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1 **The mitochondrial respiratory chain: a metabolic rheostat of innate immune**
2 **cell-mediated antibacterial responses**

3

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15

16 **Abstract**

17

18 Upon microbial infection, cells of the innate immune system undergo profound
19 metabolic reprogramming in order to eradicate pathogens, promote inflammation,
20 and eventually restore tissue homeostasis. Mitochondria are at the core of these
21 adaptations, given their dual role as metabolic hubs and innate immune signaling
22 platforms. The mitochondrial electron transport chain (ETC) is very well
23 characterized at the genetic, molecular, structural, and biochemical level. In
24 contrast, the role for mitochondrial ETC and metabolites beyond fulfilling cellular
25 ATP synthesis in innate immune cell biology was not understood until recently.
26 Here we discuss the latest advances in our understanding of immune functions of
27 mitochondria and particularly the mitochondrial respiratory chain.

28

29

30 **Keywords** Electron transport chain, metabolism, innate immunity, bacterial
31 infection, pattern-recognition receptors

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1 **Introduction: of the importance of metabolism in the innate immune system.**

2

3 The immune system of jawed vertebrates can be conceptually divided into two
4 branches; the innate and the adaptive immune system (Litman et al., 2010).
5 Although evolutionarily distinct, the innate and adaptive immune system are
6 functionally closely intertwined (Boehm, 2012). Professional phagocytes, such as
7 macrophages or dendritic cells (DCs) continuously probe their environment for
8 microbial invaders. They express a wide array of 'pattern recognition receptors'
9 (PRRs), a large group of germline-encoded immune receptors that detect highly
10 conserved molecules in microorganisms, called 'pathogen associated molecular
11 patterns' (PAMPs) (Janeway, 1989). Ligation of PRRs, such as Toll-like receptors
12 (TLRs), leads to rapid activation of intracellular signaling cascades eliciting pro-
13 inflammatory and antimicrobial responses (**Figure 1**). Upon activation, some
14 members of the NOD-like protein (NLR) family of PRRs can assemble into large
15 cytosolic protein complexes, termed inflammasomes (Martinon et al., 2009). In
16 antigen presenting cells (APCs) such as DCs, PRR activation promotes antigen
17 processing and presentation to cells from the adaptive arm of immunity (i.e T cells
18 and B cells), upregulation of co-stimulatory molecules and cytokine production.
19 Other professional phagocytes including macrophages, monocytes and neutrophils
20 show a high capacity to engulf and destroy microbes such as bacteria.
21 Nevertheless, they also have the capacity to present antigen and produce various
22 cytokines and chemokines upon PRR activation to further tailor immune responses
23 mediated by adaptive immune cells such as T cells or B cells. Thus, PRRs represent
24 a molecular link between innate and adaptive immunity (Iwasaki and Medzhitov,
25 2010).

26 Microbes can trigger distinct and specific set of PRRs. The nature of the
27 PRRs engaged during infection shapes the innate immune responses generated by
28 skewing innate immune cells differentiation, which rely on complex molecular
29 events. These events are largely viewed as a succession of phosphorylation events
30 eventually leading to controlled gene transcription. However, early observations
31 noted a profound switch to glucose fermentation upon activation of human and
32 murine professional phagocytes (Chance et al., 1955; Newsholme et al., 1986; Oren
33 et al., n.d.; Sbarra and Karnovsky, 1960, 1959) and recent studies have further

1 delineated the complex metabolic reprogramming that takes place upon immune
2 cell activation (see other reviews in this issue of *Mitochondrion*) including
3 macrophages and dendritic cells (Gleeson and Sheedy, 2016; O'Neill and Pearce,
4 2015). As the main metabolic hubs of animal cells, mitochondria are naturally
5 positioned at the center of these 'immunometabolic' regulations. Intriguingly,
6 mitochondria also serve as critical signaling platforms for PRRs including RIG-I like
7 receptors (RLR), or NLR protein 3 (NLRP3). Moreover, release of mitochondria-
8 derived molecules during infection can activate PRRs (Krüger et al., 2015; Shimada
9 et al., 2012; Zhou et al., 2011). Hence, intricate molecular interactions between
10 mitochondria and immune signaling pathways unfold during infection. In contrast,
11 potential interactions between mitochondria or mitochondrial-derived
12 metabolites and invading pathogens are less well studied. Innate immune cell
13 differentiation thus results from specific combinations of signaling events and
14 metabolic adaptations that are dictated by the biochemical composition and
15 infectious features of the microbe encountered. Mitochondria have been found to
16 play important function in both PRR signaling and metabolism of innate immune
17 cells but whether these two faces of mitochondria are interconnected is only
18 started to be understood.

19 A growing number of studies delineates the metabolic events that occur
20 during innate immune cell activation placing mitochondria at the crossroads of
21 immune signaling and metabolic adaptations. These metabolic pathways generate
22 reductive intermediates that are eventually oxidized in the electron transport
23 chain (ETC). While this process was generally considered as a means to supply
24 sufficient ATP to resting professional phagocytes and APCs, it is becoming
25 increasingly clear that the ETC's contribution in innate immunity extends beyond
26 ATP synthesis when cells become activated. Important progress has been made in
27 our understanding of metabolic adaptation in cells of the adaptive immune system
28 and T cell in particular. We strongly encourage the readers willing to understand
29 the metabolic aspects of adaptive immune cells to refer to comprehensive reviews
30 published in this issue of *Mitochondrion* and elsewhere (Buck et al., 2015; Mills et
31 al., 2017). Here we review recent works highlighting the unexpected antibacterial
32 innate immune functions of the mitochondrial respiratory chain and mitochondrial
33 metabolism.

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Metabolic flux variations upon microbial encounter

Microbial encounters initiate complex activation and differentiation programs in phagocytes, which critically shape the nature of the immune response that will be generated. In the middle of last century, several laboratories made the observation that the phagocytes change their metabolic state upon activation raising the possibility that those metabolic adaptations would be part of this differentiation program (Chance et al., 1955; Oren et al., n.d.; Sbarra and Karnovsky, 1960, 1959). Changes in metabolic pathways upon immune cell activation have been reviewed elsewhere (Gleeson and Sheedy, 2016; Mills et al., 2017; O'Neill et al., 2016), and we will only provide a brief overview of the main metabolic adjustments found in macrophages upon microbial stimulation. The majority of studies investigating metabolic changes in macrophages have used selected microbial ligands, most commonly TLR4 agonist lipopolysaccharides (LPS) as a proxy for bacterial infection.

