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Corso di Laurea in Biologia

ELABORATO DI LAUREA

ROLE OF MITOCHONDRIAL K^+ HOMEOSTASIS IN THE CONTROL OF THE INFLAMMATORY PROCESS

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SUMMARY

1. ABSTRACT

The immune system defends the organism in different ways: one of these is innate immunity. In macrophages, the assembly of the NLRP3 inflammasome is a crucial step in the inflammation process: it mediates the release of different cytokines like Il-1β, promoting pyroptosis. The inflammasome activation is a two-step mechanism that involves many different signals. According to recent data, the homeostasis of cations fluxes, particularly K^+ and Ca^{2+}, may have a role in its activation.

Mitochondrial Ca^{2+} signalling regulates different pathways inside the cells, in particular autophagy, metabolism, and cell death. Ca^{2+} enters the mitochondrial matrix thanks to the presence of the mitochondrial calcium uniporter complex (MCU). Moreover, the MitoK_{ATP} channel regulates K^+ flux across the inner mitochondrial membrane and controls the matrix water intake.

Taking for granted the influence of these ions in the inflammatory process, the aim of this work is to demonstrate the role of mitochondrial Ca^{2+} and K^+ homeostasis in the control of NLRP3 inflammasome induction. We characterized macrophages of Mito$K^{-/-}$ mouse model to unveil the influence of MitoK_{ATP} in the control of inflammasome induction. In addition, we took advantage of a chemical inhibitor of mitochondrial Ca^{2+} uptake to verify whether mitochondrial K^+ and Ca^{2+} fluxes could act synergistically in the control of inflammasome formation. Our experiments demonstrate that alterations in mitochondrial K^+ and Ca^{2+} homeostasis modulate inflammasome response.

2. RIASSUNTO

Il sistema immunitario difende l'organismo in diversi modi: uno di questi è l'immunità innata. Nei macrofagi l'assemblaggio dell'inflammasoma NLRP3 è uno step cruciale nel processo di infiammazione: esso media il rilascio di diverse citochine infiammatorie, come l'IL-1β e IL-18, promuovendo la piroptosi. L'attivazione dell'inflammasoma è un meccanismo a due step che coinvolge molteplici segnali. In accordo con dati recenti, l'omeostasi mitocondriale di Ca^{2+} e K^+, potrebbe avere un ruolo nell'attivazione dell'inflammasoma.

Il Ca^{2+} mitocondriale regola diverse vie di segnale nelle nostre cellule, in particolare l'autofagia, il metabolismo e la morte cellulare. Il Ca^{2+} entra nella matrice mitocondriale grazie alla presenza dell'uniporto del calcio mitocondriale (MCU). Inoltre, il canale MitoK_{ATP} permette il flusso di K^+ attraverso la membrana mitocondriale interna regolando l'ingresso di acqua nella matrice.

Dando per appurata l'influenza che questi ioni svolgono nel processo di infiammazione, lo scopo di questo lavoro è dimostrare il ruolo svolto dall'omeostasi mitocondriale di Ca^{2+} e K^+ nel controllo dell'induzione dell'inflammasoma NLRP3. In particolare, abbiamo caratterizzato il modello murino Mito$K^{-/-}$ per comprendere l'influenza del canale MitoK_{ATP} nel controllo dell'induzione dell'inflammasoma. Inoltre, è stato utilizzato un inibitore chimico dell'ingresso di Ca^{2+} mitocondriale per verificare se i flussi mitocondriali di K^+ e Ca^{2+} possano agire sinergicamente nel controllo della formazione dell'inflammasoma. Abbiamo quindi dimostrato che l'alterazione dell'omeostasi di K^+ e Ca^{2+} a livello mitocondriale è in grado di modulare la risposta dell'inflammasoma.

3. INTRODUCTION

3.1 NLRP3 inflammasome formation

The immune system defends the organism from pathogens' attacks. The pressure of natural selection creates new challenges and solutions that the immune system faces. The immune system has a lot of different responses to defend its organism. Immunity is divided into two principal ways: innate and acquired.

Typically, the immune system first unleashes a response using innate immunity; shortly after, the articulated response from the acquired immunity will start to operate. While innate immunity is unspecific and unleashes the response immediately; acquired immunity generates through immunological memory and is antigen dependent. In a typical immune response, innate immunity needs to respond using cells neutrophils and macrophages because they are the first to interact with pathogens to avoid infection and illness (Nicholson, 2016).

A specific component of the innate immune system is the inflammasome. The inflammasome is a protein complex able to trigger many immunological responses, including pyroptosis, cell death caused by bacterial pathogens. Studying this complex can enlighten about diseases and possible targets useful in therapeutics (Franchi et al., 2009).

The most studied type of this complex is the NLRP3 inflammasome. This protein complex is composed of three different parts: an adaptor (ASC), an effector (caspase 1), and, most importantly, a sensor (NLRP3). NLRP3 is an intracellular sensor that recognizes various microbial patterns, bacteria, and endogenous or exogenous irritants or toxic compounds (Swanson et al., 2019).

NLRP3 protein is composed of different parts: a central NACHT domain which has ATPase activity responsible for the assembly and functioning of the inflammasome, an N-terminal pyrin domain (PYD), and a C-terminal leucine-rich repeat (LRR) domain which detects a variety of cellular stresses and is responsible for the autoinhibition of the complex preventing the activation of the inflammasome.

Instead, the ASC adaptor consists of two domains: the CARD, which interacts with the CARD equivalent domain of pro-caspase 1, and a PYD domain which reacts similarly to a homophilic bond with the PYD domain present in the NLRP3 protein. Thus, they generate a helical ASC filament formation creating a polymerization complex called ASC speck (Cai et al., 2014).

When ASC assembles, it can recruit pro-caspase-1 by CARD homophilic interaction inducing the pro-caspase-1 self-cleavage and activation. The caspase 1 effector comprises the CARD domain, a p20 domain, and a smaller one called p10. The caspase 1 is the active protease released from the inflammasome. After the cut of the CARD domain, the tetramer loses its proteolytical function (Boucher et al., 2018).

To re-establish the physiological environment after a pathogen attack, the inflammasome employs many different proteins like inflammatory cytokines such

as IL-1β, IL-18 that are processed and released in the extracellular environment. Cytokines direct the inflammatory response by modulating it with a complex network of interactions. In particular, pro- and anti-inflammatory cytokines enhance or inhibit the immunological response of the inflammasome (Chen et al., 2018).

Interleukin-1β (IL-1β) is a proinflammatory cytokine produced by macrophages after the activation process. It is initially synthesized as an inactive precursor protein (proIL-1β) and needs cleavage to be transformed into its active form. The cleavage process is acted by caspase-1; it cuts proIL-1β in the intracellular space processing it into the active form, which is released in the extracellular environment (Dinarello, 1998).

