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## Commentary

## Mitochondria: A target for bacteria


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## ABSTRACT

Eukaryotic cells developed strategies to detect and eradicate infections. The innate immune system, which is the first line of defence against invading pathogens, relies on the recognition of molecular patterns conserved among pathogens. Pathogen associated molecular pattern binding to pattern recognition receptor triggers the activation of several signalling pathways leading to the establishment of a pro-inflammatory state required to control the infection.

In addition, pathogens evolved to subvert those responses (with passive and active strategies) allowing their entry and persistence in the host cells and tissues. Indeed, several bacteria actively manipulate immune system or interfere with the cell fate for their own benefit. One can imagine that bacterial effectors can potentially manipulate every single organelle in the cell. However, the multiple functions fulfilled by mitochondria especially their involvement in the regulation of innate immune response, make mitochondria a target of choice for bacterial pathogens as they are not only a key component of the central metabolism through ATP production and synthesis of various biomolecules but they also take part to cell signalling through ROS production and control of calcium homeostasis as well as the control of cell survival/programmed cell death. Furthermore, considering that mitochondria derived from an ancestral bacterial endosymbiosis, it is not surprising that a special connection does exist between this organelle and bacteria. In this review, we will discuss different mitochondrial functions that are affected during bacterial infection as well as different strategies developed by bacterial pathogens to subvert functions related to calcium homeostasis, maintenance of redox status and mitochondrial morphology.

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**Abbreviations:** A/A, ammonia/ammonium; AIM2, absent in melanoma 2; AMPK, AMP-activated protein kinase; APC, antigen presenting cell; CpG-ODN, CpG-oligodeoxynucleotide; DAMP, damages-associated molecular pattern; DC, dendritic cells; DRP1, dynamin-related protein 1; ECSIT, evolutionarily conserved signalling intermediate in Toll pathways; Eis, enhanced intracellular survival; ER, endoplasmic reticulum; ERR $\alpha$ , estrogen-related receptor alpha; ETC, electron transport chain; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GPX, glutathione peroxidase; HIF-1 $\alpha$ , hypoxia-inducible factor-1 alpha; IFN $\beta$ /IFN $\gamma$ , interferon-beta/interferon-gamma; IL-4, interleukin-4; IRF, interferon regulatory factor; JNK, Jun N-terminal kinase; LLO, listeriolysin; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MAVS, mitochondrial antiviral signalling protein; MCU, mitochondrial calcium uniporter; MDA-5, melanoma differentiation-associated gene-5; mtDAMP, mitochondrial DAMP; mtDNA, mitochondrial DNA; mtROS, mitochondrial ROS; MyD88, myeloid differentiation primary response gene 88; NFAT5, nuclear factor of activated T-cells 5; NF $\kappa$ B, nuclear factor-kappa B; NLR, NOD-like receptor; NOX, NADPH oxidase; OMM, outer mitochondrial membrane; OPA1, optic atrophy 1; OXPHOS, oxidative phosphorylation; PAMP, pathogen-associated molecular pattern; PGC-1 $\beta$ , PPAR gamma coactivator-1 beta; PI3K, phosphoinositide 3-kinase; PMN, polymorphonuclear neutrophil; PPA, propionic acid; PPAR, peroxisome proliferator-activated receptor; PPP, pentose phosphate pathway; PRR, pattern recognition receptor; PRX, peroxyredoxins; RLR, RIG-I like receptor; ROS, reactive oxygen species; SAM, sorting and assembly machinery; SERCA, sarco/endoplasmic reticulum calcium ATPase; SLO, streptolysin; SOD, superoxide dismutase; STAT6, signal transducer and activator of transcription 6; STING, stimulator of interferon genes; TXSS, type X secretion system; TACE, TNF $\alpha$ -converting enzyme; TCA, tricarboxylic acid; TcdB, *Clostridium difficile* toxin B; TCR, T-cell receptor; TFAM, mitochondrial transcription factor A; TIM, translocase of the inner membrane; TLR, Toll-like receptor; TNF $\alpha$ , tumour-necrosis factor alpha; TOM, translocase of the outer membrane; TRAF6, TNF $\alpha$  receptor-associated factor 6; UCP-2, uncoupling protein-2; VacA, vacuolating cytotoxin A; VDAC, voltage-dependent anion channel.

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## 1. Introduction

Mitochondria are dynamic organelles with a morphology controlled by fusion and fission events [1] that evolved from endosymbiotic  $\alpha$ -proteobacteria belonging to *Rickettsia* gender [2]. They still present many similarities with prokaryotic cells such as a double membrane, the ability to produce ATP through the generation of a proton gradient generated across the inner membrane or the fact that they have their own genome and bacterial type ribosomes [2]. The bacterial origin of mitochondria is further supported by the fact that various antibiotics, especially bactericidal ones such as quinolones, aminoglycosides and  $\beta$ -lactams are also able to induce mitochondrial dysfunction and reactive oxygen species (ROS) production [3].

The mitochondrial DNA (mtDNA) encodes two ribosomal, 22 transfer RNA and only 13 peptides of the mitochondrial proteins involved in the oxidative phosphorylation (OXPHOS) system. Most of the mitochondrial proteome is thus encoded by the nuclear genome [4].

Mitochondrial double membrane results in the formation of four sub-compartments. Firstly, the outer mitochondrial membrane (OMM) contains numerous porins that make it passively permeable to small molecules (<5 kDa). Secondly, the inter-membrane space (IMS) sequesters numerous of proteins acting as damages-associated molecular patterns (DAMPs), such as cytochrome c, endonuclease G, apoptosis-inducing factor (AIF), and several pro-caspases, which are also recognised by pattern recognition receptors (PRR) [5]. Indeed, their release in the cytosol will induce inflammation and/or cell death. As subversion of mitochondrial death pathways has already been extensively reviewed [6], mechanisms related to apoptotic cell death will not be developed in this review. Thirdly, the inner mitochondrial membrane (IMM) contains the different complexes of the respiratory electron transport chain (complexes I–IV) as well as the Fo-F1 ATP synthase (complex V), which are responsible for ATP production by the OXPHOS. This membrane is however much more impermeable than the OMM. Furthermore, cardiolipin, a phospholipid found exclusively in inner mitochondrial membrane and bacterial plasma membrane, make the IMM less fluid [7]. Consequently, metabolites have to use a variety of selective transporters to cross the inner membrane. The surface of this membrane forms cristae to increase the ability to produce ATP in the matrix. In addition, many mitochondrial proteins encoded by the nucleus will need the import and sorting machinery present in both OMM and IMM to reach the different sub-compartments [8]. According to the compartment they reach, proteins will use different transport complexes: translocase of the outer membrane (TOM)/translocase of the inner membrane 23 (TIM23)/presequence translocase-associated motor (PAM) for the matrix, TOM/TIM23 or TOM/TIM22 for IMM, TOM/mitochondrial inter-membrane space assembly (MIA) for IMS and TOM/sorting and assembly machinery (SAM) for  $\beta$ -barrel proteins located in the OMM [8]. Finally, the matrix contains multiple copies of mtDNA organised into nucleoids as well as the machinery that is necessary to transcribe and translate mtDNA-encoded genes. Reducing agents (NADH and FADH<sub>2</sub>) are also generated in the matrix by the tricarboxylic acid (TCA) cycle and the fatty acid  $\beta$ -oxidation (FAO) [9].

Even if mitochondria still share some features with its bacterial ancestor, the organelle also acquired new characteristics such as a dynamic morphology of the mitochondrial network that affects both mitochondrial activity and function. According to cell types and functional status, mitochondria can thus shift from separated rounded/fragmented mitochondria into interconnected and elongated tubular network [1]. This very dynamic organelle thus continuously adapts the morphology and move to specific cellular sub-compartments using different components of the cytoskeleton

[1]. The mitochondrial morphology is determined by the balance between two opposing processes that occur continually in the cell: the mitochondrial fission and fusion that are mediated by large GTPases related to the dynamin superfamily [10]. The fusion occurs in two steps: first the fusion of OMM mediated by the homo-/heterodimerisation of mitofusin1/2 (MFN1/2) and then optic atrophy 1 (OPA1) that forms homodimers leading to IMM fusion. Fission process requires the recruitment of dynamin-related protein 1 (DRP1) to the outer mitochondrial membrane, where it will assemble to form a constriction ring leading to the fission. Four different receptors for DRP1 located in the outer membrane have been identified so far: mitochondrial fission 1 (FIS1), mitochondrial fission factor (MFF) and mitochondrial dynamics protein of 49 and 51 kDa (MID49 and MID51) [1]. It is important to note that mitochondrial morphology influences the mitochondrial (dys)function while mitochondrial functional status also controls the dynamics and shape of the organelle [11]. Indeed, extremely depolarised and fragmented mitochondria are degraded by mitophagy, a specific form of autophagy [1]. The best-characterised mitophagy pathway involves the recruitment of Parkin (an ubiquitin ligase) from the cytosol to the OMM by PTEN-induced putative kinase 1 (PINK1). This relocation also allows Parkin to poly-ubiquitinate proteins located in the OMM, leading to their degradation by the 26S proteasome [12].

