UNIVERSITÀ DEGLI STUDI DI MILANO

Department of Biomolecular and Pharmacological Sciences

Department of Pharmaceutical Sciences

Doctorate in Pharmacological, Experimental and Clinical Sciences

XXXIII Cycle

MOLECULAR MECHANISMS OF THE SEXUALLY DIMORPHIC INFLAMMATORY RESPONSE INDUCED BY NEURODEGENERATIVE SIGNALS

BIO/14

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A.A 2019-2020

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ABSTRACT

English

Parkinson's disease (PD) is a progressive, neurodegenerative disease characterized by motor symptoms linked to different pathogenic mechanisms, among which a deranged activation of innate immunity and neuroinflammation play a key role. The sexual dimorphism in PD, that results in men being more frequently affected than women, has been associated with genetic as well as sex steroid hormone-mediated mechanisms. New evidence suggests that the disease may start in the enteric nervous system and spread to the CNS only at later stages.

My working hypothesis is that the sexual dimorphism in PD is due to a distinct ability of macrophages of the two sexes to drive inflammatory and neuromodulatory actions, resulting in a sexually dimorphic onset and progression of PD. The aim of my research is to study the extent to which neurotoxic molecules, that are typically associated with PD pathogenesis, or neuronal signals generated in response to them, could modulate macrophage cells and induce immune, metabolic and oxidative stress responses that influence PD progression. In addition, my goal is also to assess whether these specific functions of macrophages could be different in male and female mice.

In the laboratory where I performed my PhD thesis, engineered reporter animals were available which allowed assessment of the transcriptional activity of NRF2 and NFκB, two transcription factors deeply involved in antioxidant and inflammatory responses, respectively. Primary cultures of peritoneal macrophages obtained from these mice allowed me to highlight the key function of NRF2, and not NFκB, in the macrophage response to neurotoxic and oxidant molecules, such as MPP+ and tBHQ, respectively. Moreover, I observed that the activation of NRF2 allows macrophages to acquire a protective immune phenotype through changes in cell metabolic and inflammatory functions under homeostatic as well as inflammatory conditions, which were mimicked by LPS administration to cells. In fact, the simultaneous administration of tBHQ+LPS alters the effects of each individual pathway in a target gene-specific manner. Importantly, I observed that both NRF2 and NFκB activation and function are sexually distinct in macrophages. In fact, NRF2 transcriptional activity and

NRF2-target gene expression were induced at higher levels by NRF2 activating stimuli in female macrophages. Moreover, NRF2 activation correlated also with a stronger anti-inflammatory response against LPS activity on inflammatory genes in female cells. On the other hand, NFκB activation and the expression of pro-inflammatory cytokines, such as L-1β, IL-6 and TNFα were higher in LPS-treated male compared to female cells. By demonstrating the key role for NRF2 in the sex-specific reactivity of macrophages these results indicate possible molecular and cellular mechanisms to explain the sexual differences in PD onset and progression.

Altogether, our results show that the activation of NRF2 redirects the metabolic, immune and proliferative response of peritoneal macrophages to inflammatory signals in a sex-dependent manner, with relevant consequences for the pharmacological treatment of diseases that are associated with unopposed inflammatory responses, such as neurodegenerative diseases and PD.

Italiano

La malattia di Parkinson (PD) è una patologia neurodegenerativa progressiva, caratterizzata da sintomi motori causati da diversi meccanismi patogenetici tra i quali rivestono un ruolo chiave un'eccesiva risposta immunitaria innata e fenomeni associati alla neuroinfiammazione. La malattia di Parkinson presenta un dimorfismo sessuale: gli uomini, infatti, sono più colpiti delle donne; questo effetto è stato associato, per lo meno in parte, con le azioni neuro protettive ed antiinfiammatorie degli estrogeni. Nuove ipotesi sembrano indicare che la malattia inizi a livello del sistema nervoso enterico e si propaghi nel sistema nervoso centrale soltanto nelle fasi finali.

L'ipotesi su cui si basa il mio lavoro è quella che il dimorfismo sessuale osservato nel PD sia causato dalla diversa abilità dei macrofagi dei due sessi di compiere azioni infiammatorie e neuromodulatorie, determinando così il dimorfismo sessuale osservato nell'onset e nella progressione della patologia.

Lo scopo della mia ricerca è di studiare l'intensità con cui molecole neurotossiche, che sono tipicamente associate al PD, o segnali generati dai neuroni in risposta a queste, possano modulare le funzionalità dei macrofagi ed indurre risposte di tipo immunitario, metabolico e conto lo stress ossidativo che influenzino la progressione della malattia. In aggiunta, il mio obiettivo è valutare anche se queste specifiche funzioni possano essere differenti in macrofagi isolati da animali maschi e femmine.

Nel laboratorio in cui ho svolto la mia tesi di Dottorato, erano disponibili animali reporter per valutare l'attività trascrizionale di NRF2 e NFκB, due fattori di trascrizione strettamente coinvolti nelle risposte antiossidanti ed infiammatorie, rispettivamente.

Colture primarie di macrofagi peritoneali ottenuti da questi animali hanno permesso di mettere in luce le funzioni di NRF2, e non NFκB, nei macrofagi in risposta a molecole neurotossiche ed ossidanti, quali MPP^+ e tBHQ.

Inoltre, ho osservato che l'attivazione di NRF2 permette ai macrofagi di acquisire un fenotipo immunitario positivo, ottenuto grazie a cambiamenti nel metabolismo cellulare e nelle funzioni infiammatorie, simulate tramite trattamento con LPS.

Soprattutto ho osservato come l'attivazione e la funzione sia di NRF2 che di NFκB sono sessualmente diverse nei macrofagi. Infatti, l'attività trascrizionale di NRF2 e l'espressione dei suoi geni target sono indotti a livelli più alti da stimoli attivanti NRF2 nei macrofagi isolati da animali femmina. In aggiunta, l'attivazione di NRF2 si correla anche ad una più forte risposta antinfiammatoria in seguito a trattamento con LPS nelle cellule isolate da animali femmina.

D'altra parte, l'attivazione di NFκB e l'espressione di citochine pro-infiammatorie, quali IL-1β, IL-6 e TNFα, è maggiore in maschi trattati con LPS rispetto alle femmine.

Avendo dimostrato il ruolo chiave di NRF2 nella reattività dei macrofagi in maniera sesso-specifica, questi risultati suggeriscono possibili meccanismi molecolari e cellulari che spiegano le differenze di sesso nell'onset e nella progessione della malattia di Parkinson.

Nel complesso questi risultati dimostrano che l'attivazione di NRF2 è in grado di reindirizzare la risposta metabolica, immunitaria e proliferativa di macrofagi peritoneali in risposta a stimoli infiammatori, in maniera sesso-specifica, con rilevanti conseguenze nelle patologie caratterizzate da una risposta infiammatoria squilibrata, come le malattie neurodegenerative.

INTRODUCTION

1.1 NRF2

Organisms have developed complex mechanisms in order to defend themselves against oxidative damages caused by endogenous or exogenous agents.

Nuclear factor-erythroid 2 p45-related factor 2 (NRF2, also called Nfe2l2) is a transcription factor that acts as a "sensor" of the cellular redox status through a complex transcriptional/epigenetic and post-translational network that promotes the expression of over 100 genes coding for enzymes involved in detoxification and antioxidant responses. Besides mediating stress-stimulated induction of antioxidant and detoxification genes, NRF2 contributes to the cellular adaptation to stressors by regulating the expression of regenerative molecules and intermediary metabolic enzymes.

1.1.1 Structure of NRF2 and its regulation

NRF2 is a modular protein composed of 605 aminoacides, belonging to the family of Cap'n'collar (CNC) protein, presenting a leucine zipper (bZIP) motif comprised of seven domains called Neh (NRF2- Erythroid cell-derived Homology), each with a different function[1].

Neh1 domain comprises the CNC-bZIP region that both dimerizes with small Maf proteins and binds DNA. The Neh2 domain negatively controls NRF2 because, recruits Keap1, a dimeric redox-sensitive substrate. The C-terminal Neh3 region of NRF2 is a transactivation domain that recruits chromo-ATPase/ helicase DNA-binding protein (CHD)6. The Neh4 and Neh5 regions represent transactivation domains that recruit cAMP response element-binding protein (CREB)- binding protein (CBP) and/or receptor-associated coactivator (RAC)3. The Neh6 domain negatively controls NRF2 because it recruits the dimeric b-transducin repeat-containing protein (b-TrCP), a substrate adaptor for E3 complex. The Neh7 domain mediates repression of NRF2 by the retinoid X receptor (RXR)a through a physical association between the two proteins.

The KEAP1-NRF2 system is a typical two-component system: KEAP1 as a sensor for electrophiles, and NRF2 as an effector for the coordinated activation of cytoprotective genes[2]. In basal, homeostatic conditions KEAP1 forms a

ubiquitin E3 ligase complex with CULLIN3 (CUL3) and polyubiquitinates NRF2, which marks NRF2 for rapid degradation through the proteasome system. Thus, in unstressed conditions, NRF2 is synthesized, but constantly degraded.

However upon exposure to electrophiles or ROS, the reactive cysteine residues of KEAP1 are directly modified to the nucleus, in NRF2 stabilization: nascent NRF2 directly translocate to the nucleus where it forms a heterodimer with one of the small Musculo-aponeurotic fibrosarcoma (sMAF) proteins; the heterodimer recognize the AREs (antioxidant response element) that are enhancer sequences present in the regulatory regions of NRF2 target genes and robustly activates their transcription[3]. Notably, a number of small molecules have been found to disrupt the Keap1-mediated degradation of NRF2 and cause NRF2 accumulation, and some of these molecules are approved or being developed as NRF2-inducing therapy[4].

