Review

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Mitochondrial carriers in inflammation induced by bacterial endotoxin and cytokines

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Abstract: Significant metabolic changes occur in the shift from resting to activated cellular *status* in inflammation. Thus, changes in expression of a large number of genes and extensive metabolic reprogramming gives rise to acquisition of new functions (e.g. production of cytokines, intermediates for biosynthesis, lipid mediators, PGE, ROS and NO). In this context, mitochondrial carriers, which catalyse the transport of solute across mitochondrial membrane, change their expression to transport mitochondrially produced molecules, among which citrate and succinate, to be used as intracellular signalling molecules in inflammation. This review summarises the mitochondrial carriers studied so far that are, directly or indirectly, involved in inflammation.

Keywords: expression; inflammation; metabolism; mitochondrial carrier.

Introduction

Mitochondrial carriers, a family of transport proteins (*SLC25*) localised mainly in the inner membrane of mitochondria, provide a link between metabolic reactions occurring in the cytosol and the mitochondrial matrix by catalysing the translocation of numerous solutes across the mitochondrial membrane (Palmieri, 2013). For this reason, mitochondrial carrier gene expression requires a tight regulation in different tissues and in physiological and pathological conditions (Fiore et al., 1998; Barbera et al., 2001; Iacobazzi et al., 2005, 2009a, 2013; Indiveri et al., 2011; Infantino et al., 2011a).

Metabolic pathways are usually considered as ways to generate energy and to synthesise or break-down macromolecules. However, in recent years a growing body of evidence has highlighted that metabolism is to be considered as a wide integrated system strictly interplaying with pathologies, such as inflammation or cancer. Significant metabolic changes occur in the shift from resting to activated *status* in inflammation. In resting conditions the innate immune system cells (i.e. macrophages, dendritic cells, neutrophils, and the adaptive system cells, B and T lymphocytes) are relatively inactive. Upon recognition of pathogens-associated molecular patterns these cells become activated and shift to a very active metabolic state to respond to high energy demand similar to that observed in tumour cells (Palsson-McDermott and O'Neill, 2013). Consequently, changes in the expression level of a large number of genes and an extensive metabolic reprogramming lead to acquisition of new functions, such as production of cytokines, intermediates for biosynthesis, lipid mediators, ROS and NO, as well as morphological changes, such as tissue remodelling, migration through tissues and cellular division (Pearce and Pearce, 2013).

Some metabolites are diverted from normal metabolic pathways to the inflammatory process. In a study performed on LPS-activated macrophages and U937 cells expression of the mitochondrial citrate carrier (*SLC25A1*) increases via NF-kB and STAT1 giving rise to increased levels of citrate in the cytosol (Infantino et al., 2014). Here citrate is used to produce pro-inflammatory mediators, ROS, NO and prostaglandins (Infantino et al., 2011b). Succinate, another mitochondrial metabolite transported by the dicarboxylate carrier (*SLC25A10*), has been found to be a signal that induces IL-1β via HIF-1α (Tannahill et al., 2013). Succinate and α-ketoglutarate are also endocrine-signalling molecules. Both activate orphan receptors GRP91 (renamed SUCNR1) and GRP99 (renamed OXGR1), respectively, to stimulate

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many physiological processes including inflammation, immune cell activation and blood pressure (He et al., 2004). Thus, these metabolites, apart from their role in metabolism, are intracellular signalling molecules. Metabolism significantly affects macrophages M1 and M2 phenotypes (Kelly and O'Neill, 2015). Increased glycolysis (Warburg effect) and pentose phosphate pathways are associated with M1 activation, leading to a pro-inflammatory phenotype (high NO, TNF-α, IL-1β and other cytokines), whereas increased OXPHOS is observed in M2 and related to the anti-inflammatory phenotype (high expression of anti-inflammatory IL-10 and decreased production of pro-inflammatory NO and TNF-α) (Kelly and O'Neill, 2015). It is likely that the metabolic switches in inflammation are needed for distinct functions of macrophages and it cannot be excluded that other macrophagic polarisation states with different metabolic and inflammatory signatures and different roles in defence against pathogens exist.

In the context of metabolic adaption and reprogramming it is expected that the expression of several mitochondrial carriers changes in the inflammatory process. At present only few of these carriers have been functionally tested in inflammation. This review summarises the available information about mitochondrial carriers involved in the inflammation process.

Mitochondrial carriers differently regulated in inflammation

Fifty-three mitochondrial carriers are present in humans (Table 1) (Palmieri, 2013). Based on substrate specificity, 24 subfamilies, well conserved throughout evolution, have been functionally characterised, but several other *SLC25* family members remain to be characterised (Palmieri and Pierri, 2010; Palmieri, 2013). Moreover, mutations in the *SLC25* genes have been shown to be responsible for 13 diseases, highlighting the important role of *SLC25* genes in metabolism (Palmieri, 2014; Palmieri and Monné, 2016). To our knowledge few mitochondrial carriers have been directly or indirectly demonstrated to be involved in the inflammatory process: the citrate carrier (*SLC25A1*), the adenine-nucleotide carriers (*SLC25A4*, *SLC25A5*), the uncoupling proteins (*SLC25A7*, *SLC25A8*, *SLC25A9*), the dicarboxylate carrier (*SLC25A10*), the oxoglutarate carrier (*SLC25A11*), and the aspartate/glutamate carriers AGC1 (*SLC25A12*) and AGC2 (*SLC25A13*).

The citrate carrier CIC (SLC25A1)

The citrate carrier catalyses the transport of citrate, a product of tricarboxylate (TCA) cycle, out of the mitochondrion in exchange for malate, a process that profoundly influences energy balance in the cells (Bisaccia et al., 1989, 1990; Kaplan et al., 1993; Iacobazzi and Infantino, 2014; Kolukula et al., 2014). In the cytosol, citrate is cleaved by ATP-citrate lyase to oxaloacetate and acetyl-CoA. Acetyl-CoA is used for acetylation processes and fatty acids biosynthesis; oxaloacetate is reduced to malate and then to pyruvate via malic enzyme for the production of cytosolic NADPH + H^+ (Figure 1). Thus, the cytosolic amount of citrate depends on the CIC activity and on the *SLC25A1* gene expression level. In liver and pancreatic cells *SLC25A1* gene transcription is up-regulated by FOXA1 and Sp1 (Iacobazzi et al., 2009b; Menga et al., 2013) and down-regulated by SREBP1 in the presence of polyunsaturated fatty acids (Infantino et al., 2007; Infantino et al., 2011c). Furthermore, it is regulated in different tissues and during development by epigenetic mechanisms and ZNF224 (Iacobazzi et al., 2008).