Activation of PRRs by bacterial components strongly increases phagocyte's glycolytic flux, and levels of different glycolytic intermediates are highly elevated in activated macrophages (Everts et al., 2014; Everts et al., 2012; Jha et al., 2015). Increased in glycolytic flux and lactate production is found in macrophages upon infection with different bacterial species including *Salmonella enterica* subsp. *enterica* serovar Typhimurium (*S. Typhimurium*) (Garaude et al., 2016; Palsson-McDermott et al., 2015; Tannahill et al., 2013), *Mycobacterium tuberculosis* (Mehrotra et al., 2014; Tannahill et al., 2013), or *Escherichia coli* (*E. coli*) (Garaude et al., 2016). Interestingly, this increase in glycolysis and lactate production is observed even under unlimited access to oxygen (O'Neill and Pearce, 2015). For this reason, this induction of aerobic glycolysis has been repeatedly compared to the 'Warburg effect' observed in cancer cells. Surprisingly, concomitant to this increase in glycolysis, a decrease in mitochondrial oxygen consumption was observed suggesting that glucose catabolism was diverted from its use by the mitochondria (Everts et al., 2012; Jha et al., 2015; Krawczyk et al., 2010; Tannahill et al., 2013). However, recent studies revealed that activation of TLRs others than TLR4 could increase mitochondrial oxygen consumption and engage distinct

1 metabolic programs in human monocytes (Lachmandas et al., 2016) and
2 plasmacytoid dendritic cells (D. Wu et al., 2016). Therefore, while the increased
3 glycolytic flux is clearly a hallmark of many activated innate immune cells
4 (macrophage, DCs, monocytes or neutrophils) other more subtle metabolic
5 reprogramming events are likely to unfold depending on the nature of the
6 microbial stimulus and the PRRs engaged, as well as involved cell type.

7 The pentose phosphate pathway (PPP), which diverts glycolytic
8 intermediates towards nucleotide and amino acid production, is also augmented
9 by LPS/TLR4 stimulation (Jha et al., 2015; Tannahill et al., 2013). It generates
10 reducing equivalents of NADPH, which are required for fatty acid synthesis and
11 contribute to cellular redox homeostasis in macrophage. A recent study by
12 Haschemi *et al.* highlighted some molecular details about PPP regulation and
13 functional consequences during murine macrophage activation. This study showed
14 that a substantial portion of glycolysis-derived carbons is routed towards the PPP
15 upon LPS stimulation (Haschemi et al., 2012). The authors notably identified the
16 carbohydrate kinase-like protein (CARKL) as a sedoheptulose kinase (converting
17 the PPP metabolite sedoheptulose to sedoheptulose-7-phosphate) that can repress
18 LPS-induced inflammatory cytokines production by macrophage. CARKL is
19 downregulated in LPS-treated macrophages (also called M1 macrophages or
20 inflammatory macrophages) but upregulated in IL4-activated macrophage (or M2
21 macrophage), which are essential to fight parasite infection and can modulate
22 immune responses through the production of regulatory cytokines such as IL-10.
23 Consistently, the authors found that loss of CARKL induced a mild activation in
24 resting macrophage while its over-expression sensitized macrophage to M2
25 polarization. This suggests that CARKL may exert a pivotal function in macrophage
26 polarization.

27 The role of fatty acid metabolism during professional phagocyte activation
28 is less well understood. Fatty acid oxidation (FAO) is augmented in mouse and
29 human monocytes primed with activators of the NLRP3 inflammasome, involving
30 mitochondrial NADPH oxidase 4 (NOX4)-dependent ROS production (Moon et al.,
31 2016). However, IL4-activated macrophage have been shown to use FAO contrary
32 to macrophages induced by stimulation with LPS and Interferon- γ (Huang et al.,
33 2014) . Pearce and colleagues used etomoxir, an inhibitor of carnitine-palmitoyl

1 transferase 1 (CPT1), a key enzyme in the long-chain fatty acid oxidation to show
2 that lipolysis of triacylglycerol substrates by phagocytes was important for
3 oxidative phosphorylation elevation and expression of genes that define IL-4-
4 activated macrophages. In contrast, the genetic deletion of *Cpt2*, which also
5 strongly diminished long-chain fatty acid oxidation in the mitochondria, did not
6 alter IL-4-stimulated macrophage polarization as shown by Finkel and co-workers
7 (Nomura et al., 2016). Fatty acid synthesis seems in contrast to be closely related
8 to inflammatory phenotypes in macrophages. It is augmented in LPS-stimulated
9 macrophages (Feingold et al., 2012) and fatty acid synthase is induced during
10 sepsis through a mechanism that requires mitochondrial uncoupling protein 2
11 (UCP2) (Moon et al., 2015). More work is clearly needed to further define the exact
12 contributions of FAO and fatty acid synthesis to the functions of macrophages and
13 other innate immune cells during infection (Namgaladze and Brüne, 2014).

14 While metabolism of various amino acids is likely to contribute to immune
15 function (O'Neill et al., 2016), glutamine has emerged as the most prominent
16 amino acid in macrophage and DC metabolic reprogramming. Its catabolism
17 (glutaminolysis) has been found to extensively replenish the TCA cycle and the
18 hexosamine pathway while glycolytic product pyruvate is diverted from entry into
19 the mitochondrion in murine macrophages (Jha et al., 2015; Tannahill et al., 2013).
20 In LPS-primed macrophages, glutamine is thought to contribute to succinate
21 accumulation through anaplerosis of α -ketoglutarate (Jha et al., 2015; Tannahill et
22 al., 2013) and is required for production of the inflammatory cytokine IL-1 β
23 (Wallace and Keast, 1992) and the generation of nitric oxide (NO) through arginine
24 synthesis in murine macrophages and human monocytes (Murphy and
25 Newsholmes, 1998), thereby contributing to antimicrobial functions of
26 macrophages. In addition NO can inhibit mitochondrial respiration and ATP
27 production through nitrosylation of the iron-sulfur containing ETC complex I, II
28 and IV (Beltran et al., 2000) thus accounting for OXPHOS shut down observed in
29 LPS-stimulated macrophages and DCs. Consistently, glutamine deficiency reduced
30 inflammasome activation (He et al., 2016). Glutamine-derived succinate can also
31 be used to generate nitric oxides (Bellows and Jaffe, 1999), which in turn can
32 inhibit the mitochondrial respiratory chain in LPS stimulated DCs (Everts et al.,
33 2012). However, whether glutamine catabolism can contribute to other metabolic