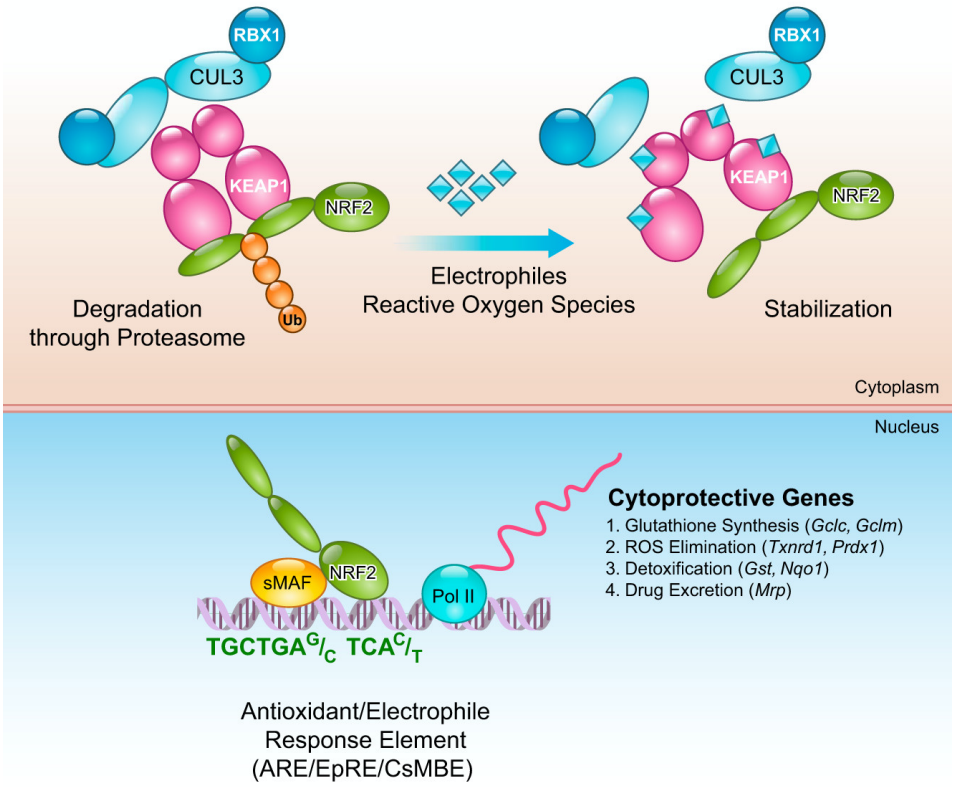


Figure 1- The KEAP1-NRF2 system[2]**.** KEAP1-NRF2 is a two-component system. In the cytoplasm, NRF2 is ubiquitinated by the KEAP1-CUL3 ubiquitin E3 ligase complex to mark it for degradation by the proteasome. When cells are ex- posed to electrophiles or reactive oxygen species, KEAP1 is modified and the KEAP1-CUL3 ubiquitin E3 ligase activity declines, which results in the stabilization of NRF2. Stabilized and accumulated NRF2 translocates to the nucleus and activates a battery of cyto- protective genes.

1.1.2 NRF2 functions

Although NRF2 is a homolog of nuclear factor-erythroid 2 p45 (NF-E2), its function is not related to hematopoiesis. Indeed, NRF2-knockout mice do not show anemia, thus suggesting that NRF2 regulates a different array of genes from NF-E2. Itoh and collaborators[5] were the first to report the similarity between the NF-E2 binding sequence and ARE. There are over 250 currently identified NRF2 target genes involved in a multitude of cellular processes, including redox regulation, phase I–III drug/xenobiotic metabolism, protein homeostasis, DNA repair, carbohydrate and lipid metabolism, iron homeostasis, transcriptional regulation, and mitochondrial function.

Perhaps the best known NRF2 function is maintaining redox homeostasis, mainly via the synthesis and redox cycling of GSH and thiol based antioxidant enzymes; for example, the catalytic and modulatory subunits (GCLM and GCLC) of glutamate cysteine ligase (GCL) areNRF2target genes, with GCL being responsible for the de novo synthesis of glutathione (GSH)[6].

Furthermore, NRF2 regulates all three phases of drugs/ xenobiotics metabolism: ARE regulatory sequence, is usually found upstream of genes encoding phase II detoxifying enzymes, regulates the induction of these genes[7], as shown by the fact that in NRF2-knockout mice there is a down-regulated expression of phase II enzymes. Phase II enzymes detoxify the intermediate metabolites generated by phase I reactions, causing a rapid excretion of toxic xenobiotics. Benzo[α]pyrene is a pro-carcinogen that is detoxified by phase II reactions after forming a highly reactive intermediate after phase I metabolism. NRF2-deficient mice are more susceptible to benzo[α]pyrene-induced tumor formation thus suggesting that NRF2 is essential for a complete phase II metabolism[8,9]. Other studies showed that the NRF2 system controls phase I-related genes, as well as phase III xenobiotic transporters, thus indicating that NRF2 is responsible for the whole process of xenobiotic metabolism. Moreover, since anti-oxidant genes, such as heme oxygenase 1 (HO-1), contain upstream ARE sequences, NRF2 can be regarded as a master regulator of the oxidative stress response[10]. Several toxic chemical stressors produce reactive oxygen species (ROS), therefore NRF2 plays a central role in

the defense against various chemical-derived stresses[11,12]. Collectively, defending against xenobiotic metabolism and providing an efficient antioxidant system, NRF2 can be considered as one of the main factors contributing to animal evolution in a changing environment. Endogenous signals, such as endoplasmic reticulum stress and autophagy impairment, are equally capable of activating the NRF2 system which provides an initial cellular defense[13]. NRF2 downstream genes are also involved in maintaining proteostasis via autophagy and the ubiquitin proteasome system: NRF2 may directly regulate mTOR, a master regulator of both protein translation and autophagy[14]. Furthermore, p62/SQSTM1, a protein that targets ubiquitylated proteins for autophagic degradation, is an NRF2 target. Functional AREs have been identified in the promoters of several transcription factors, including aryl hydrocarbon receptor (AhR), neurogenic locus notch homolog protein 1 (NOTCH1), and retinoic receptor α (RXRA), indicating that NRF2 can indirectly control the transcription of many non-ARE-containing genes. NRF2 also plays a role in DNA damage repair and preventing apo- ptosis, as p53-binding protein 1 (53BP1), DNA repair protein RAD51 homolog 1 (RAD51)[15], antiapoptotic proteins B cell lymphoma 2 (BCL2), and B cell lymphoma extra-large (BCLXL)[16,17] are transcriptionally regulated by NRF2. The diversity of NRF2 targets indicates the central role ofNRF2 in mediating cellular function.

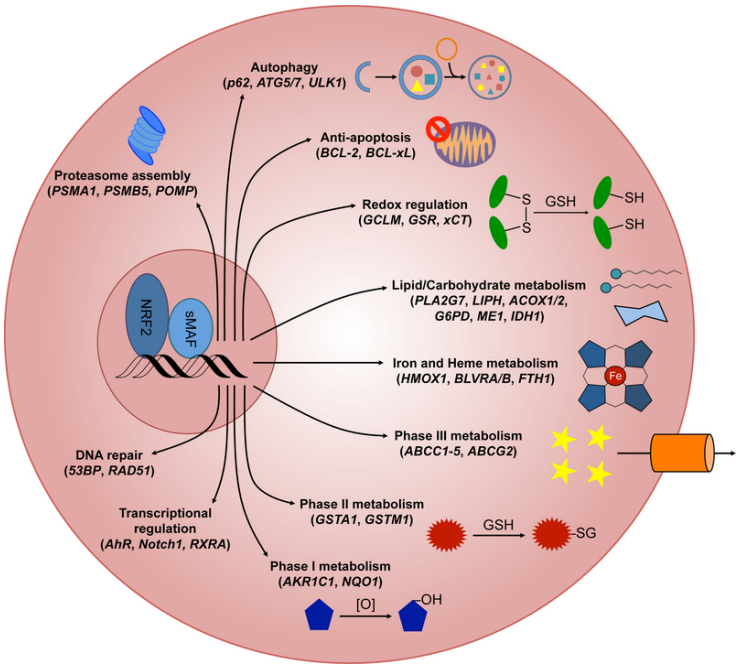


Figure 2-Cellular pathways driven by NRF2 target genes[6]**.** NRF2 heterodimerizes with sMAF proteins to initiate the transcription of antioxidant response element–containing target genes. Verified NRF2 target genes are involved in proteasome assembly, autophagy, prevention of apoptosis, maintaining redox balance, lipid and carbohydrate metabolism, heme metabolism, iron homeostasis, all three phases of drug/xenobiotic metabolism, transcriptional regulation of other transcription factors, and DNA repair.

1.1.3 NRF2 in inflammation

Inflammation is a complex process that may be caused by harmful exogenous stimuli such as pathogens or irritants, but it is also a pathological phenomenon associated with a broad spectrum of diseases. In both cases, the final aim of the inflammatory process is to eliminate the source of damage and to promote tissue repair. It is possible to distinguish between acute, that is self-limiting and beneficial to the host, and chronic inflammation that is a common cause of complications in many pathologies.

In general, both pro- and anti-inflammatory signaling pathways interact in the normal inflammatory process: macrophages and other immune are first recruited to the site of injury where they produce inflammatory mediators such as cytokines, chemokines, and prostaglandins; these mediators further recruit macrophages directly to the site of inflammation and activate multiple signal transduction cascades associated with inflammation, such as NFκB (nuclear factor kappa B), MAPK (mitogen-activated protein kinase), and JAK (janus kinase)-STAT (signal transducers and activators of transcription). On the other

hand, NRF2 contributes to the anti-inflammatory process by regulating the expression of detoxifying enzymes and antioxidant genes[18]. NRF2 has been known to attenuate inflammation, mainly because of its key role in protection against oxidative/ xenobiotic stresses: indeed, NRF2 deficiency correlates with an increase of symptoms related with inflammation in different murine models and also causes autoimmune phenotype in some murine strains[19]. Consistently, NRF2 activation in myeloid cells alleviates inflammation. In human clinical trials, one of NRF2- inducer, dimethyl fumarate (DMF), have been approved recently for the treatment of multiple sclerosis thanks to its anti-inflammatory properties. Taken together, these observations suggest that NRF2 plays a key role in controlling the inflammatory process.

The mechanism underlying NRF2 anti-inflammatory activity has been reconciled mainly to the elimination of ROS, achieved through the NRF2-mediated upregulation of numerous antioxidant genes. However, NRF2 regulates the expression of macrophage-specific genes that are not categorized as anti-oxidative stress-response genes: these insights suggest that NRF2 may also act as an anti-inflammatory in a ROS-independent manner.

Anti- inflammatory role of NRF2/Hmox1 axis

Hmox1 (or HO-1) is the rate-limiting enzyme that catalyzes the degradation of heme into carbon monoxide (CO) and free iron, and biliverdin to bilirubin; several studies demonstrated that HO-1 and its metabolites have significant anti-inflammatory effects: CO, for example, acts as an inhibitor of the NFκB pathway leading to the inhibition of pro-inflammatory cytokines, while bilirubin acts as antioxidant. Furthermore, HMOX1 directly inhibits pro-inflammatory cytokines as well as activating the anti-inflammatory cytokines.

