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Research paper

An optimized protocol for adenosine triphosphate quantification in T lymphocytes of lymphopenic patients

Thibaut Girardot [a,b,1], Julie Mouillaux [a,1], Estellie Idealisoa [a], Fanny Poujol [a], Christelle Rouget [a,b], Thomas Rimmelé [a,b], Guillaume Monneret [a,c], Julien Textoris [a,b], Fabienne Venet [a,c,*]

[a] EA 7426 (Université Claude Bernard Lyon 1 – Hospices Civils de Lyon – bioMérieux) Joint Research Unit "Pathophysiology of Injury-Induced Immunosuppression – PI3", Hôpital Edouard Herriot, Lyon, France
[b] Hospices Civils de Lyon, Anesthesia and Critical Care Medicine Department, Hôpital Edouard Herriot, Lyon, France
[c] Hospices Civils de Lyon, Immunology Laboratory, Hôpital Edouard Herriot, Lyon, France

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ABSTRACT

In several clinical contexts, the measurement of ATP concentration in T lymphocytes has been proposed as a biomarker of immune status, predictive of secondary infections. However, the use of such biomarker in lymphopenic patients requires some adaptations in the ATP dosage protocol. We used blood from healthy volunteers to determine the optimal experimental settings. We investigated technical aspects such as the type of anticoagulant for blood sampling, the effect of freeze and thaw cycles, the reagent and sample mixing sequence, and the optimal dilution buffer. We also shortened the incubation time to 8 h, and even showed that a 30 min incubation may be sufficient. To evaluate the ATP rise upon lymphocyte activation, the optimal dose of stimulant was defined to be 4 μg/mL of phytohaemagglutinin. Lastly, we determined that the number of T cells needed for this measurement was as low as 50,000, which is compatible with the existing lymphopenia in clinical settings. This optimized protocol appears ready to be assessed in lymphopenic patients to further investigate the interconnection between T lymphocyte metabolism and impaired phenotype and functions.

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1. Introduction

Warburg described 60 years ago metabolic disturbances in cancer cells (Warburg, 1956). Over the past decades, the central role of cellular metabolism in cell and organ function has become progressively understood, particularly in immune cells (Buck et al., 2015). Metabolic profile seems to underlie the polarization (Gerriets and Rathmell, 2012; Jha et al., 2015) and the level of activation (Delgoffe and Powell, 2015) of these cells.

T lymphocytes play a central role in the immune response to any type of aggression. Interestingly, preferred metabolic pathways for energy production and biosynthesis of cellular elements differ between naive, activated, and memory T cells (MacIver et al., 2013). Indeed, their effector functions are sustained by a potent activation of their metabolic machinery (Maciolek et al., 2014; Pearce et al., 2013). Consequently, a measurement of T cells metabolic profile could be a surrogate marker of lymphocyte function and global immune competence.

Adenosin-5′-tri-phosphate (ATP) is one of the most important energetic substrates. The high amount of energy needed to cover effector functions of activated T lymphocytes is supplied by an important up-regulation of the cell metabolic machinery, which can be approached by the measurement of intracellular ATP concentration. In solid organ transplantation, intra-lymphocyte ATP concentration is associated with adverse events, from acute rejection to opportunistic infections (He et al., 2013; Kobashigawa et al., 2010; Kowalski et al., 2003, 2006).

Lymphopenia is common among hospitalized patients, especially in the intensive care unit (Castelino et al., 1997; Grossbard et al., 1984), and is associated with higher mortality (Drewry et al., 2014; Heffernan et al., 2012; Vulliamy et al., 2015) and higher risk of infection (Drewry et al., 2014; Felmet et al., 2005; Gouel-Chéron et al., 2012; Rajan and Sleigh, 1997). Moreover, the small amount of blood sampled

* Corresponding author at: Laboratoire Commun de Recherche Hospices Civils de Lyon – bioMérieux, Hôpital Edouard Herriot, Pavillon P, 5ème étage, 5, place d'Arsonval, 69003 Lyon, France.
E-mail address: fabienne.venet@chu-lyon.fr (F. Venet).

[1] The two co-first authors contributed equally to the work.

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for laboratory tests limits the quantity of lymphocytes available for ATP measurement. We thus decided to adapt the protocol for ATP measurements in lymphocytes to these constraints.

2. Materials and methods

2.1. Patient population and ethical concerns

Blood from healthy donors ($n = 12$) was provided by the French blood bank institution ("Etablissement Français du Sang"). All donors gave their explicit written consent for the use of their blood for biomedical research purposes, according to local legislation. Blood samples were collected in EDTA or heparinized tubes, anonymized upon donation, and sent to the laboratory at room temperature, to be processed on the same day.

2.2. T cell purification from whole blood

Antibody-based negative selection was used to purify T lymphocytes from healthy donors' whole blood according to manufacturer's instructions. A human T cell enrichment antibody cocktail (RosetteSep™, StemCell Technologies, Grenoble, France) was added to the blood sample. This antibody cocktail contains a mix of anti-CD16 (present on NK cells, monocytes, macrophages and neutrophils), anti-CD19 (B lymphocytes), anti-CD36 (platelets, red blood cells, monocytes), anti-CD56 (NK cells), and anti-CD66b (granulocytes) antibodies. After a 10-min period of incubation, the mix was diluted with an equal volume of DPBS 1X (Dulbecco's Phosphate Buffered Saline 1X, Gibco Life Technologies-Thermo Fisher Scientific, Waltham, MA, USA), then carefully layered over a density gradient medium (Ficoll-Paque PLUS, GE Healthcare, Little Chalfon, UK) in specially designed tubes with insert (SepMate™ tubes, StemCell Technologies).

