count the cells using a cell counter and centrifuge at $800 \times g$ for 5 min.

2.2.8. Resuspend isolated NK cells at 5×10^6 cells/mL in IMDM containing 10% HS and place them in an incubator at 37°C with 5% CO_2 until the experiment is performed.

NOTE: This NK isolation protocol states cell numbers and reagent volumes adapted for the use of a 50 mL tube and a big magnet. This protocol can be scaled up (in case more PBMCs are obtained) by repeating the isolation steps in different tubes or scaled down by reducing the volume in the tube. For final volumes of 14-45 mL a 50 mL polystyrene tube is used, for volumes 4-14, a 15 mL polystyrene tube is used and for volumes 1-4 mL, a 5 mL polystyrene tube is used. The magnet is different and fit the appropriate tube in each case. The amount of antibody mix and magnetics beads can also be scaled according to the number of cells and the final volume.

2.3. NK cell population staining for flow cytometry

2.3.1. Take 0.25×10^6 cells per sample from step 2.2.8, remove the medium by centrifuging at $800 \times g$ for 5 min at room temperature and resuspend the cell pellet in 500 μ L of PBS.

2.3.2. Add viability dye according to the manufacturer's recommendation (1 μ L of DMSO-reconstituted dye to 1 mL of cell resuspension) and incubate at room temperature for 30 min.

2.3.3. Wash 2x by resuspending in 5 mL PBS and centrifuging at $800 \times g$ for 5 min, at room

temperature.

2.3.4. Stain with the corresponding anti-human antibodies in 100 μ L of IMDM containing 10% HS for 30 min on ice protected from light (see **Table 1**). All the antibodies can be combined and used in a single staining step.

2.3.5. Analyze the samples by flow cytometry to evaluate the purity of the NK cell population obtained. Use the cell gating and analysis method that have been previously described^{18,19}.

2.4. NK cells stimulation with soluble IL-15

2.4.1. Resuspend 0.75×10^6 cells from step 2.2.8 in 100 μ L of IMDM containing 10% HS in a well of a 96 well-plate (round bottom).

2.4.2 Dilute human IL-15 to 1 μ g/mL in IMDM containing 10% HS. Add 100 μ L of the diluted human IL-15 to the cells to reach a final concentration of 0.5 μ g/mL.

NOTE: 0.5 μ g/mL is a saturating concentration of IL-15. Lower concentrations of IL-15 or other cytokines such as IL-2, IL-12 or IL-18 may be tested by researchers if desired.

2.4.3 Place the cells in the incubator at 37 °C and stimulate for 48 h before performing the extracellular flux assay. Resuspend unstimulated (control) cells at the same concentration and volume in IMDM containing 10% HS without IL-15, and place them in the same incubator for 48 h.

3. Hydration of sensor cartridge

NOTE: The 96 probe tips of the sensor cartridge contain individual solid-state fluorophores for O₂ and H⁺ that need to be hydrated in order to detect O₂ and pH changes.

3.1. Turn on the analyzer and let it warm up to 37 °C.

3.2. Open the sensor cartridge package and separate the sensor cartridge from the utility plate. Add 200 μ L of the calibrant solution in each well of the utility plate and put back the sensor cartridge onto the plate, validating that the sensors are completely submerged in the solution. For optimum results incubate the cartridge overnight at 37 °C in a CO₂-free incubator that is properly humidified to prevent evaporation. Prevent bubble formation under the sensors during hydration.

NOTE: The minimum cartridge hydration time is 4 h at 37 °C in a CO₂-free incubator. Alternatively, overnight hydration of the sensor cartridge with 200 μ L of sterile water at 37 °C in a CO₂-free incubator, followed by an incubation of the sensor cartridge with 200 μ L of pre-warmed calibrant solution 45 – 60 min prior to the start of the run, can be used.

4. Extracellular flux assay

4.1. Preparation of adhesive-coated plates

NOTE: Since the measurement of metabolic parameters takes place in a microchamber formed at the bottom of the 96-well assay plate, suspension cells must first be adhered to the bottom of the well. A cell adhesive extracted from the mussel *Mytilus edulis* is employed. The manufacturer of the cell adhesive recommends a coating concentration of 1 to 5 $\mu\text{g}/\text{cm}^2$. The well of the analyzer cell culture microplate has a surface of approximately 0.110 cm^2 . Thus, for a 5 $\mu\text{g}/\text{cm}^2$ concentration, around 0.55 μg of adhesive are required. As 25 μL of the adhesive solution will be used to coat each well, the optimal adhesive solution concentration for the analyzer cell culture microplates is around 22.4 $\mu\text{g}/\text{mL}$ ($22.4 \mu\text{g}/\text{mL} \times 0.025 \text{ mL} = 0.56 \mu\text{g}$).

4.1.1. Prepare 2.5 mL of cell adhesive solution (22.4 $\mu\text{g}/\text{mL}$) in 0.1 M sodium bicarbonate, pH 8.0. Bicarbonate provides the optimum pH for cell adherent performance which, according to the manufacturer, is between 6.5 and 8.0.

4.1.2. Pipette 25 μL of the cell adhesive solution to each well of the assay plate and incubate at room temperature for 20 min. After that, remove the solution and wash 2x with 200 μL of sterile water/well. Let the wells dry by keeping the plate open for 15 minutes inside a cell culture hood.

NOTE: Coated plates may be stored for up to 1 week at 4 $^{\circ}\text{C}$.

4.2. Cell seeding in plates coated with adhesive

4.2.1. Centrifuge cells from step 2.2.8 at 200 x g for 5 min at room temperature. Remove supernatants and wash cells in warmed mitochondrial stress test medium (if a mitochondrial stress test is being performed) or glycolysis stress test medium (if a glycolysis stress test is being performed). Pellet cells again and resuspend to the preferred cell concentration in the same medium (resuspension volume will depend on the cell concentration chosen; since each well will contain 180 μL of the cell suspension, prepare 0.26×10^6 , 0.52×10^6 , 1.04×10^6 , 2.08×10^6 , 4.17×10^6 and 8.33×10^6 cells/mL cell suspensions for 0.047×10^6 , 0.094×10^6 , 0.187×10^6 , 0.375×10^6 , 0.75×10^6 and 1.5×10^6 cells per well respectively).

4.2.2. Plate 180 μL of cell suspension per well along the side of each well. A multichannel pipette is recommended. Use wells A1, A12, H1, and H12 of the analyzer culture plate as control wells for background correction. Add 180 μL of the assay medium in these wells (no cells). Additional control wells can be used if desired and if there is enough space in the plate.

NOTE: The presence of serum may cause poor cell attachment.

4.2.3. Incubate the plate for 30 min at 37 $^{\circ}\text{C}$ in a CO_2 -free incubator. Prepare 10x compounds in the meantime (see step 4.3 below).

4.2.4. Change the centrifuge settings to zero braking. Centrifuge the plate at 200 x g for 5 min. Observe the cells under the microscope to check that they form a monolayer at the bottom of the well. Transfer the plates back to the CO₂-free incubator for 25 min. For best results, total time after plating should be no greater than around 1 h.

4.3 Preparation of 10x working solutions to load into sensor cartridge

NOTE: Each of the 96 probe tips of the sensor cartridge harbors 4 ports (A, B, C and D) that can be used to inject compounds sequentially into individual wells.

4.3.1. To perform mitochondrial stress test, prepare 2.5 mL each of 10 µM oligomycin, 1 mM DNP, and a mixture of 10 µM rotenone and 10 µM antimycin A, in mitochondrial stress assay medium (use the stock solutions from step 1.2.3). Final concentrations in the well after injection will be 1 µM oligomycin, 0.1 mM DNP and 1 µM antimycin A/rotenone.

4.3.2. To perform glycolysis stress test, prepare 2.5 mL of a mixture of 10 µM rotenone and 10 µM antimycin A in glycolysis stress assay medium (use the stock solutions from step 1.2.3). Dissolve glucose in glycolysis stress test medium for a 100 mM solution and 2-deoxy-glucose (2-DG) in glycolysis stress test medium for a 500 mM solution. Final concentrations in the well after injection will be 10 mM glucose, 1 µM antimycin A/rotenone and 50 mM 2-DG.