NRF2 induces the HO-1 gene by increasing mRNA and protein expression and it is one of the classical NRF2 target genes, widely used both in vivo and in vitro studies.

HMOX1 has an impact on LPS-mediated inflammatory responses in foam cell macrophages preventing immoderate inflammation of these cells which play important role in progression of atherosclerosis[20]. It also suppresses LPS-

induced mouse BV2 microglial cells activation which protects hippocampal cells death, with impact on neuroinflammation[21].

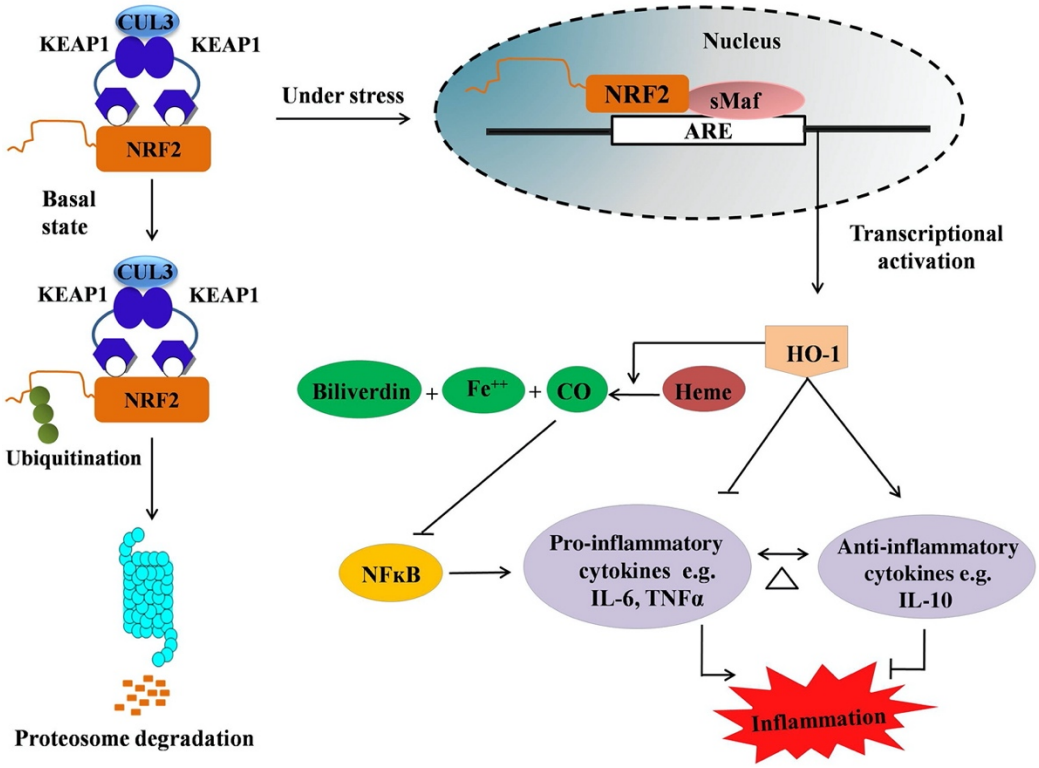


Figure 3- Anti-inflammatory effects of NRF2/ HMOX1axis[18] Under basal conditions, NRF2 binds to its repressor Keap1which leads to ubiquitination followed by proteasome degradation. During oxidative stress, free NRF2 translocate to the nucleus, where it dimerizes with members of the small Maf family and binds to ARE genes such as HO-1. UpregulatedHO-1 catalyzes the heme into CO, bilirubin, and free iron. CO acts as an inhibitor of the NFκB pathway which leads to the decreased expression of pro-inflammatory cytokines, while bilirubin also acts as antioxidant. Furthermore, HO-1 directly inhibits the proinflammatory cytokines as well as activating the anti-inflammatory cytokines, thus leads to balancing of the inflammatory process.

Inflammatory mediators and enzymes inhibited by NRF2

Overproduction of cytokines is one of the main consequences after exposure to oxidative stress, causing oxidative stress in target cells. In response to oxidative stress, trascription factor NFκB causes the overproduction of pro-inflammatory cytokines that further activate NFκB creating a self-amplifying circle that maintains itself. Activation of the NRF2/ARE system is crucial in disrupting this cycle.

It has been reported that activation of NRF2 prevents LPS-induced transcriptional upregulation of pro-inflammatory cytokines, including IL-6 and IL-

1β[4]. Sulforaphane (SFN), one of the well-known NRF2 activators, inhibits also the expression of TNF-α, COX-2 and iNOS in primary peritoneal macrophages. Furthermore, several NRF2- dependent antioxidant genes such as HO-1, NQO1, Glc block TNFα, IL-6 and inflammatory mediators; but in the case of NRF2-knockout mice the anti-inflammatory effect does not occur[22].
Moreover, it reduces inflammation by increasing the efferocytic activity of murine macrophages and inhibits MMP-9, a metalloproteinases widely present in the extracellular matrix and involved in cell migration and tumor metastasis: the down-regulation of MMP-9 in tumor cell invasion and inflammation is regulated by inhibition of NFκB pathway[23].

NRF2 and NLRP3 inflammasome activity

The NLR family, pyrin domain containing 3 (NLRP3) inflammasome is a multiprotein complex that functions as a pathogen recognition receptor (PRR) and recognizes the wide range of microbial, oxidative stress signals. When activate, NLRP3 regulates the cleavage of caspase-1 and the release of IL-1β that ultimately controls the process of cell death known as pyroptosis in order to protect host against a broad spectrum of insults[24]. However, aberrant activation of the inflammasome is associated with protein-misfolding disease such as Alzheimer's disease, Parkinson's disease, type 2 diabetes, cancer[25], gout, atherosclerosis. Recent observations revealed that NQO1, one of the classical NRF2- target gene, is able to block NLRP3 activation, caspase-1 cleavage and the consequent IL-1β generation in macrophages.
Moreover, tert-butylhydroquinone (tBHQ), a well-known NRF2 activator, negatively regulated NLRP3 transcription by activating the ARE by NRF2-dependent manner[26].

Suppression of pro-inflammatory cytokines transcription by NRF2

A very recent work revealed a novel mechanism by which NRF2 negatively controls the expression of pro-inflammatory cytokines. It was demonstrated,through chromatin immunoprecipitation (ChIP)-seq and ChIP-qPCR studies in mouse macrophages revealed that NRF2 binds to the

promoter regions of pro-inflammatory cytokines such as IL-6 and IL-1β and inhibits RNA Pol II recruitment. As a result, RNA Pol II is unable to process the transcriptional activation of IL-6 and IL-1β leading to the inhibition of gene expression[4]. Thus, contrary to the current hypothesis that NRF2 represses inflammation as a secondary consequence of anti-oxidation, these results demonstrate that NRF2 inhibits the induction of proinflammatory cytokine gene transcription. These findings indicate that NRF2 is the key regulator for the two important cytoprotective pathways, anti-inflammation and anti-oxidation.

Crosstalk between NRF2 and NFκB pathways

NFκB is a protein complex responsible for DNA transcription found in almost all types of animal cells, known as the "master regulator" of the inflammatory response; it is involved in inflammation, apoptosis, cell growth and development. Exaggerated activation of NFκB has been linked to several pathological conditions, like rheumatoid arthritis, asthma, inflammatory bowel disease and *Helicobacter Pylori*-induced gastritis[27]. It is now accepted that NFκB interferes with the Keap1/NRF2/ ARE signaling pathway at least in three different ways: first, Keap1 causes IKK (the protein responsible of NFκB nuclear traslocation) degradationthrough ubiquitination leading to inhibition of NFκB phosphorylation and ultimately activation. Second, several inflammatory mediators induced in oxidative stress reacts with Keap1 activating NRF2, starting gene transcription and simultaneously blocking NFκB activity[7,28]. Third, NFκB can combine with CBP, a competitive NRF2 transcriptional co-activator, thus inducing the expression of ARE-driven genes involved in antioxidant responses and detoxification.

It is assumed that NFκB and NRF2 pathways interact to reciprocally control the transcription or function of downstream genes and target proteins. For example, increased expression of NRF2-target gene HO-1 inhibits NFκB activity.

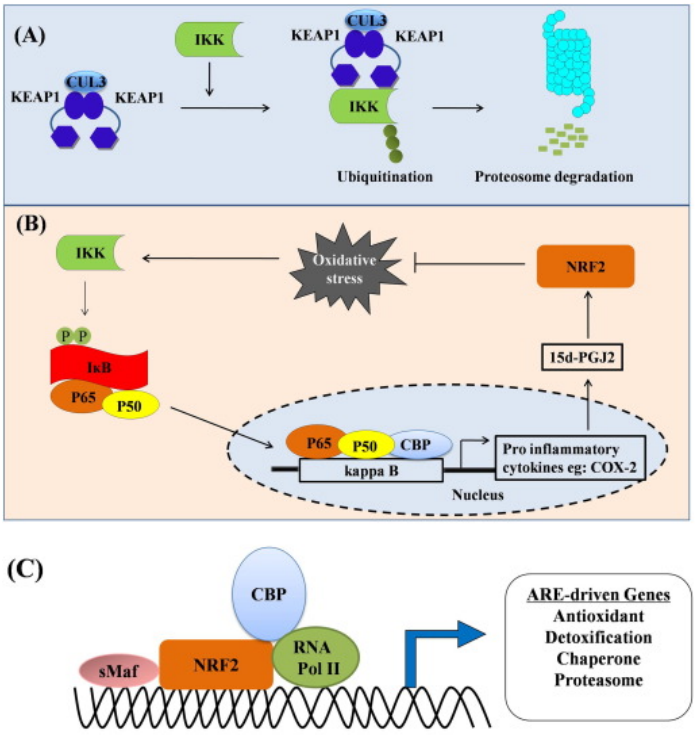


Figure 4- Crosstalk between the NRF2 and NFκB pathway[18]. (A) Keap1 directs the IKK to CUL3-mediated ubiquitination and proteasome degradation leading to the inhibition of NFκB. (B) Oxidative stress activates IKK which phosphorylates NFκB, leading to its translocation into the nucleus and activation of proinflammatory cytokines such as COX-2. The terminal product ofCOX-2 known as 15d-PGJ2 acts as an inducer of NRF2 that ultimately leads to the suppression of oxidative stress. (C) NRF2 binds with its transcriptional cofactor CBP along with small Maf proteins and other transcriptional machinery to initiate ARE-driven gene expression.