## 2. Mitochondrial targeting by bacteria

While the impact of the mitochondria functional status on the efficiency and persistence of infection and/or trafficking (for intracellular bacteria) is still poorly understood, the effects of bacteria infection on several parameters of the mitochondrial population start to be better delineated.

First, to impact mitochondria, bacterial effectors need to cross several barriers. They have first to be secreted out of the different layers of the bacterial envelope through dedicated secretion systems, then to reach (and pass through) the host plasma and organelle membranes. Several bacterial effectors, collectively called AB toxin, once secreted in the extracellular medium, are able to translocate inside the eukaryotic cell cytoplasm [13]. The B domain is responsible for the cellular tropism and often induces the receptor mediated-endocytosis [14] of the holotoxin followed by the translocation of the A domain into the cytoplasm of the targeted cell. The A domain has an activity responsible for the “toxic effect” such as the glycosyl transferase activity of the *Clostridium difficile* toxin B (TcdB) [14] or the pore forming activity of the *Helicobacter pylori* VacA toxin [15]. Moreover, in Gram-negative bacteria, some complex secretion systems are able to deliver effectors directly from the bacterial cytoplasm into host cytoplasm [16] as observed for EspF of the enteropathogenic *Escherichia coli* [6]. It is important to note that, to impact mitochondria, effectors do not necessarily have to enter host cells. Indeed, pore-forming toxins can induce mitochondrial dysfunction and organelle fragmentation just by inducing ion fluxes through the plasma membrane (e.g. listeriolysin (LLO) of *Listeria monocytogenes* [17]). Once in host cells, toxins that directly target mitochondria have to enter and reach the appropriate mitochondrial sub-compartment. They usually interact with mitochondrial translocase complex to be imported (e.g. OMM: *Neisseria* PorB [18], IMM: *H. pylori* VacA (vacuolating cytotoxin A) [15] and mitochondrial matrix: enteropathogenic *E. coli* MAP effector [19]).

As mitochondria evolved from an ancestral bacterium, some similarities are observed between proteins containing mitochondrial targeting sequence (MTS) and sequences targeting to bacterial cytoplasmic membrane such as the presence of a cleavable hydrophobic domain containing non-conserved amino

acid sequences that are composed of positively charged residues located at the N-terminal domain of the protein [20,21]. Furthermore, *Neisseria* PorB uses an evolutionary conserved “bacterial-like” machinery to reach the OMM of infected cells. Indeed, the SAM complex, the functional equivalent of  $\beta$ -barrel assembly machinery located at outer membrane of gram-negative bacteria, is composed of several subunits [18]. Even if the different subunits are essential for the insertion of eukaryotic  $\beta$ -barrel protein into the OMM, PorB insertion is exclusively dependent on the Sam50 subunit, the only subunit for which a bacterial homolog does exist [18].

### 3. Energy metabolism

In mammalian cells, ATP is mainly produced from the catabolism of glucose and fatty acids by co-regulated and interconnected pathways that involves glycolysis and  $\beta$ -oxidation, respectively, followed by TCA cycle and OXPHOS. After its entry in the cell, glucose is phosphorylated by hexokinases forming glucose-6-phosphate (G6P), which prevents the release from the cell and allows its glycolytic degradation [22]. Glycolysis is a cytosolic 10-reactions metabolic pathway leading to the conversion of G6P into two molecules of pyruvate accompanied by the production of two ATP and two NADH. The pyruvate is then imported and converted in acetyl-CoA in the mitochondrial matrix, where it will be fully oxidised into  $\text{CO}_2$  by TCA cycle, allowing the formation of more reducing molecules such as NADH and  $\text{FADH}_2$ . Reducing agents, including those generated by FAO are then oxidised, via the OXPHOS to generate a proton gradient cross the IMM. The proton-motive force of this gradient will next be used by the Fo-F1 ATP synthase (complex V) to produce ATP [22]. Besides the production of ATP, mitochondria also contribute, at least partly, to the biosynthesis of lipids [23], steroids, amino acids, nucleotides and in the assembly of iron-sulphur clusters [24].

#### 3.1. Mitochondrial metabolism and immune response

Unlike the paragraphs about mitochondrial ROS, calcium and morphology described below in which we will develop examples of direct manipulation of mitochondrial functions by bacterial effectors, the present section about metabolism will only present general and global impact of bacterial infection on cell metabolism, that affect mitochondria. Some of the examples described might thus refer to indirect effects of bacterial infection on mitochondria, taking part in a broader metabolic reprogramming during cell activation that might subsequently affect the organelle.

While the common pathway to produce energy from glucose in mammalian cells includes the OXPHOS [22], in some conditions, cells use preferentially and almost exclusively activated glycolysis to produce ATP, even in the presence of oxygen, a condition that coincides with a repression of the TCA cycle and the OXPHOS [9]. This phenomenon, called aerobic glycolysis or Warburg effect, was first described in tumour cells in which it allows to supply the anabolic demand associated with the high proliferation of these cells [25]. Indeed, the partial glucose oxidation provides precursors for the biosynthesis of carbohydrates, fatty acids and amino acids as well as nucleotides and NADPH by the pentose phosphate pathway (PPP), a pathway that also oxidises G6P [26]. As the biosynthesis of new molecules consumes both glycolysis and TCA cycle intermediates, anaplerotic reactions are required to replenish those pathways. One of those reactions is the glutaminolysis that consists in the degradation of glutamine into  $\alpha$ -ketoglutarate, an intermediate of the TCA cycle [9].

More recently, several studies have shown that the aerobic glycolysis can contribute to different physiological processes such as the maturation/differentiation and the activation of immune

cells during infection, including a severe metabolic reprogramming [22]. In fact, activated innate immune cells, including neutrophils and dendritic cells (DCs), mostly rely on glycolysis and glutaminolysis to produce their energy [22]. In neutrophils, this metabolic shift from oxidative metabolism to glycolysis is under the control of Toll-like receptor (TLR) induced hypoxia-inducible factor 1 alpha (HIF-1 $\alpha$ ) [22]. It increases the production of NADPH by the PPP, which is required to fuel the oxidative burst by NADPH oxidase (NOX) system activated in response to microbial exposure [22]. However, even if the mitochondrial respiration is not required for cell ATP supply, the mitochondrial membrane potential is involved in the temporal regulation of apoptosis induction following neutrophil activation as the elimination of activated neutrophils by this cell death pathway is crucial to prevent unnecessary extended inflammation [27]. In DCs, the reprogramming during activation is dependent on TLR-induced HIF-1 $\alpha$  and phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt/PKB) pathway and seems to be required for the antigen presentation and cytokine production [22]. This metabolic shift could also be supported by a stronger recruitment of the glycolytic enzyme hexokinase II (HKII) to the mitochondria [28].

Beside immune cell activation, metabolism is also involved in cell differentiation and polarisation. Indeed, macrophages differentiate from circulating monocytes once they infiltrate tissues. Circulating monocytes, according to the environment they encounter, are immunologically imprinted to tolerant (immunosuppression) or trained (innate immune memory which is antigen unspecific) [29]. This imprinting will determine functional fate of monocytes and macrophages, which is caused by a glycolytic switch under the control of the activation of Akt/mammalian target of rapamycin (mTOR)/HIF-1 $\alpha$  pathway [30]. Macrophages can display different phenotypes according their location and the type of stimuli they respond to. The two best phenotypes characterised are M1 and M2 macrophages [22]. The polarisation M1 (classical activation) is induced in response to different pathogen associated molecular pattern (PAMPs) such as lipopolysaccharide (LPS) and interferon-gamma ( $\text{IFN}\gamma$ ). On the other hand, the M2 polarisation (alternative activation) is developed in response to interleukins-4 and -13 (IL-4 and IL-13), two cytokines. M1 macrophages take part in the acute phase of inflammation and are more efficient to eliminate bacteria while M2 macrophages are mainly involved in the resolution phase of inflammation and healing process [31]. Classical macrophage activation in response to LPS exposure is linked to the activation of glycolysis [32]. In addition, in LPS-stimulated macrophages, glutaminolysis-derived  $\alpha$ -ketoglutarate leads to the accumulation of succinate, which, once exported from the mitochondria, is able to stabilise HIF-1 $\alpha$ , the master regulator of metabolic switch associated with macrophage classical activation [32]. However, mitochondrial metabolism is also involved in M1 macrophage functions as mtROS take part in their bactericidal activity and pro-inflammatory cytokine secretion (see Section 4.1.1).