4.3.3. Warm solutions to 37 °C, check pH and readjust to 7.4 if required. Load compounds prepared in step 4.3.1. (for a mitochondrial stress test) or 4.3.2 (for a glycolysis stress test) into ports A, B and C of the hydrated sensor cartridge (from step 3.2) using a multichannel micropipettor and the port-loading guides provided with the cartridge, as shown in Table 2.

NOTE: To ensure proper injection in all wells during the assay, each series of ports that are used (e.g., all ports A) must contain the same injection volume across the entire sensor cartridge. This applies to background correction wells and even to those wells not used in the experiment.

4.3.4. Incubate the loaded sensor cartridge at 37 °C in a CO₂-free incubator while setting up the program.

4.4. Setting up extracellular flux assay protocols

4.4.1. Open the extracellular flux analyzer software, and using the **Group Definitions** and **Plate Map** tabs indicate groups of wells that have similar conditions (e.g., wells with the same number of cells, or wells with either resting cells or IL-15-stimulated cells). Also, indicate background correction wells (by default A1, A12, H1, and H12 will be set, but additional wells can be used) and empty wells.

4.4.2. Set up the program described in Table 3 in the software using the **Protocol** tab.

4.4.3. Begin the program using the **Run Assay** tab. Place the sensor cartridge (hydrated and

loaded with 10x compounds) and utility plate onto the tray. After the calibration step (15 – 20 min), when prompted, replace the calibrant plate for the assay plate (without lid) with attached cells. After this, the run is fully automated (the machine will perform all measurements and injections).

NOTE: It is possible to perform a mitochondrial stress test and a glycolytic stress test in the same plate, as long as the specific compounds are loaded into the proper ports (oligomycin, DNP and antimycin/rotenone in ports A, B and C respectively of the wells where a mitochondrial stress test is performed; glucose, antimycin/rotenone and 2-DG in ports A, B and C respectively of the wells where a glycolysis stress test is performed), the same volumes for injections are used in each series of ports and wells for each test are properly identified using the **Group Definitions** and **Plate Map** tabs of the software.

4.4.4. After the completion of the run, retrieve the data and analyze them using the software.

5. Cell number determination

NOTE: Results can be normalized to account for possible differences in cell number. Two major approaches described below can be used.

5.1. DNA content determination

5.1.1. Remove the remaining assay medium with a pipette from each well. When aspirating medium, take care not to disturb the cells. The plate can be stored at -20 °C until analysis.

NOTE: The freezing step is important for the efficient cell lysis and DNA content determination.

5.1.2. Prepare a 1x solution of cell proliferation assay cell-lysis buffer (20x) in distilled water (200 µL/well).

5.1.3. Add the cell proliferation assay dye stock solution (400x) into the 1x cell-lysis buffer. For the detection of 50 to 50.000 cells per well, use 1x cell proliferation assay dye. For higher cell numbers, using the cell proliferation assay dye at a final concentration higher than 1x is recommended. In this case, use the cell proliferation assay dye at a 5x final concentration (dilute the cell proliferation assay stock solution 80-fold into 1x cell-lysis buffer).

5.1.4. Add 200 µL of the cell proliferation assay reagent to each well. Incubate the samples for 2–5 min at room temperature. Protect from light.

5.1.5. Measure the fluorescence of the samples at excitation: 480 nm; emission: 520 nm using a fluorescence microplate reader according to manufacturer's recommendations.

5.2. Protein content determination

5.2.1. Carefully, aspirate completely the assay medium from each well without touching the cells and freeze the cells at -20 °C for at least 1 h. Alternatively, the cells can be kept frozen for longer times (up to 1 week) until the analysis is performed.

5.2.2. Add 50 µL of radioimmunoprecipitation assay (RIPA) lysis medium supplemented with 1x protease inhibitors (from a 100x stock solution) to each well. Place the plate on a shaker for 5 min at room temperature, and then incubate the plate on ice for 30 min for a complete cell lysis.

5.2.3. Centrifuge the plate for 5 min at 200 x g at room temperature. This step will pellet cellular debris to prevent interference with the protein measurement.

5.2.4. Measure protein concentration by bicinchoninic acid (BCA) assay according to manufacturer's recommendations.

REPRESENTATIVE RESULTS:

Isolation of NK cells from peripheral blood provides a pure and viable population

The extracellular flux assay is based on the measurement of H⁺ and O₂ concentration in the well and will not distinguish among different populations of cells or their viability. For this reason, obtaining a highly pure and viable population of the cell of interest was the key step to succeed in these experiments.

The isolation of NK cells from peripheral blood was performed as stated in section 2. In order to assess the purity and viability of the NK cells obtained, small aliquots from PMBCs and the isolated NK cell population were stained and analyzed by flow cytometry (**Figure 2A**). Mononuclear cells were gated in the plot showing forward (FSC-A) versus side scatter area (SSC-A). Within this population, single cells were gated along the diagonal in the plot showing FSC-A versus forward scatter height (FSC-H). Within the singlet populations, viability was assessed, and found to be higher than 98% in both, PMBCs and NK cell populations. The purity of the NK cell population isolated was established by double staining against CD3 (present in T cells, the dominant population among PMBCs) and CD56 or NKp46 (**Figure 2B**). NK cells are defined as the population negative for CD3 and positive for CD56 or NKp46. According to these criteria, the purity of the NK cells was around 88%, which represents an 18-fold enrichment on NK cells compared to those present in the PMBCs population.

OCR and ECAR values are dependent on cell number

For the mitochondrial stress test, cells were metabolically perturbed by the addition of three different compounds: oligomycin, DNP and antimycin A + rotenone. For every cell type, the number of cells per well were carefully optimized for an extracellular flux assay experiment. **Figure 3A** shows representative plots of mitochondrial oxygen consumption rate (OCR) using several human NK cell numbers (0.75×10^6 , 0.375×10^6 , 0.187×10^6 , 0.094×10^6 and 0.047×10^6). All measurements were done in triplicate. Cell numbers correlate linearly with the amount of DNA or protein in the well (**Figure 3B**). As expected, high cell numbers displayed higher OCR values, whereas fewer than 0.187×10^6 cells per well did not provide robust results. On the other

hand, higher cell numbers (1.5×10^6) were not optimal, as upon addition of DNP, the oxygen concentration in the well was totally depleted in each cycle (**Figure 3C**), which precludes the accurate calculation of the OCR. The concentration of uncoupler must be titrated carefully for each cell type, as not adding sufficient DNP results in submaximal OCR, whereas adding too much may inhibit maximal OCR as well. In human NK cells, 100 μ M DNP was found to be the optimal dose (**Figure 3D**). Other uncouplers such as carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP) or carbonyl cyanide m-chlorophenyl hydrazine (CCCP) may be used instead of DNP but would need to be titrated for each cell type as well.

For the glycolysis stress test, after a baseline measurement of the ECAR, glucose-starved cells were perturbed by the following compounds: glucose, antimycin A + rotenone and 2-DG. **Figure 3E** shows representative plots of ECAR using several NK cells numbers (0.75×10^6 , 0.375×10^6 , 0.187×10^6 , 0.094×10^6 and 0.047×10^6). All measurements were done in triplicate. The glycolysis stress assay was most successful at the highest plating density, whereas fewer than 0.187×10^6 cells per well did not provide robust results.

IL-15 treatment increases OCR and ECAR basal and maximal values

The optimal seeding density of 0.75×10^6 cells per well was used for subsequent experiments. NK cells were cultured in the presence or absence of saturating concentrations of the cytokine IL-15 for 48 h, after which time their viability was found to be $93.7 \pm 4.8\%$ and $85.7 \pm 12.0\%$, respectively. **Figure 4A,B** shows a typical mitochondrial stress test experiment with 0.75×10^6 NK cells per well. In this test, oligomycin leads to a dramatic decrease in oxygen consumption (**Figure 4A**) and to an increase in ECAR that represents a switch to glycolysis to try to maintain cellular ATP levels (**Figure 4B**). Activation of the NK cell by IL-15 caused an increase in both mitochondrial oxygen consumption and extracellular acidification. This result was consistent when several human subjects were compared (**Figure 4C**). Basal, maximal and ATP-linked respiration, but not proton leak or non-mitochondrial respiration, increased with IL-15 (**Figure 4C,D**). Also, the OCR/ECAR rate decreased, indicating a trend to shift to a glycolytic metabolism after IL-15 stimulation (**Figure 4E**).