The CIC is the most studied mitochondrial carrier in inflammation (Infantino et al., 2011b, 2014; Iacobazzi and Infantino, 2014; Palmieri et al., 2015). Transport of citrate via the CIC and citrate metabolism appear to be critical for the activation of macrophages (Infantino et al., 2011b; Iacobazzi and Infantino, 2014). In LPS-activated macrophages expression of the *SLC25A1* gene is induced at the transcriptional level by two pro-inflammatory cytokines, tumour necrosis factor-α (TNFα) and interferon-γ (IFNγ), through NF-kB and STAT1 transcription factors, respectively (Infantino et al., 2014) (Figure 1). Under these conditions citrate is preferentially removed from the TCA cycle and used for the *de novo* synthesis of fatty acids to respond to the cellular increase in biosynthetic demand. The increased acetyl-CoA level is necessary for TNFα or IFNγ to induce nitric oxide and prostaglandin production. Acetyl-CoA is also a source of acetyl groups for the acetylation of histones (Wellen et al., 2009) (Figure 1). Inhibition of histone acetylation through ATP-citrate lyase silencing suppresses expression of the glycolytic enzymes hexokinase, phosphofructokinase-1 and lactate dehydrogenase A resulting in a decrease of glucose consumption. Inhibition of ATP-citrate lyase also decreases the production of NO and ROS (Infantino et al., 2013). Oxaloacetate, the other product of citrate metabolism, is converted to malate and then to pyruvate generating NADPH, which serves in both iNOS

Table 1: The SLC25A mitochondrial carrier family. For more detailed informations about SLC family, please visit: http://www.bioparadigms.org.

Gene name	Protein name	Aliases	Main substrates
SLC25A1	citrate carrier	CIC, CTP	citrate, isocitrate, malate
SLC25A2	ornithine carrier	ORC2, ORNT2	ornithine, basic amino acids
SLC25A3	phosphate carrier	PiC, PTP, PHC	phosphate
SLC25A4	ADP/ATP carrier 1	AAC1, ANT1, T1	ADP, ATP
SLC25A5	ADP/ATP carrier 2	AAC2, ANT2, T2	ADP, ATP
SLC25A6	ADP/ATP carrier 3	AAC3, ANT3, T3	ADP, ATP
SLC25A7	uncopupling protein 1	UCP1	H+
SLC25A8	uncopupling protein 2	UCP2	H+, C4 metabolites
SLC25A9	uncopupling protein 3	UCP3	?
SLC25A10	dicarboxylates carrier	DIC	succinate, malate, phosphate, sulphate, thiosulpahte
SLC25A11	malate/oxoglutarate carrier	OGC	malate, 2-oxoglutarate
SLC25A12	aspartate/glutamate carrier 1	AGC1, aralar	aspartate, glutamate
SLC25A13	aspartate glutamate carrier 2	AGC2, citrin, aralar2	aspartate, glutamate
SLC25A14	uncopupling protein 5	UCP5	?
SLC25A15	ornithine carrier 1	ORC1, ORNT1, HHH	ornithine, basic amino acids
SLC25A16	grave's disease carrier	GDC,	?
SLC25A17	peroxisomal cofactor carrier	PMP34,ANC1	NAD/FAD/CoA Peroxisome
SLC25A18	glutamate carrier 2	GC2	glutamate
SLC25A19	thiamine-pyrophosphate carrier	TPC, DNC, MCPHA	thiamine-pyrophosphate,thiamine-monophosphate, (deoxy)nucleotides
SLC25A20	carnitine carrier	CAC, CACT	carnitine, acylcarnitine
SLC25A21	2-oxoadipate carrier	ODC	2-oxoadipate, 2-oxoglutarate
SLC25A22	glutamate carrier 1	GC1	glutamate
SLC25A23	ATP-Mg/Pi carrier	APC2, ScaMC-3	ATP-Mg, Pi, ADP, ATP
SLC25A24	ATP-Mg/Pi carrier	APC1, ScaMC-1	ATP-Mg, Pi, ADP, ATP
SLC25A25	ATP-Mg/Pi carrier	APC3, ScaMC-2	ATP-Mg, Pi, ADP, ATP
SLC25A26	S-adenosyl methionine carrier	SAMC	S-adenosyl methionine, S-adenosyl homocysteine
SLC25A27	uncoupling protein 4	UCP4	?
SLC25A28	mitoferrin-2	MFRN2	?
SLC25A29	basic amino acids carrier	C14orf69, ORNT3	basic amino acids (arginine, lysine, to a lesser extent ornithine)
SLC25A30	kidney mitochondrial carrier protein (uncoupling protein 6)	KMCP (UCP6)	?
SLC25A31	ADP/ATP carrier 4	AAC4, ANT4	ADP, ATP
SLC25A32	mitochondrial folate carrier	MFT	?
SLC25A33	pyrimidine nucleotide carrier1	PNC1	uracil, thymine, and cytosine (deoxy)nucleoside di-and triphosphates, guanine nucleotides
SLC25A34	?	?	?
SLC25A35	?	?	?
SLC25A36	pyrimidine carrier2	PNC2	cytosine and uracil(deoxy)nucleoside mono-, di-, and triphosphates and guanine nucleotides
SLC25A37	mitoferrin-1	MFRN1	?
SLC25A38	?	?	?
SLC25A39	?	CGI-69	?
SLC25A40	?	MCFP	?

