

Microglia activation: a role for mitochondrial DNA?

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Microglial cells and perivascular macrophages are the only resident immune cells of the brain parenchyma and act as innate immune sentinels in the central nervous system (CNS). Microglial cells are vital for the maintenance of CNS homeostasis thanks to their strict interaction with neurons. When the homeostasis of the microenvironment is disrupted, microglia can alter their phenotype acquiring pro- or anti-inflammatory function to defend the brain. On the other hand, the excessive activation of proinflammatory microglia in response to primary neurodegeneration, axonal degeneration, and additional peripheral activation processes linked to systemic inflammation can trigger or maintain chronic inflammation. Therefore, under such conditions, the proinflammatory phenotype of microglia could be harmful and associated with the pathogenesis of neurological disease characterized by inflammation, such as neurodegenerative diseases, demyelinating diseases, CNS trauma, and epilepsy. Despite the numerous studies on that field, the primary stimuli that provoke and maintain such inflammation, as well as the biological pathways and mechanisms that cause detrimental actions of microglia are still a subject of debate. Microglia can sense cellular damage and stress by recognizing the damage-associated molecular patterns (DAMPs) through the pattern recognition receptors (PRRs). Several lines of evidence, obtained from studies in humans and animal models, suggest that DAMPs could play a relevant role in the pathogenesis of several neurodegenerative diseases (Gong et al., 2020). The category of DAMPs includes several molecules, some of them can be released from damaged mitochondria (the so-called mitochondrial DAMPs, mtDAMPs), such as N-formyl peptides, cardiolipin, the mitochondrial transcription factor A (TFAM), succinate, adenosine triphosphate, and mitochondrial DNA (mtDNA). Damaged cells accumulate dysfunctional mitochondria that trigger processes such as cell senescence, apoptosis, or necrosis. In all of these cases, mtDAMPs can be released in the extracellular space and could be recognized through different pattern recognition receptors by innate immune cells recruited to remove cellular debris of dying cells. Recently, increasing attention has been paid to mtDNA, as DAMP able to strongly stimulate cells through Toll-like receptor (TLR) 9 contributing to inflammation even in the absence of infection (sterile inflammation) (Riley et al., 2020). After an extensive cell injury, several mitochondrial products, including mtDNA, can enter the bloodstream or cerebrospinal fluid (CSF), where they are

recognized by the innate immune system and evoke a local or systemic response. The cell-free mtDNA is stable and resistant to nuclease digestion, more than genomic DNA, and could be detected in blood or CSF. Even in healthy people, mtDNA is present at relatively high levels in the blood and easily measurable. Over the past few years, there has been a growing interest in mtDNA as a potential biomarker as its levels are increased in several physiopathological conditions characterized by chronic inflammation (Cossarizza et al., 2011; Pinti et al., 2014; Nasi et al., 2016), including neurodegenerative diseases such as multiple sclerosis (MS) (Nasi et al., 2020a). Interestingly, mtDNA levels were found higher also in CSF from people with MS but not in people affected by Parkinson's disease or Alzheimer's disease (Gambardella et al., 2019). Parkinson's disease and Alzheimer's disease are characterized by a loss of neuronal mitochondria (where probably the low levels of mtDNA in the CSF come from) followed by neuronal death, while MS is characterized by a strong inflammatory response in which mtDNA could be released into the CSF. Thus, MS represents a valuable model of neuro-inflammation, in which mtDAMPs could have a prominent role. On the other hand, the neuro-inflammation itself is strictly associated with mitochondrial dysfunction that could trigger a vicious circle: dysfunctional mitochondria can induce inflammation and inflammation induces mitochondrial dysfunction followed by the further release of mtDAMPs. However, the triggers by which mtDAMPs are released are still unknown, as well as the precise role of mtDNA and mtDAMPs in patients with MS has poorly been investigated.

So starting at such observations, we studied the effects of three mtDAMPs (mtDNA, N-formyl-Met-Leu-Phe and cardiolipin) on microglia, finding an increase of the reactive oxygen species (ROS) production in HMC3, a human microglial cell line, treated with mtDNA and cardiolipin (Nasi et al., 2020b). MtDNA-induced ROS production could have a role in the activation of the microglia, acting as secondary messengers and influencing the nuclear factor kappa-light-chain-enhancer of activated B cells and mitogen-activated protein kinase signaling pathways, that result in the synthesis of proinflammatory cytokines (Simpson and Oliver, 2020). Moreover, ROS generation, especially from mitochondria, is one of the first identified triggers of activation of the NLR family pyrin domain containing 3 (NLRP3) inflammasome, a multiprotein complex formed by procaspase-1 and ASC (the adaptor molecule