Figure 4F,G shows a typical glycolysis stress test experiment with 0.75×10^6 NK cells per well. Addition of glucose triggered a huge increase in ECAR due to glycolysis activation, while subsequent addition of antimycin A and rotenone drove compensatory glycolysis, and 2-DG caused an inhibition of the pathway and a decrease of ECAR to the minimum (**Figure 4F**). In parallel, glucose consistently caused a slight decrease in oxygen consumption, possibly by the Crabtree effect²⁰, while antimycin A and rotenone fully blocked the respiration and 2-DG does not provide any further effects (**Figure 4G**). Activation of both extracellular acidification and mitochondrial oxygen consumption with IL-15 could be observed as well in this test, and again this is consistent when using cells from several donors (**Figure 4H**).

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic of Extracellular flux assays. (A) Schematic of glycolysis, the tricarboxylic acid cycle (TCA) and the electron transport chain (ETC). Inhibitors of different steps are written in red.

The electron flux in the ETC is represented in green with the NADH as donor and the O₂ as final acceptor. (B) Glycolysis stress test schematic profile representing the extracellular acidification rate (ECAR) versus time. (C) Mitochondrial stress test schematic profile representing the oxygen consumption rate (OCR) versus time.

Figure 2: Natural Killer (NK) cells purified from peripheral mononuclear cells (PBMCs). (A) Gating strategy followed to assess purity and viability of NK cells (right column) versus the total of PBMCs (left column). From the gate of mononuclear cells (top panels), single cells were selected (middle panels) and viability was measured (bottom panels). (B) Live cells were stained for CD3 and CD56 or NKp46. The numbers in the panels indicate the percentage of cells in the selected regions. The results are representative of three independent experiments.

Figure 3: Optimization of the mitochondrial and glucose stress tests in activated human NK cells. (A) Mitochondrial Stress test: OCR for each plating density (0.75×10^6 , 0.375×10^6 , 0.187×10^6 , 0.094×10^6 and 0.047×10^6) is shown. Each data point represents the mean of 3 wells with standard deviation. Results are representative of 3 independent experiments. (B) DNA (upper panel) and protein (lower panel) levels in the well at different plating densities. Results are representative of at least three independent experiments. Each data point represents the mean of 3 wells with standard deviation. Results are representative of 3 independent experiments. (C) Raw oxygen level traces in wells containing no cells (black trace), 0.75×10^6 cells (blue trace) or 1.5×10^6 cells (green trace). In the green trace after addition of DNP, oxygen is completely depleted in every cycle. (D) The spare respiratory capacity of 0.75×10^6 cells was tested in the presence of several DNP concentration. (E) Glycolysis Stress Test: ECAR for each plating density (0.75×10^6 , 0.375×10^6 , 0.187×10^6 , 0.094×10^6 and 0.047×10^6) is shown. Each data point represents the mean of 3 wells with standard deviation. Results are representative of 3 independent experiments.

Figure 4: Detection of metabolic changes after NK cell activation by IL-15. Mitochondrial Stress test: OCR (A) and ECAR (B) plots are shown for 0.75×10^6 resting or IL-15 activated cells. Each data point represents the mean of 3 wells with standard deviation. Results are representative of 5 independent experiments. Basal and Maximal Respiration changes for individual human donor are shown in (C), while ATP-linked respiration, proton leak, non-mitochondrial respiration (NMR) are shown in (D) and the OCR/ECAR ratio is shown in (E). Glycolysis Stress test: ECAR (F) and OCR (G) plots are shown for 0.75×10^6 resting or IL-15 activated cells. Each data point represents the mean of 3 wells with standard deviation. Results are representative of 5 independent experiments. Basal and Maximal extracellular acidification changes for individual human donor are shown in (H). ns: not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure 5: Contribution of the TCA and glycolysis on ECAR. The first measurements of ECAR correspond largely to acidification due to the CO₂ produced in the TCA cycle. After addition of glucose to fuel glycolysis the contribution of TCA-derived acidification to ECAR decreases. Injection of Antimycin A and Rotenone blocks the TCA, and glycolysis compensates the increase in ATP demand by increasing to its maximal level (compensatory glycolysis). Blockade of glycolysis by 2-DG diminishes ECAR to minimal levels, corresponding to acidification that is not attributed

to glycolysis or respiratory activity as well as any residual glycolysis not fully inhibited by 2-DG (post 2-DG-acidification).

Table 1: Reagents and antibodies used for flow cytometry

Table 2: Compound loading

Table 3: Program layout

DISCUSSION:

In this paper, we have established a protocol for efficiently isolating and culturing pure and viable primary human NK cells from peripheral blood. We have also optimized the conditions for the measurement of the metabolic activity of these NK cells assessed by oxygen consumption rate and extracellular acidification rate by using an extracellular flux analyzer. Compared to other respirometric methods, the extracellular flux analyzer is fast, requires small numbers of cell, and allows high throughput screenings. However, its reagents are expensive, and injections of compounds are limited to just four. Metabolic remodeling and activation of glycolysis and oxidative phosphorylation by cytokines in NK cell is essential for robust NK cell responses²¹, and the techniques describe here allow the study of the metabolic profile of NK cells in real time. This protocol could be extended to cells activated by other cytokines, such as IL-2, IL-12 and IL-18, or antibodies that bind to activating receptors. We have addressed several key steps that are often overlooked, such as plating the cells at the optimal confluence or using the optimal concentration of uncoupler to stimulate maximal oxygen consumption. Nevertheless, we recommend checking the actual O₂ level curves (**Figure 3C**), if other stimuli different to IL-15 are given to the cells, to make sure that O₂ is not being depleted in the wells. If that was the case, the cell number should be decreased until a linear O₂ consumption is observed to prevent an underestimation of the real OCR.

Basal respiration reflects the basal metabolic state of the cell, which is largely controlled by the activity of the mitochondrial ATP synthase⁸, and is an indication of the basal ATP demand (for protein synthesis, cytoskeleton dynamics, ATPases such as the Na⁺/K⁺-ATPase, etc). Typically, in the presence of sufficient substrates this parameter increases in metabolically active or stressed cells. Addition of oligomycin, which blocks the ATP synthase, reveals ATP-linked respiration and the proton leak, which can happen across the lipid bilayer or proteins of the inner mitochondrial membrane, but can also be inducible through proteins such as the Uncoupling Proteins (UCPs)⁹, implicated in the regulation of adaptive thermogenesis and thus converting mitochondrial energy potential to heat in brown adipocytes. Maximal respiration is determined by factors including the availability of substrates for the mitochondrial respiratory chain and the amount and activity of respiratory complexes. Mitochondrial respiratory complexes and their activity can also be modified by posttranslational modifications such as acetylation or phosphorylation^{22,23}. This parameter can also indicate the health of the cells and their capacity for responding to acute insults. Under situations of mitochondrial dysfunction, maximal respiration decreases, and this limits the capacity of the cell to respond to changes in ATP demand and leads to cell death²⁴.

It is very important to remark that, for every time point of the mitochondrial and glycolysis stress tests, ECAR is the sum of glycolysis-derived acidification (via generation of lactate + H^+) and respiratory-derived acidification (via generation of CO_2 , which dissolves in H_2O to generate HCO_3^- and H^+), and importantly, respiratory-derived acidification may account for a substantial proportion of the total ECAR in some cell types²⁵. For interested researchers, there are published protocols to calculate actual glycolytic rates. To that end, oxygen consumption rates during the glycolytic stress test (**Figure 4G**) can be used to calculate the proton production rate by respiration at each time point, and that value can be subtracted from the total proton production rate, which is obtained using ECAR values and the buffering power of the medium^{7,25,26}.