1.1.4 NRF2 in metabolism

NRF2 affects multiple aspects of the intermediary metabolism: it is either directly involved in the regulation of several key metabolic genes or it affects their expression indirectly through crosstalk with other transcription factors. Notably NRF2 regulates enzymes that catalyze rate-limiting steps or that are situated at branching points in major metabolic pathways, thus influencing the synthesis of carbohydrates, nucleic acids, lipid and amino acids[29].

NRF2 and NADPH synthesis and utilization

NRF2 regulates all four NADPH-generating enzymes, G6PD, PGD, ME1 and IDH1. Importantly NADPH is required for the biosynthesis of lipids and nucleotides, and this may contribute to increase cell growth in tumor where NRF2 in upregulated.

NRF2 and the pentose phosphate pathway (PPP)

The branching point enzyme G6PD, controlled by NRF2, determines flux of glucose either glycolysis or the PPP, with the latter favoring biosynthetic reactions. Moreover, NRF2 also influences the nonoxidative phase of PPP, by positively regulating the expression of transaldolase 1 (TALDO1) and transketolase (TKT), thereby directing carbon flux toward the PPP. Although TALDO1 has a functional ARE in its promotor and it is classified as a direct target gene of NRF2, the expression of G6PD, PGD and TKT appears to be indirectly regulated by NRF2. The expression of pyruvate kinase (PK) is decreased upon NRF2 activation; since PK regulate the final step of glycolysis it is expected that a decrease in its activity would favor build up pf glycolytic intermediate and their channeling through the PPP and into the synthesis of nucleic acids, amino acids and phospholipids.

NRF2 and the de novo *purine biosynthesis pathway*

Two major products of the PPP, ribose-5-phosphate and erythrose-4-phosphate are essential for the biosynthesis of nucleotides and aromatic amino acids, respectively. In proliferating cells NRF2 enhances the expression of phosphoribosyl pyrophosphate amidotransferase (PPAT), which catalyzes the rate-limiting step in the de novo purine biosynthetic pathway, and of methylenetetrahydrofolate dehydrogenase $(MTHFD)_{2,}$ an enzyme that provide one-carbon for purine biosynthesis. Indeed, purine, but not pyrimidine, biosynthesis is affected by the activity of NRF2.

NRF2 and glutamine metabolism

Analogously to the role NRF2 plays in directing glucose along anabolic pathways in proliferating cells, the CNC-bZIP factor also contributes to glutamine metabolism. Glutamine is the obligatory nitrogen donor for the biosynthesis of nucleotides and nonessential amino acids. In turn, the glutamate generated by glutaminase may be deaminated to form the the TCA cycle intermediate a-ketoglutarate. It has been found that when NRF2 is activated, the carbon flux from glutamine is directed towards GSH biosynthesis and the TCA cycle. This is largely due to enhanced NRF2-dependent

expression of GCLC and GCLM as well as induction of ME1, which accelerates the oxidative decarboxylation of malate to give pyruvate that feeds into the TCA.

NRF2 and lipid metabolism

It has been demonstrated that, in mice lung, NRF2 positively regulates several lipases. Furthermore, in mouse liver, NRF2 negatively regulates many genes encoding enzymes involved in lipid biosynthesis, fatty acid desaturation and fatty acid transport, but the mechanism involved is presently unknown.

In the mouse, hepatic mRNA levels of ATP-citrate lyase (ACL), acetyl-CoA carboxylase (ACC)1, fatty acid synthase (FAS), stearoyl CoA desaturase (SCD)1, and fatty acid elongases are downregulated by both genetic and pharmacological activation of NRF2.

In addition to the negative effects of NRF2 fatty acid synthesis, NRF2-mediated downregulation of SCD1 and ACC1 will decrease levels of malonyl-CoA, thus increasing fatty acid oxidation. Consequently, when NRF2 is activated, the import of fatty acids into mitochondria for b-oxidation will increase because malonyl-CoA levels are low, whereas it will decrease when the NRF2 activity is impaired.

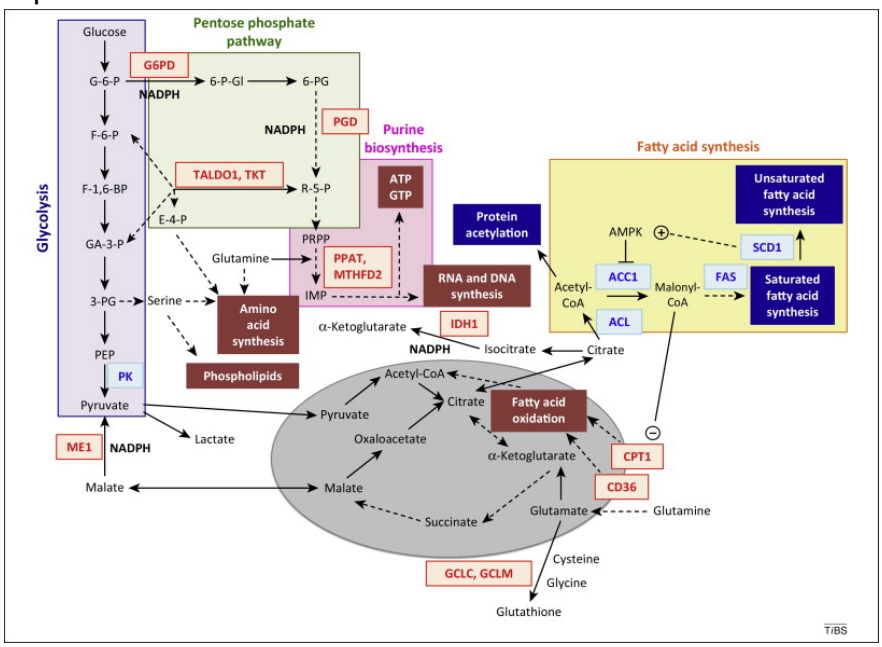


Figure 5- Regulation of intermediary metabolism by NRF2[29].The enzymes in the red and the blue colors indicate positive and negative regulation by NRF2, respectively. The mitochondrion is shown in gray, with the mitochondrial fatty acid oxidation (β-oxidation) pathway indicated in the red-brown box. The light purple box symbolizes glycolysis, the green box – the pentose phosphate pathway, the pink box – *de novo* purine biosynthesis, and the yellow box – lipid biosynthesis.

1.1.5 NRF2 in neurodegeneration

Similar to cancer, the role of NRF2 in neurodegenerative diseases is complex. Interestingly, reduced expression of NRF2 is associated with an age-related decline in neural stem cell function[30], and the NRF2 response to oxidative stress also diminishes with age[31]. During neurodegeneration, NRF2 can be activated or suppressed depending on the affected cell type and stage of disease. For example, some studies have indicated that NQO1 and HMOX1 are decreased in the brains of Alzheimer's disease (AD) patients, whereas others indicate that NRF2 remains confined to the cytosol, resulting in decreased target gene expression[32]. NRF2 and NQO1 expression is elevated in infiltrating macrophages and astrocytes found in active, but not inactive, MS lesions, whereas NQO1, HMOX1, and PRDX levels are consistently elevated and nuclear localization of NRF2 is observed in the substantia nigra of Parkinson's disease (PD) patients[32]. In contrast, NRF2 protein levels are decreased in the primary motor cortex and spinal cord of patients with amyotrophic lateral sclerosis[33]. These discrepancies could be specific to cell type and brain region but may also occur as a result of the stage of disease investigated. Since the responsiveness of the NRF2 pathway decreases with age, NRF2 activation could occur in the early stages but decline during later stages of disease. Some pharmacological activators of NRF2 improve neurodegenerative phenotypes. Bardoxolone-methylamide has been shown to improve memory and decrease amyloid-β plaque formation in transgenic AD mice[34]. Similarly, puerarin, improved the AD phenotype[35]. 3H-1,2-dithiole-3-thione (natural, dithiolethione, D3T) and bardoxolone-ethylamide/trifluoroethylamide protected wild-type, but not NRF2−/−, mice from MPTP-induced PD[36,37]. DMF is the only current NRF2 activator to make it through phase II clinical trials for the treatment of MS; it is curious that DMF is the only drug in this group to be approved by the US Food and Drug Administration, despite not being the most potent activator ofNRF2, which could be a result of NRF2-independent immunomodulation[38], synergistic effects with other pathways, or the degree ofNRF2 activation being critical depending on the disease context.

1.2 Parkinson's disease

Parkinson's disease (PD) is the second most common neurodegenerative disease after Alzheimer's disease. It is characterized by a slow and progressive selective degeneration of dopaminergic neurons of the substantia nigra. Yet, other neurons beside dopaminergic ones are affected although to a lesser extent[39]. This loss of dopaminergic neurons is responsible of most of the motor symptoms of PD among which there are rigidity, slowness of movement, bradykinesia and tremor at rest. There are no effective therapies for PD, current treatment aims to alleviate motor symptoms by restoring neurotransmission of dopamine with levo-dopa, a precursor of dopamine, or with dopamine agonists. Patients with PD also present non-motor symptoms such as gastrointestinal impairments, hysosmia, sleep disturbance and cognitive defects like depression and dementia.

In addition to neuronal loss the disorder is characterized by proteinaceous inclusions, the so-called Lewy bodies. The presence of this intracellular aggregates of α-synuclein set the definitive diagnosis of PD post-mortem.

Despite intensive research, the etiology of PD is presently unknown as well as the role of this protein inclusions is not fully understood.

Although a variety of possible pathogenetic mechanisms have been proposed over the years, including excessive release of oxygen free radicals during enzymatic dopamine breakdown, impairment of mitochondrial function, loss of trophic support, abnormal kinase activity, disruption of calcium homeostasis, dysfunction of protein degradation and neuroinflammation, the pathogenesis of PD is still largely uncertain.

The traditional view of (PD) as a primary disorder of dopaminergic neurons in the substantia nigra has been reconsidered in recent years. In fact, Lewy pathology is much more extensive and affects other non-dopaminergic neurons like neurons in the locus coerulus, neurons of the olfactory bulb and cholinergic neurons as well as the peripheral nervous system[40].