Table 1 (continued)

Gene name	Protein name	Aliases	Main substrates
SLC25A41	ATP-Mg/Pi carrier	APC4, SCaMC-3L	ATP-Mg, Pi, ADP, ATP
SLC25A42	CoA/PAP carrier	CoAPC	CoA, adenosine3′,5′ -diphosphate
SLC25A43	?	?	?
SLC25A44	?	?	?
SLC25A45	?	?	?
SLC25A46	?	?	?
SLC25A47	?	C14orf68, HDMCP	?
SLC25A48	?	?	?
SLC25A49	mitochondrial carrier homologue 1	?	?
SLC25A50	mitochondrial carrier homologue 2	MTCH2	?
SLC25A51	mitochondrial carrier triple repeat 1	MCART1	?
SLC25A52	mitochondrial carrier triple repeat 2	MCART2	?
SLC25A53	mitochondrialcarriertriple repeat 6	MCART6	?

catalysed NO production and NADPH oxidase-catalysed ROS generation. NADPH is also required for the conversion of arginine to NO and citrulline (Figure 1). NO can also inhibit the OXPHOS through nitrosylation of components of respiratory chain subunits; ROS can stabilise HIF-1α, promote glycolysis and sustain transcription of the pro-inflammatory cytokines (Calvani et al., 2012) (Figure 1). The central role of the mitochondrial citrate export pathway via *SLC25A1* as inflammatory signal is supported by additional evidence obtained with dendritic cells. Genetic silencing of *SLC25A1* and inhibition of acetyl-CoA-carboxylase lead to decreased fatty acid biosynthesis that limits membrane production necessary for the expansion of endoplasmic reticulum (ER) and Golgi. Consequently, ER protein synthesis decreases resulting in reduction of cytokines needed for T-cell activation by dendritic cells (Everts et al., 2014).

Recently, acetylation of CIC was demonstrated to be a key event that increases the activity of the carrier itself (Palmieri et al., 2015). CIC is acetylated in activated primary human macrophages and U937 cells and the level of CIC acetylation is higher in glucose-deprived compared to normal glucose medium. NADPH demand necessary for NADPH oxidase activity, typical of M1 macrophages, is usually met by activation of the pentose phosphate pathway. In conditions in which glutamine, and not glucose, is available as an energy source, it is unclear how activated cells sustain NADPH production. It is likely that the efflux of citrate from mitochondria may serve to respond to the cellular demand of NADPH. Acetylated CIC catalyses a faster transport of citrate into the cytosol. In this way activated cells can sustain NADPH synthesis in the absence of glucose by channeling a portion of citrate to isocitrate dehydrogenase without depauperating ATP-citrate lyase of its substrate. Macrophage plasma membrane NADPH oxidase is a potent source of very high concentrations of ROS and hydrogen peroxide employed for the killing of sequestered pathogens (Wang et al., 2007). Altogether these results highlight the essential role of 'the citrate pathway in inflammation' that is actively induced in LPS-activated macrophages.

Very recently, a link between citrate pathway activation and oxidative stress in Down syndrome (DS) has been reported (Convertini et al., 2016). Indeed, inhibition of citrate pathway leads to a significant reduction of ROS as well as lipid peroxidation levels, common landmarks of subjects with DS (Brooksbank and Balazs 1984), suggesting that mechanisms other than imbalance in the SOD1/catalase (Feaster et al., 1977) are also involved in the pro-oxidant *status* in the DS.

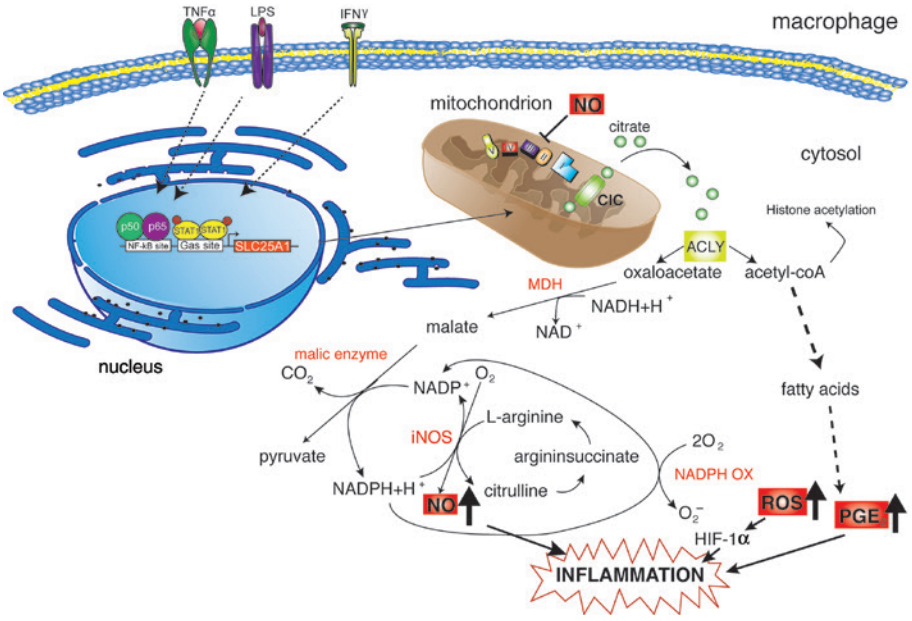

Figure 1: Role of citrate and CIC in cytokine-induced inflammation.
Tumour necrosis factor-α (TNFα) and Lipoplysaccharide (LPS) increase the CIC gene expression through NF-kB signalling. Interferon-γ (IFNγ) acts through STAT transcription factors. These signals induce an increase of CIC expression resulting in an increased citrate transport outside mitochondria. In the cytosol citrate is metabolised to acetyl-CoA and oxalacetate by citrate lyase. Acetyl-CoA is used in the fatty acids biosynthesis and acetylation processes. Oxaloacetate is converted to malate and then to pyruvate generating NADPH which serves in both iNOS catalysed NO production and NADPH oxidase-catalysed ROS generation. Upward arrows indicate up-regulation. Green rectangle and green circle indicate the citrate carrier and the citrate molecule, respectively. Coloured shapes with roman numerals indicate the respiratory chain. ACLY, ATP-citrate lyase; CIC, citrate carrier; HIF-1α, hypoxia-inducible factor-1α; iNOS, inducible nitric oxide synthase; MDH, malic dehydrogenase; NADPHOX, NADPH oxidase; PGE_2, prostaglandin E_2.