An important modification of this protocol compared to the manufacturer's recommendation is the second injection of the glycolysis stress test. Antimycin A and rotenone are preferred to oligomycin, for several reasons that have already been described⁷: 1) some cells display a high glycolytic capacity that may fully meet the basal ATP demand of the cell, even in the absence of oxidative phosphorylation; 2) blocking the ETC with antimycin A and rotenone artificially increases the ATP demand of the cell, as it causes ATP hydrolysis by the ATP synthase (the mitochondria become an ATP sink), which reverses to pump protons in an attempt to recover the mitochondrial membrane potential that collapses after respiratory inhibition; 3) blocking the ETC prevents respiratory acidification of the medium (respiratory CO_2 generated in the TCA cycle that is converted to HCO_3^- and H^+) which can confound the ECAR results. Thus, observed maximal ECAR with antimycin A and rotenone is only attributable to glycolysis. Interestingly, it has been reported that the ECAR observed with respiratory inhibitors alone may still be submaximal⁷, and an additional mechanism to increase cellular ATP demand (and therefore maximal ECAR) has been described: the addition of the ionophore monensin, which increase the import of Na^+ into the cell²⁷ and stimulates the rate of ATP hydrolysis by the plasma membrane Na^+/K^+ -ATPase, to the mixture of antimycin A and rotenone⁷.

A second important concept is that we do not recommend the subtraction of the non-glycolytic acidification, which probably corresponds to respiratory CO_2 that is converted to HCO_3^- and H^+ , from the entire trace to calculate glycolytic parameters as recommended by the manufacturer, as this amount changes considerably during each stage of the Glycolysis Stress Test (it is highest in the basal state, decreases after addition of glucose due to the Crabtree effect²⁰, and is virtually abolished with antimycin A and rotenone) (**Figure 5**).

To summarize, this protocol shows a simple and efficient tool to test the metabolic activity of human natural killer cells with an extracellular flux analyzer. This method can be used to interrogate the metabolic state in these cells, which is known to change with cell activation by cytokine stimulation or under certain pathological conditions, including obesity, cancer or viral infections²⁸.

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DISCLOSURES:

The authors declare that they have no competing financial interests.

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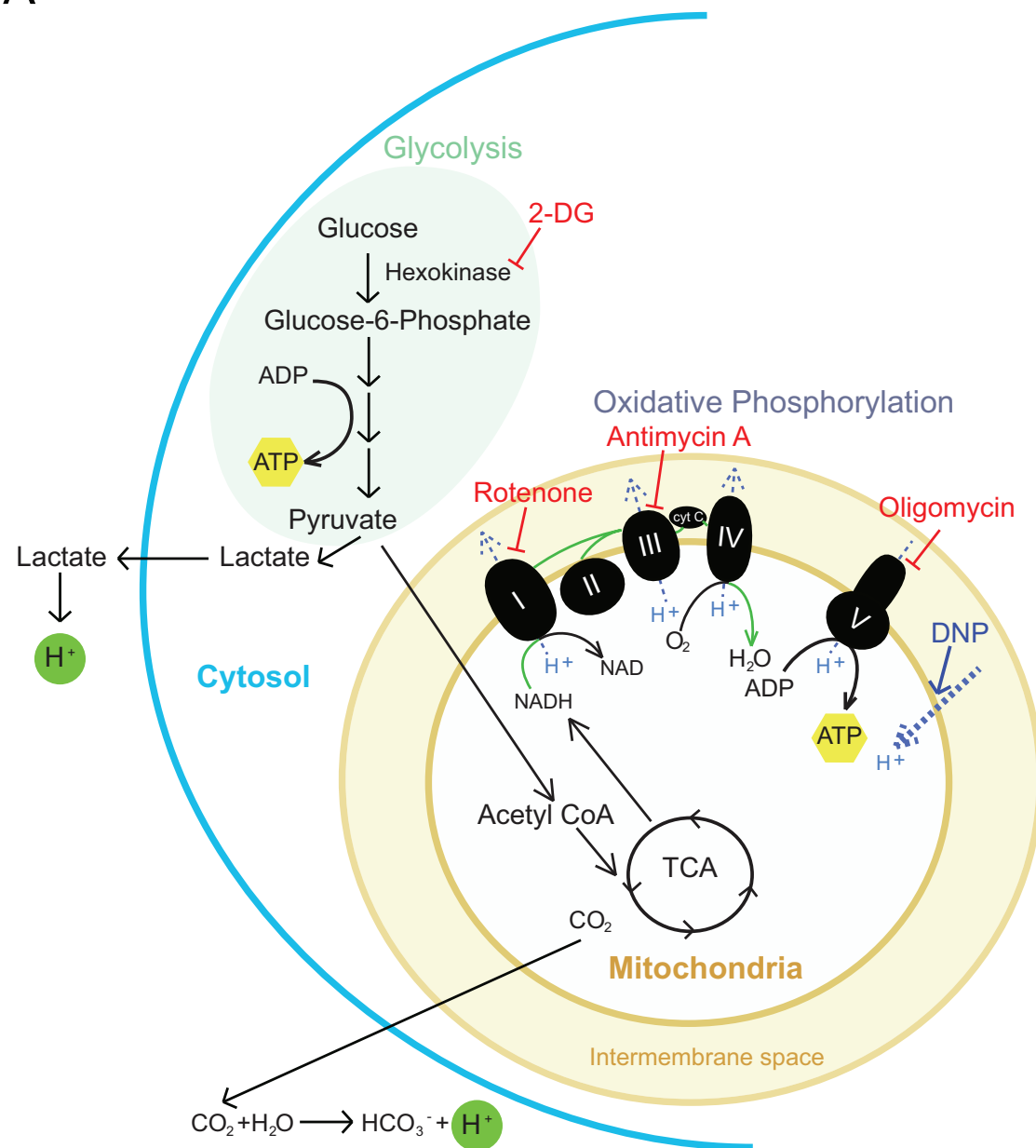
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Figure 1

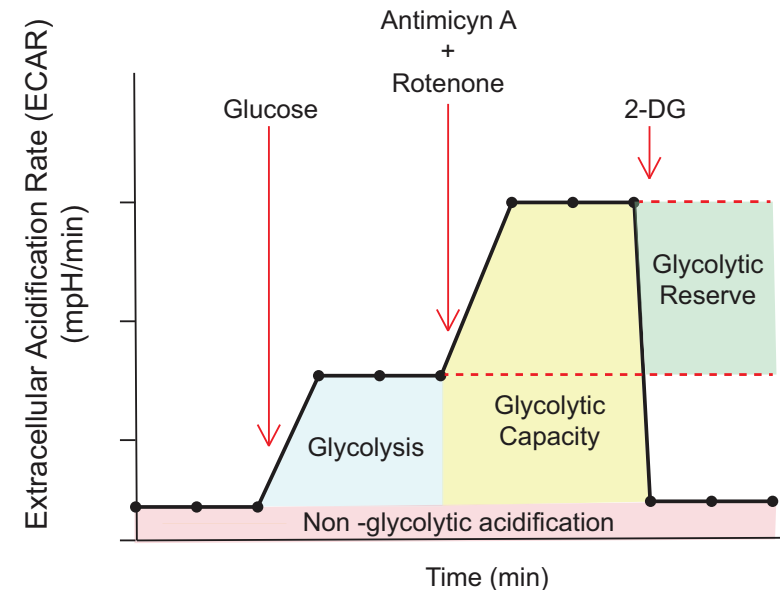
[Click here to access/download;Figure;Figure 1.eps](#)