1 pathways in phagocytes still needs to be further documents. A recent study
2 nonetheless revealed some interesting features of the use of glutamine in
3 macrophages. Liu et al. showed that α -ketoglutarate produced by glutaminolysis
4 accumulates in IL-4-stimulated macrophages in which it induces Jumonji domain-
5 containing protein 3 (Jmjd3)-dependent epigenetic reprogramming and instructs
6 cells to augment FAO. In contrast, α -ketoglutarate represses the NF- κ B pathway in
7 LPS-primed macrophages through prolyl-hydroxylase (PHD)-dependent post-
8 translational modification of IKK β . The ratio between glutamine-derived α -
9 ketoglutarate and succinate controls macrophage differentiation. A high α -
10 ketoglutarate/succinate ratio promotes IL-4-induced macrophage differentiation,
11 whereas a low ratio supports inflammatory macrophage generation. Hence,
12 glutamine metabolism is a critical a key metabolic checkpoint of macrophage fate
13 and endotoxin tolerance (Liu et al., 2017).

14

15 **Adaptations of the mitochondrial Electron Transport Chain (ETC) during** 16 **microbial infection**

17

18 *ETC adaptations as a response to fuel source fluctuations*

19 Most catabolic processes converge on the mitochondrial ETC by supplying
20 electrons through the reductive equivalents of nicotinamide adenine dinucleotide
21 (NADH) and flavin adenine dinucleotide (FADH). The ETC comprises two electron
22 carriers (coenzyme Q [CoQ]/ubiquinone and cytochrome c) and four respiratory
23 complexes (complex I-IV [CI-CIV]). Respiratory complexes I, III, and IV can
24 assemble as large molecular supercomplexes (SCs) in the mitochondrial inner
25 membrane (for a comprehensive review see (Enriquez, 2016)). The transfer of
26 electrons through the respiratory complexes generates a H⁺ gradient that is used
27 by the H⁺-ATP synthase (CV) to generate ATP. The supramolecular organization of
28 the electron transport chain and its functional implications are a subject of
29 passionate debate (Cogliati et al., 2016; Lapuente-Brun et al., n.d.; Mourier et al.,
30 2014; Pérez-Pérez et al., 2016). Nevertheless, recent studies have provided
31 detailed insights into the sequential assembly of the respiratory chain (Cogliati et
32 al., 2016; Guerrero-Castillo et al., 2016; Mick et al., 2012; Stroud et al., 2016) and
33 resolved the structure of the so-called respirasome (a supercomplex composed of

1 CI, a dimer CIII and CIV) (Gu et al., 2016; Letts et al., 2016; M. Wu et al., 2016) and
2 CI (Fiedorczuk et al., 2016). The dynamic assembly of respiratory complexes into
3 supercomplexes may provide a structural environment favorable to assembly of CI
4 (Enriquez, 2016; Genova and Lenaz, 2014; Lapuente-Brun et al., n.d.), stabilize the
5 individual respiratory complexes (Acín-Pérez et al., 2004), prevent ROS production
6 by sequestering reactive radicals (Maranzana et al., 2013), or organize electron
7 flux to optimize the use of available substrate required for ATP synthesis and
8 oxygen consumption (Guarás et al., 2016; Lapuente-Brun et al., n.d.). Switch in the
9 nature of the fuel that feed cellular metabolism alters the intramitochondrial ratios
10 of FADH₂ and NADH (herein F/N ratios) (Speijer, 2016), which may require
11 adjustment of respiratory chain super-assembly. The predicted F/N ratio is lowest
12 for glucose oxidative breakdown with a ratio of 0.2 (one molecule of FADH₂
13 formed per five molecules of NADH), but is high (0.5) for saturated long-chain fatty
14 acids with one molecule of FADH₂ per two NADH molecules (Speijer, 2011).
15 Interestingly, fasting modifies SCs distribution and ETC activity (Lapuente-Brun et
16 al., n.d.), and shifting nutrient substrate from glucose to fatty acids can
17 downregulate CI content (Guarás et al., 2016). Mechanistically, it was proposed
18 that accumulation of the reduced form of CoQ can generate a reverse electron
19 transfer leading to oxidation of CI subunits (Chouchani et al., 2016). The resulting
20 degradation of CI is likely to modify the stoichiometry of SCs to adjust the electron
21 flow within the ETC (Guarás et al., 2016). Nutrient availability also regulates
22 mitochondrial dynamics. However, the natural occurrence and functional roles
23 supercomplexes still remain to be fully established.

24

25 ***Architectural changes of the ETC and their role in innate immune responses***

26 Given the profound metabolic changes during activation of innate immune cells, it
27 could be assumed that the architecture and the activity of the ETC would also vary
28 in such conditions. A first indication came from observations in macrophages
29 infected with *Listeria monocytogenes*, an intracellular Gram-positive pathogen that
30 causes listeriosis. It was found that *Listeria* infection causes transient
31 mitochondrial network fragmentation (Stavru et al., 2011). These alterations
32 require pore-forming toxin listeriolysin O (LLO), but are independent of dynamin-
33 like protein 1 (Drp1) or Opa1, two well-known proteins involved in mitochondrial