Among these peripheral autonomic neuronal circuits, the enteric nervous system (ENS) has received great attention. A recent autopsy survey has shown that almost all patients with PD display Lewy pathology within their ENS.

Furthermore, Braak suggested that these lesions in enteric neurons develop early in the course of disease, prior to the appearance of pathology in substantia nigra neurons, and therefore that the ENS could be critically involved in the pathophysiology of the disease[41,42].

1.2.1 Gut-to brain hypothesis

A growing body of evidence suggest that early on in PD, the peripheral nervous system and organs other than the brain are affected by neuropathological and neuroinflammatory events. Distinct non-motor symptoms, such as dysphagia, constipation and gastroesophageal reflux experienced by PD patients[43], have raised interest on the role of ENS. Accumulation of α-synuclein in the ENS can occur 20 years before the onset of motor symptoms, associated with degenerative changes in the CNS. The spread from the ENS to the CNS was proposed to occur via the DMV and the intermediolateral nucleus of the spinal cord [44–46]. Very little is known about the mechanisms that may promote this propagation. For example, a prion-like cell-to-cell progression along nerve bundles of the vagus nerve and spinal cord has been suggested[47,48], which, similar to prion disorder, could involve immune pathways as well[49].

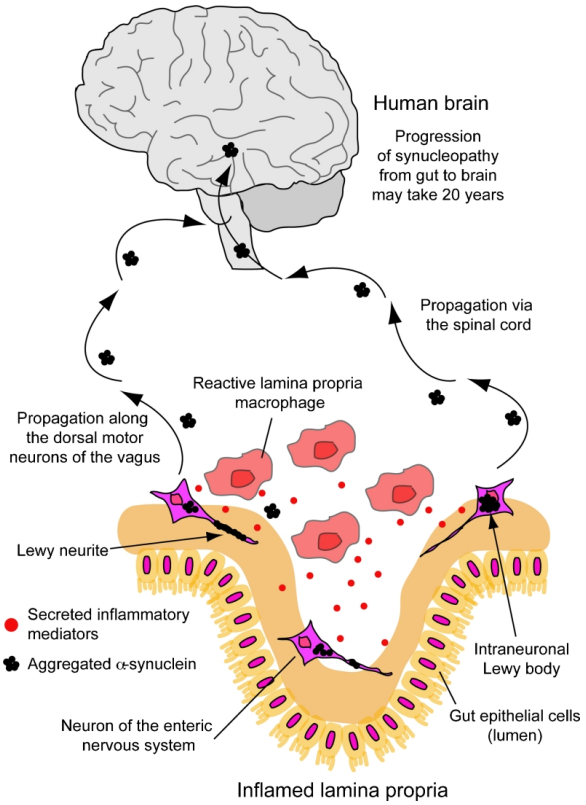


Figure 6- Possible spreading of synucleinopathy from the enteric nervous system to the brain[50]. Macrophages in the lamina propria become reactive upon natural or induced immune challenges. These cells react with secretion of inflammatory mediators which can harm the surrounding tissue and may induce accumulation of α-syn in enteric nerves. Aggregated α-syn may be released by damaged nerve cells, which further activating local macrophages. Cell-to-cell transmission could further contribute to the progression of synucleinopathy, which would eventually propagate from the enteric nervous system via nuclei in vagus and spinal cord.

This finds some clinical support in a study of patients with early stage diagnosed PD, where staining in the ENS correlated with com- promised intestinal barrier integrity. Possible drivers of the observed pathology may have been bacterial and environmental toxins, resulting in increased oxidative stress most likely produced by macrophages in the luminal wall. Yet in another study, patients with prolonged inflammation due to chronic inflammatory bowel disease did not display colonic synucleinopathy[51]. These findings in human patients indicate that acute local immune effects and certain forms of chronic intragastric inflammation may contribute differently to synucleinopathy in the GI.

Some transgenic mice expressing human α–synuclein shows early aggregates of α–synuclein in the ENS and express motor deficits prior to detectable pathology in the CNS[52,53]. In other studies, chronic intragastric administration of the mitochondrial toxin rotenone to rats was reported to induce a progressive

accumulation of endogenous α-synuclein starting in the ENS and reaching the brain along the vagal and spinal cord nerve connections to the substantia nigra[54]. In support to a direct link between PD and gut immune status, it was found that the gene LRRK2, the major genetic cause for familiar PD, is located in the risk region for Crohn's disease that is an autoimmune chronic inflammatory disease. In the brain, neuronal overexpression of LRRK2 accelerated the development of neuropathology in α-synuclein transgenic mice, whereas its ablation suppresses neuronal aggregation and cytotoxicity of α-synuclein[55]. In the peripheral immune system, LRRK2 is upregulated in macrophages under inflammatory conditions which promotes the production of ROS, phagocytosis, and killing of bacteria[56]. The link between genetic risk for PD and GI immune mechanisms for LRRK2 is intriguing and warrants further studies to identify possible associations with progression of α-synuclein aggregation from the ENS to the brain. The intestines contain the largest pool of tissue macrophages in humans. Thereby, the intestines share physiological similarities with the immune system of the olfactory epithelium. Macrophages in both locations demonstrate a two-edged sword: on one hand, they maintain tissue homeostasis by active phagocytosis[45,57]; on the other hand, they bear the potential to release an arsenal of detrimental cytokines, chemokines, and ROS[58,59]. Thus, depending on the circumstances, GI macrophages have the potential to harm the surrounding tissue and may play a role in promoting the accumulation of α-syn in enteric nerves, leading to altered gut motility and constipation and, in later stages, to propagation of the pathology to the brain. If this indeed constitutes an etiological trigger in PD, then it could be relevant for developing earlier treatment and diagnosis of the disease.

1.2.2 Sexual dimorphism in PD

Epidemiological, clinical and biological data suggest the existence of a sexual dimorphism in PD. Together with aging, male sex is the strongest risk factor for PD. The risk to develop PD is 1.5-fold greater in men than in women at all ages[60]: women show lower risk of develop the disease ant their age at onset tends to be higher[61]: in fact age at onset in women occurs about two years later

in women compared to men in the majority of studies. These differences are likely mediated by sex steroids hormones, estrogens in particular: indeed, increase of reproductive lifespan is positively associated with a delay in age of onset, suggesting that increased circulating estrogen levels may act as protective agents[62]. Earlier observations have described an inverse correlation between estrogen levels and severity of PD symptoms[63], and the incidence and prevalence of PD is higher in postmenopausal than in premenopausal women of the same age[64]. Moreover, a significant correlation between age at PD onset and menopause have been reported, as well as an increased risk of PD in conditions causing reduced cumulative exposure to endogenous estrogens, such as early menopause and reduced fertile life length[65].

Estrogen replacement therapy is associated with reduction of symptoms severity or lesser risk of developing PD[66]. By contrast, the risk of PD is higher in women with surgical menopause[67] or undergoing ovariectomy prior to menopause[68].

Thus, exposure to estrogen activity during lifetime may modify brain sensitivity to degeneration, influencing disease onset.

The neuroprotective activity of estrogens has been widely reported in a broad variety of neuronal cell system, including nigrostriatal dopaminergic neurons and also in glial cells,through genomic and non-genomic mechanisms[69]. Positive influence of estrogens on neurodegeneration has been reported by several studies, caused by their anti-inflammatory and anti-apoptotic effects[70]. Another mechanism that may account for estrogens positive role in PD may involve estrogens effects on glial reactivity, since glial reactivity is particularly relevant in the progression of neuronal damage[39,71–73].

Microglia, the macrophage population residing in the brain, can be polarized in response to signals from the microenvironment towards a M1, pro-inflammatory and cytotoxic phenotype, or M2, neuroprotective, phenotype, that although represent two extremes of a spectrum of phenotypes that microglia can acquire in response to different stimulation. Prolonged microglia activation, as occurs in PD in response to neuronal death and oxidative stress, results in M1

inflammatory response[74]; the resulting cytokines overproduction further contributes to neuronal death sustaining degeneration in PD.

Ovariectomy, and the consequent decrement in estrogen receptor- alpha (ERα) levels, increases the expression of neuroinflammatory markers that may be reverted by estrogen replacement therapy[75].

Classical murine PD models have been used to explore the modulatory effects of estrogens in glial activation associated with neurodegeneration and their neuroprotective effects. In mice treated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a neurotoxic compound widely used to replicate PD features, estrogens were able to counteract damages induced by the toxin through direct action on dopaminergic neurons orthrough modulation of astroglia and microglial activation[76,77].

Oxidative stress, that is another hallmark of PD, and mitochondrial functions are also sexually dimorphic in specific brain areas; females have increased antioxidant defenses and respiratory chain activity compared to males, thus with lower mitochondrial ROS production and oxidative damage as a consequence of the higher expression of mitochondrial proteins and antioxidant enzymes (eg, paraoxynase-2 or thioredoxin)[60].

1.2.3 Role of neuroinflammation

Neuroinflammation is a common pathological marker in many central nervous system (CNS) neurodegenerative diseases; it results in disturbances of CNS homeostasis ultimately leading to neuronal death.

Data from post-mortem studies provided for the first time evidence for neuroinflammatory process in PD disease: in 1988, it was reported the presence of activated microglia within the substantia nigra of patients with PD at post-mortem[78]. Further biochemical analysis reveals higher levels of proinflammatory cytokines such as IL-1β, TNFα, IFN-γ in the midbrain of PD patients.

Emerging data indicated that sustained inflammatory response, glial cell activation and T cell infiltration play key role in the degeneration of dopaminergic neurons and are common features of both human PD patients and animal models of PD; for instance microglial activation has been described

after 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) injection in mice[79] and monkeys[59,80]. Furthermore, injection with LPS, a bacterial endotoxin able to activate microglial cells, can selectively kills dopaminergic neurons in animals after intranigral[81] or systemic[82] injection. Further studies on dopaminergic neuronal cultures treated with LPS, demonstrated that neuroinflammation was associated with dopaminergic neuronal death and accumulation of cytoplasmatic inclusions of α-synuclein in nigral neurons which was not the case of α -synuclein null mice[83].

Altogether, evidence on microglia activation from animal models, whether chronic or acute, supports its role in dopamine cell loss.

Under homeostatic conditions, microglia are maintained in a quiescent state by a variety of immunomodulatory mediators, like CXCL1, CD200, CD47 and neuronal cell adhesion molecules[84]. Interestingly, microglia express almost exclusively in the CNS receptors for these molecules, indicating the critical role of neuron-microglia interactions in the control of neuroinflammation. For instance, CXCL1-CX3CR1 deficiency in vivo results in increased susceptibility to neurotoxicity induced by LPS and consequent dopaminergic loss[85].