Recessive mutations in *SLC25A1* gene encoding the mitochondrial citrate carrier (Edvardson et al., 2013; Nota et al., 2013; Chaouch et al., 2014) cause a rare inherited metabolic disorder, combined 2-hydroxyglutaric aciduria (2-HGA), characterised by epileptic encephalopathy, respiratory insufficiency, developmental arrest and early death. The origin of elevated 2-HG due to *SLC25A1* deficiency is still unclear, but has been attributed to accumulation of citrate and other tricarboxylic acid (TCA) cycle intermediates in the mitochondria including α-ketoglutarate, which in turn is converted to 2-HG (Kranendijk et al., 2012) (see section The oxoglutarate carrier OGC). Specific studies on inflammatory response have not been performed in these patients. However, it is expected that the severity of the clinical findings could be accounted for the key role played by *SLC25A1* in carbohydrate and lipid metabolism and in promoting mitochondrial function (Palmieri 2013). On one hand, accumulation of mitochondrial citrate affects the integrity of TCA cycle as well as mitochondrial inner membrane potential, on the other hand, cytosolic citrate deficiency affects glycolysis, which proceeds unchecked and impairs lipogenesis. Thus, it could be speculated that the citrate-dependent pro-inflammatory mediators biosynthesis should be also affected. Another genetic defects involving the *SLC25A1* carrier is the congenital disorder 22q11.2 deletion syndrome (22qDS) (Napoli et al. 2015). These patients show a metabolic shift from oxidative phosphorylation to glycolysis (higher lactate/pyruvate ratios) accompanied by an increase in reductive carboxylation of α-ketoglutarate (increased concentrations of 2-hydroxyglutaric acid, cholesterol, and fatty acids) (Napoli et al., 2015).

The adenine-nucleotide carriers AAC (SLC25A4, SLC25A5)

Four isoforms of the ADP/ATP carrier, differently expressed in tissues, have been found in humans. AAC1 is predominantly expressed in heart and skeletal muscle (Levy et al., 2000), AAC2 is more abundant in proliferating tissues (Stepien et al., 1992; Chevrollier et al., 2005), AAC3 is ubiquitously expressed and AAC4 is specifically expressed in testis (Dolce et al., 2005). These carriers catalyse the

specific translocation of ATP^{4-} outside mitochondria in exchange with cytosolic ADP^{3-} (Dolce et al., 2005). AAC has also been reported to be a component of the mitochondrial permeability transition pore (MTP), along with voltage-dependent anion channel (VDAC) in the outer membrane and cyclophyllin D in the matrix (Woodfield et al., 1998). The involvement of AAC as member of MTP in inflammation could be related to the ROS production. Decreased ADP levels induce an increase of the mitochondrial membrane potential, which lowers the respiratory rate leading to stimulation of ROS production due to the highly reduced state of the components of respiratory chain. This control also includes hexokinase, a phosphorylating enzyme located on the surface of mitochondria (da-Silva et al., 2004; Santiago et al., 2008). A fraction of ATP transported outside mitochondria is immediately used by hexokinase to phosphorylate glucose leading to generation of ADP. Thus, a steady stream of ADP is always available to ensure that coupled respiration remains efficient (Mailloux and Harper, 2011).

Upon systemic inflammation of leukocytes induced by bacterial endotoxins, a widespread suppression of the expression of genes involved in energy production, including *SLC25A5*, VDAC, OXPHOS subunits, pyruvate dehydrogenase, was found suggesting that leukocytes exposed to inflammatory stimuli may have an altered capacity to sustain subsequent immune challenges (Calvano et al., 2005). It is known that leukocytes are active secretory cells that devote a substantial amount of energy expenditure to protein synthesis (Buttgereit and Brand, 1995). Furthemore, it is worth noting that *SLC25A5* is succinylated in LPS-induced macrophages, which suggests that different factors contribute to modulate the expression level of bioenergetically-relevant genes in inflammation (see section on The dicarboxylate carrier).

Recently, interesting studies have been performed on *SLC25A4* (AAC1) expression in H9c2 cells, derived from myocardial myoblast (Pan et al., 2015). H9c2 cells treated with TNF-α and LPS showed a reduction of AAC1 protein levels in a dose-dependent manner. In the same study AAC1 was found to be down-regulated significantly in the inflamed heart in a murine model of systemic inflammation induced by cecal ligation and puncture (CLP). In the H9c2 cell line *SLC25A4* was silenced by siRNA resulting in decreased respiration and increased membrane potential, TNF-α-induced NF-kB reporter gene activity, expression of IL-6 and TNF-α as well as increased swollen mitochondria and ROS production (Pan et al., 2015). Thus, a vicious cycle between AAC1 down-regulation and NF-kB, and subsequent production of cytokines exacerbates the inflammation process.

The uncoupling proteins UCPs (SLC25A7, SLC25A8, SLC25A9)

The UCPs subfamily of mitochondrial carriers consists of five members (UCP1-5) differently expressed in human tissues. UCP1 is specific to brown adipose tissue; UCP2 is ubiquitous and highly expressed in organs that are involved in immune defence or rich in macrophages; UCP3 is specific to skeletal muscle; UCP4 and UCP5 (originally described as brain mitochondrial carrier protein-1, BMCP1) are expressed predominantly in brain (Bouillaud et al., 2016). UCP1 acts as uncoupling protein to dissipate energy as heat in mammalian brown fat (Bouillaud et al., 1984). At variance with UCP1, the physiological role for the other members of UCP subfamily is still unclear. The involvement of UCP2 in inflammation is related to the controls of ROS production and macrophage activity (Arsenijevic et al., 2000). UCP2 is expressed in most of the cells involved in inflammation and immune system, such as macrophages, dendritic cells, mastocytes, neutrophils, B and T lymphocytes (Rousset et al., 2006; Emre and Nübel, 2010). In resting conditions UCP2 is highly expressed and the energetic need is satisfied by glucose, glutamine and fatty acids. Upon stimulation with LPS, UCP2 is rapidly down-regulated by the MAPK pathways resulting in an increase of mitochondrial ROS levels that act as feedback signals stimulating the ERK and p38 pathways (Emre et al., 2007) (Figure 2). Metabolically the cellular use of glucose is increased, whereas glutamine oxidation is unchanged, and fatty acid utilisation is directed away from mitochondrial oxidation (Emre et al., 2007) (Figure 2). Decrease of UCP2 levels promotes not only the mitochondrial ROS-dependent MAPK signalling, but also expression of inducible form of the NO synthase, NO production, and release of pro-inflammatory cytokines in macrophage (Arsenijevic et al., 2000; Kizaki et al., 2002; Lee et al., 2005; Rousset et al. 2006; Emre et al., 2007).