1 fusion/fission dynamics (Stavru et al., 2013). In contrast, the obligate intracellular
2 bacterium *Chlamydia trachomatis* maintains mitochondrial network integrity by
3 inhibiting Drp1-induced mitochondrial fragmentation (Chowdhury et al., 2017).
4 Mitochondrial dynamics play a particularly important role in antiviral immunity. It
5 was observed that RIG-I (a member of the RIG-I-like receptors or RLR), its
6 downstream adaptors mitochondrial antiviral signaling (MAVS) and mitochondria
7 localize around centers of viral replication upon infection by single-stranded RNA
8 Sendai virus or 5'ppp-RNA detection (Onoguchi et al., 2010). MAVS is a RLR-
9 signaling adaptor that translocates at the mitochondria upon viral infection to
10 form a mitochondrial antiviral signaling platform and induce type-I interferons
11 (IFN) essential for fighting viral infection (West et al., 2011b). Consistent with the
12 link between mitochondrial dynamics and MAVS signaling, both mitofusin proteins
13 (MFN) 1 and 2, two well-characterized fusion regulators, can interact with MAVS
14 (Castanier et al., 2010; Yasukawa et al., 2009). Although the respective role of
15 MFN1 and MFN2 in RLR signaling and the formation of MAVS platform is still not
16 clear, other proteins involved in mitochondrial dynamics have been found to
17 interact with proteins of the RLR/MAVS pathway during viral infection (West et al.,
18 2011b) supporting the notion that mitochondrial dynamics is closely linked to
19 innate immune signaling. However, whether this also occurs upon bacterial
20 infection is still poorly appreciated. Additional research should clarify the role of
21 mitochondrial dynamics related protein in innate immune signaling and their
22 contribution to phagocyte-mediated antibacterial responses. Nevertheless, taken
23 together those studies suggested that the alterations of the mitochondrial network
24 induced by bacterial infection and inflammation are likely to affect the
25 bioenergetics and immune signaling of innate immune cells. Accordingly,
26 significant changes in the expression of mitochondrial carriers including the
27 dicarboxylate carrier DIC, the citrate carriers CIC or the adenine-nucleotide
28 carriers ACC have been associated with inflammation (Iacobazzi et al., 2017).
29 Adaptations of the ETC are therefore likely to occur to meet the strong energetic
30 requirements and metabolic adjustments of activated immune cells.

31 It was originally postulated that the overall mitochondrial oxygen
32 consumption by the respiratory chain of macrophages, DCs and monocytes is
33 reduced following TLR- (Krawczyk et al., 2010; Tannahill et al., 2013) and Dectin-1

1 (Cheng et al., 2014) activation. Thus it has been general wisdom that during
2 infections, the ETC might serve other roles than sustaining metabolic fluxes and
3 generating ATP, which would then mainly arise from glycolysis (Mills et al., 2017).
4 However, an increase in glucose-dependent oxygen consumption was observed
5 within few hours after LPS stimulation of DCs (Bart Everts et al., 2014; Everts et al.,
6 2012) suggesting that mitochondrial respiration is required for innate immune cell
7 activation. In fact, phagocytosis of living *Escherichia coli* by macrophages can the
8 maximal respiration capacity and induces changes in the architecture of the ETC
9 within two hours post-infection leading to a decrease in the abundance of CI and
10 CI-containing SCs (Garaude et al., 2016). Consequently, CI activity is decreased
11 upon *E. coli* infection. However, the overall mitochondrial respiratory capacity is
12 maintained at least for several hours after bacterial stimulation. This is explained
13 by an increase in mitochondrial glycerol-3-phosphate dehydrogenase activity,
14 which allows mitochondrial recycling of cytosolic NADH and supply of electrons to
15 the ETC (Garaude et al., 2016). CII activity is also strongly increased and
16 contributes to mitochondrial respiration and ATP synthesis during bacterial
17 infection (Garaude et al., 2016). Interestingly, the observed ETC adaptations were
18 absent in macrophages stimulated with TLR4 agonist LPS or with heat killed
19 bacteria. We have previously shown that murine phagocytes possess the intrinsic
20 capacity to discriminate viable from dead bacteria through the recognition of
21 specialized PAMPs, which we refer to as *vita*-PAMPs (Sander et al., 2012). Bacterial
22 RNA was identified as the first *vita*-PAMP, recognition of which signals microbial
23 viability to the immune system and elicits pronounced immune responses. A
24 signature response of macrophages and DC to live bacteria or bacterial RNA is the
25 activation of the NLRP3 inflammasome. When we investigated the ETC adaptations
26 following bacterial stimulation we found that bacterial RNA or live bacteria, but
27 not their killed counterparts, initiated ETC adaptations through activation of the
28 phagosomal NADPH oxidase and the ROS-dependent tyrosine kinase Fgr, which
29 was shown to phosphorylate the tyrosine 604 of the CII subunit succinate
30 dehydrogenase subunit A (SDHA) (Acin-Perez et al., 2014; Garaude et al., 2016).
31 The partner phosphatase of Fgr was recently identified (Nath et al., 2015),
32 highlighting the potential of pharmaceutical manipulation of post-translational
33 modifications of CII activity to control inflammation and other metabolic-related

1 diseases (Bezawork-Geleta et al., 2017). Hence, we demonstrate that specific
2 innate immune signals that convey an increased level of infectious threat to the
3 immune system induce profound metabolic alterations beyond the well-described
4 glycolytic switch (Garaude et al., 2016).

5 This study suggested that innate immune receptor-mediated endosomal
6 ROS production during bacterial infection directly increases mitochondrial ETC
7 capacity but also enhance mitochondrial CII-dependent ATP synthesis, challenging
8 the idea that ATP demand is mostly covered by anaerobic glycolysis. Nevertheless,
9 it remains to be fully established whether metabolic flux modulation and the
10 subsequent changes in the proportion of reductive equivalents NADH and FADH₂
11 contribute to those adaptations. First evidence came from a recent study by O'Neill
12 and colleagues. They found that LPS/TLR4-activated murine macrophages
13 accumulate succinate (Mills et al., 2016). As a consequence, TLR4-induced
14 succinate oxidation by succinate dehydrogenase (SDH - which is also CII) and
15 elevation of the mitochondrial membrane potential drives a reverse electron
16 transfer (RET) that promotes mitochondrial ROS production by CI. This was
17 dampened by the over-expression of an alternative oxidase (AOX) from *Ciona*
18 *intestinalis*, which restores the normal flow of electrons through CoQ thereby
19 avoiding RET (El-Khoury et al., 2014). It is tentative to speculate here that upon
20 LPS stimulation, the increase ATP synthesis through glycolysis allows RET to occur
21 assuming that mitochondrial ETC is not uncoupled. This would place ATP demand
22 as an important driver of mitochondrial changes in LPS-activated phagocytes. This
23 study also confirmed the importance of CII for macrophage activation upon innate
24 immune receptor stimulation, and placed CI at the core of immune signaling.
25 Complex I deficiency is a well-known cause of rare neurological and metabolic
26 disorders. However, a recent study by Jin *et al* based on the deletion of the CI
27 subunit Ndufs4 highlighted a key function of CI in macrophage differentiation and
28 contribution to inflammatory responses (Jin et al., 2014). The global Ndufs4 loss
29 results in lethal encephalomyopathy (Kruse et al., 2008) and decreases bone
30 resorption and increases bone mass (Jin et al., 2014), while Ndfus4-deficient
31 macrophages exhibit a pro-inflammatory phenotype, which can be attenuated by
32 deletion of TLR2 and TLR4 again highlighting the relationship between innate
33 immune receptors and the ETC (Jin et al., 2014). Together with the observation

1 that Gram-negative bacterial infections leads to a decrease in CI (Garaude et al.,
2 2016) and that LPS stimulation induce RET and ROS production at CI (Mills et al.,
3 2016), the study of Jin et al. further suggests that CI functions as a regulator of
4 inflammatory responses by macrophages and that its downregulation or
5 dysregulation act as an inflammatory signal.