Figure 1

A



B



C

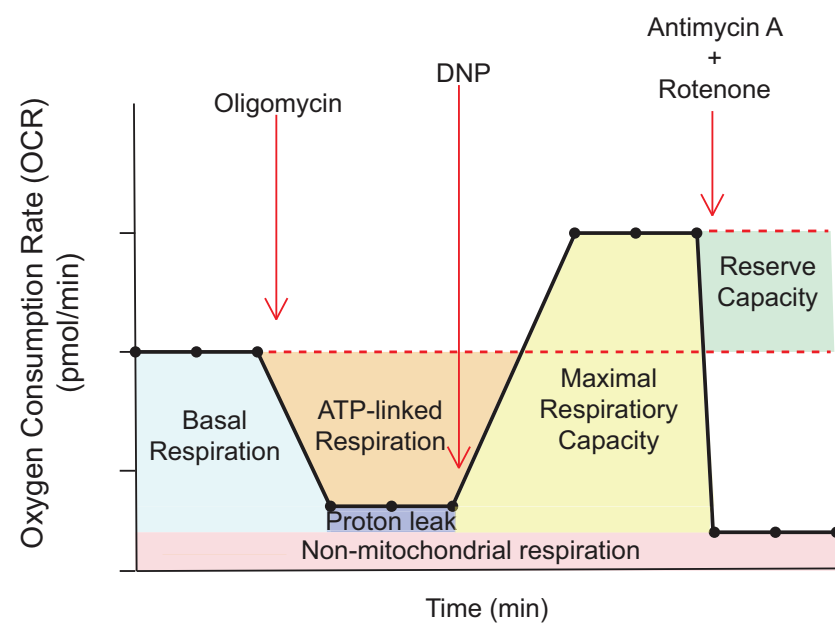
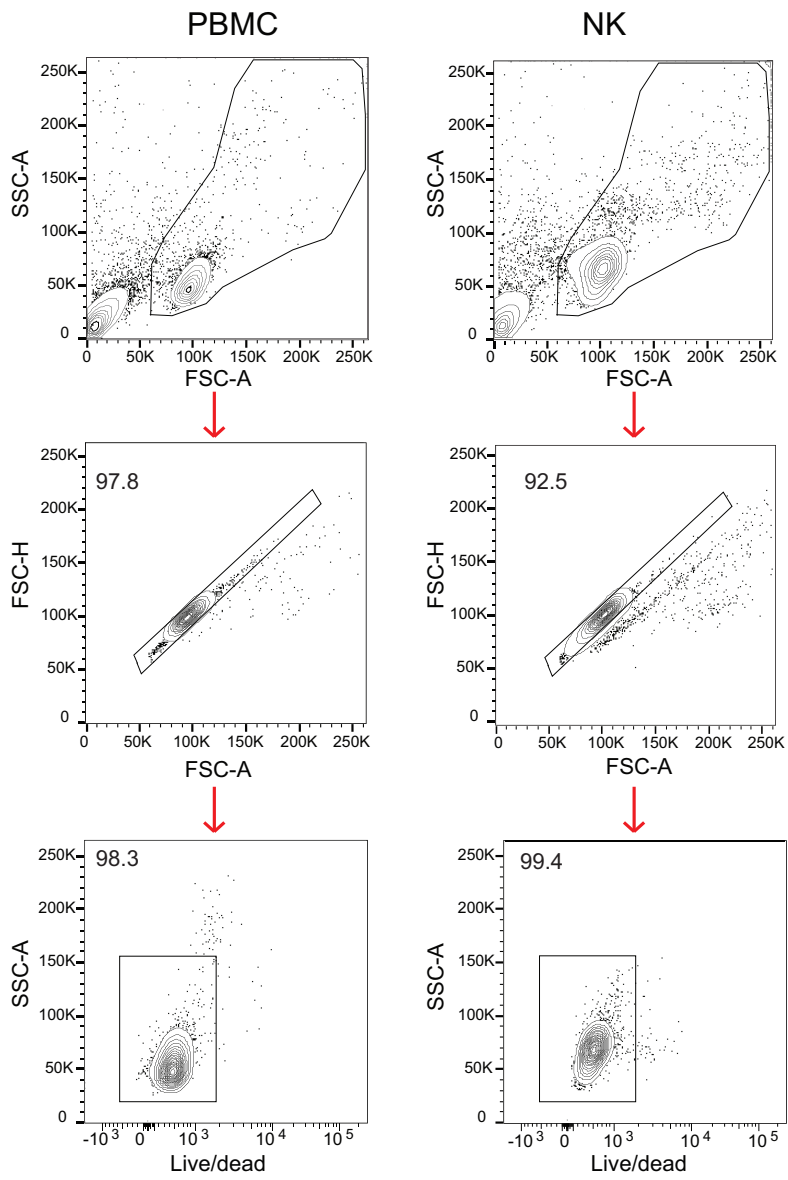