The inflammasome activation is a two-step mechanism modulated by many different signals (Fig. 1). The first signal, called priming, aims to activate the transcription of monomeric components of the inflammasome. This signal activates the cascade of events which triggers the expression of the transcription factor NF-kB. It is provided by bacteria or other pathogens ligands (for example, LPS) which bind to the toll-like receptors (TLR), nod-like receptors (NLR), or cytokine receptors. The second signal is the activation signal, which is provided by extracellular ATP, nigericin, or other pore-forming molecules. Also, multiple cellular or molecular events, including ionic flux, mitochondrial dysfunctions or reactive oxygen species (ROS), can activate the inflammasome. In general, cellular stressors cause the definitive activation of the inflammasome, promoting inflammation. Both signals are necessary for the activation of the inflammasome. These signals promote the release of gasdermin D (GSDMD), which is cleaved by caspase 1. After the cleavage, the N-terminal domain creates pores in the cell membrane, causing pyroptosis, a pro-inflammatory cell death (Kelley et al., 2019).

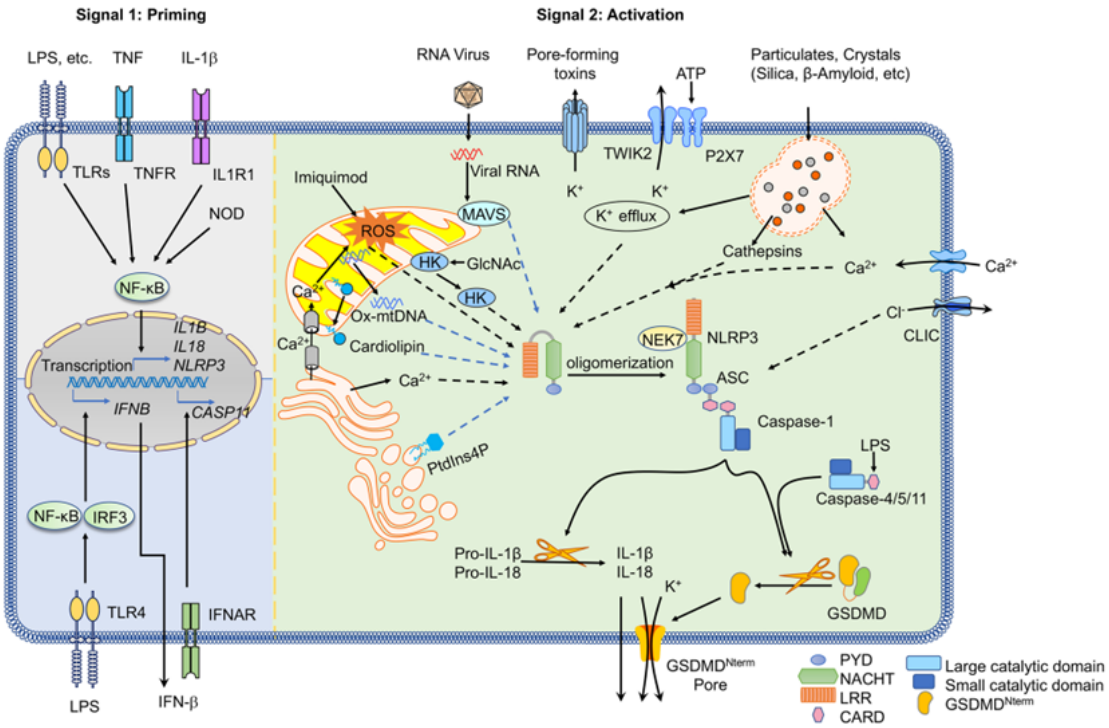

Figure 1: ***The NLRP3 inflammasome induction is caused by the simultaneous presence of two signals.***

Signal 1 (on the left; called priming) is supplied by cytokines or pathogen-associated molecular patterns (PAMPs). The priming causes the transcriptional up-regulation of the NLRP3 inflammasome constituent parts'. Signal 2 (on the right; called activation) is supplied by any of the several PAMPs or damage-associated molecular patterns (DAMPs), such as debris and ATP, which trigger many upstream signalling events. These involve K^+ efflux, Ca^+ flux, lysosome disruption, and mitochondrial reactive oxygen species (mtROS) production. When the inflammasome is complete, it activates caspase 1, which cleaves pro-IL-1β and pro-IL-18 in succession, generating IL-1β and IL-18. In addition, gasdermin D (GSDMD) is cleaved and placed into the membrane; this insertion causes the formation of pores and induces pyroptosis (Swanson et al., 2019).

3.2 Mitochondrial K^+ signalling and its regulation

The regulation of mitochondrial volume is crucial in cellular physiology. Mitochondria change shape and volume based on different cytosolic events: these circumstances are modulated by networks of proteins and, most importantly, by ion transport across the membrane, particularly K^+. In addition, mitochondria are susceptible to swelling, a particular event caused by a distortion in K^+ influx. This event is one of the fundamental features which describes the pathological state of mitochondria and induces apoptosis. In particular, the osmotic swelling of the matrix is caused by an exceeding in the K^+ influx rate originated by the opening of K^+ channels or leaks through the inner mitochondrial membrane. There are a multitude of K^+ channels that modulate K^+ homeostasis in the mitochondrial environment. The K^+-H^+ exchanger regulates the matrix pH and the matrix volume. Also, voltage-dependent membrane channels participate in this regulation process (Nowikovsky et al., 2009).

In particular, in this work, we will focus on MitoK_{ATP} channel. This channel has been described as a protein complex that mediates the transport of K^+ across the inner

mitochondrial membrane. It is composed of two different subunits: the channel-forming subunit, MitoK_{ATP}, and a regulatory subunit which carries the ATP-binding domain, called Mito$_{SUR}$ (Fig. 2) (Paggio et al., 2019). The MitoK_{ATP} complex is identified as a potential mechanism for describing ATP availability, but also a remarkable mechanism in the contribution of maintaining the homeostatic control of cellular metabolism under stress conditions. In particular, MitoK_{ATP} overexpression induces mitochondrial perturbation with modifications in its structure and function, in particular swelling. Contrarily to MitoK_{ATP} overexpression, the silencing of this channel causes a difference in the morphology of the mitochondria. In particular, the cells interested in this process show enlarged cristae. In conclusion, MitoK_{ATP} plays a crucial role in controlling mitochondrial physiology and has potential effects on several pathological processes (Paggio et al., 2019).