6

7 **4- The mitochondrial Electron Transport Chain (ETC): an immune rheostat** 8 **during infection (~600words)**

9

10 ***ETC byproducts as innate immune signals***

11 Several byproducts of mitochondrial activity have been implicated in inflammatory
12 processes (**Figure 2**). Shadel and co-workers recently found that perturbation of
13 oxidative phosphorylation by moderate mitochondrial DNA (mtDNA) stress
14 engages cytosolic signaling to initiate antiviral immune responses (West et al.,
15 2015). Heterozygous 'transcription factor A, mitochondrial' (TFAM) deficiency
16 leads to aberrant packaging of mtDNA and induces its escape to the cytosol where
17 it is sensed by the innate immune receptor cGAS, which promotes STING-IRF3-
18 dependent induction of interferon-stimulated genes. Mitochondrial ROS (mtROS)
19 are generated as a byproduct of ETC activity or dysfunction, and mtROS have also
20 been identified as a critical stimulator of cytosolic immune receptors [8].
21 Phagocytosis of microorganisms like bacteria elicits a respiratory burst leading to
22 the generation of high amounts of ROS, which critically contribute to bacterial
23 clearance (Lambeth, 2004). Although ROS generated by the phagosomal NADPH
24 oxidase are considered the main component of the antimicrobial response in
25 phagocytes, mtROS have recently emerged as important contributors to the
26 bactericidal capacity and immune signaling in innate immune cells (West et al.,
27 2011a). As many as 10 possible sites of ROS production have been proposed in
28 mitochondria (Quinlan et al., 2013) with CI being one of the most relevant for
29 macrophages (Kelly et al., 2015; Mills et al., 2016). In phagocytes, expression of
30 mitochondrial uncoupling protein 2 (UCP2) dampens mtROS production
31 (Arsenijevic et al., 2000) and TLR4 engagement decreased UCP2 expression in LPS-
32 primed murine macrophages (Kizaki et al., 2002). Consequently, *Ucp2*^{-/-} mice are
33 more resistant to infection by intracellular pathogens *L. monocytogenes* (Rousset et

1 al., 2006) and *Toxoplasma gondii* (Arsenijevic et al., 2000), and UCP2-deficient
2 macrophages exhibit increased killing of *S. Typhimurium* (Arsenijevic et al., 2000).
3 Finally, a recent study by Kawataba and colleagues shows that treatment of mouse
4 embryonic fibroblasts (MEFs) with chemical uncoupling molecules that decrease
5 mitochondrial membrane potential lowers type IFN production (Koshiba et al.,
6 2011). Precisely, they observed that the increased abundance of UCP-2 leads to
7 proton leakage from mitochondria that impairs MAVS-dependent signaling. This
8 suggests that the regulation of the mitochondrial ETC could directly influence the
9 outcomes of innate immune signaling. Importantly, these studies highlights the
10 notion that induction of RET, CI-mediated ROS production and subsequent
11 increase in antimicrobial capacity and inflammatory responses by activated
12 macrophages all require dampening of mitochondrial uncoupling mechanisms.

13 In further support of the important role of mitochondrial byproducts, West
14 *et al.* observed that mitochondria co-localize and potentially interact with bacteria-
15 containing phagosomes (West et al., 2011a). They proposed that mitochondrial
16 ROS production is induced by cell surface TLRs (e.g. TLR2 or TLR4), in a process
17 that requires the TLR-adaptor TRAF6 (TNF receptor associated factor 6) and the
18 mitochondrial protein ECSIT (evolutionary conserved signaling intermediate),
19 which was originally described as a CI assembly factor (Vogel et al., 2007). Hence,
20 accumulating evidence suggests a direct interaction between mitochondria, or
21 their metabolic byproducts and invading pathogens. Aside from their proposed
22 bactericidal role, mtROS have been reported to be involved in the activation of key
23 innate immune modules. NLRP3 is a member of the cytosolic Nod-like receptors
24 (NLRs) family. Upon activation, NLRP3 assembles into a large cytosolic protein
25 complex termed the inflammasome. Inflammasomes serve as a platform for
26 autocatalytic activation of pro-inflammatory caspases, most prominently Caspase-
27 1, which processes inflammatory cytokines IL-1 β and IL-18 (Martinon et al., 2009).
28 The NLRP3-inflammasome is activated by a variety of endogenous and exogenous
29 stimuli during bacterial infection including bacterial pore-forming toxins or
30 bacterial nucleic acids (Martinon et al., 2009; Sander et al., 2012). Similar to the
31 observed changes in ETC SC composition, NLRP3 is also activated by bacterial RNA
32 (Kanneganti et al., 2006; Sander et al., 2012). ROS production is common to many
33 activators of NLRP3 inflammasome and mtROS seem to be critically involved in

1 NLRP3 inflammasome activation. ETC inhibition promotes mtROS generation,
2 which increases NLRP3 activation and IL-1 β secretion (Zhou et al., 2011).
3 Accordingly, removal of dysfunctional ROS-producing mitochondria by directed
4 autophagy (mitophagy) inhibits NLRP3 activation and IL-1 β release (Nakahira et
5 al., 2011). However, the precise molecular mechanisms that links mtROS to NLRP3
6 inflammasome activation remain unresolved. Macrophages deficient for the
7 cytosolic superoxide dismutase 1 (SOD1) produce less IL-1 β due to the inhibition
8 of caspase-1 by oxidation and glutathionylation of specific cysteines (Meissner et
9 al., 2008). In addition, mtROS generation may be dispensable for some NLRP3
10 activators. The oxazolidinone antibiotics linezolid, used in the treatment of
11 methicillin-resistant *S. aureus* infections, can activate the NLRP3 inflammasome
12 independently of ROS (Ament et al., 2002). In this context, NLRP3 activation occurs
13 via its binding to the mitochondrial-specific phospholipid cardiolipin (Iyer et al.,
14 2013). In addition to their role in NLRP3 inflammasome activation, mtROS also
15 regulate LPS-mediated production of different cytokines including proIL-1b (Kelly
16 et al., 2015; Mills et al., 2016; Tannahill et al., 2013) by stabilizing hypoxia-
17 inducible factor 1a (HIF1a) (Tannahill et al., 2013). HIF1a has thus emerged as a
18 key regulator of metabolic reprogramming in innate immune cells as exemplified
19 by its important role in human monocyte functional adaptations occurring in
20 sepsis (Shalova et al., 2015).