An important question concerns the mechanism by which UCP2 modulates ROS production and triggers signalling cascade in inflammation. UCP2 was proposed as a mediator of mitochondrial respiration uncoupling resulting in lowering the mitochondrial membrane potential (Nègre-Salvayre et al., 1997; Gustafsson et al., 2004). In acute increase of ROS production UCP2 induces proton conductance, providing a negative feedback loop to limit further mitochondrial ROS formation (Echtay et al., 2002). Mailloux et al., 2011 proposed a glutathione-related mechanism by which UCP2 might control ROS production. According to this mechanism, UCP2 reactive cysteine residues can be conjugated with glutathione in a

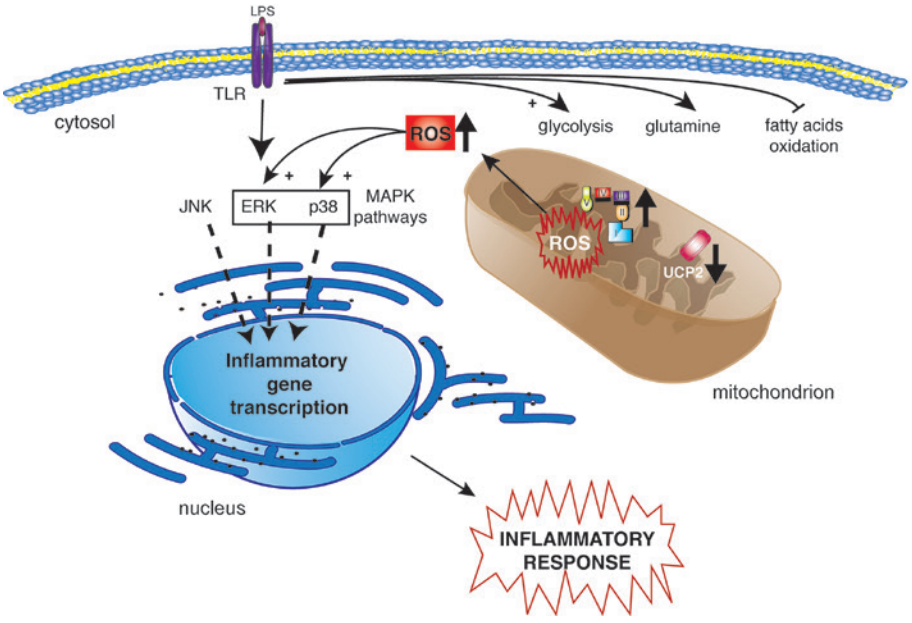

Figure 2: UCP2 down-regulation and generation of ROS in LPS-activated macrophages.
LPS binding increases glycolysis, inhibits fatty acid oxidation and activates MAPK pathways. UCP2 is down-regulated via the JNK and p38 pathways to increase mitochondrial ROS production. Mitochondrial ROS generation, as a feedback signal, stimulates the ERK and p38 pathways to induce expression of inflammatory genes. Downward and upward arrows indicate down-regulation and up-regulation, respectively; '+' and interrupted arrow indicate increase and decrease, respectively. Red rectangle indicates the carrier. Coloured shapes with roman numerals indicate the respiratory chain. TLR, toll-like receptor.

process of glutathionylation/deglutathionylation. When mitochondrial ROS are maintained in tolerable levels and the cellular redox state is normal UCP2 is glutathionylated. On the contrary, increase of ROS levels causes a deglutathionylation inducing increased state 4 respiration (the proton leak dependent respiration) (Mailloux and Harper, 2011).

Alternative studies have highlighted a metabolic role for UCP2, in particular in pathophysiological conditions (Stuart et al., 2001; Nedergaard and Cannon, 2003). UCP2 can be implicated in the choice of the substrate oxidised in mitochondria. (Pecqueur et al., 2008; Bouillaud, 2009; Emre and Nübel, 2010). Recently, UCP2 has been found to transport C4 metabolites from mitochondria to the cytosol (Vozza et al., 2014). UCP2 negatively controls the oxidation of acetyl-CoA-producing substrates via the Krebs cycle, thus lowering the redox pressure on the respiratory chain, the ATP/ADP ratio and ROS production. The export of C4 metabolites is of particular interest in cancer cells, where UCP2 is highly expressed (Ayyasamy et al., 2011) and the glutamine metabolism increases to supply the mitochondrial C4 metabolites (aspartate, oxalacetate) needed for the synthesis of macromolcules. It is noteworthy that the metabolic role is indipendent of previously proposed uncoupling activity for UCP2. However, the metabolic role does not preclude the possibility that the activation of the uncoupling activity could be of physiological relevance.

Similarly to UCP2, other UCPs have been associated with protection from ROS (Echtay et al., 2003). UCP1 (*SLC25A7*) overexpression suppresses the TNFα-induced mitochondrial ROS production in cultured human hepatoma (Huh7) cells (Imoto et al., 2006), and in rat muscle the UCP3 (*SLC25A9*) protein content protects from ROS production (Vidal-Puig et al., 2000).

The dicarboxylate carrier DIC (SLC25A10)

The dicarboxylate carrier (DIC) transports the dicarboxylate metabolites malate and succinate, as well as phosphate and sulphur containing molecules (e.g. sulphite, sulphate and thiosulphate) (Palmieri and Monné, 2016), supplying substrates for OXPHOS, gluconeogenesis, ureogenesis and sulphur metabolism. Thus, it contributes to sustain the balance of TCA cycle substrates between mitochondria and the cytosol (Mizuarai et al., 2005). The expression of *SLC25A10* significantly increases by about four times in LPS-stimulated U937 cells and macrophages (Iacobazzi et al. unpublished work, 2016). Although

extensive and specific studies have not been performed on *SLC25A10* in inflammation yet, interesting clues come from cancer cells that show a metabolic condition similar to that of inflammation (Zhou et al., 2015). Similar to the situation in inflammatory U937 cells and macrophages, *SLC25A10* is up-regulated in cancer cells (Zhou et al., 2015). Down-regulation of its expression changes the growth properties to a less malignant phenotype in A549 cells, which is likely due to increased glutamine dependency and sensitivity to oxidative stress. As a metabolic consequence of *SLC25A10* down-regulation, there is a shift from glycolysis to OXPHOS, which is observed in reduced level of lactate dehydrogenase and HIF-1α, as well as increased glutamate dehydrogenase expression (Zhou et al., 2015).