Figure 2

Figure 2

A



B

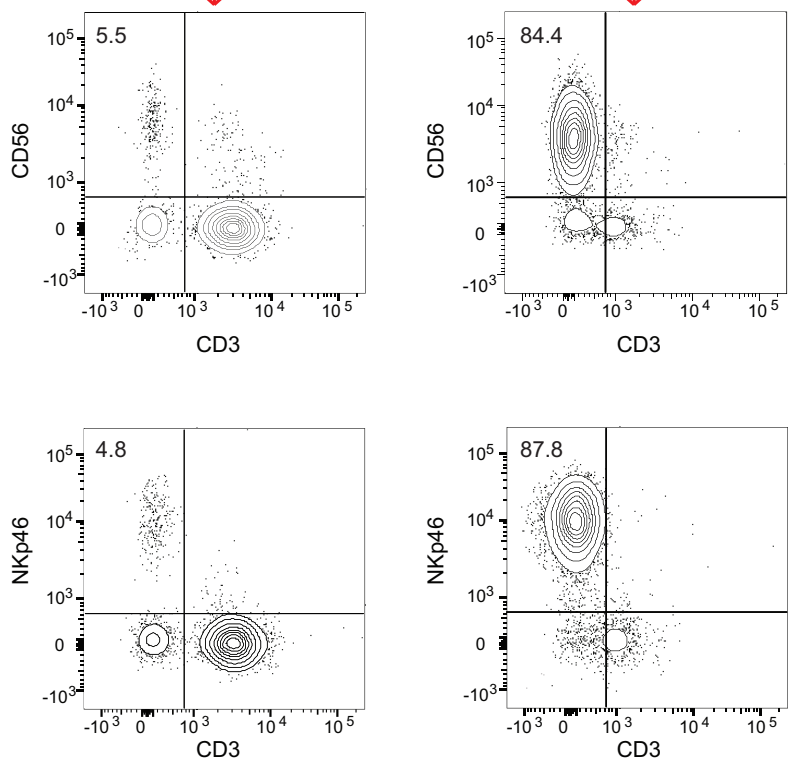


Figure 3

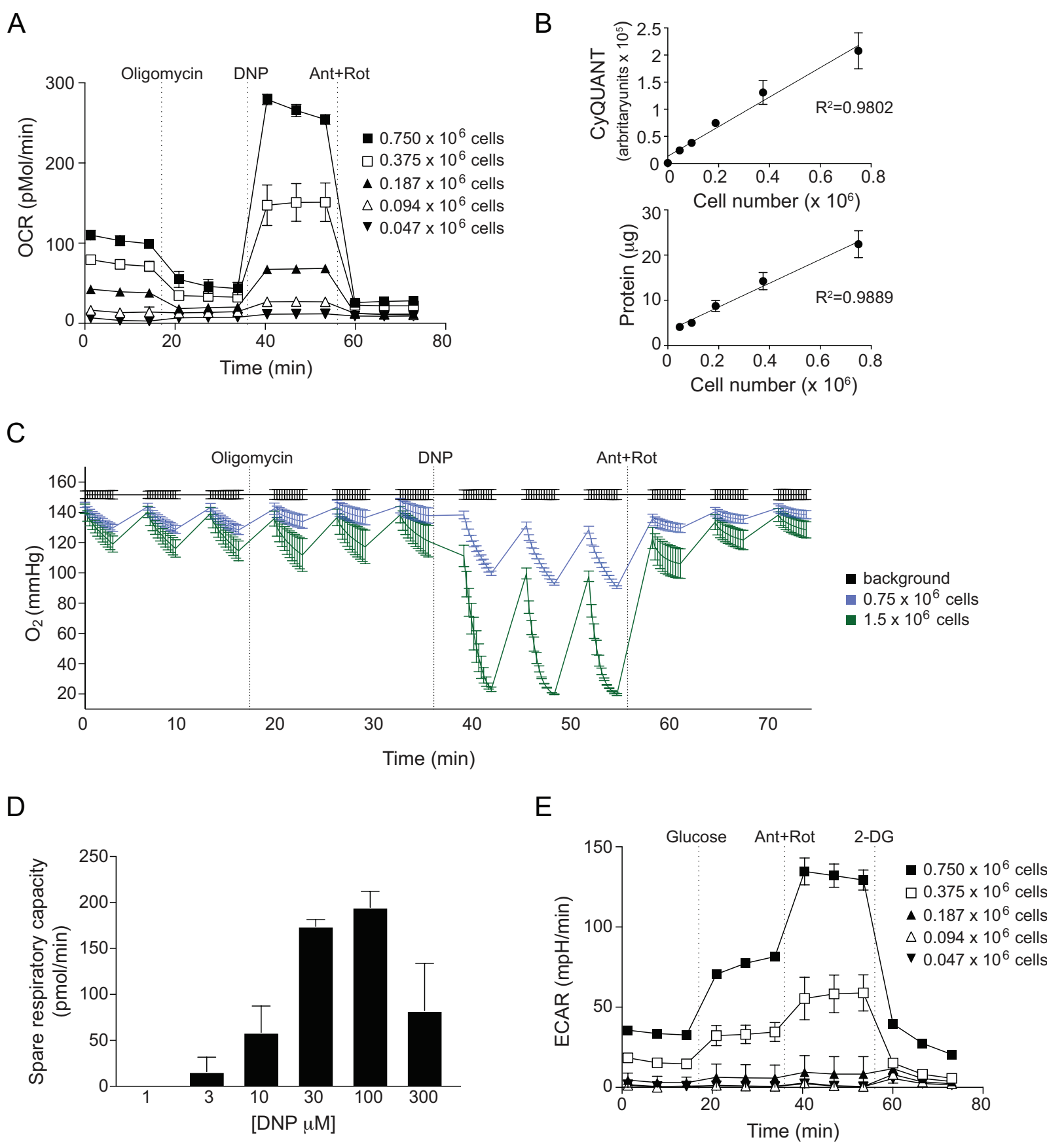


Figure 4

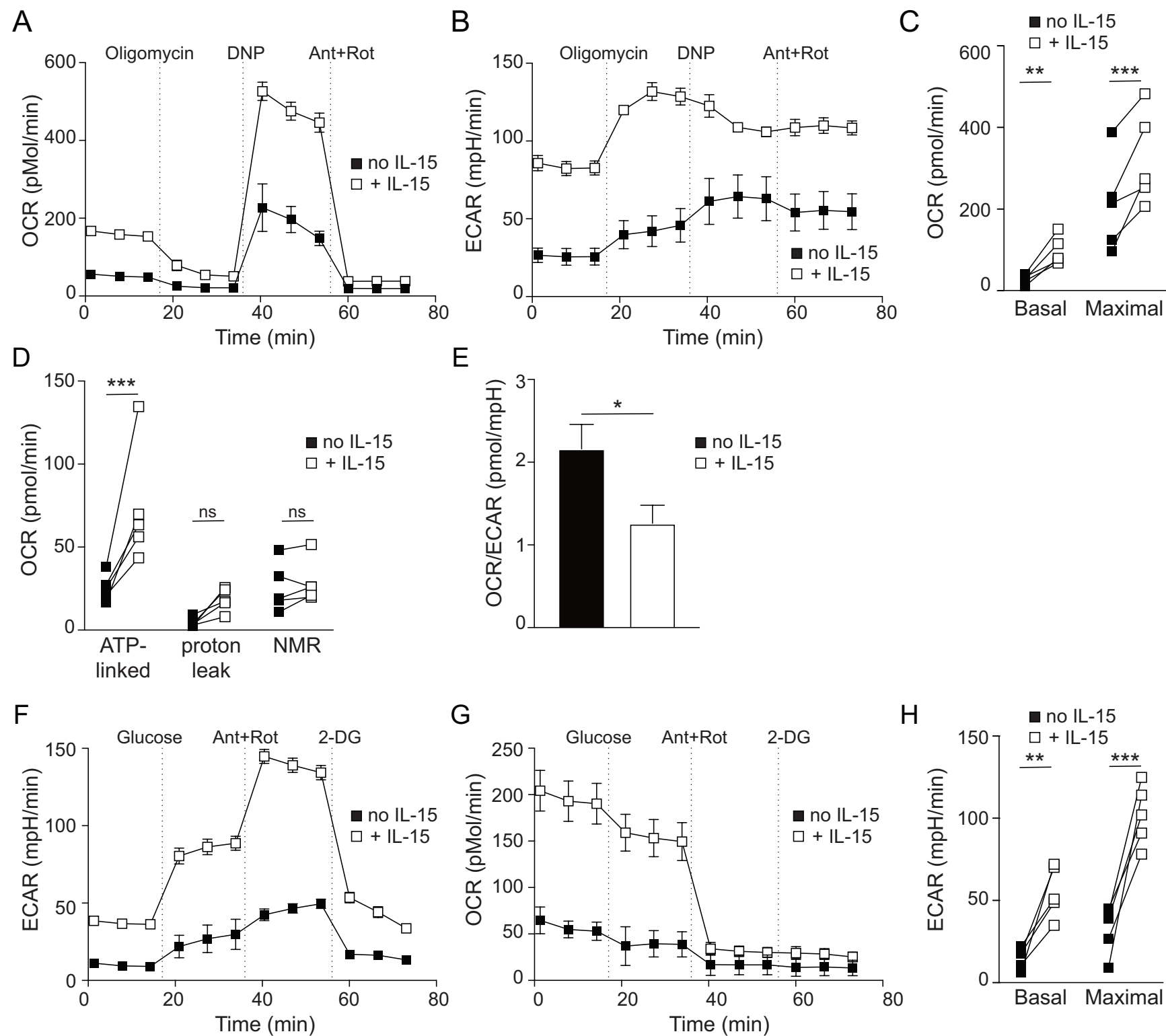
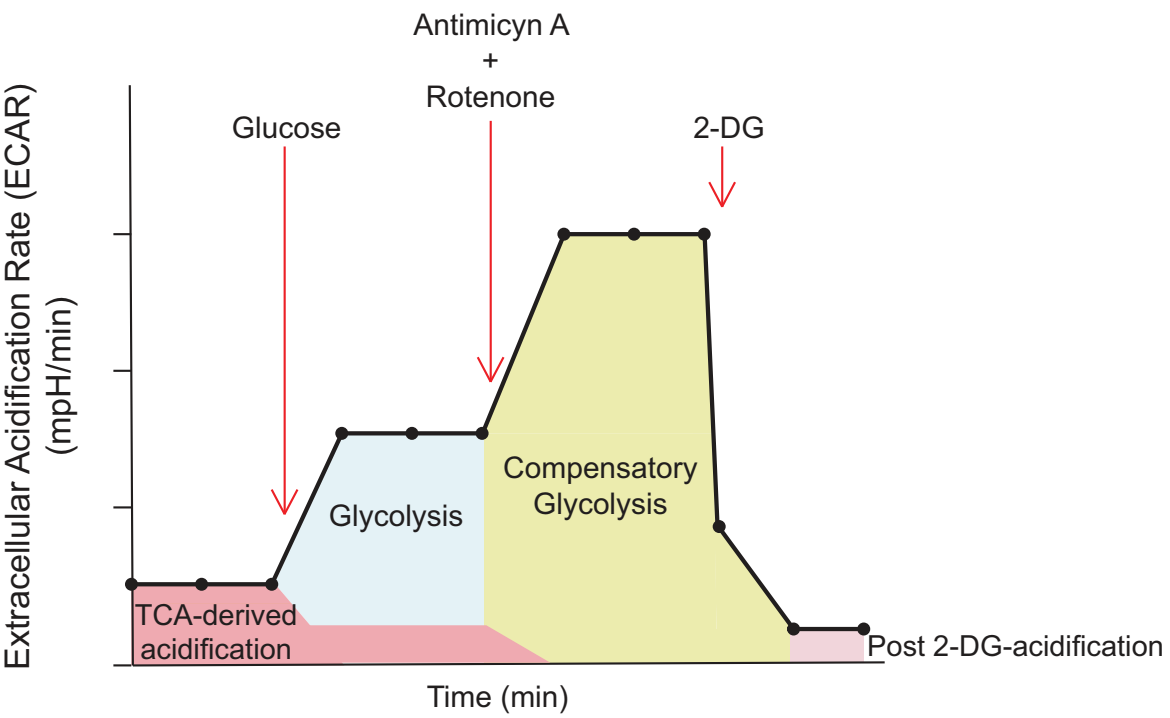
[Click here to access/download;figure;Figure 4.eps](#)