While M1 macrophages mainly rely on aerobic glycolysis to acquire their bactericidal activity, M2 macrophage activation is supported by FAO and mitochondrial oxidative metabolism. This metabolic programme is regulated by signal transducer and activator of transcription 6 (STAT6), a master regulator of Th2 polarisation in the immune response. This transcription factor controls the expression of peroxisome proliferator-activated receptors (PPARs) and PPAR gamma coactivator-1 beta (PGC-1 $\beta$ ), well-known regulators that promote mitochondrial biogenesis to sustain this activation [33].

Adaptive immune cells, undergo several phases after an activating/challenge stimulus. Indeed, T lymphocytes, after their activation by the DC-presented antigen recognition, will proliferate into clonal populations and then establish an antigen specific memory [22]. At the basal state,  $\text{CD4}^+$  T-cells produce energy using



the oxidation of glucose and fatty acids and the OXPHOS pathways [34]. When activated, they also switch from oxidative metabolism to glycolysis and glutaminolysis. The glycolytic switch is also induced in a PI3K/Akt-dependent pathway, in response to CD28 binding [35]. Additionally, T-cell receptor (TCR) binding, activates extracellular signal regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) pathway, that in turn, induces glutamine uptake and promote the expression of catabolic enzymes of the glutaminolysis [36]. Metabolism might also play an important role in the activation of CD4<sup>+</sup> T-lymphocytes by controlling the expression of key signalling molecules, such IL-2 and IFN $\gamma$ . The mechanism would involve glyceraldehyde 3-phosphate dehydrogenase (GAPDH), one of the enzymes of the glycolysis, that binds the 3'-untranslated region (UTR) of IL-2 and IFN $\gamma$  mRNAs leading to the inhibition of their translation. When glycolysis is activated in T cells, GAPDH is required to promote the glycolytic flux and, consequently, is not able to bind these mRNAs anymore, allowing the synthesis of IL-2 and IFN $\gamma$  by activated CD4<sup>+</sup> T cells [37].

Memory T cells are also modulated by metabolism. For instance, CD8<sup>+</sup> T cells are able to enter in quiescent state to participate in the memory response, allowing faster and stronger activation in response to subsequent pathogen exposure. Unlike activated T cells, memory T cells produce their energy by the mitochondrial oxidative metabolism and especially by the FAO [38]. For example, TNF $\alpha$  receptor-associated factor 6 (TRAF6) induces FAO and mitochondrial biogenesis required for the establishment of a memory response against *L. monocytogenes* and this process is dependent on AMP-activated protein kinase (AMPK) signalling [38]. The importance of metabolism for T-cells during bacterial infection has recently and extensively been addressed [39].

This shift from oxidative metabolism to glycolysis observed during immune cell activation seems to be a general response to microbial exposure as it is also observed in various tissues such as liver cells from infected mice exposed to the pathogenic bacteria *Salmonella enterica* serovar typhimurium (*S. typhimurium*), lung cells from *Mycobacterium tuberculosis*-infected mice, intestinal epithelia from *L. monocytogenes*-infected mice and HepG2 cells infected by *Chlamydia pneumoniae*. In all these models, it was also shown that the metabolic reprogramming was under the control of HIF-1 $\alpha$  [9].

### 3.2. Manipulation of mitochondrial metabolism by bacterial pathogens

*Legionella pneumophila* is known to secrete a mitochondrial carrier protein during infection. This effector, targeted to the inner mitochondrial membrane, is able to transport ATP from the matrix to the IMS. However, the mechanism by which this effector supports *L. pneumophila* infection still needs to be determined [40]. As Eisenreich and collaborators have recently and extensively reviewed the multiple facets by which metabolism could be affected/manipulated by bacterial pathogens [9], we will focus in the rest of the review on mitochondria-associated cell signalling and mitochondrial messenger production affected by bacteria.

## 4. Mitochondria-associated cell signalling

### 4.1. mtROS and mitochondria

Mitochondrial respiration is responsible for the production of mtROS as metabolic by-products. Indeed, mtROS are generated through the monovalent reduction of a molecule of O<sub>2</sub> by electrons leaking from the mitochondrial electron transport chain (ETC) [41] (Fig. 1A). mtROS abundance is however tightly regulated at two different levels: their production and their degradation. First, the quantity of mtROS produced depends on the mitochondrial

respiration rate. Indeed, an increase in mtROS production is observed when the proton-motive force is enhanced [41]. Additionally, it has been suggested that uncoupling protein 2 (UCP2), a ubiquitously expressed uncoupling protein, is involved in the regulation of mtROS production [42,43] as, when activated, it reduces the proton gradient across the IMM. However, it is not clear whether UCP2 down-regulates mtROS production only through its putative uncoupling activity or by the other mechanism such as the regulation of the glutathione system [44].

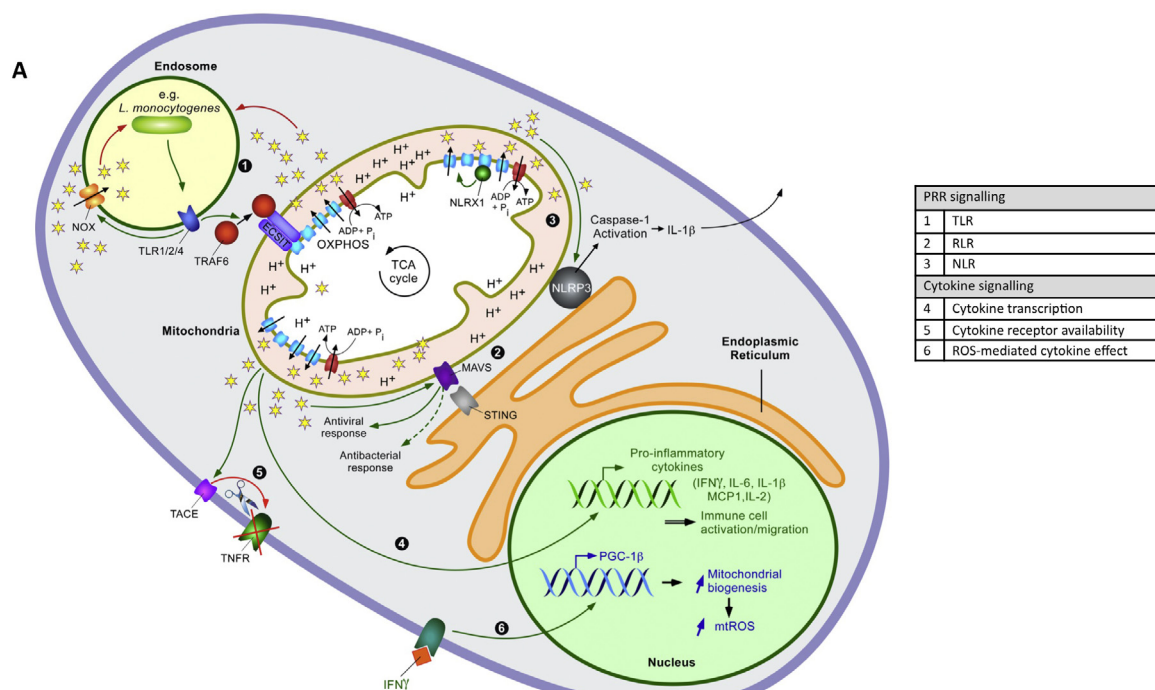
mtROS degradation is also tightly regulated. Indeed, O<sub>2</sub><sup>•(-)</sup> are converted in H<sub>2</sub>O<sub>2</sub> by superoxide dismutases (SODs). The three members of this enzyme family display different locations in the cell: SOD1 located in the mitochondrial IMS and cytosol, SOD2 in the mitochondrial matrix and SOD3 is an extracellular form. H<sub>2</sub>O<sub>2</sub> is then able to diffuse freely across the mitochondrial membrane to reach to cytosol. In addition to SODs, there are other enzymes that are able to detoxify H<sub>2</sub>O<sub>2</sub>: peroxyredoxins (PRXs), glutathione peroxidases (GPXs) and catalase. On the one hand, GPXs are thought to scavenge mtROS when found at high levels to prevent damages in the organelle and keep the concentration of mtROS in the range of concentrations that is compatible with ROS signalling function. On the other hand, PRXs, which have a high substrate affinity and are highly expressed, are thought to be involved in the termination of mtROS signalling that occurs at low concentrations [41]. An important point that needs to be taken into account in the modulation of mtROS signalling is the mitochondrial location itself. Indeed, mtROS are short-lived molecules, so their efficiency depends on the proximity between the site of production and the site of targets [41].

In all of the studies mentioned in this review, it is assumed that mtROS are only generated in response to bacteria exposure. However, it is important to note that most of the studies referenced below detected and/or quantified (mitochondrial) ROS content using older technologies based on fluorescent probes such as dihydroethidium (DHE and MitoSox, its mitochondrial targeted form) or dichlorodihydrofluorescein (DCF) probes. These probes, even if commonly used and accepted, present some disadvantages (when compared to more recent methods) such as a lack of specificity or even an artificial induction of ROS production when too high concentrations are used as they can react with oxygen and thus artificially elevate ROS abundance. In the future, studies to analyse ROS in cells exposed to bacteria should opt for new detection methods such as fluorescent protein-based redox probes. For additional information, Dikalov and Harrison recently reviewed the advantages and limits of various old and recent methods for ROS detection [45].