The activity of the MitoK_{ATP} channel is widely distributed among tissues. It has been identified in the mitochondria of liver, skeletal muscle, smooth muscle, fibroblasts, lymphocytes, heart, brain, and kidney. This channel is thought to be responsible for the cytoprotection process in different tissue components; one of these particular processes is the reduction of ROS in tissues (Szewczyk et al., 2018).

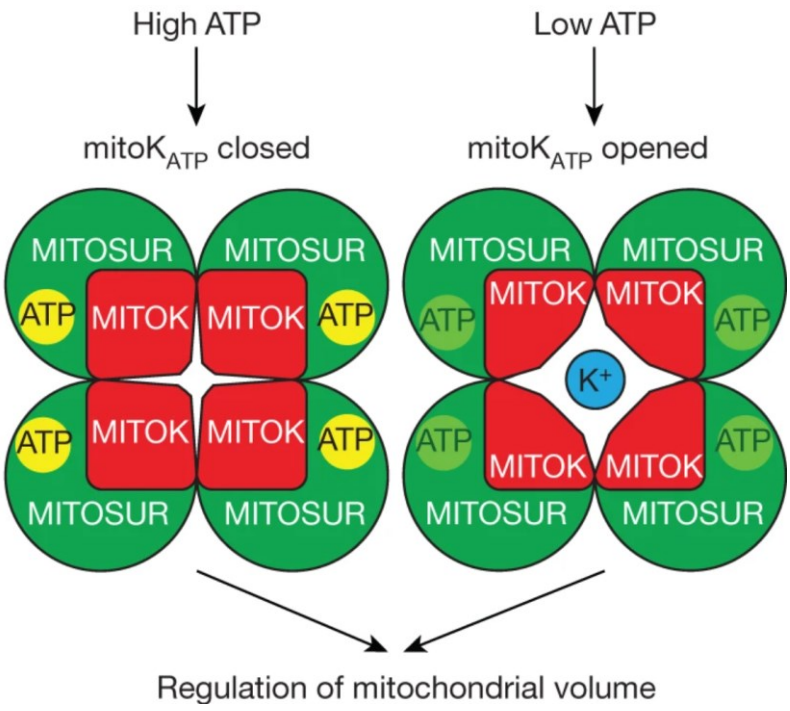

Figure 2: ***Visual representation of the MitoK_{ATP} channel.***

The MitoK_{ATP} channel is a tetramer composed of four of the channel-forming subunits (MitoK_{ATP}) and four of the regulatory subunits which carries the ATP-binding domain (Mito$_{SUR}$). An increase in intracellular levels of ATP will close the channel inhibiting K^+ influx; a decrease in ATP levels will enhance the K^+ uptake in the mitochondrial matrix (Paggio et al., 2019).

3.3 Role of mitochondrial cations (K^+ and Ca^{2+}) in the inflammasome activation

A universal mechanism for NLRP3 inflammasome activation remains undiscovered. A recent work has shown that mitochondrial dysfunction and K^+ efflux are fundamental events in this process (Yabal et al., 2019).

K^+ efflux plays a key role in IL-1β maturation and its release: K^+ ionophores such as lasalocid, A204, and nigericin mediate the release and the activation of this cytokine in macrophages treated and conditioned with LPS, the first signal, responsible for the priming phase. These experiments demonstrate that K^+ efflux is required for cleaving pro-IL-1β into physiologically active IL-1β (Koumangoye, 2022).

Moreover, in 2013, Muñoz-Planillo and coworkers demonstrated that a drop in intracellular K^+ is sufficient and necessary for activating the NLRP3 inflammasome, cleaving caspase-1 and releasing IL-1β. These experiments provide a model describing how K^+ efflux can activate the NLRP3 inflammasome without the priming phase (Muñoz-Planillo et al., 2013). However, the investigation regarding K^+ efflux in the activation of NLRP3 inflammasome is still in progress.

K^+ is not the only cation involved in the NLRP3 inflammasome activation, also Ca^{2+} mobilization is considered to be a critical upstream event in the NLRP3 activation. This event happens either by the opening of the plasma membrane channels or the discharge of endoplasmic reticulum Ca^{2+} vesicular storage. Furthermore, K^+ efflux can manage Ca^{2+} flux by acting like a counter ion. Additionally, NLRP3 inflammasome activation induced by ionophores, such as nigericin, has been discovered to be dependent on both Ca^{2+} flux and K^+ efflux. Ca^{2+} influx and K^+ efflux are often coordinated to guarantee the NLRP3 inflammasome activation (Swanson et al., 2019).

In particular, it has been demonstrated that the accumulation of Ca^{2+} at a mitochondrial level can regulate the inflammasome activation in cystic fibrosis epithelial cells. In particular, the MCU takes part in the inflammasome process: its silencing diminishes the inflammatory process in these cells (Rimessi et al., 2015). The molecular mechanism involved in this process is the regulation of autophagy by mitochondrial Ca^{2+} uptake. In particular, KB-R7943, a MCU inhibitor, can correct the autophagy response in cystic fibrosis cells during *Pseudomonas aeruginosa* infection (Rimessi et al., 2020).

Moreover, our laboratory has proven that MCUb, the dominant negative subunit of the MCU channel, is responsible for the anti-inflammatory profile during skeletal muscle regeneration. $MCUb^{-/-}$ macrophages show an upregulation of inflammation, indicating that an increased mitochondrial Ca^{2+} uptake is able to enhance the overall inflammation status (Feno et al., 2021) .

In addition, another work describes mitochondrial Ca^+ signaling as a crucial factor in the process of phagocytosis, but not important in the inflammasome induction. Specifically, mitochondrial Ca^+ uptake seems not involved in the nigericin/ATP-

induced activation of the inflammasome, but only in the specific process of phagocytosis-dependent NLRP3 inflammasome activation (Dong et al., 2022).

Thus, further experiments are needed to clarify the role of mitochondrial Ca^{2+} uptake in the regulation of the NLRP3 inflammasome induction.

4. AIM

Until today the molecular pathways which regulate NLRP3 inflammasome induction have not been completely verified. Emerging research has underlined evidence about how mitochondrial cations play a key role in the signalling pathways which result in the induction of the inflammasome. In this work, we studied in detail how Ca^{2+} and K^{+} are necessarily in the activation phase of the inflammasome and how they work synergistically to obtain the inflammatory response. We investigated whether the homeostasis of Ca^{2+} and K^{+} fluxes is altered and the precise effect in the NLRP3 inflammasome in bone marrow derived macrophages (BMDMs). In detail, we used primary culture of BMDMs from WT and Mito$K^{-/-}$ mouse models to check whether mitochondrial K^{+} signaling can alter the inflammatory response by altering the inflammasome cytokines release. We induced the NLRP3 inflammasome by supplying different stimuli necessary for the transcription of its components and activation. We then checked whether K^{+} and Ca^{2+} fluxes work synergistically for the inflammasome activation by treating BMDMs with MCUi-11, a chemical inhibitor of mitochondrial Ca^{2+} uptake. These results represent relevant discoveries to unveil the precise pathways which lead to inflammasome activation.