Extensive studies have investigated the role of succinate as a signalling molecule in inflammation (Mills and O'Neill, 2014). Despite the overall decrease of TCA cycle and mitochondrial respiration in inflammatory condition, succinate levels accumulated in macrophages induced by LPS (Tannahill et al., 2013), which suggests that succinate is mainly derived from other sources than the TCA cycle, such as glutamine metabolism via anaplerosis (Owen et al., 2002) through α-ketoglutarate as well as from the γ-aminobutyric acid (GABA) shunt (Tannahill et al., 2013) (Figure 3). In the cytosol, accumulation of succinate induces activation of HIF-1α in normoxia directly by inhibition of prolyl hydroxylase, a member of super-family of α-ketoglutarate-dependent hydroxylases, or indirectly by induction of ROS production (Tannahill et al., 2013) (Figure 3). Succinate could also be involved in remodelling the epigenome and altering gene expression by inhibiting α-ketoglutarate-utilising Jumonji (JmjC) domain-containing histone demethylases (Tsukada et al., 2006), and the TET (Ten-eleven-translocation) family of 5-methylcytosine (5mC) hydroxylases (Tahiliani et al., 2009) leading to DNA hypermethylation (Figure 4). In addition, patients harbouring mutations in succinate dehydrogenase (Killian et al., 2013; Letouzè et al., 2013) show increased HIF-1α activity and high succinate level, which indicates an important similarity between inflammation and cancer. It could be hypothesised that the inflammatory process may have tumourigenic effect by increasing succinate levels. Interestingly, fumarate hydratase mutations

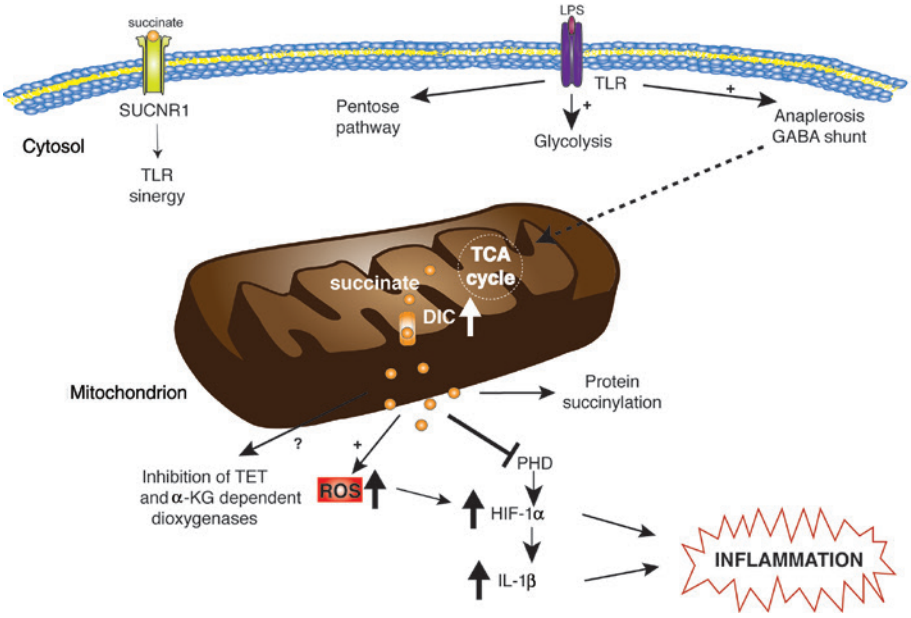

Figure 3: Role of succinate and DIC in macrophage activation by LPS.
Binding of LPS to TLR leads to an increase of glycolysis and pentose pathway, and a decrease of TCA cycle. Succinate level increases via anaplerosis through α-ketoglutarate and GABA shunt; it is transported by DIC outside mitochondria into cytosol where it acts as signalling molecule. It inhibits the prolyl hydroxylase activity leading to an increase and stabilisation of HIF-1α and other pro-inflammatory genes, and induces succinylation of proteins. It could also inhibit TET and α-ketoglutarate dioxygenases enzymes. Succinate can also act as an extracellular signalling molecule which binds to SUCNR1 receptor sustaining cytokines production. Downward and upward arrows indicate down-regulation and up-regulation, respectively; '+' indicates stimulation, and '?' indicates that the pathway has to be tested in inflammation. Orange rectangle and orange circle indicate the dicarboxylate carrier and the succinate molecule, respectively. DIC, dicarboxylate carrier; GABA, γ-aminobutiric acid; HIF-1α, hypoxia-inducible factor-1α; IL-1β, interleukin-1β; PHD, prolyl hydroxylase; TLR, toll-like receptor; SUCNR1, succinate receptor 1; TET, Ten-eleven-translocation.