#### 4.1.1. mtROS and immunity

**4.1.1.1. mtROS and PRR.** mtROS have been reported to be involved in the clearance of different intracellular pathogens such as *L. monocytogenes*, *S. typhimurium* or *Toxoplasma gondii* [43,46–48]. During infection, mtROS production is induced in response to PRR activation. PRR-induced signalling leads to the activation of nuclear factor-kappa B (NF $\kappa$ B) and interferon regulatory factor (IRF) pathways, that both promote the expression of pro-inflammatory cytokines and chemokines as well as type I interferon [49]. Those mediators are part of an arsenal required for the development of an adequate immune response leading to the eradication of pathogens [49]. PRR family contains three different classes of receptors sensing different PAMPs/DAMPs leading to the development of an effective immune response: TLR, Rig-I like receptor (RLR) and NOD-like receptor (NRL) [49].

**TLR** are receptors located at the plasma membrane or at the endosome/endoplasmic reticulum (ER) membranes that can sense PAMPs associated with bacteria, viruses, fungi and parasites



## B Examples of bacterial pathogens modulating mtROS

Intracellular				Extracellular	
<i>M. tuberculosis</i>	<i>C. trachomatis</i>	<i>E. chaffeensis</i>	<i>P. aeruginosa</i>	<i>H. pylori</i> / Heat killed <i>E. coli</i>	ASD-associated microbiome ( <i>Clostridia</i> , <i>Bacteroidetes</i> and <i>Desulfovibrio</i> )
Eis	T3SS unknown effector	T4SS mitochondrial-targeted effector	LsfA	LPS	PPA and SCFA
Macrophages	Epithelial cells	Monocytes/Macrophages	Macrophages/Lung	Gastric epithelial cells / Liver cells	Gut and Brain
Acetylation of MKP7 Decrease of JNK phosphorylation Decrease of ROS production	Cytosolic K <sup>+</sup> efflux NOX ROS production NLRX1 activation mtROS production NLRP3 activation	Up-regulation of MnSOD Increase in ROS detoxification	ROS detoxification (impact on mtROS: unknown)	TLR4-induced (mt)ROS production (mt)DNA damages induction	Cytoplasmic accumulation Intracellular acidification Carnitine-dependent mitochondrial dysfunction and mtROS production
AUTOPHAGY INHIBITION	CASPASE-1-MEDIATED BACTERIAL GROWTH	APOPTOSIS INHIBITION	BACTERIAL SURVIVAL	INFECTION-ASSOCIATED ONCOGENESIS	ASD-RELATED BEHAVIOUR

**Fig. 1.** Mitochondria-associated redox signalling in host–pathogen relationship. (A) mtROS and immune response. mtROS (yellow stars) are produced as by-products of mitochondrial respiration. They are involved in PRR regulation. (1) Activation of TLR1/2/4 leads to mtROS production in a TRAF6/ECSIT-dependent manner. These mtROS participate to bacterial killing such as *L. monocytogenes* [46,47]. (2) mtROS promote RLR signalling by activating MAVS dependent pathway. MAVS signalling is primarily involved in antiviral response (full arrow) but it can also take part in bacterial-induced interferon- $\beta$  production (e.g. during *L. pneumophila* infection) (dotted arrow) [53,56]. (3) NLRX1 is able to promote mtROS production [120] while mtROS can induce NLRP3 [58]. mtROS can also modulate cytokine signalling (4) by inducing their transcription [69,70], (5) by modulating their receptor availability [71] or (6) by taking part to the cytokine-induced effect [46]. Green arrows: positive effect; red arrows: detrimental effect. (B) Examples of bacterial pathogens manipulating mtROS production: *M. tuberculosis* [73], *C. trachomatis* [76], *E. chaffeensis* [84], *P. aeruginosa* [83], *H. pylori* [79], heat killed *E. coli* [80] and ASD-associated microbiome [82]. The table summarises the bacterial specie, the effector involved, the target cell/tissue, the effector impact on host cell and the final phenotype observed. Abbreviations: ECSIT (evolutionarily conserved signalling intermediate in Toll pathways), FAO (fatty acid  $\beta$ -oxidation), IFN $\gamma$  (interferon gamma), IL-1 $\beta$ /2/6 (interleukin-1 $\beta$ /2/6), MAVS (mitochondrial antiviral signalling protein), MCP1 (monocyte chemoattractant protein-1), mtROS (mitochondria reactive oxygen species), NLR (NOD-like receptor), OXPHOS (oxidative phosphorylation), PGC-1 $\beta$  (peroxisome proliferator-activated receptor gamma coactivator 1- $\beta$ ), STING (stimulator of interferon genes), TACE (TNF $\alpha$  converting enzyme), TCA (tricarboxylic acid), TLR (Toll-like receptor), TNFR (TNF $\alpha$  receptor) and TRAF6 (TNF receptor-associated factor 6).

[49]. In LPS-stimulated macrophages, UCP2 expression is down-regulated through cell signalling dependent on Jun N-terminal kinase (JNK)/p38 pathways, promoting mtROS production followed by a subsequent MAPK activation and the onset of respiratory burst [43]. More recently, it was shown that, in RAW264.7 macrophages, TLR signalling is able to trigger the mtROS production and the recruitment of mitochondria around the phagosome [47]. Indeed, in response to TLR1/2/4 binding, TRAF6 is translocated at the mitochondria where it interacts with evolutionarily conserved signalling intermediate in Toll pathways (ECSIT), a protein that contributes to the ETC complex assembly. TRAF6 interaction with ECSIT then promotes its accumulation at the mitochondrial surface, triggering an increase in mtROS production [47] (Fig. 1A).

It is important to note that while TLRs induce pro-inflammatory responses in order to eradicate invading pathogens, they also take part in resolution of this inflammation/oxidative stress as in order to maintain mitochondria integrity and to prevent cell death, host cells are able to respond by the activation of a transcriptional

programme leading to the expression of genes involved in anti-inflammatory and anti-oxidant responses such as IL-10 and heme oxygenase-1 (HO-1), respectively. This process is accompanied by a stimulation of mitochondria biogenesis [50]. For example, a LPS-mediated TLR4 challenge might also lead to the development of a cell response aiming to recover from energy impairment [51]. It is known that, in macrophages, the early effect of a LPS stimulation of TLR4 is mitochondrial dysfunction, which, if prolonged, can be detrimental for the host cell. TLR4, by its interaction with myeloid differentiation primary responses gene 88 (MyD88) and TIR-domain-containing adapter-inducing interferon- $\beta$  (TRIF), is able to activate the pro-survival PI3K/Akt pathway leading to the expression of the mitochondrial transcription factor A (TFAM). This signalling pathway leads, *in fine*, to an increase in the abundance and activity of two different subunits of the respiratory chain complex IV, cytochrome oxidase-1 and -4 (COX1 and COX4), encoded by the mitochondrial and nuclear genomes, respectively [51].

Alternatively, mtROS can also regulate TLR signalling. For example, nuclear factor of activated T-cells 5 (NFAT5) is a

transcription factor activated in response to different stimuli such as LPS-induced TLR4 signalling or NaCl induced-osmotic shock [52]. A recent study showed that these stimuli lead to a ROS-dependent expression inhibition of a subset of NFAT5 target genes [52]. In addition, these authors have shown that ROS origin seems to be crucial to qualitatively select genes regulated by this factor as cytosolic ROS (generated by the xanthine oxidase) will promote a pro-inflammatory response (IL-6 transcription) while mtROS induce hypertonic response [52].

**RLR** are cytoplasmic receptors binding mostly virus-associated PAMPs such as double stranded RNA [49]. Mitochondria are also known to provide a platform for signalling induced by two different RLR: RIG-I and melanoma differentiation-associated gene-5 (MDA-5). RIG-I or MDA5 has been shown to signal through mitochondrial antiviral signalling protein (MAVS), a nuclear encoded protein located in OMM [53]. When activated, MAVS interacts with stimulator of interferon genes (STING) located in the ER membrane and TRAF3/6 leading to IRF3/7 and NFκB activation [49].

The expression of RLR might also be regulated and controlled by mtROS; cells deficient for autophagy protein-5 (ATG5<sup>-/-</sup> cells), that contain high mtROS concentrations, up-regulate RLR expression as well as active signalling and are also more resistant to viral infection [54] (Fig. 1A). However, mtROS are not the only mitochondrial feature to regulate MAVS pathways. Indeed, mitochondrial dynamics and membrane potential are also required for efficient MAVS signalling [53]. Furthermore, different mitochondrial proteins have been reported to act as activators such as TOM70 or inhibitors such as mitochondrial E3 ubiquitin protein ligase 1 (MUL1) of MAVS signalling [53].