21

22 ***TCA cycle-derived metabolites are essential components of immune responses***

23 Mitochondrial-derived metabolites generated in the Krebs cycle have important
24 inflammatory signaling functions (Mills et al., 2017), and many of these
25 metabolites accumulate in macrophages upon PRR ligation (Jha et al., 2015).
26 Accumulation of succinate is associated with an enhanced production of pro-IL-1 β
27 and diminution of IL-10 in LPS-stimulated macrophages (Mills et al., 2016;
28 Tannahill et al., 2013). Several mechanisms have been proposed to explain how
29 succinate regulates cytokine production. First, succinate is structurally similar to
30 α -ketoglutarate and has been shown to inhibit dioxygenases including the HIF1a
31 regulators prolyl 4-hydroxylases (PHD) (Hewitson et al., 2007; Selak et al., 2005).
32 Second, succinate oxidation by CII and subsequent mtROS generation from CI leads
33 to HIF-1a activation, which in turn induces *IL1B* transcription. Consequently, the

1 pharmacological inhibition of CII inhibits LPS- or bacteria-induced production of
2 IL-1 β and boosts IL-10 expression (Garaude et al., 2016; Mills et al., 2016).
3 Fumarate, another TCA cycle metabolite and enzymatic product of CII also
4 accumulates in activated innate immune cells. Work from Netea and co-workers
5 has previously demonstrated that fumarate accumulates in monocytes activated by
6 Beta-glucans due to replenishment of the Krebs cycle through glutaminolysis (Arts
7 et al., 2016). In this context, fumarate was shown to inhibit KDM5 histone
8 demethylases leading to epigenetic imprinting and altered cytokine expression
9 profiles, a hall-mark of the recently proposed form of innate immune memory, also
10 referred to as trained immunity (Arts et al., 2016; Netea et al., 2016). The link
11 between metabolic adaptations and epigenetic imprinting has long since been
12 noted in metabolic disorders, and it will be exciting to further investigate its role in
13 immunity. Interestingly, fumarate also exerts direct antibacterial capacities. We
14 could show that fumarate impairs the growth of *E. coli* and *S. Typhimurium*
15 (Garaude et al., 2016). Dimethyl-fumarate (DMF) is an approved drug for the
16 treatment of multiple sclerosis and psoriasis (Al-Jaderi and Maghazachi, 2016) but
17 its potential as an antimicrobial drug has not been explored so far. Taken together,
18 these studies place CII activation and subsequent fumarate production at the
19 crossroads of many inflammatory signaling pathways, and moreover deliver
20 further evidence for a direct interaction between mitochondrial molecules and
21 invading pathogens. Another important TCA cycle metabolite driving inflammatory
22 responses is citrate. The accumulation of citrate in TLR-activated innate immune
23 cells is thought to support fatty acid biosynthesis, which is essential for the
24 production of proinflammatory cytokine in DCs (B Everts et al., 2014). Citrate
25 accumulation is likely caused by a decreased isocitrate dehydrogenase expression
26 in LPS-activated cells. This also leads to accumulation of itaconate by
27 decarboxylation of cis-aconitate, a citrate derivative and substrate of isocitrate
28 dehydrogenase, by 'immune responsive gene-1' (IRG1). TLR4 activation strongly
29 enhances IRG-1 expression in macrophages leading to increased production of
30 itaconate (Michelucci et al., 2013; Naujoks et al., 2016). Recent studies have shown
31 that itaconate limits the deleterious effects of excessive ROS production caused by
32 sustained SDH (CII) activity (Lampropoulou et al., 2016). Pre-treatment of
33 macrophages with itaconate prior to LPS stimulation inhibits SDH, increases

1 succinate levels, limits HIF-1a expression, and reduces inflammatory cytokine
2 production. Similar to fumarate, itaconate possesses direct antimicrobial effects.
3 IRG-1 dependent production of itaconate was shown to inhibit *in vitro* growth of
4 several bacterial pathogens including *Legionella pneumophila*, *S. enterica* or *M.*
5 *tuberculosis* (Michelucci et al., 2013; Naujoks et al., 2016). The expression of IRG-1
6 is in turn regulated by interferons, which are produced upon PRR activation. The
7 antibacterial effects of itaconate may be at least partially explained by the
8 inhibition of the glyoxylate shunt, an essential pathway in fatty acid- and acetate-
9 dependent bacteria, which is absent in mammals. It was thus proposed that
10 itaconate impairs microbial metabolism while sparing host cells from the
11 potentially detrimental induction of metabolism-dependent inflammatory
12 responses.

13

14 **Concluding remarks (~150words)**

15 Recent work revisiting old observations on metabolic reprogramming of
16 phagocytes during infection and inflammation has placed mitochondria, and
17 particularly the ETC, at the center of the newly discovered immuno-metabolic
18 adaptations. The mitochondrial respiratory chain has the capacity to adjust its
19 architecture and activity in response to innate immune signals, and thereby
20 regulate immune responses. These findings may provide novel options for
21 controlling immune responses. Manipulation of specific features of mitochondrial
22 metabolism could represent a valid strategy to fine-tune immune responses. It is
23 becoming clear that mitochondrial-derived metabolic intermediates, redox
24 molecules, and ROS play essential roles in antimicrobial immune responses. More
25 research is needed to deepen our understanding of the intricate cross-regulations
26 between the mitochondrial respiratory chain and innate immune responses during
27 infection.