The tubes were centrifuged at 1200*g* for 10 min at room temperature (RT), leading to the isolation of a ring enriched with T lymphocytes localized in the plasma-Ficoll interface, which was collected by pipetting. Residual red blood cells were lysed with Versalyse™ (Beckman Coulter, Brea, CA, USA).

2.3. Cell count and purity verification after purification procedure

At the end of the purification process, cells were counted with a flow cytometer (Navios, Beckman Coulter) using LDS (LDS 751, Molecular Probes, Life Technologies, Carlsbad, CA, USA) and calibration beads (Flow-Count™ Fluorospheres, Beckman Coulter), which concentration is known.

Quality of T cell purification was systematically controlled. Purified cells were labeled with an anti-human CD3-Pacific Blue antibody (IOTest®, Beckman Coulter) and LDS. Sample was then processed in a flow cytometer (Navios, Beckman Coulter), and double positive (CD3+/LDS+) cells were gated. The mean sample purity was 93.6 ± 3.5% of T lymphocytes.

2.4. Ex vivo culture and stimulation

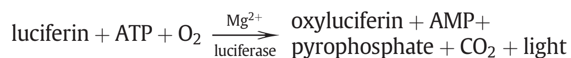
Purified T cells were diluted in RPMI (Roswell Park Memorial Institute) complete medium to achieve a concentration of 1.10^6 cells/mL. RPMI complete medium is a RPMI 1640 medium (Eurobio, Les Ulis, France), supplemented with 10% human AB serum (Life Technologies), 200 μg/mL amphotericin B (Gibco), 1000 IU/mL penicillin (Eurobio), 1000 μg/mL streptomycin (Eurobio), and 200 mM L-glutamine (Eurobio).

The obtained cellular solution was then distributed into two wells of a low-binding Costar® 24-wells cell culture plate (Corning®, Corning, NY, USA). One well was added with PHA (phytohaemagglutinin, Remel-Oxoid, Dardilly, France) at concentrations ranging from 0.2 to 4 μg/mL, or with anti-CD2/CD3/CD28 antibody-coated beads (T cell activation/expansion kit, Miltenyi Biotec, Bergisch Gladbach, Germany) at concentrations ranging from 0.5 to 2 beads per cell, to represent the stimulated condition, while the other well represented the unstimulated condition. Finally, the cell culture plate was incubated in standard conditions (37 °C, 5% CO_2) for a duration ranging from 0.5 to 24 h.

Every cell culture experiments presented in this study were performed on freshly purified cells. The effect of a freeze and thaw cycle on T cell capacity to produce ATP has never been evaluated in this study.

2.5. ATP quantitative determination

After incubation, cells were aliquoted to different cell numbers (ranging from 25,000 to 100,000), then lysed (IP lysis buffer, Pierce, Thermo Fisher Scientific). An ATP determination kit (Molecular Probes, Invitrogen, Carlsbad, CA, USA) based on a bioluminescent method was used for measurement of intra-lymphocyte ATP concentration. In the presence of ATP, firefly luciferase catalyzes the conversion of its substrate D-luciferin into oxyluciferin, producing light in an ATP-proportional manner, as shown in the reaction equation below.



$$\text{luciferin} + \text{ATP} + O_2 \xrightarrow[\text{luciferase}]{Mg^{2+}} \begin{array}{l}\text{oxyluciferin} + \text{AMP} + \\ \text{pyrophosphate} + CO_2 + \text{light}\end{array}$$

A reaction mix prepared according to manufacturer's instructions was mixed with the samples. The emitted light was measured with a spectrophotometer (Victor™ X4 Multilabel Plate Reader, Perkin Elmer, Waltham, MA, USA) at a wavelength of 560 nm for 1 s. Samples and standard curves were always processed in duplicates. Means of duplicates were used to calculate ATP concentration in samples and standards. The mean measured luminescence of the blank, corresponding to the background luminescence, was subtracted to all measurements. ATP concentrations were calculated from the standard curve (ranging from 0 to 1000 nM of ATP), generated from the ATP standard according to manufacturer's instructions.

2.6. Results analysis

Unless specified, results are expressed as means of the duplicates for each donor or points of the standard curves. Statistical analysis was performed with R Studio® software (RStudio Inc., Boston, MA, USA). Comparisons between groups used non parametric tests, such as Wilcoxon, Kruskal-Wallis and post-hoc Nemenyi tests, when appropriate. Paired tests were performed for matched data. Repeated measures ANOVA test was used to investigate buffer effect across time. Differences with p-values lower than 0.05 were considered significant.

3. Results

3.1. Effect of blood anticoagulant and sample stability

3.1.1. EDTA vs heparinized tubes

We initially investigated whether the use of EDTA or heparinized blood sample could significantly influence the result, by activating cells or by interfering with one reagent. We compared T cell response of two healthy donors – one sample in EDTA tube and another in heparinized tube for each donor. T cells were purified separately and stimulated or not with PHA for 24 h. For each conditions, T cells were aliquoted with four different cell numbers – 25,000, 50,000, 75,000 and 100,000. Although these results need to be confirmed on additional samples, Wilcoxon signed rank test showed no significant difference between the two anticoagulants, neither in unstimulated ($p = 0.469$) nor in stimulated ($p = 0.109$) samples (Fig. 1). To note, none of the other experiments presented thereafter were performed on heparinized tubes.

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