It has been proposed that activated microglia may be beneficial to the host, at least in the early phases of neurodegenerative process: for example, suppression of M2 microglial polarization dramatically causes microglial overreaction and exacerbates dopamine neuronal loss in PD model[86], highlighting the protective role of M2 microglia in this process.

However, long-term activation of microglia in the PD brain significantly up-regulates the expression of a large series of pro-inflammatory cytokines including IL-1β, TNFα, IFN-γ and IL-6 that accelerates neuronal loss[87,88]. Progression of the disease is associated with the release of molecules like α–synuclein, ATP and metalloproteinase-3 (MMP3) from degenerating dopaminergic neurons that further enhances microglia activation, thus amplifying the neuroinflammatory response resulting in a deteriorating of the neurodegenerative process[88,89], forming a vicious and self-maintaining cycle of neurodegeneration.

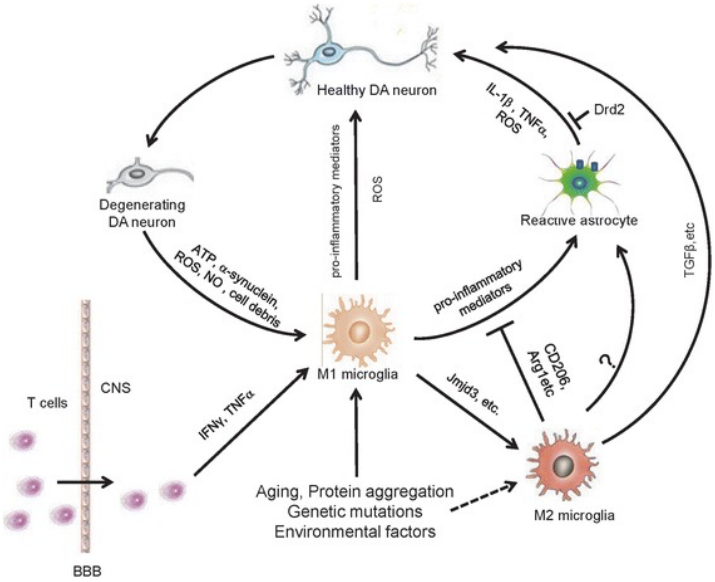


Figure 7- Diagrammatic representation of inflammatory mechanisms involved in PD pathogenesis[84]**.** Microglia become activated M1 phenotype in PD under pathological conditions such as protein aggregation, gene mutations, environmental factors and cytokines released from infiltrated T cells. The pro-inflammatory mediators from M1 microglia activate astrocytes leading to elevated production of proinflammatory factors, nitric oxide and superoxide radical, contributing to degeneration of DA neurons. The molecules released from degenerative DA neurons can further cause activation of glia and enhanced inflammatory response. At certain stage of PD, subpopulation of microglia may become activated M2 phenotype releasing anti-inflammatory factors, including TGF-β, and exert a neuroprotective effect in PD

1.3 Macrophages

Macrophages were initially identified in the late 19th Century by Elie Metchnikoff, who first described their phagocytic activity, winning the Nobel Prize Physiology or Medicine for his discovery of phagocytosis and making official the birth of Immunology[90].

Macrophages, the most plastic cells of the haematopoietic system, are a heterogeneous population of immune cells that is present in all tissue ,showing great functional diversity, and play a key role in maintaining tissue integrity and homeostasis; they show an high phagocytic activity and are able to recognize and respond to a broad spectrum of stimuli, including tissue damage and infection[91,92].

The specialized role played by macrophage residing in different tissues has been highlighted also by a global trascriptome analysis of purified macrophages from different tissue, called the Immunological Genome Project [93].

In 1968 Van Furth and Cohn demonstrated that tissue macrophages derived from blood monocytes developed from bone marrow precursors[94]. For decades it was believed that tissue-resident macrophages are continuously repopulated

by blood circulating monocytes which arose from progenitors in the adult bone marrow[95]. Nowadays it has been accepted that the contribute of circulating monocytes to macrophages population is restricted to specific tissue such as the gut and the dermis; instead, many tissue-resident macrophages population arise from embryonic precursor and maintain themselves locally throughout adulthood.

Beside their origin, macrophage can be classified according to their inflammatory state and according to this criterion it is possible to divide these cells into two main group: the “M1-M2 paradigm”, even if these paradigm has been recently questioned since it appears too bipolar given the complexity and the dynamism of macrophages polarization[96].

Thus, macrophages represent an heterogenous population of cells, able to constantly shift their phenotype in order to respond to many challenges and stimuli and they should be considered as different subtypes of cells according to their different origins.

1.3.1 Macrophage origin and functions

Hematopoiesis occurs in several waves in embryonic development. Initially is limited to the yolk sac (YS), which can produce macrophages and primitive nucleated erythrocytes. Definitive multi-lineage hematopoiesis, on the other hand, depends on hematopoietic stem cells (HSCs) that are specified before E9.5 during a small-time window. After E10.5, HSCs colonize the fetal liver (FL), which serves as the intermediate site of definitive hematopoiesis. In the perinatal period, the bone marrow (BM) replaces the FL as the main hematopoietic organ, giving rise to all hematopoietic lineage[97]. During embryonic development, macrophages can be derived from primitive hematopoiesis or definitive hematopoiesis, the latterthrough FL monocyte intermediates.

Evidence indicates that exist at least three lineages of macrophages that originate at different stages of development and persist in the adulthood.

The first lineage involves a series of progenitors in temporal succession. Primitive progenitors of macrophages originate from early and late erythro-

myeloid progenitors (EMPs) generated in yolk sac that can arise macrophages that do not have a monocytic precursors.

Second lineage of macrophages derives from yolk sac progenitors. Tissue resident populations of macrophages (F4/80 high) in skin, spleen, pancreas, liver, brain and lung originate from yolk sac progenitors, as demonstrating by studies using ablation of c-Myb-dependent bone marrow hematopoiesis and next transplantation with different bone marrow[98].

The third lineage arises from fetal liver; as occur for Langerhans cells that have a mixed origin from yolk sac and fetal liver [99].

The master regulator of macrophage lineage is macrophage colony-stimulating factor 1 receptor (CSF1R), a transmembrane tyrosine kinase receptor, involved in macrophages differentiation: its ablation leads to a severe depletion of macrophages in several tissues, such as brain, ovaries, bone and skin[100].

Despite the great functional diversity shown by macrophages from different tissue, these cells are required for the maintenance of tissue homeostasis and they have roles in almost every aspect of an organism's biology, from development, repair to immune responses to pathogens. Resident macrophages regulate tissue homeostasis by acting as sentinels, able to quickly respond to changes in physiology as well as challenges from outside[101].

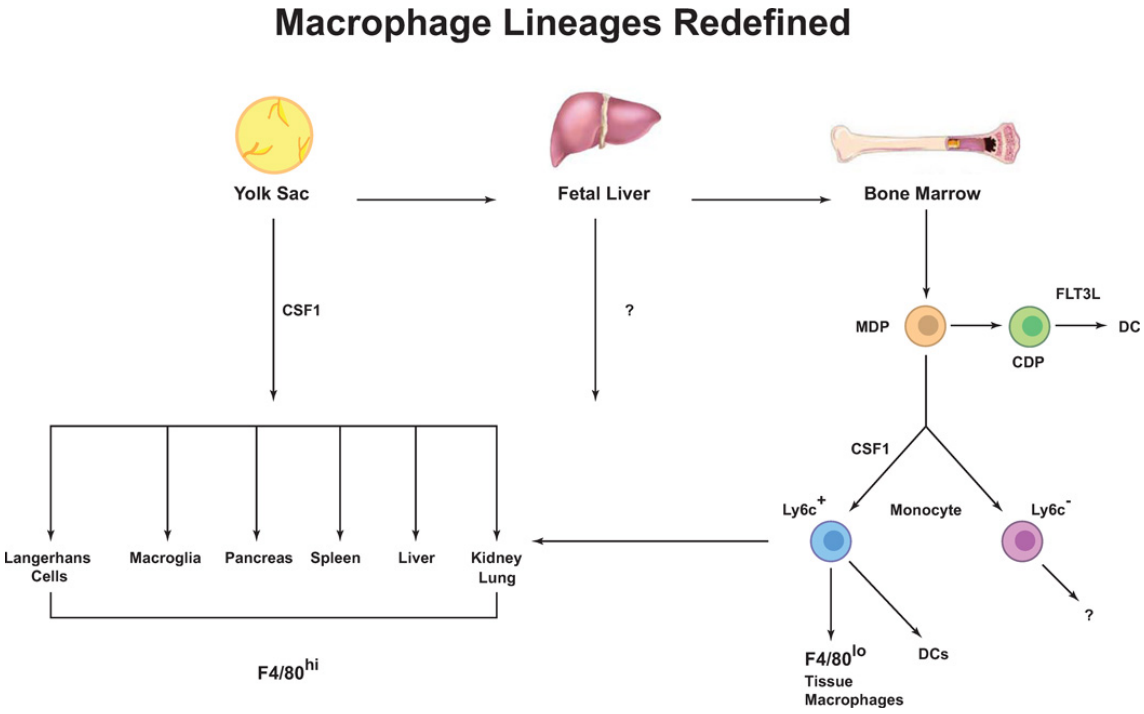


Figure 8- Macrophage lineages in mice[101]. The mononuclear phagocytic system in adults derives from at least three sources. The first is the yolk sac, which produces progenitors that populate all tissues and that have progeny that persist throughout life as F4/80 bright resident macrophages. These lineages are mainly regulated by CSF1R and its ligands, IL- 34 and CSF1. The second is the fetal liver, and this is less well defined but seems to contribute to the production of adult Langerhans cells, perhaps through a progenitor that is derived from the yolk sac. The third lineage derives from the bone marrow (BM) to give circulating monocytes and their progeny F4/80low macrophages, and dendritic cells (DCs). In this case the Ly6c1 monocytes give rise to the classic Steinman dendritic cells under the regulation of FLT3, and these are continuously replenished. Other macrophages that are F4/80low also emanate from Ly6c1 monocytes, and in some cases—such as in kidney and lung—they co-exist with those derived from the yolk sac to give chimeric organs. The exact role of the patrolling Ly6c– macrophages, and the contribution of fetal liver to adult tissue macrophages, remain unclear. CDP committed dendritic cell progenitor; MDP, monocyte dendritic cell progenitor.