Interestingly, the mitochondrial protein ECSIT is not only involved in the regulation of TLR signalling as it also participates in RLR induced signalling. In fact, it has been shown that tripartite motif 59 (TRIM59) is able to inhibit RLR-induced IRF and NFκB activation by its interaction with ECSIT [55]. While type I interferon is typically considered as an antiviral cytokine [56], it has been shown that the gene encoding this cytokine is also induced in response to bacterial infection [56]. For example, it has been shown that RIG-I/MDA-5/MAVS pathway is involved in the induction of IFNβ in response to *L. pneumophila*, a facultative intracellular bacterium (Fig. 1A). Furthermore, authors showed that SdhA, a bacterial effector translocated in host macrophages during *L. pneumophila* infection, is able to inhibit the IFNβ production probably by inhibiting the MAVS protein [56].

**NLR** is the third type of PRRs. In response to the detection of cytosolic PAMPs/DAMPs, NLRs can induce a caspase 1-dependent maturation of pro-inflammatory cytokines such as IL-1β and IL-18 [49]. The most-described family member is NLRP3 and recent studies showed that mitochondria play a crucial role in the activation of NLRP3 inflammasome by both the production of mtROS (Fig. 1A) and the mtDNA release out of the organelle [57,58]. NLRP3 activation requires the combination of two different signals. The priming signal, coming from TLR4 and cytokines receptors, that either induces NLRP3 transcriptional up-regulation [59] and/or NLRP3 deubiquitination, in a mtROS-dependent manner [60]. The second signal, which leads the oligomerisation, can be triggered by different PAMPs and DAMPs, including extracellular ATP, lysosomal destabilisation, (mt)ROS, oxidised mtDNA and pore forming toxins-induced ions fluxes such as K<sup>+</sup> efflux, a mechanism used by several bacteria such as *L. monocytogenes* or *Staphylococcus aureus* to activate NLRP3 inflammasome [61]. The molecular mechanisms by which those different signals activate NLRP3 are still poorly understood [61]. However, NLRP3 activation seems to depend on a NLRP3 translocation from ER to mitochondria, in a MAVS-dependent manner [62] [58]. In macrophages, mitochondrial dysfunction caused by NLRP3

inducers (such as nigericin and monosodium urate) leads to a decrease in NAD<sup>+</sup> concentration, which in turn leads to a decrease in sirtuin2 (SIRT2), a NAD<sup>+</sup>-dependent deacetylase, activity leading to the accumulation of acetylated α-tubulin that takes part in dynein-dependent mitochondrial transport by microtubules [63]. These authors suggest that cytoskeleton-dependent transport of mitochondria would promote NLRP3 activation by apposing mitochondrial apoptosis-associated speck-like protein containing a CARD (ASC) and NLRP3 (on the ER) [63]. No matter what the molecular mechanism of activation is, it seems that mitochondrial dysfunction-mediated NLRP3 activation is also required to control the infection of several facultative or obligate intracellular bacteria such as *Brucella abortus* [64] or *C. pneumoniae* [65]. Interestingly, some bacterial pathogens are also able to modulate NLR activation as it is known that NLRP3 can be inhibited by toxins secreted by several bacteria such as *Yersinia enterocolitica* (YopE and YopT), *Yersinia pseudotuberculosis* (YopK), *M. tuberculosis* (Zmp1) and *Streptococcus pneumoniae* (pneumolysin). These toxins can either block inflammasome oligomerisation, prevent ligand to be recognised or modulate NFκB/MAPK signalling to impair IL-1β expression and activation [66].

Finally, bacteria activate other inflammasome such as absent in melanoma-2 (AIM2) inflammasome, which recognise cytosolic double stranded DNA. It has recently been shown that mtROS might potentiate indirect activation of AIM2 inflammasome in response to *Francisella* infection [67]. These authors showed that mtROS might be involved in the regulation of AIM2 activation by promoting bacterial DNA release. Indeed, *Francisella novicida* (a non-virulent strain) is susceptible to mtROS induced-membrane damages, while *Francisella tularensis* (a pathogenic strain) is not, leading to a differential DNA release in the cytosol and a differential AIM2 activation [67].

**4.1.1.2. mtROS induction during infection.** As mtROS production in response to infections is dependent on respiration, different strategies can be developed to increase the respiratory flow: (1) promoting the mitochondrial biogenesis and (2) enhancing OXPHOS activity through FAO fuelling. Indeed, those hypotheses were verified in infection models. First, in IFNγ-stimulated macrophages, the activation of oestrogen-related receptor alpha (ERRα) and PGC-1β, two key regulators of mitochondria biogenesis [50], leads to an increase in the expression of several OXPHOS components [46]. Second, a recent study on *S. typhimurium* infection suggests that mtROS production in response to bacterial infection depends on FAO [68]. Indeed, the activation of glucocorticoid receptor and the stimulation of Janus kinase (JAK)/STAT signalling pathway promote a CCAAT enhancer binding protein (CEB/P) and STAT3-dependent transcription of immuno responsive gene-1 (*Irg1*) encoding a mitochondrial protein that regulates the mitochondrial uptake and FAO, and is required (but not sufficient) for mtROS production by murine macrophages in response to *S. typhimurium* infection [68].

**4.1.1.3. mtROS and cytokine signalling.** mtROS are known as modulators of cytokine signalling involved in immunity, as demonstrated in various models [41]. The first level of regulation is the modulation of cytokine synthesis. Indeed, an increase in mtROS abundance is observed in the spleen of *L. monocytogenes*-infected mice invalidated for UCP-2 (Ucp-2<sup>-/-</sup> mice), a phenomenon that is accompanied with both an increase in expression of pro-inflammatory cytokines such as IFNγ, IL-6, IL-1β and monocyte chemotactic protein-1 (MCP1) and a delay of infected animal death [69] (Fig. 1A). Additionally, in T lymphocytes activation, mtROS generated in response to TCR-mediated Ca<sup>2+</sup> influx, allow the activation of the transcription factor NFAT and subsequent IL-2 production [70]. Secondly, mtROS are not only



able to regulate cytokine expression but also control their signalling efficiency by indirectly limiting the availability of their receptor. For example, TNF receptor-1 (TNFR1) binding leads to a  $\text{Ca}^{2+}$ -dependent mtROS production [71]. Subsequently, mtROS are able to activate the protease TNF- $\alpha$  converting enzyme (TACE) leading to the receptor shedding which limits further or prevent uncontrolled inflammation [71] (Fig. 1A). Finally, mtROS can also take part in the cytokine-induced downstream signalling as it has been shown, in *L. monocytogenes* infected-macrophages, that bactericidal effect of IFN $\gamma$  is dependent on PGC-1 $\beta$ /ERR $\alpha$  induced mtROS [46] (Fig. 1A). The importance of mtROS in the regulation of a proper immune response signalling was recently supported by the observation that splenocytes, isolated from Ucp-2 $^{-/-}$  mice, that contain high mtROS concentrations, failed to produce immunoglobulin G (IgG) in response to LPS stimulation both in vitro and in vivo [72].

In summary, it seems that ROS can participate to the immune response in different ways: ROS can induce bacterial damages leading to antimicrobial activity or are able to modulate infected eukaryote cell signalling.

#### 4.1.2. Modulation of redox signalling by bacteria

Considering the deleterious effects of ROS for bacteria in infected cells, it is not surprising that several bacteria developed strategies to limit their production. For example, *M. tuberculosis* is known to decrease ROS production by the N-acetyltransferase activity of the effector enhanced intracellular survival (Eis). Indeed, when cells are infected with mutant *Mycobacteria* that do not express Eis, an increase in JNK dependent-ROS production accompanied by the stimulation of autophagy and an inflammatory response is observed [73]. It seems that Eis down-regulates the LPS-mediated phosphorylation of JNK by acetylating and activating MAPK phosphatase-7 (MKP7), a JNK-specific phosphatase [74] (Fig. 1B).