Figure 5



Reagent	Working Concentration
Mouse anti-human CD3 BV711	50 ng/ml
Mouse anti-human CD56 PE	1/50 dilution
Mouse anti-human NkP46 PE	1/50 dilution
Viability Dye	1/1000 dilution

Clone (for antibodies)	Final staining volume
UCHT1	100 µl
B159	100 µl
9/E2	100 µl
	500 µl

Mitochondrial Stress Test

Port	Volume	Compound	10x Stock
A	20 µl	oligomycin	10 µM
B	22 µl	DNP	1 mM
C	25 µl	antimycin A + rotenone	10 µM each

Glycolysis Stress Test

Port	Volume	Compound	10x Stock
A	20 µl	glucose	100 mM
B	22 µl	antimycin A + rotenone	10 µM each
C	25 µl	2-DG	500 mM

Final Concentration in the assay

1 μ M

0.1 mM

1 μ M each

Final Concentration in the assay

10 mM

1 μ M each

50 mM

Step	Loop				Repeat (times)
	Mix	Wait	Measure		
Calibration		—			
Equilibration		—			
Baseline readings	3 minutes	0 minutes	3 minutes		3
End loop		—			
Inject Port A		—			
Measurements	3 minutes	0 minutes	3 minutes		3
End loop		—			
Inject Port B		—			
Measurements	3 minutes	0 minutes	3 minutes		3
End loop		—			
Inject Port C		—			
Measurements	3 minutes	0 minutes	3 minutes		3
End loop		—			
End Program		—			

Name of Material/ Equipment	Company	Catalog Number
2-Deoxy-D-glucose (2-DG)	MilliporeSigma	D8375-5G
2,4-Dinitrophenol (2,4-DNP)	MilliporeSigma	D198501
96 Well Cell Culture Plate/ Round bottom with Lid	Costar	3799
Antimycin A	MilliporeSigma	A8674
BD FACSDIVA Software	BD Biosciences	
BD LSR Fortessa	BD Biosciences	
Cell-Tak	Corning	354240
CyQUANT cell proliferation assay	ThermoFisher Scientific	C7026
EasySep Human CD3 Positive Selection Kit II	Stemcell technologies	17851
EasySep Human NK cell Enrichment Kit	Stemcell technologies	19055
EasySep Magnet	Stemcell technologies	18001
EDTA 0.5 M, pH 8	Quality Biological	10128-446
FACS tubes	Falcon-Fisher Scientific	352235
Falcon 50 ml Conical tubes	Falcon-Fisher Scientific	14-432-22
Fetal Calf Serum (FCS)	Gibco	10437-028
FlowJo Software	BD Biosciences	
Glucose	MilliporeSigma	G8270
Halt Protease Inhibitor Cocktail	ThermoFisher Scientific	78429
Human IL-15	Peprotech	200-15-50ug
Human serum (HS)	Valley Biomedical	9C0539
IMDM	Gibco	12440053
L-Glutamine (200 mM)	ThermoFisher Scientific	25030-081
LIVE/DEAD Fixable Aqua Dead Cell Stain Kit	ThermoFisher Scientific	L34965
LSM	mpbio	50494X
Mouse anti-human CD3 BV711	BD Biosciences	563725
Mouse anti-human CD56 PE	BD Pharmingen	555516
Mouse anti-human NKp46 PE	BD Pharmingen	557991
Oligomycin	MilliporeSigma	75351
PBS pH 7.4	Gibco	10010-023
Pierce BCA Protein Assay Kit	ThermoFisher Scientific	23225
RIPA Buffer	Boston BioProducts	BP-115
Rotenone	MilliporeSigma	R8875

Seahorse Wave Controller Software	Agilent	
Seahorse Wave Desktop Software	Agilent	
Seahorse XF Base Medium	Agilent	102353-100
Seahorse XFe96 Analyzer	Agilent	
Seahorse XFe96 FluxPak	Agilent	102416-100
Sodium bicarbonate	MilliporeSigma	S5761
Sodium pyruvate (100 mM)	ThermoFisher Scientific	11360-070

Comments/Description

Glycolysis stress test injector compound
ETC uncoupler / mitochondrial stress test injector compound
NK cell culture
Complex III inhibitor / glycolysis and mitochondrial stress test injector compound
Flow data acquisition
Flow data acquisition
Cell adhesive
Cell proliferation Assay for DNA quantification. Contains cell-lysis buffer and CyQUANT GR dye
NK cell isolation from PBMCs
NK cell isolation from PBMCs
NK cell isolation from PBMCs
NK cell separation buffer
Flow cytometry experiment
NK cell separation
NK cell separation buffer
Flow data analysis
Component of mitochondrial stress test medium. Glycolysis stress test injector compound
Protease inhibitor 100X. Use in RIPA lysis buffer
NK cell stimulation
NK cell culture medium supplement
NK cell culture medium
Component of stress test media
Viability dye for flow cytometry staining
PBMCs separation from human blood
T cell flow cytometry staining
NK flow cytometry staining
NK flow cytometry staining
Complex V inhibitor / mitochondrial stress test injector compound
NK cell separation buffer
For determination of protein concentration
Cell lysis
Complex II inhibitor / glycolysis and mitochondrial stress test injector compound

Controller for the Seahorse XFe96 Analyzer

For data analysis

Extracellular Flux assay base medium

Extracellular Flux Analyzer

Includes 20 XF96 cell culture plates, 18 XFe96 sensor cartridges, loading guides for transferring compounds to the assay cartridge, and 1 bottle

To prepare the Cell-Tak solution

Component of mitochondrial stress test medium

e of calibrant solution (500 ml).

Dr. Bajaj, Review Editor of the Journal of Visualized Experiments,

Thank you for your input to our article “Analysis of Natural Killer cell metabolism” JoVE61466R1. Here we provide a detailed reply to the editorial comments:

1. Line 58:

Editor comment: “Most of the details which do not directly discuss the representative result obtained by using the protocol above can be moved to the introduction. Figures talking about the introduction can be referenced in the introduction section. However please refer figures in order.

Also claims in this section need citations.”

Reply: This text has been moved from Results to the Introduction and citations have been added (references 7 and 8)

2. Line 132:

Editor comment: “Citation?”

Reply: We have rephrased the sentence “Here we show that this is a valid method for the study of metabolic changes in resting and IL-15 activated human NK cells”

3. Line 134:

Editor comment: “Citation?”

Reply: Reference 17 has been added.

4. Line 143:

Editor comment: “Please highlight complete sentences throughout.”

Reply: This has been done.

5. Line 227:

Editor comment: “The pellet above was resuspended in something?”

Reply: The pellet is resuspended in NK separation Buffer and now it has been clearly stated.

“2.2.1. Count the cells from 2.1.6 and resuspend them in NK Separation Buffer (1×10^8 PBMCs/mL).

2.2.2. Take 10 ml of the cell resuspension (10^9 PBMCs) and place them into a 50 mL tube.”

6. Line 258:

Editor comment: “Do you remove the medium before resuspending?”

Reply: The medium is removed by centrifugation. It is stated in the text now “2.3.1. Take 0.25×10^6 cells per sample from step 2.2.8, remove the medium by centrifuging at $800 \times g$ for 5 min at room temperature and resuspend the cell pellet in 500 μ L of PBS.”

7. Line 264:

Editor comment: “How?”

Reply: It is explained now “Wash 2x by resuspending in 5 mL PBS and centrifuging at $800 \times g$ for 5 min, at room temperature.”