5. MATERIALS AND METHODS

5.1 Animal model

Adult mice (8 weeks old) are used to perform bone marrow derived macrophages (BMDMs) extraction. With this purpose, *in vivo* experiments are performed according to the Italian Law "Decreto Legislativo" n° 26/2014.

Wildtype and MitoK$^{-/-}$ animals, already available in our laboratory, were utilized. For the mouse model generation strategy see (Paggio et al., 2019).

5.2 Bone Marrow Derived Macrophages Extraction

The mouse is sacrificed by cervical dislocation. Therefore, femurs and tibias from both paws are harvested and stored in RPMI 1640 medium (10% FBS, 1% PS). The bone marrow is washed and collected by injecting approximately 5 ml of RPMI 1640 medium per bone, then it is centrifugated for 5 min at 400 xg at room temperature to pellet the cells. The supernatant is removed, and the pellet, containing both erythrocytes and leukocytes, is resuspended in 5 ml of eBioscienceTM 1X RBC buffer to eliminate erythrocytes through osmotic lysis. After 5 min of incubation, the solution is diluted in 5 ml of RPMI medium to inactivate the lysis process and filtered with a 70 µm filter. Next, the solution is centrifuged at 400 xg for 5 min at room temperature and the pellet is resuspended in 10 ml of RPMI 1640. Macrophage Colony Stimulating Factor (MCSF) (20 ng/ml) is added to RPMI 1640 to induce proliferation and survival of monocytes and their differentiation into macrophages. Cells are counted with the Cell Counter device (Thermo Fisher Scientific) using Trypan Blue (1:1) to estimate the number of dead cells in a viable population. 500000 monocytes are plated in a 100 mm petri dish, and subsequently incubated at 37°C with 5% di CO_2.

After 5 days, BMDMs are detached using 2mM EDTA PBS (phosphate buffer solution). 500000 macrophages are plated per well into 12 well plates.

5.3 Inflammasome Activation

NLRP3 inflammasome is induced by combining different types of stimuli to mimic the biological priming and activation stages. These different signals are required to trigger the transcription of NLRP3 inflammasome components (first signal - priming) and its activation (second signal). In detail, BMDMs are incubated with lipopolysaccharide (LPS) 100 ng/ml for 3 hours, for the "priming" phase. Then, LPS is removed and ATP 5mM is added for 45 min, for the "activation" phase. Finally, supernatants and cells are collected to evaluate inflammasome activation through the IL-1β ELISA technique on BMDMs supernatants and Western blotting on cell lysates.

5.4 SDS-PAGE and Western Blotting

5.4.1 Protein extraction

BMDMs are harvested after inflammasome activation and resuspended in a proper volume of RIPA buffer (125 mM NaCl, 25 mM TRIS-Cl pH 7.4, 1 mM EGTA-TRIS pH 7.4, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS). Complete EDTA-free protease inhibitor mixture (Roche) and phosphoSTOP to block phosphatases (Roche) are added to the lysis buffer. To remove cell debris, crude extracts are centrifuged at 15000 xg for 10 min. Then, proteins were measured using the BCA Protein Assay Kit (Thermo Fisher Scientific).

5.4.2 BCA protein assay

Protein lysates are quantified using the BCA (Bicinchonic Acid) protein assay. This assay is used to quantify protein concentration by using a colorimetric chemical reaction. Under alkaline conditions, Cu^{2+} binds proteins through peptide bonds and it is reduced into Cu^{+}. Subsequently, the BCA reacts with Cu^{+} giving a purple-coloured product. Acid and copper complex (BCA-Cu^{+}) absorbs linearly at 563 nm. To determine the precise protein concentration of the sample, the calibration curve is created using absorbance values from serial dilutions of bovine serum albumin (BSA) (Tab. 1). Two measurements are performed for each sample, to minimize potential measurement errors.

BSA STANDARD	***FINAL BSA CONCENTRATION (µg/ml)***	***PROTEINS (µg)***
A	2 (µg/ml)	50 µg
B	1,5 (µg/ml)	37,5 µg
C	1 (µg/ml)	25 µg
D	750 (µg/ml)	17,75 µg
E	500 (µg/ml)	12,5 µg
F	250 (µg/ml)	6,25 µg
G	125 (µg/ml)	3,125 µg
H	25 (µg/ml)	0,625 µg
I	0 (µg/ml)	0 µ

Table 1: Calibration line for BCA assay is performed with scalar dilutions of bovine serum albumin (BSA).

5.4.3 Western Blotting

Western blotting technique is used to separate and quantify protein levels. A mixture of proteins is separated by molecular weight through gel electrophoresis and quantified upon incubation with specific antibodies.

The loading solution for each sample is composed by 30 ug of proteins, LDS sample buffer (Thermo Fisher), 100mM DTT to reduce disulphide bridges and H_2O.

Then, protein samples are denatured for 5 min at 95°C and loaded on a gradient 4-12% Bis-Tris NuPage gel (Thermo Fisher).

A 120 V electric field is applied for 2h with MOPS 1X Running Buffer (Thermo Fisher). Proteins are negatively charged; thus, they will migrate in the gel pores from cathode to anode based on their molecular weight.

After electrophoretic separation, the proteins in polyacrylamide gel are transferred into a nitrocellulose membrane, in semidry conditions under the effect of an electric field. The nitrocellulose membrane is placed in a sandwich, between the gel and the positive catode; from the bottom layer to the top layer: a sponge, therefore the nitrocellulose membrane, then the gel and lastly another sponge layer. The sandwich is wet in the transfer buffer (50 ml of transfer buffer 20X, 200 ml of methanol, H_2O to bring the volume to 1L) and placed into the electroblotting semi-dry apparatus (Bio-Rad) with a constant voltage of 23 V for 40 min. The proteins migrate from the gel to the nitrocellulose membrane.