28

29

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31

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11 **Disclosure statement**

12 The authors declare no competing financial interests

15 **Figure legends**

17 **Figure 1:** *Schematic view of pattern recognition receptor signaling and*
18 *mitochondria in antibacterial immunity.*

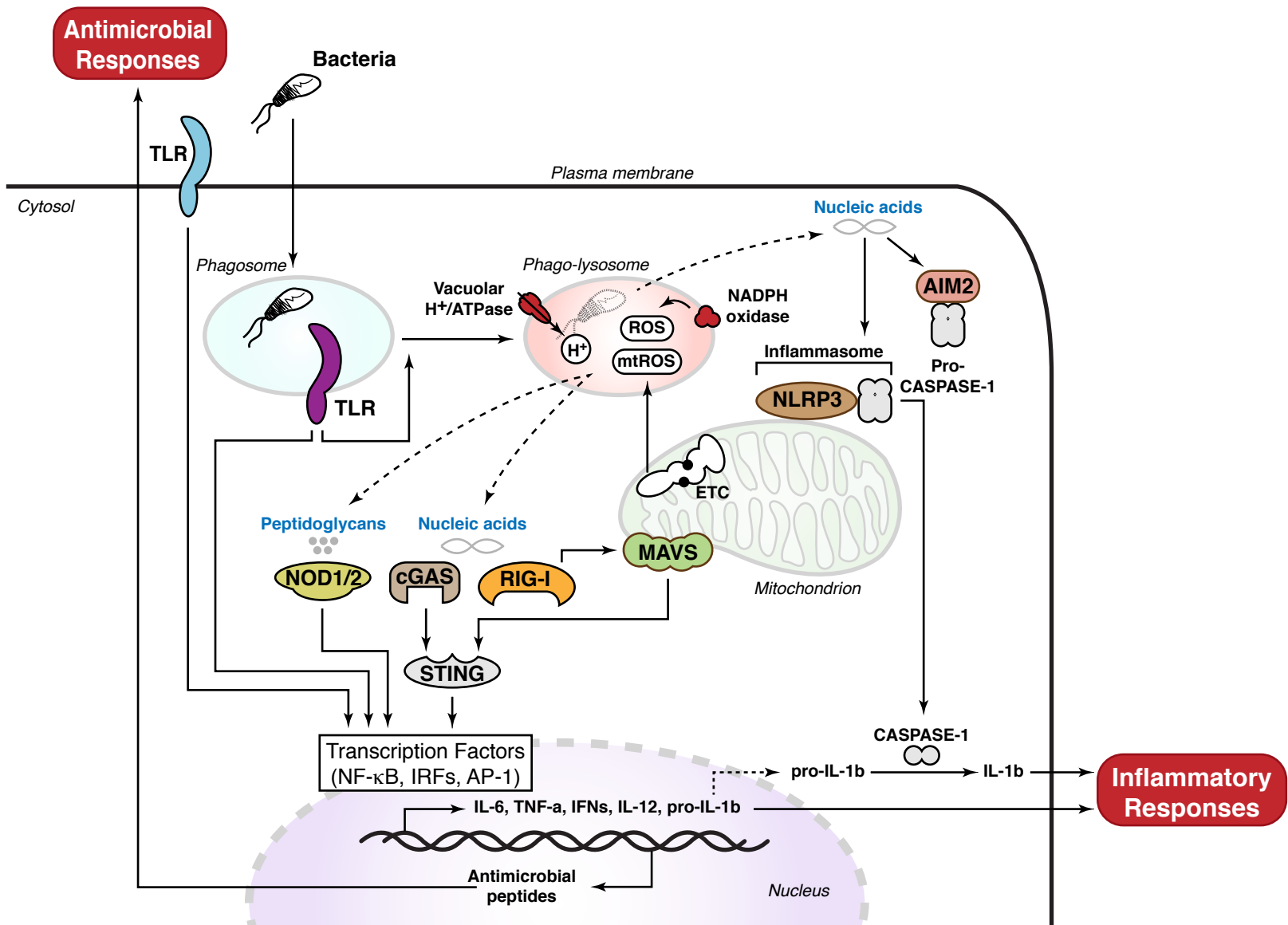
19 Professional phagocytes express a vast array of pattern recognition receptors
20 (PRRs), including Toll-like receptors (TLRs) located on the cell surfaces and in the
21 endosomal compartment, whereas as NOD-like receptors (NLRs) and other PRRs
22 survey the cytosol. Upon ligation, TLRs and other PRRs including NOD1 and -2
23 activate various signaling cascades generally converging on NF- κ B, MAPK, and
24 IRFs, leading to massive transcriptional reprogramming and the production of
25 cytokines like interleukin (IL)-6, tumor necrosis factor (TNF), pro-IL-1 β and/or
26 type-I interferons (IFNs). Upon activation, several NLRs assemble into large
27 multimeric protein complexes termed inflammasomes, which catalyze the
28 activation of Caspase-1. Active Caspase-1 in turn cleaves the proforms of IL-1 β and
29 IL-18 leading to the release of active IL-1 β . Phagocytosis of bacteria leads to the
30 activation of surface-, endosomal, and cytosolic PRRs. Induction of phagosomal
31 ROS by TLRs contributes to bacterial killing. Mitochondria colocalize with bacteria-
32 containing phagosomes, and mitochondrial (mt) ROS may further promote
33 endosomal PRR signaling. Degradation of internalized bacteria and subsequent

1 release of bacterial nucleic acids leads to the activation of cytosolic and endosomal
2 PRRs including TLR7/8, TLR9, cGAS, and RIG-I, all of which induce activate IRFs
3 and induce the production of type-I IFNs. Bacterial nucleic acids also activate the
4 NLRP3- and AIM2- inflammasomes. NLRP3 and other PRRS, as well as PRR adaptor
5 proteins like MAVS localize to mitochondria.