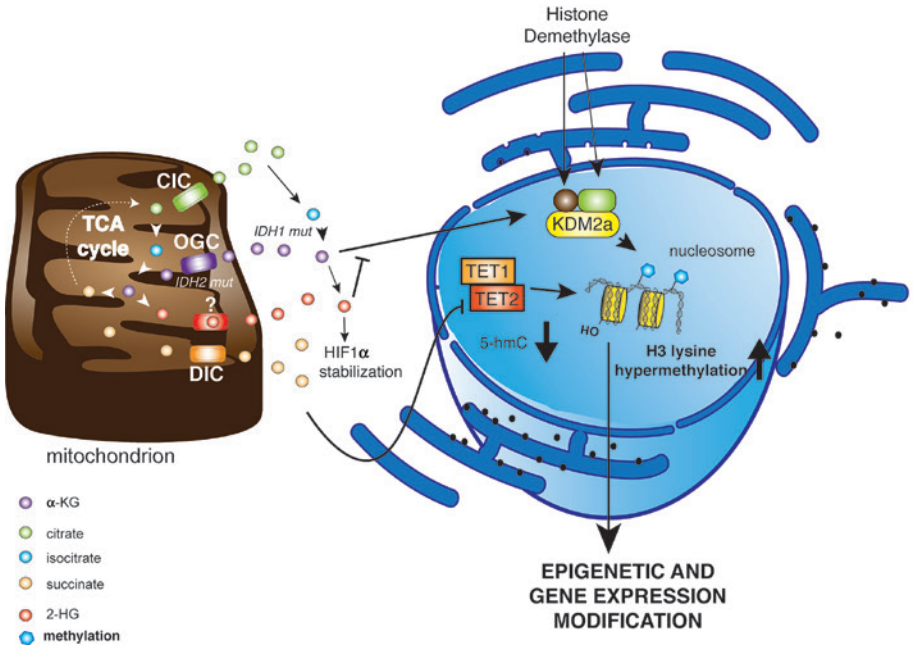

Figure 4: Role of 2-Hydroxyglutarate (2-HG) and succinate as α-KG antagonists to inhibit α-KG-dependent dioxygenases, including Jumonji domain (JMJD) family KDMs, and TET family of 5-methyl-cytosine hydroxylases.
This disregulation contributes to aberrant regulation of gene expression. Specific carriers still unknown are indicated with '?'. Green, violet, red and orange rectangles indicate the CIC, OGC, unknown and DIC carriers, respectively. Green, blue, violet, red, orange circles indicate the citrate, isocitrate, α-ketoglutarate, 2-hydroxyglutarate and succinate molecules, respectively. CIC, citrate carrier; OGC, oxoglutarate carrier; 2-HG, 2-hydroxyglutarate, α-KG, α-ketoglutarate ; 5-hmC, 5-hydroxymethylcytosine; IDH1, isocitrate dehydrogenase 1; IDH2, isocitrate dehydrogenase 2; KDM2a, lysine-specific demethylase 2a; mut, mutations; TET, Ten-eleven-translocation.

cause accumulation of another TCA cycle intermediate, fumarate (Xiao et al., 2012), which could also function as competitive inhibitor of α-KG-dependent dioxygenase. At present, no mammalian mitochondrial carrier tested for fumarate transport activity has been identified. High levels of succinate increase succinylation of several proteins affecting their function in energy metabolism (Tannahill et al., 2013) (Figure 3). Among them two mitochondrial carriers, the glutamate carrier (*SLC25A22*) and the adenine nucleotide carrier (*SLC25A5*), have been found to be succinylated in inflammatory conditions associated with reduced OXPHOS. Moreover, succinate acts also as a ligand of SUCNR1, a succinate receptor highly expressed in kidney, liver, spleen small intestine (He et al., 2004), and dendritic cells (Rubic et al., 2008). HEK293 cells stably expressing SUCNR1 display inositol-triphosphate accumulation, calcium mobilisation, ERK phosphorylation, calcium-dependent NO and PGE_2 production under succinate stimulus (He et al., 2004).

Altogether these features indicate that succinate is an important signalling molecule in innate immune cells, and *SLC25A10* may represent a target to regulate succinate availability for reprogramming metabolism in inflammation and other pathologies including cancer.

The oxoglutarate carrier OGC (SLC25A11)

The oxoglutarate carrier (OGC) catalyses the transport of oxoglutarate in electroneutral exchange for some other dicarboxylates, among which malate is bound with the highest affinity (Bisaccia et al., 1985; Indiveri et al., 1987; Runswick et al., 1990; Monné et al., 2013). The activity as a glutathione transporter in mitochondria was ascribed to OGC (together with DIC) based on observations in low- and high-affinity transport systems reported in previous studies (Kurosawa et al., 1990; Mårtensson et al., 1990). Recently on the contrary, by using fused membrane vesicles of *Lactococcus lactis* overexpressing OGC and DIC, no transport of GSH by these carriers was observed in any tested condition (Booty et al., 2015).

Although transport of the canonical substrates (malate and oxoglutarate for OGC, and malate and phosphate for DIC) can be measured readily, an excess of glutathione did not compete for substrate uptake nor could transport of glutathione be measured directly. Thus, neither *SLC25A11* nor *SLC25A10* can be considered a carrier of glutathione (Booty et al., 2015). However, it

is not excluded that a relationship between OGC and/or DIC and glutathione may exist. In fact, neuronal cell lines (NSC34) stably overexpressing OGC show an increased mitochondrial GSH level, leading to neuronal resistance to oxidative stress induced by hydrogen peroxide and to nitrosoactive stressors, induced by sodium nitroprusside (Wilkins et al., 2012).

An interesting finding related to OGC comes from cancer cells. Mutations in cytoplasmic isocitrate dehydrogenase 1 and mitochondrial isocitrate dehydrogenase 2 lead to accumulation of α-ketoglutarate that is converted into 2-hydroxyglutarate (2-HG) (Dang et al., 2010). The increased level of 2-HG competitively inhibits α-KG binding to several histone demethylases, including KDM2a, and TET1 and TET2 hydroxymethylases, which decreases the levels of 5-hydroxymethylcytosine (Figure 4). It is noteworthy that, even though 2-HG and α-KG share a very similar structure, whether 2-HG is transported by the OGC carrier or other carriers is not known. Finally, 2-HG also helps to stabilise HIF-1α, partially by decreasing levels of the HIF-1α antagonist endostatin, which results in increased VEGF signalling, a driver of increased angiogenesis in human cancers (Prensner and Chinnaiyan, 2011).

These findings highlight the importance of crosstalk between mitochondria and cytosolic pathways in the regulation of inflammation (Saitoh and Akira, 2010).

The aspartate/glutamate carriers AGC1 (SLC25A12) and AGC2 (SLC25A13)

Two isoforms of this carrier, AGC1 and AGC2, are present in humans (Palmieri, 2013). Differently expressed in tissues (Palmieri, 2013), they catalyse the electrogenic exchange of aspartate for glutamate plus H^+, a transport that is regulated by the concentration of free Ca^{2+} in the cytosol (Palmieri et al., 2001). AGC1 and AGC2 play an important role in the malate/aspartate shuttle transferring the reducing equivalents of NADH from the cytosol to the mitochondria. Aspartate is also required in the urea cycle, gluconeogenesis from lactate, as well as for purine and pyrimidine synthesis (Palmieri and Monné, 2016). The expression of *SLC25A12* was investigated in the context of neuroinflammation (Menga et al., 2015); since this carrier is the main isoform in the brain and aspartate is used to synthesise N-acetyl-aspartate needed in the myelinisation process (Sakurai et al., 2010). Upon treatment with a combination of pro-inflammatory cytokines TNFα, IFNγ, and IL-1β a pronounced decrease of *SLC25A12* mRNA and protein in neuronal cells was observed (Menga et al., 2015). It should be noted that the cytokines-mediated down-regulation of AGC1 is exacerbated by prolonged pro-inflammatory stimuli. Down-regulation is mediated by CREB phosphorylation, a well-known transcriptional factor regulating several genes encoding proteins involved in energy metabolism (Menga et al., 2015).