In addition to peculiar phenotypes acquired in the different tissue, macrophage share some common features that can be summarized in the following ones:

- **Phagocytosis:** phagocytosis represents the primary function of macrophages, they are able to recognize and engulf pathogens, toxic substances and cellular debris. This process plays a key role in the turnover of old or damaged cells (for example, erythrocytes) and especially for the immune response.
- **Secretion**: macrophages are able to produce several biologically active molecules endowed with autocrine and paracrine functions, like cytokines (IL-1, IL-12, INF-α, TNF), proteolytic enzymes (such as plasminogen activators, collagenase and elastase) and chemokines.

- **Antigen presentation to CD4+ T lymphocytes:** macrophages acts as Antigen-Presenting-Cells (APC): they can capture, process and present antigen on their membrane linked with MHC Class II causing the activation of other immune cells.

1.3.2 Tissue-resident macrophage populations.

Macrophages populate the majority of tissue in the organism, where they adopt peculiar phenotypes and play specific and different functions. Tissue-resident macrophages can be defined as “sentinels” thanks to their ability to start and modulate immune responses following infections or tissue damage; moreover, they play a key role in maintaining the homeostasis of the tissue.

It has been observed a marked difference in gene expression profile of macrophages residing in the different tissue, suggesting a distinct transcriptional program for each population, in accordance with the different tasks performed: for example, only spleen resident macrophages express Spi-C gene, essential in the recycling of iron from old red blood cells and Gata6 is selectively expressed by peritoneal macrophages.

This means that, depending on the organ, these cells can play specialized tasks: microglia, for example, regulates neuronal plasticity in steady state conditions, while alveolar macrophages engulf and eliminate surfactants proteins, preventing proteinase excess[102,103]. Tissue microenvironment is also crucial in modulating tissue-resident macrophages phenotype and each anatomical niche provides peculiar signals, able to orchestrate their differentiation, regulate and maintain their specialized functions.

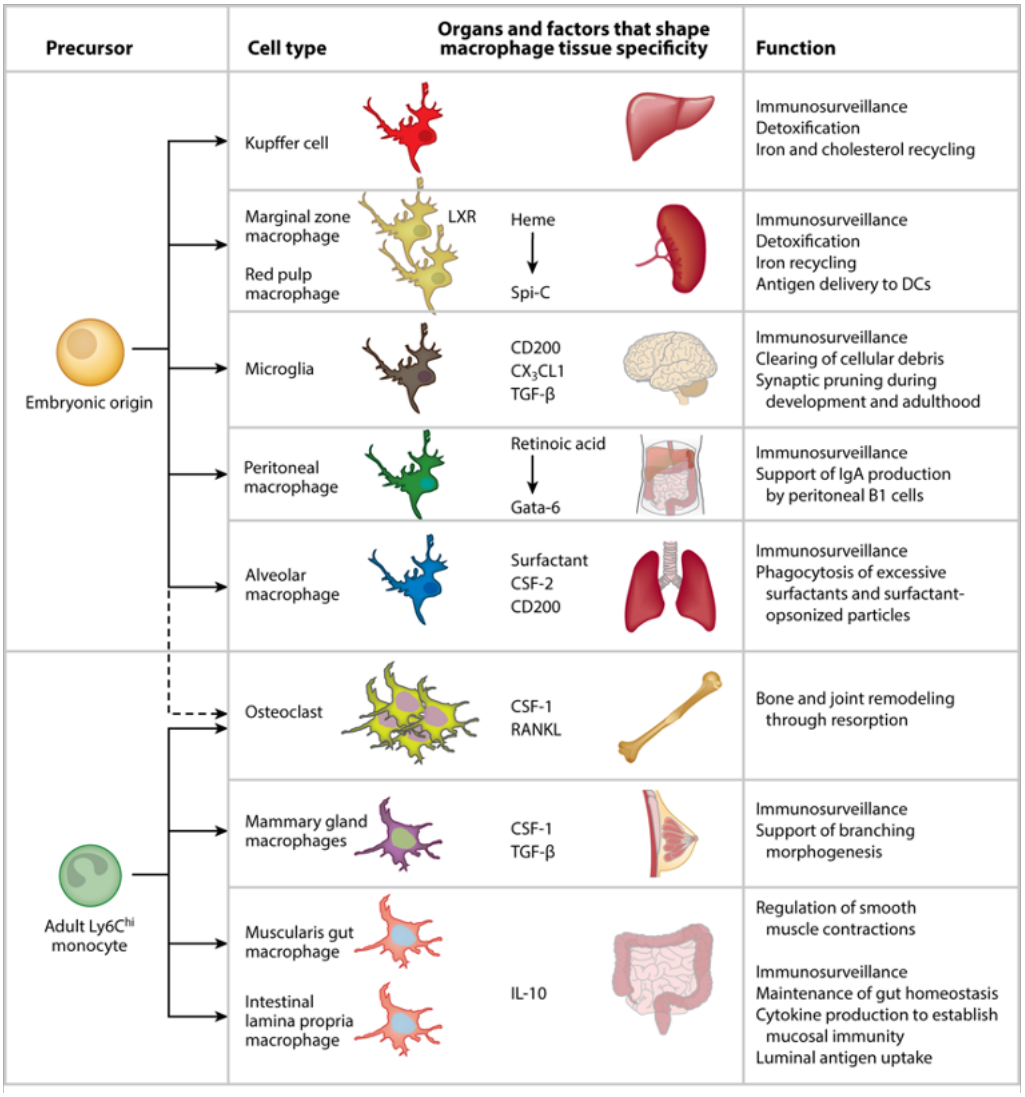


Figure 9- Tissue-resident macrophages population, modified from [104]
Factors imprinting tissue macrophage fate and maintaining specific tissue macrophage features. In their target organs, tissue-resident macrophages of either embryonic or adult monocytic origin are exposed to tissue-specific factors, which influence their development, polarization, and function. This includes the induction of an acquired deactivation state through exposure to factors such as TGF-β, CX3CL1, IL-10, and CD200, which might prevent early, premature immune activation. Other factors, such as retinoic acid, which induces expression of Gata-6 in peritoneal macrophages, or surfactants in the lung, contribute to the proliferation, migration, and activation behavior of the cells. An additional example is heme, which was shown to induce Spi-C expression by red pulp macrophages.

Peritoneal macrophages

The peritoneum is the largest serous membrane of the human body, presenting an unique structure and peculiar functions[105]. In the peritoneal cavity (PerC) coexist a variety of immune cells, such as eosinophilis, neutrophilis, B cells, dendritic cells and macrophages, that represent 30-35% of the cells.

Peritoneal macrophages are the best studied population of macrophages and using them as source of cells it has been possible to reach most of current knowledge about macrophage biology, functions and specialization.

Only recently, studies performed in mice have revealed that in the peritoneal cavity coexist two different subpopulations of peritoneal macrophages that are phenotypically and functionally heterogeneous[106].

On the basis of their size, these subpopulations are defined as Large Peritoneal Macrophages (LPMs) and Small Peritoneal Macrophages (SPMs). Both subpopulations show phagocytic activity *in vivo* and express macrophage typical surface markers, although they show different levels of these markers:

- **F4/80:** only LPMs express high levels of these glycoprotein belonging to the family of *Epidermal Growth Factor* (EFG), expressed by macrophages belonging to different tissues, while SPMs present low levels of F4/80.
- **Cd11b:** only LPMs show high levels of this integrin that is expressed not only by macrophages but also by other cells type like dendritic cells.
- **MHC II** (major histocompatibility complex II): LPMs express low levels of this complex, in contrast with SPMs.
- **GATA6:** only LPMs express this transcription factor.

SPMs and LPMs show a different morphology and phenotype: indeed, LPMs display a classical morphology with prominent vacuolization and abundant cytoplasm, whereas SPMs have a morphology that resembles DCs with dendrites.

Moreover, the analysis of a complex panel of other surface molecules between the two subtypes demonstrated that they differ in the expression of a wide variety of markers, resulting in different response patterns following treatment with classical macrophage stimuli (like lipopolysaccharide, LPS).

Surface molecule	LPMs	SPMs
F4/80	+++	+
CD11b	+++	+
CD11c	+	−
MHC-II	+	++
GR1	+	−
Ly6C	−	−
c-kit	−	−
CD62L	−	++
Dectin-1	+	++
DC-Sign	−	++
TLR4	++	+
CD80	++	+
CD86	+++	+
CD40	++	+
12/15-LOX	+	−
TIM4	+	−

Table 1- Phenotypic profile of SPMs and LPMs[107]

As mentioned before, both LPMs and SPMs show phagocytic activity *in vivo* but SPMs seem to be more efficient in phagocytosis; furthermore, LPMs produce more NO in response to LPS *in vitro*.

Even the origin and development of the two subtypes is different: LPMs derive from yolk sac progenitors and are able to proliferate *in situ*, whereas SPMs derive from circulating monocyte, and their number strongly increase under inflammatory conditions.

Under homeostatic conditions, peritoneal cavity includes a wide variety of immune cells and the two subpopulations of macrophages represent about 30-35 percentage of total cells. In presence of an inflammatory or infectious signals lead to a dramatic change in the number and in the percentage of each subpopulation. Modifications in the composition of the peritoneal cavity include the disappearance of LPMs, the increase in the number of SPMs and a massive recruitment of circulating monocytes.

These changes occur during hypersensitivity reactions and during the process of acute inflammation; the increase in the number of SPMs and monocytes is to be correlated with the renewal and improvement of the immune conditions of the peritoneal cavity. We can conclude that the SPMs and their precursor (circulating inflammatory monocyte) are the main population present in

stimulated peritoneal cavity, in fact this subpopulation show a pro-inflammatory functional profile while LPMs seem have a role in the maintenance of the peritoneal cavity in physiological conditions[107].