If ROS are primarily a host defence mechanism, they can also be detrimental for the host cells. The ROS dual effect in infected cells is well illustrated in *M. tuberculosis*-infected macrophages. Indeed, tumour necrosis factor alpha (TNF $\alpha$ ) binding to its receptor triggers mtROS production, which initially take part in bactericidal activity [75]. However, in this model, mtROS will rapidly induce necrosis, which results in bacterial release in the permissive extracellular environment [75]. Some bacteria even manipulate host cell to promote mtROS production. Indeed, *Chlamydia trachomatis*, an intracellular bacterium responsible for a severe sexually transmissible disease, induces ROS production to promote its growth [76]. Indeed, once inside the epithelial cell, the bacteria secrete effectors into the cytoplasm through the type 3 secretion system (T3SS), which leads to a potassium exit from the cytoplasm. This potassium efflux leads to ROS production by the NOX system, which in turns, diffuse to the mitochondrial matrix and activate NLRX1 to produce more ROS in the form of mtROS [76] (Fig. 1B). ROS induction in *C. trachomatis* infected cells takes part in the activation of caspase-1 by the NLRP3 inflammasome, a process required for its intracellular growth [76,77].

mtROS can also directly cause the symptoms associated with the disease. For example, during *H. pylori* infection, redox signalling takes part in the inflammation and oncogenesis of the gastric mucosae observed in patients [78], a phenotype that could be explained by the mtROS production triggered by the pathogen and its ability to inhibit DNA repair machinery causing nuclear and mitochondrial genomic instability [78] (Fig. 1B). Indeed, mtDNA, even in the nucleoid organisation, is particularly sensitive to this genomic instability considering its proximity to the site of mtROS production and the absence of histone complexes [79]. TLR4 dependent mtROS accumulation also mediates mtDNA damages as observed in heat killed *E. coli* injected mice [80] (Fig. 1B).

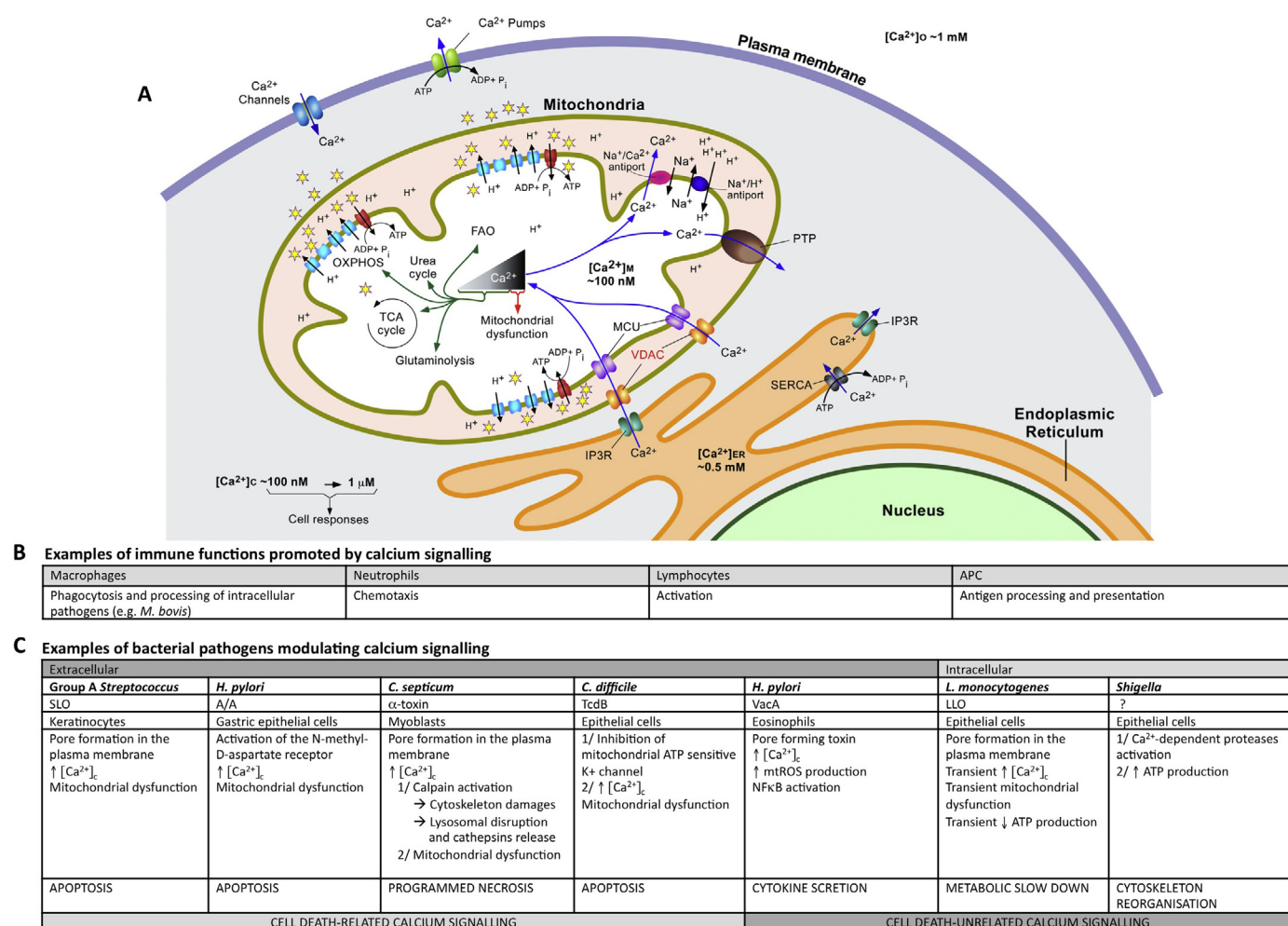
One can also mention that pathogenic bacteria are not the only ones to induce mtROS production. Indeed, fermentation products of the commensal gut microbiome, such as short chain fatty acids (SCFA), are also known to induce mtROS production [81]. It is interesting to note that propionic acid (PPA) overproduction, a metabolic product of bacterial fermentation from commensal bacteria of patients with ASD (*Autism Spectrum Disorders*) such as *Clostridia*, *Bacterioidetes* and *Desulfovibrio* might also be involved in the induction of mitochondrial dysfunction observed in patient gut and brain [82]. PPA is a low molecular weight organic acid that can accumulate in the cytosol causing intracellular acidification and subsequent carnitine dependent-mitochondrial dysfunction that impairs fatty acid metabolism [82] (Fig. 1B).

As mentioned previously, regulation of ROS content occurs at two different levels: their production and their scavenging/detoxification rate. Hereafter are two examples of bacteria whose virulence is dependent on the modulation ROS content by interfering with their detoxification. Firstly, most of bacteria possess their own ROS detoxification enzymes. However, it was unclear if those enzymes take part in the bacterial virulence during infection. A recent study analysed the potential implication LsfA, a 1-cys PRX of the opportunistic pathogen *Pseudomonas aeruginosa* during infection. It was shown that LsfA is involved in the bacterial resistance to NOX burst occurring in infected macrophages [83]. Even if no data are available about the impact of those enzymes on mitochondrial ROS, it is reasonable to think that they might be involved in their detoxification as well as it appears that the mtROS participate to bacterial killing in infected macrophages [84] (Fig. 1B). It would thus be interesting to study the impact of those bacterial enzymes on the mitochondrial ROS cytotoxicity. Secondly, it is crucial for some bacteria to prevent programme cell death of their host cell. For example, *Ehrlichia chaffeensis* secretes a mitochondrial-targeted effector during monocyte/macrophage infection. This bacterial toxin seems to up-regulate the mitochondrial detoxification enzyme MnSOD/SOD2, which results in a lower ROS content and an inhibition of apoptosis [84] (Fig. 1B). However, a more systematic analysis and screen of the different antioxidant enzymes would be necessary to address the origin and mechanisms leading to the altered ROS production in response to bacterial pathogens.

#### 4.2. Calcium signalling and mitochondria

Free calcium bivalent cation is a key signalling molecule in eukaryotic cells. In basal state, cytosolic and mitochondrial calcium concentrations ( $[\text{Ca}^{2+}]_c$ ;  $[\text{Ca}^{2+}]_{mt}$ , respectively) are very low (ranging between 100 and 200 nM) when compared with millimolar concentration found in the extracellular fluids [85] (Fig. 2A). This gradient concentration is maintained by the action of several ligand and/or voltage-dependent channels (uniporters and antiporters) and ATP-consuming pumps for calcium located in the plasma membrane and membranes of organelles such as mainly the ER and mitochondria [86]. In response to various stimuli, channels located in the plasma membrane or the ER membrane do open, allowing calcium (in)fluxes or release, that leads to a sudden (and often transient) 10-fold increase in the cytosolic calcium concentration ( $>1 \mu\text{M}$ ) (Fig. 2A). These changes in calcium concentration can be translated into different cell responses such as muscle contraction or vesicle secretion depending on cell type and conditions [85]. Even if the major intracellular calcium store is the ER (a calcium reservoir in which calcium concentration reaches 0.5 mM), mitochondria also cycle and/or accumulate calcium in the matrix [86] (Fig. 2A). In conclusion, mitochondria regulate calcium signalling by taking and releasing  $\text{Ca}^{2+}$  locally, allowing changes in concentration in microdomains [86]. Indeed, when calcium concentration increases in the cytosol, calcium also