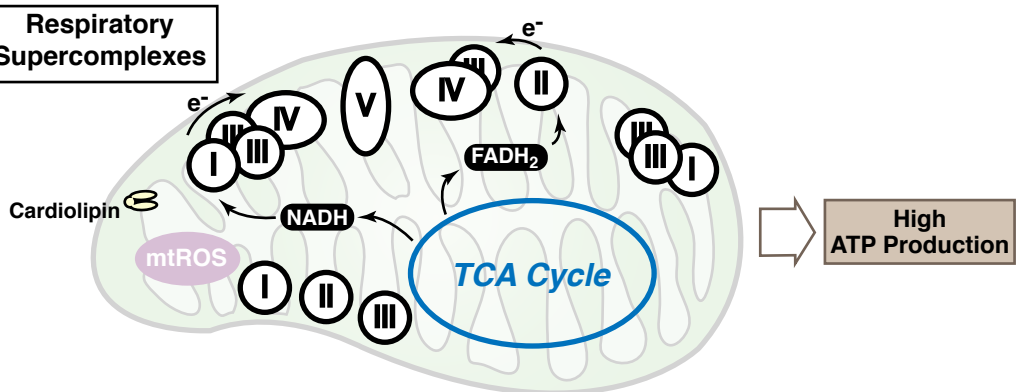
6
7 **Figure 2:** *The mitochondrial electron transport chain (ETC) is at the core of innate*
8 *immune responses against bacteria.* In macrophage, TCA cycle produces reductive
9 equivalents of NADH and FADH₂ that provide electrons to the ETC, which in turn
10 produces sufficient ATP to fulfill cellular energetic demands of resting cells. A
11 significant proportion of the ETC is assembled in supercomplexes (SCs) including
12 the respirasome (CI/CII₂/CIV). Mitochondrial ROS (mtROS) production is low in
13 resting macrophage. The engagement of innate immune receptors including Toll-
14 like receptors (TLRs) and NOD-like receptors (NLRs) during infection by live
15 Gram-negative bacteria is thought to divert mitochondrial metabolism from
16 energy production towards anabolic and signaling functions. Levels of SCs are
17 decreased, probably due to destabilization of the complex I (CI) and is associated
18 with a decrease in CI activity. In turn, CII and glycerol-3-phosphate dehydrogenase
19 (G3PDH, here signalized as G) activities are augmented and provide electrons to
20 the ETC and generate reverse electron transfer, accounting for CI destabilization
21 and mitochondrial ROS (mtROS) production. MtROS can then activate hypoxia-
22 induced factor 1a (HIF1 α) to increase expression of the pro-inflammatory cytokine
23 pro-IL-1b, and contribute to NLRP3 activation, which assembles the inflammasome
24 to convert pro-IL-1 β into the mature biologically active cytokine IL-1 β . MtROS can
25 also promote the recruitment of mitochondrial anti-viral signaling protein (MAVS)
26 to the mitochondrial outer membrane where it interacts with stimulator of inferno
27 genes (STING) to stimulate the production of antimicrobial Type-I interferons.
28 MtROS also exert direct antibacterial properties when released in the microbe-
29 containing phagosomes. Cardiolipin translocation from the inner membrane to the
30 outer membrane in damaged mitochondria – as it can be induced by different
31 bacterial infection – provides a docking site for NLRP3. microbes that trigger
32 mitochondrial damages. Reprogramming of the tricarboxylic acid (TCA) cycle lead
33 the accumulation of citrate, which can be metabolized into itaconate thereby

1 contributing to antibacterial capacities of macrophages. Fumarate also
2 accumulates due to increased CII activity and has been show to possess
3 antibacterial properties.

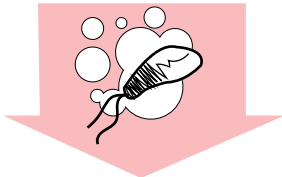
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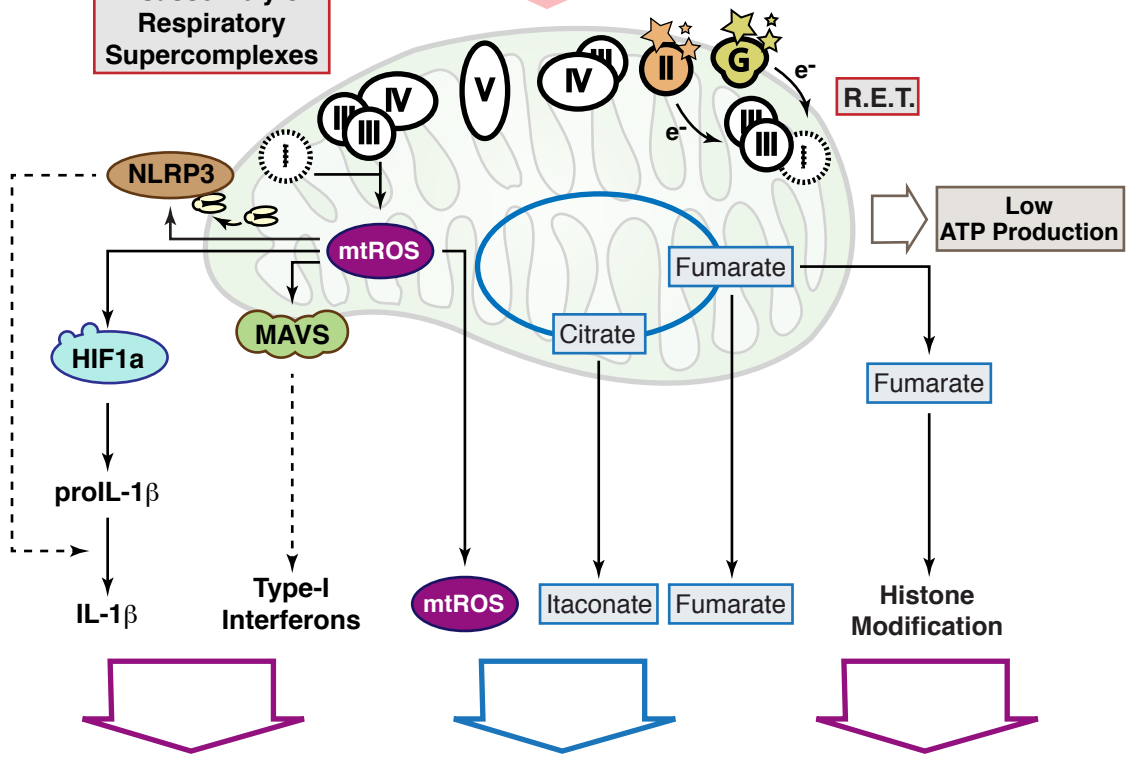
Respiratory Supercomplexes



Bacterial Infection



Disassembly of Respiratory Supercomplexes



Immune and Inflammatory Responses

Antibacterial Properties

Immune and Inflammatory Responses