Surprisingly, in LPS-activated macrophages AGC1 mRNA expression increased (three fold) and a much higher increase (eight fold) was found for the AGC2 mRNA expression with respect to macrophages not treated with LPS (Iacobazzi et al. unpublished work, 2016). Although the reason for this difference in expression is not known, it is clear that the malate-aspartate shuttle is very active in LPS-activated macrophages and that the contribution of AGC2 to the shuttle is greater than that of AGC1. NAD^+ is emerging as a regulator of inflammation by acting through sirtuins, a class of NAD^+-dependent deacetylases, which influence chromatin structure and protein function (He et al., 2012). In this context, what is the role of AGCs? Due to their role in transferring the reducing equivalents, it is likely that changes in the NAD^+/NADH ratio can change the redox state of the cell and alter the activity of sirtuins and other enzymes with subsequent effects on signalling cascades and gene expression. For example, a decrease of NAD^+ concentration following LPS stimulation impairs Sirt1 function and enhances inflammatory NF-kB dependent signals (Yeung et al., 2004). Activators of NLRP3 (NLR family, pyrin domain containing 3) inflammasome inhibit mitochondrial functions, reduce NAD^+ levels and impair Sirt2 function (Misawa et al., 2013). Sirt3 interacts with some TCA cycle enzymes, such as succinate dehydrogenase and isocitrate dehydrogenase (Schlicker et al., 2008).

Patients with AGC1 and AGC2 deficiency have been identified (Palmieri, 2014). The main features of the AGC1 deficiency, caused by mutation in the *SLC25A12* gene, are severe global hypomyelination and marked reduction of *N*-acetyl aspartate in the brain (Wibom et al., 2009, Falk et al., 2014). AGC2 deficiency, due to mutations in the *SLC25A13* gene, can cause neonatal intrahepatic cholestasis or adult-onset type II citrullinemia (CTLN2) (Kobayashi et al. 1999; Saheki and Kobayashi, 2002). At present it is not known whether and to what extent deficiency of these carriers causes change in production of pro-inflammatory molecules.

Conclusion and perspective

The data discussed in this review highlight the relationship between intermediary metabolism and

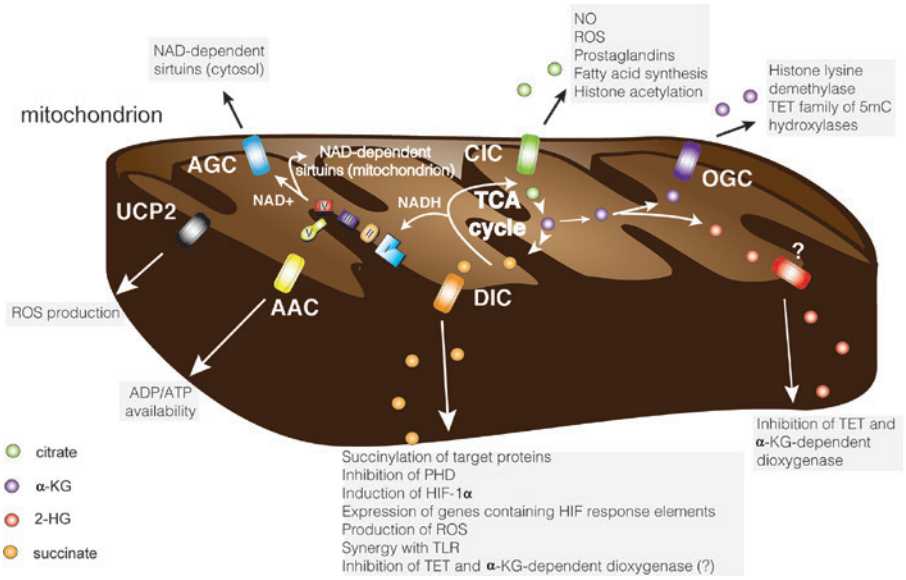

Figure 5: Overview of the mitochondrial carriers and related TCA cycle intermediates as inflammatory signals discussed in the corresponding sections of the text.
Green, violet, red, orange, yellow, black and blue rectangles indicate the CIC, OGC, unknown, DIC, AAC, UCP and AGC carriers, respectively. Green, violet, red, orange circles indicate the citrate, isocitrate, α-ketoglutarate, 2-hydroxyglutarate and succinate, respectively. Coloured shapes indicate the respiratory chain. Specific carrier still unknown is indicated with '?'. AAC, adenine-nucleotide carrier; AGC, aspartate/glutamate carrier; CIC, citrate carrier; DIC, dicarboxylate carrier; OGC, oxoglutarate carrier; UCP2, uncoupling protein 2.

inflammation. The expression of some mitochondrial carriers is modulated to meet the metabolic changes associated with inflammation finalised to pro-inflammatory molecules, such as ROS and NO production. Thus, mitochondrial metabolites, such as citrate and succinate, are not simply products of metabolism, but also act as intracellular signalling in LPS action. Therefore, the metabolic pathways and metabolites should be considered from a new point of view. In particular, the Krebs cycle intermediates may represent a novel class of regulators acting as key signals in different biological processes. In the context of inflammation the role of mitochondrial specific carriers and related transported metabolite is now beginning to be understood. The carriers examples treated in this review (Figure 5) are part of broader picture. It is very likely that the shift in metabolism that occurs in inflammation has specific consequences on the alteration of TCA cycle intermediates levels, epigenetic modifications, and gene expression. Thus, the involvement of other mitochondrial carriers and related metabolites is expected. For example, the glutamine and 2-HG carriers are still unknown; whether inhibition of α-KG dependent dioxygenase and TET by 2-HG and succinate is a common event in inflammation needs to be further investigated. Therefore, different questions remain still open. Beyond the specific involvement of the metabolites described in this review, which other mitochondrially derived molecules are involved in the inflammation process? Which mechanism(s) address(es) mitochondria toward inflammation or apoptosis? Do other mitochondrial carriers play a role in these mechanisms? Due to the importance of anti-oxidant defense, what determines the preferential usage of a particular carrier in a particular organ or cell type? Is there an organ- or cell-specific mitochondrial carrier for glutathione? To what extent do specific alterations in the TCA cycle and cellular redox status affect the phenotype of macrophages?

In conclusion, there is clear evidence about the involvement of mitochondrial carriers and related metabolites in inflammation. Understanding the mechanisms that induce inflammation through mitochondrial players may help identifying new therapeutic targets for treatment of inflammatory disease as well as cancer.

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