Then, the membrane is colored with Red Ponceau solution (Ponceaux red S 0,1% in Acetic Acid 5%) to evaluate the transfer efficacy. Subsequently, the membrane is washed in TBS-Tween solution (TBS 1X, 1 % Tween). Next, the membrane is blocked with a solution of 5% milk in TBS Tween to prevent non-specific bindings. Finally, the membrane is incubated with the primary antibodies at 4°C overnight under agitation. Primary and secondary antibodies used are shown in the next table (Tab 2):

Primary Ab	***Dilution***	***Secondary Antibody***	***Dilution***
GRP75 (Santa Cruz)	1:1000 in milk	HRP Anti-Mouse Ig	1:5000 in milk
MITO-K (*Sigma*)	1:1000 in milk	HRP Anti-Mouse Ig	1:5000 in milk

Table 2: Primary and secondary antibodies used in the Western Blotting

After the primary antibody incubation, the membrane is washed three times in TBS-Tween and it is incubated with the secondary antibody labelled with HRP (horseradish peroxidase) enzyme for 1 hour at room temperature. At the end, the membrane is washed three times in TBS-Tween. Then, the chemiluminescent reaction is developed by chemiluminescent kit (SuperSignal™ West Pico PLUS, Chemiluminescent Substrate, Thermo Fisher). To develop the chemiluminescent reaction, the nitrocellulose membrane is added to a solution with the SUPERSIGNAL WEST PICO reagent containing luminol and Hydrogen peroxide. The secondary antibody binds a hydrogen peroxidase which catalyses a chemiluminescent reaction, in which luminol is oxidized by the peroxidase and emits photons.

Finally, the signal is visualized using the UVITEC image capturing equipment. The intensity of the light signal is proportional to the amount of the protein bound to the primary antibody, for this reason it is possible to quantitatively analyse the various protein samples.

5.5 IL-1β Elisa analysis

The ELISA technique quantitatively determines the mouse IL-1β concentration in cell culture supernatants. All the analyses are performed using the Quantikine ELISA kit (R&D system) following manufacturer's instructions (Fig. 3). The samples come from the BMDMs supernatants upon inflammasome activation.

The 96 wells micro-plate is precoated with IL-1β monoclonal antibody. Standards, controls and samples are loaded in duplicate and incubated for 2 hours at room temperature to allow the binding between IL-1β and the immobilized antibody. Therefore, the wells are washed to delete all unbound substances.

Then, a polyclonal antibody conjugated with HRP enzyme is added to the wells, to bind specifically mouse IL-1β. After washing away any unbound substances, the substrate solution is added to trigger an enzymatic reaction. When the substrate solution is added to the wells, a blue coloration develops in proportion to the amount of IL-1β present in the samples.

Finally, the stop solution is added, turning the colour of the wells to yellow.

The optical density is read out using Envision microplate reader (Perkin Elmer) sets to 450 nm and 570 nm.

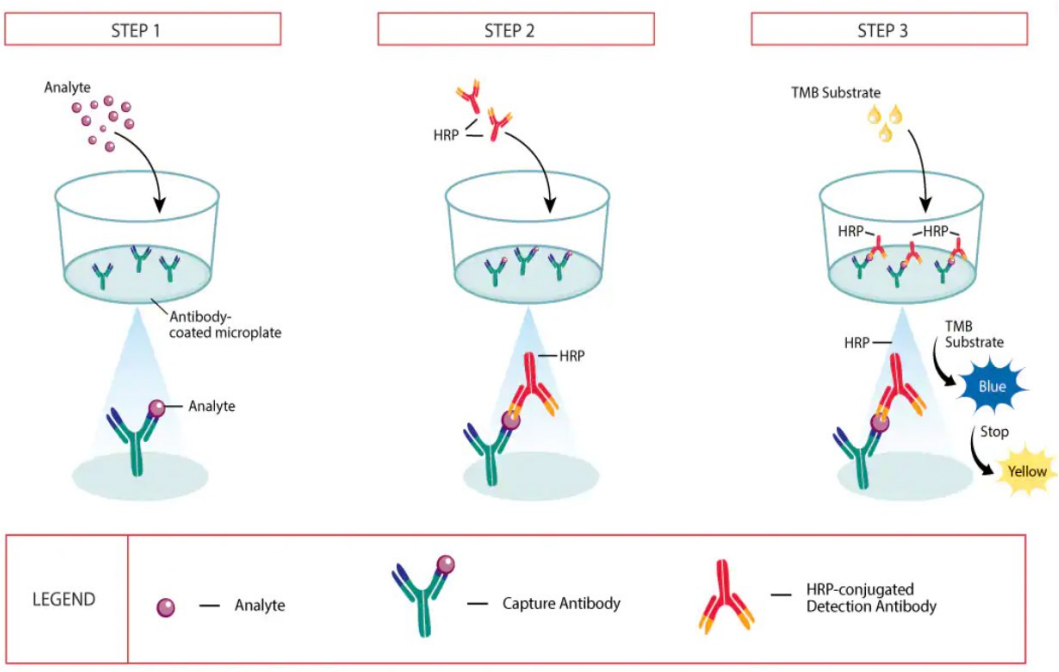

Figure 3: ***Supernatants from BMDMs are analysed with IL-1β ELISA technique.***

Every microplate is pre-coated with capture antibody. Samples or standards are added and any analyte present is bound by the immobilized antibody. A second antibody, which needs to be HRP-labeled, is added and binds to the captured analyte. This antibody is used for detection purpose. Subsequently, the substrate solution is added to the wells and a blue coloration evolves in proportion to the amount of IL-1β present in the sample. Finally, the color development is stopped by adding a reagent, turning the blue coloration in the wells to a yellowish one. The absorbance of the color is measured at 450 nm. (Quantikine ELISA Kits | R&D Systems, a Bio-Techne Brand, n.d.) (https://www.bio-techne.com/reagents/elisakits/quantikine).

The different absorbance values of the standards are used to construct a calibration line, used to calculate the concentration of IL-1β of the samples. The realization of the calibration curve is shown in the following table (Tab. 3).

	DILUTIONS	***CONCENTRATION (pg/ml)***
A	400 µl of standard	800 pg/ml
B	200 µl of solution A + 200 µl of RD5-16	400 pg/ml
C	200 µl of solution B + 200 µl of RD5-16	200 pg/ml
D	200 µl of solution C + 200 µl of RD5-16	100 pg/ml
E	200 µl of solution D + 200 µl of RD5-16	50 pg/ml
F	200 µl of solution E + 200 µl of RD5-16	25 pg/ml
G	200 µl of solution F + 200 µl of RD5-16	12,5 pg/ml
H	200 µl of RD5-16	0 pg/ml

Table 3: Serial dilutions of IL-1β to get a calibration line to quantify the IL-1β levels.

6. RESULTS

6.1 MitoK ablation reduces NLRP3 inflammasome formation

Since emerging evidence has demonstrated that high mitochondrial K^+ levels promote IL-1β release, we have investigated the role of mitochondrial K^+ flux in the inflammasome induction, (Muñoz-Planillo et al., 2013). Thus, we verified whether a decrease in mitochondrial K^+ levels is able to decrease the inflammatory response. We took advantage of MitoK$^{-/-}$ mouse model, already available in our laboratory (Paggio et al., 2019).