8. Line 276:

Editor comment: “From which step? 2.2.8?”

Reply: Step 2.2.8. was added to the text “Resuspend 0.75×10^6 cells from step 2.2.8 in 100 μ L of IMDM containing 10% HS in a well of a 96 well-plate (round bottom).”

9. Line 297:

Editor comment: “Please reword as it matches with previously published literature.”

Reply: The sentence has been rephrased “Open the sensor cartridge package and separate the sensor cartridge from the utility plate. Add 200 μ L of calibrant solution in each well of the utility plate and put back the sensor cartridge onto the plate, validating that the sensors are completely submerged in the solution.”

10. Line 326:

Editor comment: “Please reword as it matches with previously published literature.”

Reply: The step has been rephrased “4.1.2. Pipet 25 µl of the cell adhesive solution to each well of the assay plate and incubate at room temperature for 20 minutes. After that, remove the solution and wash 2x with 200 µl of sterile water per well. Let the wells dry by keeping the plate open for 15 minutes inside a cell culture hood.”

11. Line 335:

Editor comment: “Please bring out cohesivity in the protocol. Cells from which step? Volume and number of cells used in this assay etc.”

Reply: This information has been added

Editor comment: “What is the appropriate assay medium?”

Reply: This information has been added

Editor comment: “Please instead include your preferred cell concentration. What is volume of the medium?”

Reply: This information has been added

“4.2.1. Centrifuge cells from step 2.2.8 at $200 \times g$ for 5 min at room temperature. Remove supernatants and wash cells in warmed mitochondrial stress test medium (if a mitochondrial stress test is being performed) or glycolysis stress test medium (if a glycolysis stress test is being performed). Pellet cells again and resuspend to the preferred cell concentration in the same medium (resuspension volume will depend on the cell concentration chosen; since each well will contain 180 µL of the cell suspension, prepare 0.26×10^6 , 0.52×10^6 , 1.04×10^6 , 2.08×10^6 , 4.17×10^6 and 8.33×10^6 cells/mL cell suspensions for 0.047×10^6 , 0.094×10^6 , 0.187×10^6 , 0.375×10^6 , 0.75×10^6 and 1.5×10^6 cells per well respectively).”

12. Line 351:

Editor comment: “Wasn’t this already prepared in the reagent preparation section? So redundant here.”

Reply: In the reagent preparation section we describe how to prepare the stock solutions. This refers to working 10x solutions. This is clarified in the text now. Line 182 “1.2.3. Prepare [stock solutions](#) for reagents: Oligomycin (ATP synthase inhibitor), 10 mM stock solution in DMSO; 2,4-dinitrophenol (DNP, uncoupler), 1 M stock solution in DMSO; antimycin A (complex III inhibitor), 10 mM stock solution in DMSO; rotenone (complex I inhibitor), 10 mM stock solution in DMSO. Make 30 µL aliquots of all reagents and store at -20 °C. “

13. Line 359:

Editor comment: “wasn’t this done initially?”

Reply: In the reagent preparation section we describe how to prepare the stock solutions. This refers to working 10x solutions. This is clarified in the text now “**4.3 Preparation of 10x working solutions to load into sensor cartridge.**”

14. Line 364:

Editor comment: “Is the working solutions of the compounds prepared before?”

Reply: To prepare this 10x working solutions, use the stock solutions from 1.2.3.

“4.3.1. For the mitochondrial stress test, prepare 2.5 mL each of 10 μ M oligomycin, 1 mM DNP, and a mixture of 10 μ M rotenone and 10 μ M antimycin A, in mitochondrial stress assay medium (use the stock solutions from step 1.2.3). Final concentrations in the well after injection will be 1 μ M oligomycin, 0.1 mM DNP and 1 μ M antimycin A/rotenone.

4.3.2. For the glycolysis stress test, prepare 2.5 mL of a mixture of 10 μ M rotenone and 10 μ M antimycin A in glycolysis stress assay medium (use the stock solutions from step 1.2.3). Dissolve glucose in glycolysis stress test medium for a 100 mM solution and 2-deoxy-glucose (2-DG) in glycolysis stress test medium for a 500 mM solution. Final concentrations in the well after injection will be 10 mM glucose, 1 μ M antimycin A/rotenone and 50 mM 2-DG.”

15. Line 375:

Editor comment: “Please check this and bring out clarity? Solutions from 4.3.2 have different concentrations.” “How do you identify appropriate cartridge? Please spell out the details. Do you have different port for different assay? Do you load each compound in different ports? “

Reply: Hydrated cartridge comes from step 3.2. Loading of the ports is shown in table 2. “4.3.3. Warm 10x solutions to 37 °C, check pH and readjust to 7.4 if required. Load compounds prepared in step 4.3.1. (for a mitochondrial stress test) or 4.3.2 (for a glycolysis stress test) into ports A, B and C of the hydrated sensor cartridge (from step 3.2) using a multichannel micropipettor and the port-loading guides provided with the cartridge, as shown in **Table 2.**”

16. Line 384:

Editor comment: “After loading with loading solution? “

Reply: After loading ports A, B, and C with compounds. “4 Incubate the loaded sensor cartridge at 37 °C in a CO₂-free incubator while setting up the program.”

17. Line 389:

Editor comment: “Please include an example of condition used in your experiment?”

Reply: This information has been added to the text

“4.4.1. Open the extracellular flux analyzer software, and using the *Group Definitions* and *Plate Map* tabs indicate groups of wells that have similar conditions (for example, wells with the same number of cells, or wells with either resting cells or IL-15-stimulated cells). Also, indicate background correction wells (by default A1, A12, H1, and H12 will be set, but additional wells can be used) and empty wells.”

18. Line 400:

Editor comment: “Few steps are missing here, what happens after you put the assay plate? When and how do you inject the compounds in the plate? Which port has what compound etc. For how long is this done?”

Reply: The run is fully automated. This is indicated now.

“4.4.3 Begin the program using the *Run Assay* tab. Place the sensor cartridge (hydrated and loaded with 10x compounds) and utility plate onto the tray when prompted. After the calibration step (15 – 20 min), replace the calibrant plate for the assay plate (without lid) with attached cells. After this, the run is fully automated (the machine will perform measurements and injections).

NOTE: It is possible to perform a mitochondrial stress test and a glycolytic stress test in the same plate, as long as the specific compounds are loaded into the proper ports (oligomycin, DNP and antimycin/rotenone in ports A, B and C respectively of the wells where a mitochondrial stress test is performed; glucose, antimycin/rotenone and 2-DG in ports A, B and C respectively of the wells where a glycolysis stress test is performed), the same volumes for injections are used in each series of ports and wells for each test are properly identified using the *Group Definitions* and *Plate Map* tabs of the software.

4.4.4. After the completion of the run, retrieve the data and analyze them using the software.”

19. Line 434:

Editor comment: “Which reagent?”

Reply: The cell proliferation assay reagent

“5.1.4. Add 200 µL of the cell proliferation assay reagent to each well.”

20. Line 440:

Editor comment: "Please reword this part as it matches with the previously published literature. Other option is to just include citation instead."

Reply: This part has been reworded

"5.2. Protein content determination

5.2.1. Carefully, aspirate completely the assay medium from each well without touching the cells and freeze the cells at -20°C for at least 1 hour. Alternatively, the cells can be kept frozen for longer times (up to 1 week) until the analysis is performed.

5.2.2. Add 50 µL of radioimmunoprecipitation assay (RIPA) lysis medium supplemented with 1x protease inhibitors (from a 100x stock solution) to each well. Place the plate on a shaker for 5 min at room temperature, and then incubate the plate on ice for 30 min for a complete cell lysis.

5.2.3. Centrifuge the plate for 5 min at 200 x *g* at room temperature. This step will pellet cellular debris to prevent interference with the protein measurement.

5.2.4. Measure protein concentration by bicinchoninic acid (BCA) assay according to manufacturer's recommendations."

21. Line 571:

Editor comment: "Please label the x-axis."

Reply: The x-axis in Figure 5 has been labeled as "Time(min)".