**Fig. 2.** Mitochondria-associated calcium signalling in host–pathogen relationship. (A) Mitochondrial calcium regulation: The very low basal cytosolic calcium concentration is maintained by the action of several channels and ATPases pumps located either at the plasma, ER and mitochondrial membranes. Calcium release from the mitochondrial matrix is mediated by sodium exchange from the IMM to the matrix, which in turn is exported from the matrix by the sodium/proton exchange (calcium fluxes are shown in blue). Changes in cytosolic calcium concentration can lead to the activation of signalling pathways. At moderate concentrations, calcium promotes several mitochondrial functions (green arrows) such as TCA cycle, OXPHOS (and subsequent mtROS production (yellow stars)), FAO, glutaminolysis, and urea cycle [89]. However, at high concentrations, calcium induces mitochondrial dysfunction (red arrows), PTP opening and cell death [89]. (B) Examples of immune functions modulated by calcium signalling: macrophage phagocytosis [92], neutrophils chemotaxis [93], lymphocytes activation [95] and antigen processing and presentation by APCs [94]. (C) Examples of bacterial pathogens modulating calcium signalling: Group A *Streptococcus* [98], *C. difficile* [101], *H. pylori* [99,102], *C. septicum* [100], *L. monocytogenes* [102] and *Shigella* [103]. The table summarises the bacterial species, the effector involved, the target cell/tissue, the effector impact on host cell and the final phenotype observed. Abbreviations: A/A (ammonia/ammonium), APC (antigen presenting cell), FAO (fatty acid  $\beta$ -oxidation), IP3R (inositol-3-phosphate receptor), MCU (mitochondrial calcium uniporter), OXPHOS (oxidative phosphorylation), PTP (permeability transition pore), SERCA (sarco/endoplasmic reticulum calcium ATPase), SLO (streptolysin O), TCA (tricarboxylic acid), TcdB (*Clostridium difficile* Toxin B), VacA (vacuolating cytotoxin A) and VDAC (voltage-dependent anion channel).

accumulates in mitochondria that act as a “calcium buffer”. To reach the mitochondrial matrix, cytosolic calcium has to cross both mitochondrial membranes. The ions cross the OMM by the protein voltage-dependent anion channel-1 (VDAC1) also known as the mitochondrial porin that functions as a gatekeeper for the entry and exit of mitochondrial metabolites) and the IMM by the recently identified mitochondrial calcium uniporter (MCU), a highly specific transporter [87] (Fig. 2A). In some conditions, calcium export might be mediated by the PTP, even if this is still controversial [88] but calcium efflux could also be mediated by the exchange with sodium, which, in turn, is pumped outside of the matrix by a sodium/proton exchange [89] (Fig. 2A).

Calcium homeostasis is not only regulated by mitochondrial bioenergetics and redox status but also contributes to maintain several functions in mitochondria (Fig. 2A). Calcium, ATP and ROS concentrations are thus tightly interconnected and their regulation involves several crosstalks [89]. Firstly, calcium entry in the mitochondrial matrix is dependent on the mitochondrial

membrane potential. Secondly, calcium entry and accumulation into the matrix can be modified by ROS [89] while its positive effect on TCA and respiratory chain can modulate ROS formation [89]. Mitochondrial activity is dependent on calcium concentration as mitochondrial calcium stimulates the TCA cycle and OXPHOS through positive allosteric regulation of 3 enzymes (pyruvate dehydrogenase, isocitrate dehydrogenase and  $\alpha$ -ketoglutarate dehydrogenase) [89]. Calcium also stimulates enzymes belonging to (Cytochrome c oxidase, Fo-F1 ATP synthase) or regulates (glycerophosphate dehydrogenase and adenine nucleotide translocase) the OXPHOS [89]. Moderate increase in  $[Ca^{2+}]_{mt}$  therefore results in a higher ATP production [89]. However, calcium also regulates mtROS production. Indeed, increased calcium not only boosts the respiratory chain and thus the associated electron leakage [90], but also might dissociate cytochrome c from the IMM cardiolipin leading to an increase in electron leakage [89]. Calcium also regulates other mitochondrial functions than bioenergetics such as urea cycle [89] and the glutaminolysis [90].

If physiological range of calcium concentrations has a positive impact on different mitochondrial functions, sustained high concentration of this ion is detrimental for mitochondria and can induce mitochondrial dysfunction [89]. It is well described that calcium overload leads to an increase in the IMM permeability that can result to cell death [91].

#### 4.2.1. Calcium signalling and immunity

Mitochondria associated calcium signalling is involved in the regulation of the immune response. Calcium signalling promotes *Mycobacterium bovis* phagocytosis and processing in macrophages [92] (Fig. 2B). Secondly, mitochondrial UCP2 down-regulates the polymorphonuclear neutrophils (PMN) chemotaxis by increasing the  $[Ca^{2+}]_c$  also responsible for the increased expression of  $\beta_2$ -integrins allowing the initial firm adhesion of PMN to endothelial cells [93] (Fig. 2B). Finally, calcium also regulates adaptive immunity by promoting T lymphocytes activation by two mechanisms [94]. On the one hand, calcium controls the antigen processing and presentation in antigen presenting cells (APCs), a step that is required to get T lymphocytes activated (Fig. 2B). Indeed, it has been shown that the inhibition of Fo-F1 ATP synthase and MCU, both inhibit the antigen processing [94]. On the other hand, calcium can regulate T-cell activation itself. When exposed to an appropriate stimulus, mitochondria relocate near the immunological synapse to regulate the  $Ca^{2+}$  fluxes and provide local energy required for T-cell activation and cytokine secretion [95,96] (Fig. 2B).

The importance of calcium signalling during intracellular bacterial infection is now emerging. Interestingly, a recent screening (using a library of 640 drugs targeting various host cell functions (host-directed antimicrobial drugs)) devoted to test drugs able to make THP-1 cells resistant to four different bacteria (*B. abortus*, *Coxiella burnetii*, *L. pneumophila* and *Rickettsia conorii*) revealed that most of these drugs target either G protein-coupled receptors, membrane cholesterol distribution and intracellular calcium signalling [97]. The fact that numerous drugs modulating calcium signalling in infected cells were able to protect THP-1 cells against four different bacteria species that display different lifestyles emphasises the central role of calcium signalling during bacterial infections. In the following paragraph, recent mechanisms by which bacteria exert an impact on calcium homeostasis and calcium signalling will be discussed.

#### 4.2.2. Subversion of calcium homeostasis by bacteria

Bacterial pathogens can manipulate calcium signalling in eukaryotic cells in order to modulate mitochondria-dependent apoptosis. Group A *Streptococcus* (GAP), extracellular bacteria found on oropharynx and damaged skin, have to reach deeper tissues to cause infection [98]. The mechanism used by GAP to achieve this goal is to secrete streptolysin O (SLO), a pore-forming molecule that induces premature differentiation and apoptosis of keratinocytes. Those pores increase the permeability of the plasma membrane and allow calcium influx in the cytosol, leading to ER stress and mitochondrial dysfunction that subsequently triggers apoptosis [98] (Fig. 2C).

*H. pylori* infection leads to gastric cell death caused by the ammonia/ammonium (A/A) produced by the bacteria [99]. These authors showed that A/A-associated cytotoxicity was mediated by the activation of a N-methyl-D-aspartate receptor, which leads to calcium entry. Cytosolic calcium is thus first taken by the ER, mediated by the sarco/endoplasmic reticulum calcium ATPase (SERCA) pumps, and then transferred to mitochondria. Calcium also induces the transcription of cell death effectors such as BAK and BAX and activates calpain and cathepsin B, which both take part in A/A cytotoxicity. The consequences of these changes result in OMM damage, ATP depletion and finally cell death [99] (Fig. 2C).

*Clostridium septicum* is a commensal gut bacterium that can sporadically cause necrosis of the human skeletal muscle cells. These bacteria secrete  $\alpha$ -toxin in the extracellular environment that inserts into myoblast plasma membrane, forms a  $Ca^{2+}$  permeable channel, and triggers a calcium influx [100]. Subsequent calpain activation and release of cathepsins from lysosomes then modify cytoskeleton organisation. In these conditions, the increase in cytosolic calcium concentration also leads to mitochondrial dysfunction, a decrease in ATP production as well as nuclear DNA damages, and other hallmarks of necrosis [100] (Fig. 2C).

*C. difficile* is an extracellular bacterium responsible for pseudo-membranous colitis. *C. difficile* secretes toxins (TcdA and TcdB) that will disrupt the epithelial barrier. A recent study showed that TcdB seems to induce apoptosis in epithelial through the inhibition of the mitochondrial ATP sensitive potassium channel, which accompanied with an increase in  $[Ca^{2+}]_c$ , leads to mitochondrial membrane hyper-polarisation and apoptosis [101] (Fig. 2C).