Firstly, we confirmed the absence of MitoK$_{ATP}$ protein in BMDMs from MitoK$^{-/-}$, comparing to WT mice by Western Blotting (Fig. 4).

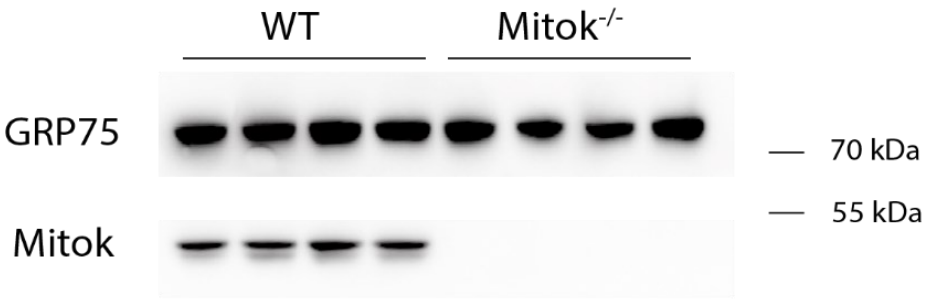

Figure 4: ***MitoK is not expressed in MitoK$^{-/-}$ BMDMs comparing to WT BMDMs***

MitoK is not expressed in MitoK$^{-/-}$ BMDMs compared to WT BMDMs. GRP75 is used as a loading control.

NLRP3 inflammasome is induced by the combination of diverse stimuli that mirror the biological priming and activation stages. In particular, lipopolysaccharide (LPS) is used to mimic the priming phase. LPS is a glycolipid present in the outer layer of Gram-negative bacteria, and it can bind the Toll-like Receptor 4 (TLR4), activating the NF-kB signaling cascade. NF-kB enters the nucleus promoting inflammasome components transcription.

To mimic the second signal, BMDMs are treated with adenosine triphosphate (ATP) 5mM for 45 min. ATP binds the P2X7R purinergic receptors, producing a rapid intracellular Ca^{2+} efflux, activating the inflammasome.

The time flow of NLRP3 inflammasome assay is shown in Figure 5A.

The NLRP3 inflammasome activation was evaluated through the measurement of IL-1β concentration in the culture BMDMs supernatants, through ELISA technique. In detail, we wanted to verify whether a reduction of mitochondrial K^+ levels is able to decrease the IL-1β release. IL-1β levels were quantified after LPS and ATP treatments, comparing MitoK$^{-/-}$ and WT mice (Fig. 5B).

Firstly, the combination of the first (LPS) and the second signal (ATP) is mandatory to activate the NLRP3 inflammasome. On the contrary, if BMDMs have been stimulated only with LPS (first signal), or without any stimuli (null), no IL-1β is detected.

Importantly, the IL-1β levels were significantly reduced in MitoK$^{-/-}$ BMDMs, stimulated with LPS and ATP, compared to control; thus, these results

demonstrate that the NLRP3 inflammasome activation is inhibited in MitoK$^{-/-}$ BMDMs.

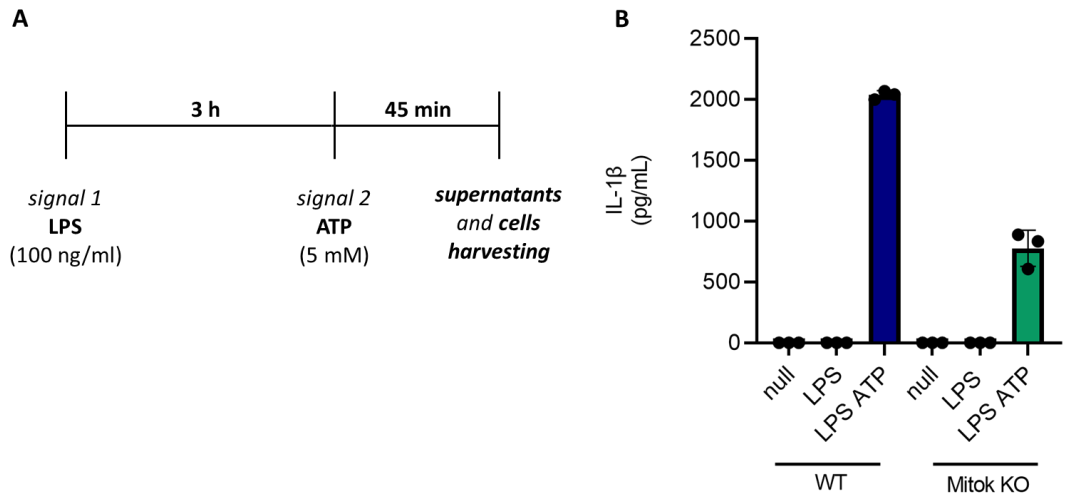

Figure 5: ***MitoK $^{-/-}$ BMDMs show decreased IL-1β levels release***

(A) Time flow of NLRP3 inflammasome induction. (B) IL-1b release is decreased in Mitok-/- BMDMs compared to WT.

6.2 Mitochondrial Ca^{2+} and K^{+} signals are synergistically involved in the control of NLRP3 inflammasome formation

Emerging evidence has shown that both mitochondrial Ca^{2+} and K^{+} are involved in NLRP3 inflammasome activation. In detail, the inhibition of mitochondrial Ca^{2+} uptake reduces the inflammatory response (Rimessi et al., 2020; Sebag, 2018). Thus, we want to verify whether the mitochondrial Ca^{2+} and K^{+} signals synergistically interact to decrease NLRP3 inflammasome activation.

Specifically, to inhibit the mitochondrial Ca^{2+} uptake, we took advantage of the MCU-i11, which is a chemical inhibitor of the MCU channel, by the binding MICU1, a regulatory subunit of the complex (Di Marco et al., 2020).

The time flow of NLRP3 inflammasome assay is shown in Figure 6A.

Since we hypothesized that mitochondrial cations signals have an active role in the second phase of the inflammasome induction, BMDMs were treated with the MCU-i11 immediately before the addition of the second signal (10') or 30 min before. This last condition was used to be sure to give sufficient time to the inhibitor to act.

IL-1β levels were measured in MitoK$^{-/-}$ and control BMDMs, treated or not with MCU-i11, even 30 and 10 min before ATP administration (Fig. 6B).