apoptosis-associated speck-like protein containing a CARD) able to cleave and release the mature form of the proinflammatory cytokines interleukin (IL)-1 β and IL-18. The activation of the inflammasome platform could also lead to the formation of pores in the plasma membrane causing a cell death called pyroptosis. Moreover, mtDNA elicits the activation of NLRP3 inflammasome also inside the cell leading to the release of proinflammatory molecules. Indeed, dysfunctional mitochondria lead to a condition of oxidative stress and a loss of their integrity accompanied by the intracellular release of their content. As early as in the cytoplasm, the increased amount of ROS can induce oxidative base lesions in mtDNA. Thus, oxidized mtDNA may trigger an inflammatory response by binding the TLR9 present on the endo-lysosomal membrane and causing the activation of NLRP3 and stimulator of interferon genes pathway. Inflammasome activation can eventually result in cell death and tissue damage, leading to the release of other DAMPs that can, in turn, activate more inflammasome platform. Thereby, the significant release of proinflammatory cytokines propagates a vicious cycle of inflammation that plays a critical role in the development and the progression of MS. The key point is that the release of these mediators, which should have a role in preventing further damage to the brain parenchyma, may be toxic to neurons and other glial cells. It follows that the release of proinflammatory cytokines is a cornerstone event, in CNS as much as in the periphery, where they contribute to the systemic activation (**Figure 1**). To add another piece to this complex puzzle, we found that free mtDNA and proinflammatory cytokines (tumor necrosis factor- α , IL-6, IL-1 β , interferon- γ , and IL-8) are increased in the plasma of patients with progressive MS compared to healthy subjects (Nasi et al., 2020a). To our knowledge, no data are available on the plasmatic levels of mtDNA in relapsing-remitting MS patients. Proinflammatory cytokines produced in the periphery could cross the blood-brain barrier (BBB) and bind microglial receptors stimulating the activation of NLRP3 and the shifting toward a proinflammatory phenotype (Garaschuk, 2021).

The source and the form of cell-free mtDNA remain important issues to be addressed, since different forms can have different effects on the innate immune response. Indeed, mtDNA could be released or actively ejected. Studies on mtDNA as DAMP released after cell death have been performed using the purified mtDNA to stimulate cells in *in vitro* experiments. Otherwise, mtDNA internalization could be conveyed by TFAM through the receptor for advanced glycation end products promoting also its recognition by TLR9. It should be noted that, in the presence of interferon- γ , TFAM itself or other mitochondrial proteins are also able to provoke IL-6 secretion from primary human microglia (Little et al.,

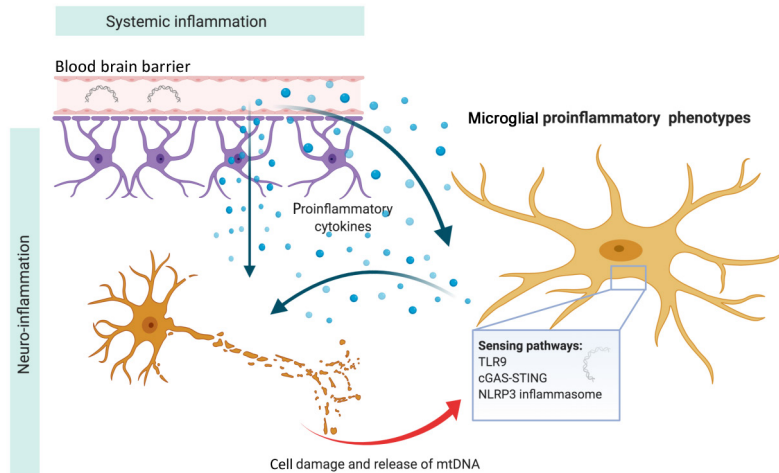


Figure 1 | The release of proinflammatory cytokines at peripheral (systemic inflammation) and central (neuroinflammation) level contributes to the neurodegeneration processes damaging neurons and maintaining the inflammation through the microglia activation.

One of the mechanisms that could trigger and maintain this inflammatory status is the release of mtDNA from damaged cells. MtDNA could be sensed from microglial cells through different pathways promoting their proinflammatory phenotype and perpetuating a vicious circle of cytokines release. Created with BioRender.com. cGAS-STING: Cyclic GMP-AMP synthase-stimulator of interferon genes; mtDNA: mitochondrial DNA; NLRP3: NLR family pyrin domain containing 3; TLR9: toll-like receptor 9.

2014). Moreover, mtDNA fragments could be transported and released by extracellular vesicles from astrocytes even in response to oxidative stress. This field has not been well studied and the effects of mtDNA contained in extracellular vesicles are still unknown.

Finally, mtDNA could derive from extracellular traps (ETs), a DNA fibrous scaffold released from several human cell types from blood or tissues, such as neutrophils, eosinophils, and basophils. In different pathological conditions neutrophils, through the release of neutrophils, can damage the BBB and the surrounding neurons. Recent studies have demonstrated that ETs are also produced by monocytes/macrophages and lymphocytes that can readily cross the BBB during inflammation. This release of nuclear or mtDNA is ROS-dependent and is caused by different inflammatory mediators, such as IL-8 and tumor necrosis factor- α (that are increased in progressive MS patients). Indeed, ROS inhibition hinders the release of ETs, resulting in a significant reduction in the secretion of several cytokines (Costanza et al., 2019). The role of ETs as an effective antimicrobial first-line protection is well-documented, but there is increasing evidence that this mechanism occurs in various clinical settings even in the absence of microbial infections and that they are probably also associated with pathophysiological conditions. For example, ETs could lead to the activation of NLRP3 inflammasome and proinflammatory macrophages. Higher circulating ETs have been found in serum from relapsing-remitting MS patients, and resulted correlated with relapses and higher monocyte counts in whole blood. Conversely, ETs have not been detected in the CNS of MS patients. It is reasonable to assume that the systemic activation driven by proinflammatory cytokines could involve this

mechanism contributing to the circulating levels of mtDNA.

The biological effects of the ROS increase induced by mtDNA stimulation are still to be clarified, as well as the contribution of inflammasome activation to trigger/maintain the inflammatory process in microglia. So, it stands to reason that mtDNA could also have a role in shifting functions of macrophages/microglia by altering their phenotype mainly through the activation of NLRP3 inflammasome. Further *in vitro* and *in vivo* studies are needed to deeply understand the capacity of mtDNA to trigger and/or maintain a proinflammatory status in the CNS and periphery in MS as well as in other neurodegenerative diseases.

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