While severe and sustained changes in calcium concentration in response to infection might lead, *in fine*, to cell death, some pathogens do subvert calcium signalling in a cell death-independent manner. For example, in eosinophils exposed to purified VacA, a pore forming toxin secreted by *H. pylori*, the toxin is able to induce  $Ca^{2+}$  release from intracellular stores. This increase in cytosolic calcium concentration leads to an increase in mtROS production, subsequent NF $\kappa$ B activation and chemokine production responsible for the recruitment of inflammatory cells in the gastric mucosae observed in infected patients [102] (Fig. 2C). Another illustration is given for LLO, a bacterial toxin secreted by *L. monocytogenes* during infection, is able to oligomerise to form pores in the plasma membrane of infected HeLa cells. The calcium influx and the metabolite leakage mediated by these pores induce a transient membrane potential disruption, mitochondrial fragmentation, and a decrease in the ATP production. The mitochondrial fragmentation might thus induce a “transient metabolic slow down” required for the establishment of the bacterial replicative niche [17] (Fig. 2C). Eventually, *Shigella* is an intracellular bacterium known to induce actin re-organisation during infection. A recent study showed that such cytoskeleton alterations limit the diffusion of small solutes and therefore lead to confinement of calcium signalling at bacterial entry sites [103]. This microenvironment promotes a long-lasting calcium signalling taking part in calcium dependent-protease activation, known to be involved in *Shigella* infection. This calcium rich environment also allows the induction ATP production required for the actin re-organisation by mitochondria that are trapped in the polymerised actin, while it prevents the spreading of apoptotic signalling associated with sustained and largely diffused high calcium concentrations at early stages of the infection [103] (Fig. 2C).

#### 4.3. mtDAMPs

As mentioned in Section 1, in addition to PAMPs, PRR also recognise DAMPs, molecular patterns that are sequestered in healthy tissues but exposed/released when cells are damaged, in response to trauma or necrosis for instance [49]. As mitochondria still display some prokaryote-related features, several mitochondrial components could be recognised as mitochondrial DAMPs (mtDAMPs).

Firstly, mtDNA, which like the bacterial genome is enriched in unmethylated CpGs [104] is able to activate TLR9 [105]. For example, injection of endogenously oxidised mtDNA (but not nuclear DNA) in mice leads to the development of arthritis in mice, a phenotype that could be supported by the fact that oxidised mtDNA induces NF $\kappa$ B-dependent TNF $\alpha$  production by monocytes/macrophages [106]. In addition, mtDNA are able to stimulate

neutrophils in response to TLR9 binding and activation [105]. However, it seems that, according to cell type, unmethylated CpG-oligodeoxynucleotide (CpG-ODN) is able to promote a pro- or anti-inflammatory response [107]. In immune cells such as macrophages, a chaperone Unc93b1 is able to shuttle TLR9 from the ER to the lysosomes, where receptor processing and binding by CpG-ODN leads to the classical MyD88-dependent pro-inflammatory response [107]. However, in non-immune cells, mitochondria take part in the development of an anti-inflammatory response. While in neurons and cardiomyocytes, Unc93b1 is less abundant and TLR9 is mainly found in the ER [107]. Consequently, TLR9 binding by CpG-ODN triggers a different signalling in those cell types. Indeed, in the ER, TLR9 interacts with SERCA2 (a calcium ATPase pump), decreases its activity limiting the calcium (re)entry in the ER lumen and subsequently reduces the calcium transfer to the mitochondria. A higher AMP/ATP ratio associated with lower mitochondrial matrix calcium concentration also leads to AMPK activation, a condition known to promote autophagy and cell survival [107,108].

Secondly, similar to prokaryotes, mitochondrial translation is initiated by the addition of N-formyl methionine [109]. Consequently, mitochondrial N-formyl peptides can trigger inflammation. They are recognised by formyl peptide receptor-1 (FPR-1) in PMN, which leads to their activation in a MAPK-dependent manner [110]. The same group also recently showed that mtDAMPs are able to promote PMN adherence to endothelial cells (EC) and enhance systemic endothelium permeability [111]. Additionally, other mitochondrial proteins, such as the mitochondrial transcription factor TFAM are also able to act as mtDAMPs [112]. TFAM release by necrotic cell potentiates cells to produce CXCL8 chemokine ligand-8 (CXCL-8) in response to N-formyl-peptide exposure [112]. In addition, some mitochondrial proteins, such as heat shock proteins of 60 and 70 kDa (HSP60 and HSP70) are still able to trigger inflammation via TLR4 activation in macrophages, leading to the production of IL-6 and TNF $\alpha$ , when they are released in the blood stream for example after surgery [113,114]. Furthermore, mitochondria extracts from H<sub>2</sub>O<sub>2</sub>-stressed THP-1 cells induce stronger TLR4-dependent inflammatory response when compared with mitochondrial extracts of control cells [115]. Eventually, very recently, a study showed that in response to trauma induced-cell injury, mtDAMPs released in the extracellular fluids could activate  $\gamma\delta$ -T lymphocytes (assessed by an increase in TLR expression and cytokines production), which are known to be involved in healing processes [116].

As mentioned in Section 1, mitochondrial morphology and functions are tightly related. It is thus not surprising that several bacterial pathogens can alter mitochondrial morphology during infection to manipulate mitochondrial functions.

## 5. Mitochondrial dynamics

In recent years, several bacterial effectors have been reported to induce or inhibit mitochondrial fragmentation to control different cell responses. While not exhaustive, here are few examples of recently characterised effectors known to modulate mitochondrial morphology during bacterial infection. First, as described in Section 4.2.2, subversion of calcium homeostasis by bacteria during infection, *L. monocytogenes* secretes a pore-forming toxin called LLO. Pores formed at the plasma membrane by this bacterial effector allow calcium influx in the cytoplasm leading to mitochondrial fragmentation [17]. These authors show that alterations of mitochondrial morphology occur rapidly after (or even before) bacterial entry, are transient and accompanied with a decrease in ATP production. They also hypothesised that mitochondrial fragmentation observed during *L. monocytogenes* infection might mediate a “metabolic slow-down” required for

the establishment of the bacterial replication niche [17] but this remains to be experimentally demonstrated. The mechanism involved in the LLO-induced fragmentation is not clear as LLO could induce an atypical fission mediated by a DRP1- and OPA1-independent mechanism [117]. Secondly, VacA is also a pore-forming toxin secreted during *H. pylori* infection that localises, at least partially, at the mitochondria. VacA would induce activation and recruitment of DRP1 resulting in mitochondrial fragmentation in infected cells. DRP1-induced fragmentation also leads to BAX activation, cytochrome c release and finally programmed cell death [118]. The molecular mechanisms by which VacA induces DRP1-mediated mitochondrial fragmentation and the connection between mitochondrial fragmentation and BAX activation remain to be determined [118]. Finally, *Vibrio cholerae* secretes VopE, a T3SS-secreted effector, that inhibits mitochondrial network reorganisation [119]. It was shown that in cells infected with *V. cholerae* depleted for the gene encoding this effector, some T3SS-associated components induce mitochondrial peri-nuclear clustering that leads to MAVS aggregation and induction of NF $\kappa$ B signalling. The mitochondrial clustering could be mediated by Miro1 and Miro2 (two Rho GTPases) that sense calcium fluxes changes in response to *V. cholerae* infection. However, in cells infected with the WT bacteria, VopE, which acts as a specific GTPase activating protein (GAP), is able to physically and directly interact with Miro1 and Miro2 keeping them in a GDP-bound form allowing the inhibition of mitochondrial clustering and subsequent pro-inflammatory signalling. Thus, changes in mitochondrial morphology during *V. cholerae* infection seem to inhibit the development of an efficient immune response [119].

## 6. Perspectives

If the impact and effect of infectious pathogens on mitochondria are relatively well studied, it is now imperative to assess the effect of mitochondrial activity and metabolic status during infection. Systematic studies to analyse the effect of boosted or impaired mitochondrial functions on infection efficiency, intracellular trafficking (for facultative or obligate bacteria), bacterial resistance or sensitivity to intra- and extracellular defence mechanisms should be developed in the future to better understand the numerous and tightly regulated cross talks between a host and its bacterial pathogens. In addition, mechanisms by which bacteria affects, directly or indirectly, mitochondria still need to be determined as many bacterial effectors are still most likely to be identified. Eventually, the origin of increased mtROS production detected in response to bacterial infection still need to be addressed by modern and robust approaches to detect ROS, especially that so far, few elements allow to determine whether the changes in mtROS abundance in response to bacterial pathogens result from a true increase in their production or a reduction of antioxidant capacity.

## 7. Conclusion

As we have seen, mitochondria are very important organelle allowing many functions in the immune systems, especially through metabolic control, calcium homeostasis and mtROS production and degradation [89]. Many of these mitochondrial controls help the host to defend against the pathogens. However, many bacteria (both intracellular and extracellular) have also evolved and developed several types of effectors and mechanisms that target and alter the mitochondria to circumvent or avoid mitochondria-dependent mechanisms, such as mtROS production, that could be deleterious or harmful for the pathogenic bacteria.

Finally, the expected outcome of future studies should bring a better knowledge of effectors and mechanisms that affect mitochondria as well as a detailed picture of the importance of



this organelle in infection, data that could also probably help to identify new host mitochondrial targets and the development of innovative medications to fight bacterial pathogens.

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