As previously shown (Fig. 5B), in MitoK$^{-/-}$ BMDMs the NLRP3 inflammasome activation is reduced compared to WT BMDMs (Fig. 6B). Moreover, MCU-i11 is able to decrease IL-1β levels both in MitoK$^{-/-}$ and WT BMDMs, compared to control (Fig. 6B). In detail, NLPR3 inflammasome activation is reduced when MCU-i11 is

administered 30 and 10 minutes before the second signal. Finally, MitoK$^{-/-}$ BMDMs treated with MCU-i11 show a stronger reduction of IL-1β levels, compared to control. These data demonstrate that the concomitant inhibition of mitochondrial Ca^{2+} and K^{+} fluxes decreases the inflammatory response. Thus, suggesting a synergistic role played by these two cations in the NLRP3 activation.

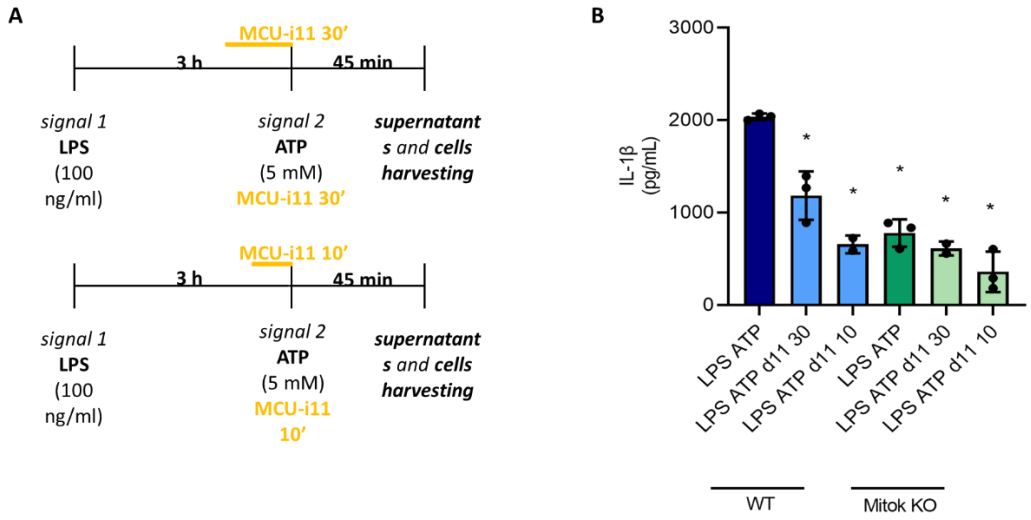

Figure 6: ***MitoK $^{-/-}$ BMDMs show decreased IL-1β levels release upon MCU-i11 administration.***

(A) Time flow of NLRP3 inflammasome induction. (B) IL-1b release is decreased in Mitok-/- BMDMs, upon MCU-i11 administration compared to WT BMDMs. MCU-i11 is able to inhibit the inflammasome activation when it is administered 30' or 10' before ATP.

7. DISCUSSION

This work aims to demonstrate the role of mitochondrial Ca^{2+} and K^+ fluxes in the NLRP3 inflammasome activation.

The innate immunity is the first response against pathogens and the NLRP3 inflammasome is one of the protein complexes that takes part in this process. The NLRP3 inflammasome is triggered by a multitude of signals, in particular cation fluxes (such as Ca^{2+} and K^+) play a crucial role in the activation of this complex.

K^+ efflux plays a key role in IL-1β maturation. In particular, emerging evidences show that high mitochondrial K^+ levels mediate inflammasome activation (Koumangoye, 2022; Muñoz-Planillo et al., 2013).

As previously stated, K^+ is not the only cation involved in the NLRP3 inflammasome activation, also Ca^{2+} mobilization is considered to be a critical upstream event in the NLRP3 activation.

Recent works have been showed that mitochondrial Ca^{2+} signalling participates in the NLRP3 inflammasome activation. In detail, the inhibition of mitochondrial Ca^{2+} uptake reduces the inflammatory response (Rimessi et al., 2020; Sebag, 2018).

To demonstrate that both cations work synergistically to induce the inflammatory response, we took advantage of two different strategies. To modulate the mitochondrial K^+ fluxes we took advantage of the MitoK$^{-/-}$ mouse model, already available in our laboratory (Paggio et al., 2019). In addition, to decrease mitochondrial Ca^{2+} uptake we used a chemical inhibitor, MCUi-11, recently discovered in our laboratory (Di Marco et al., 2020).

To get this conclusion, firstly, we induced the NLRP3 inflammasome in MitoK$^{-/-}$ BMDMs, to understand whether the inhibition of mitochondrial K^+ flux could alter the inflammasome activation. Then, we administered MCU-i11 to MitoK$^{-/-}$ BMDMs, to verify whether the mitochondrial Ca^{2+} and K^+ signals play a synergistic role in modulating the inflammatory cascade.

The NLRP3 inflammasome was induced in MitoK$^{-/-}$ and WT BMDMs by the administration of LPS for the priming phase and ATP as a second signal . The combination of the first and the second signal is mandatory to assemble and activate NLRP3 inflammasome.

We showed that the inflammasome activation is significantly reduced in MitoK$^{-/-}$ BMDMs compared to controls. This result confirms that mitochondrial K^+ homeostasis is involved in NLRP3 inflammasome activation and its inhibition is able to counteract the development of inflammatory responses.

Then, we used the chemical inhibitor MCU-i11 to investigate whether the decrease in mitochondrial Ca^{2+} uptake could alter the inflammatory response. Specifically, we want to investigate how mitochondrial Ca^{2+} and K^+ fluxes interact to regulate the inflammatory response.

In detail, the combination of mitochondrial Ca^{2+} and K^+ signals was investigated in MitoK$^{-/-}$ BMDMs upon MCU-i11 treatment at different time points.

As expected, the NLRP3 inflammasome activation is reduced in MitoK$^{-/-}$ BMDMs compared to WT. Moreover, MCU-i11 is able to decrease IL-1β levels both in MitoK$^{-/-}$ and WT BMDMs, compared to control. Finally, MitoK$^{-/-}$ BMDMs show a significant decrease in the IL-1β levels compared to WT, when MCU-i11 is administered 30 or 10 minutes before the second signal.

These results confirm that mitochondrial K^+ and Ca^{2+} fluxes work synergistically in the activation of NLRP3 inflammasome and that their modulation is able to counteract the inflammatory response.

These data strongly suggest that, in terms of pharmacological discoveries, MCU and MitoK_{ATP} channels should be taken into consideration as good candidates to modulate the inflammatory response in different disease conditions.

8. BIBLIOGRAPHY

Boucher, D., Monteleone, M., Coll, R. C., Chen, K. W., Ross, C. M., Teo, J. L., Gomez, G. A., Holley, C. L., Bierschenk, D., Stacey, K. J., Yap, A. S., Bezbradica, J. S., & Schroder, K. (2018). Caspase-1 self-cleavage is an intrinsic mechanism to terminate inflammasome activity. *Journal of Experimental Medicine*, *215*(3), 827–840. https://doi.org/10.1084/jem.20172222

Cai, X., Chen, J., Xu, H., Liu, S., Jiang, Q.-X., Halfmann, R., & Chen, Z. J. (2014). Prion-like Polymerization Underlies Signal Transduction in Antiviral Immune Defense and Inflammasome Activation. *Cell*, *156*(6), 1207–1222. https://doi.org/10.1016/j.cell.2014.01.063

Chen, L., Deng, H., Cui, H., Fang, J., Zuo, Z., Deng, J., Li, Y., Wang, X., & Zhao, L. (2018). Inflammatory responses and inflammation-associated diseases in organs. *Oncotarget*, *9*(6), 7204–7218. https://doi.org/10.18632/oncotarget.23208

Di Marco, G., Vallese, F., Jourde, B., Bergsdorf, C., Sturlese, M., De Mario, A., Techer-Etienne, V., Haasen, D., Oberhauser, B., Schleeger, S., Minetti, G., Moro, S., Rizzuto, R., De Stefani, D., Fornaro, M., & Mammucari, C. (2020). A High-Throughput Screening Identifies MICU1 Targeting Compounds. *Cell Reports*, *30*(7), 2321-2331.e6. https://doi.org/10.1016/j.celrep.2020.01.081

Dinarello, C. A. (1998). Interleukin-1β, Interleukin-18, and the Interleukin-1β Converting Enzyme [a]. *Annals of the New York Academy of Sciences*, *856*(1), 1–11. https://doi.org/10.1111/j.1749-6632.1998.tb08307.x

Dong, H., Zhao, B., Chen, J., Liu, Z., Li, X., Li, L., & Wen, H. (2022). Mitochondrial calcium uniporter promotes phagocytosis-dependent activation of the NLRP3 inflammasome. *Proceedings of the National Academy of Sciences*, *119*(26). https://doi.org/10.1073/pnas.2123247119

Feno, S., Munari, F., Reane, D. V., Gissi, R., Hoang, D.-H., Castegna, A., Chazaud, B., Viola, A., Rizzuto, R., & Raffaello, A. (2021). The dominant-negative mitochondrial calcium uniporter subunit MCUb drives macrophage polarization during skeletal muscle regeneration. *Science Signaling*, *14*(707). https://doi.org/10.1126/scisignal.abf3838

Franchi, L., Eigenbrod, T., Muñoz-Planillo, R., & Nuñez, G. (2009). The inflammasome: a caspase-1-activation platform that regulates immune responses and disease pathogenesis. *Nature Immunology*, *10*(3), 241–247. https://doi.org/10.1038/ni.1703

Kelley, N., Jeltema, D., Duan, Y., & He, Y. (2019). The NLRP3 Inflammasome: An Overview of Mechanisms of Activation and Regulation. *International Journal of Molecular Sciences*, *20*(13), 3328. https://doi.org/10.3390/ijms20133328

Koumangoye, R. (2022). The role of Cl^- and K^+ efflux in NLRP3 inflammasome and innate immune response activation. *American Journal of Physiology-Cell Physiology*, *322*(4), C645–C652. https://doi.org/10.1152/ajpcell.00421.2021

Muñoz-Planillo, R., Kuffa, P., Martínez-Colón, G., Smith, B. L., Rajendiran, T. M., & Núñez, G. (2013). K+ Efflux Is the Common Trigger of NLRP3 Inflammasome Activation by Bacterial Toxins and Particulate Matter. *Immunity*, *38*(6), 1142–1153. https://doi.org/10.1016/j.immuni.2013.05.016

Nicholson, L. B. (2016). The immune system. *Essays in Biochemistry*, *60*(3), 275–301. https://doi.org/10.1042/EBC20160017

Nowikovsky, K., Schweyen, R. J., & Bernardi, P. (2009). Pathophysiology of mitochondrial volume homeostasis: Potassium transport and permeability transition. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, *1787*(5), 345–350. https://doi.org/10.1016/j.bbabio.2008.10.006

Paggio, A., Checchetto, V., Campo, A., Menabò, R., Di Marco, G., Di Lisa, F., Szabo, I., Rizzuto, R., & De Stefani, D. (2019). Identification of an ATP-sensitive potassium channel in mitochondria. *Nature*, *572*(7771), 609–613. https://doi.org/10.1038/S41586-019-1498-3

Quantikine ELISA Kits | R&D Systems, a Bio-Techne Brand. (n.d.). Retrieved June 6, 2023, from https://www.bio-techne.com/reagents/elisa-kits/quantikine

Rimessi, A., Bezzerri, V., Patergnani, S., Marchi, S., Cabrini, G., & Pinton, P. (2015). Mitochondrial Ca2+-dependent NLRP3 activation exacerbates the Pseudomonas aeruginosa-driven inflammatory response in cystic fibrosis. *Nature Communications*, *6*(1), 6201. https://doi.org/10.1038/ncomms7201

Rimessi, A., Pozzato, C., Carparelli, L., Rossi, A., Ranucci, S., De Fino, I., Cigana, C., Talarico, A., Wieckowski, M. R., Ribeiro, C. M. P., Trapella, C., Rossi, G., Cabrini, G., Bragonzi, A., & Pinton, P. (2020). Pharmacological modulation of mitochondrial calcium uniporter controls lung inflammation in cystic fibrosis. *Science Advances*, *6*(19). https://doi.org/10.1126/sciadv.aax9093

Sebag, J. (2018). Posterior Vitreous Detachment. *Ophthalmology*, *125*(9), 1384–1385. https://doi.org/10.1016/j.ophtha.2018.05.018

Swanson, K. V., Deng, M., & Ting, J. P.-Y. (2019). The NLRP3 inflammasome: molecular activation and regulation to therapeutics. *Nature Reviews Immunology*, *19*(8), 477–489. https://doi.org/10.1038/s41577-019-0165-0

Szewczyk, A., Bednarczyk, P., Jędraszko, J., Kampa, R. P., Koprowski, P., Krajewska, M., Kucman, S., Kulawiak, B., Laskowski, M., Rotko, D., Sęk, A., Walewska, A., Żochowska, M., & Wrzosek, A. (2018). Mitochondrialne kanały potasowe: podsumowanie. *Postępy Biochemii*, *64*(3), 196–212. https://doi.org/10.18388/pb.2018_132

Yabal, M., Calleja, D. J., Simpson, D. S., & Lawlor, K. E. (2019). Stressing out the mitochondria: Mechanistic insights into NLRP3 inflammasome activation. *Journal of Leukocyte Biology*, *105*(2), 377–399. https://doi.org/10.1002/JLB.MR0318-124R