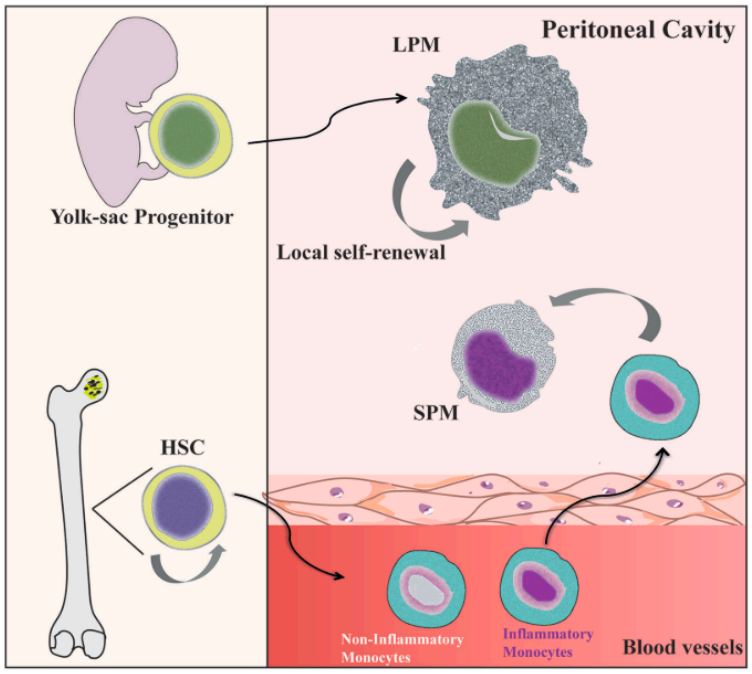


Figure 10- Distinct origin of peritoneal macrophages subsets[107]. SPMs are generated from hematopoietic stem cells (HSC) in the bone marrow (BM) by differentiation of inflammatory blood monocytes. However, LPMs appear to be originated from progenitors from yolk sac and independent of hematopoietic progenitors. Local proliferation of LPMs ensures homeostatic maintenance by self-renewal.

Microglia

Microglia are myeloid cells that populate the parenchyma of the central nervous system (CNS), representing 5%-12% of the total number of cells depending on the brain region[57,108] and were firstly identified in 1919[109,110].

Microglia derive from yolk sac progenitors that colonize the neuroepithelium in early stage of embryogenesis[98], acquiring their definitive characteristics, in terms of numbers and phenotype, immediately after birth; although a peak of microglia proliferation has been demonstrated at early postnatal stages[111,112].

Microglia population is maintained locally by self-renewal, without the contribution of bone-marrow-derived progenitors[98,113,114], even though the mechanisms that regulate microglia proliferation in steady state are unclear. Some studies reported the involvement of transcription factor PU.1, CSF1 and IL-34 in the regulation of microglia proliferative pathway[115].

Microglia physiological activity includes most of the biological properties that are typical for peripheral macrophages, although their developmental origin and anatomical distribution allows these cells to perform distinctive immune and neuromodulatory functions in the CNS. Through their physical and biochemical interactions with neurons, microglia are able to sense and remodel neuronal activity, support neurogenesis, and maintain CNS homeostasis. Microglia constantly scan the microenvironment to detect and remove neurotoxic substances or inflammatory mediators. Microglia phagocytic activity is essential during development (pruning microglia), removing supernumerary synapses in some neuronal pathways[116].

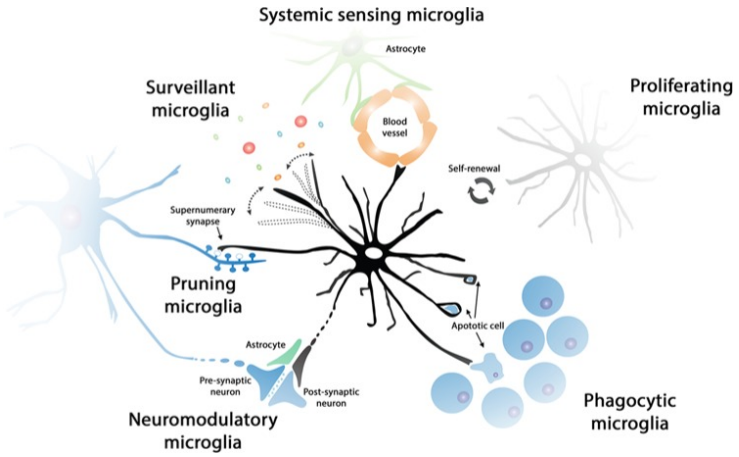


Figure 11- Functions of microglia in the healthy brain[117]. The population of microglial cells is maintained by self-renewal. Surveillant microglia sense and remodel neuronal activity, support neurogenesis, and maintain CNS homeostasis.

1.3.3 Macrophage polarization.

Macrophage polarization is a dynamic process that allows macrophages to adopt a specific phenotype, characterized by specific factors and peculiar biological activity in response to stimuli of their microenvironment and signals arising from different tissue.

In normal, unstimulated conditions macrophages are present in all tissue in a quiescent state, acting as “immune sentinels”, performing tasks aimed at maintaining tissue homeostasis. In response to specific signals typically

produced by innate immune cells, they can undergo to activation leading to long-term alterations in macrophages.

According to the current classification, macrophages are grouped in two main groups, however these classes represents the two extremes in a much complex series of phenotype that macrophage can assume: the “classical” activation (M1) and the “alternative” activation (M2) [118,119].

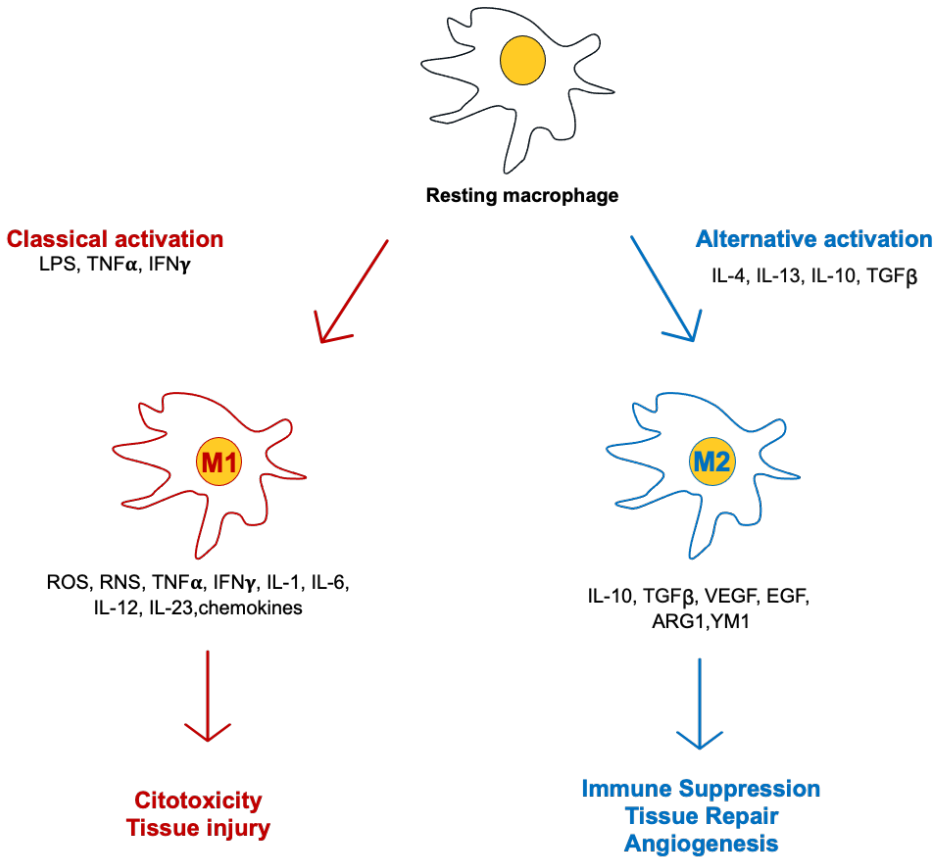


Figure 12- Schematic representation of macrophage polarization.

M1 Macrophage Polarization

M1 macrophages participate in the first phases of the inflammatory response and they are in charge of the elimination of invading microorganismthrough the production of reactive oxidative species (O2- OH-), nitrate (LIKE NO-, NO2-, ONOO-), inflammatory cytokines (IL-1β, TNFα, IL-6) and chemokines. Since this antimicrobic activity has been identified first, it has been called “classical” activation.

However, classical activation can often lead to tissue damage as a collateral damage since there is no discrimination between microbic targets and host tissue; so, even if this response represents a key event in the defense against external insults, inflammatory activation needs to be strictly regulated in order to eliminate its causative events without causing damage to the organism itself. Macrophages classical activation is triggered after the recognition of molecules associated with microorganism, like the bacterial lipopolysaccharide (LPS), a component of Gram-negative bacterial wall and a ligand of TLR4. Helper Th-1 cells, CD8 + cytotoxic T cells (Tc1) and Natural Killer (NK) cells too can activate macrophages by producing interferon-γ (IFN-γ), as well as molecules associated to cell damage, such as intracellular proteins or nucleic acids. Classically activated macrophages show an elevated ability in antigen presentation, an increased production of pro-inflammatory cytokines such as IL-1, IL-6, IL-1β, TNFα and toxic mediators such as nitric oxide (NO); furthermore, they show an higher phagocytic activity mediated by the complement[120]. Commonly, M1 macrophages are associated with pathological states characterized by low grade inflammatory states such as atherosclerosis and type-2 diabetes[121] and are involved in the immunopathology that occurs during several autoimmune diseases, such as rheumatoid arthritis[122] and inflammatory bowel disease[123]; in fact silencing genes that are involved in classical activation, like TLR4 gene, results in disease improvement in some murine models.

M2 Macrophage Polarization

Alternative or M2 macrophage activation usually takes place in a later time point when compared to M1 activation, and it has anti-inflammatory effects helping with tissue repair.

M2 macrophages are able to block M1 macrophages pro-inflammatory properties and they produce important factors able to repair damaged tissues, helping also with angiogenesis[124]; they are involved also in the regulation of immune response and in tumor progression[125]. Many molecules induce M2 polarization like glucocorticoids, cytokines produced by Th2 cells like IL-4, IL-

13, IL-33 and IL-21 or by TGFβ,Also CSFs seem to be important in promoting M2 phenotype[126,127].

M2 phenotype is also necessary in controlling extracellular parasites, including helminths, protozoa and fungi, as well as in the states allergic and increasing susceptibility to other pathogens.
Many proteins involved in repair, healing, angiogenesis and cells proliferation are produced by M2 macrophages and they act as markers of M2 polarization; among them there are Arginase 1 (Arg1), Ym1, Fizz1 and Vegfα.
Macrophages displaying and M2 phenotype can be further divided in subgroups, as demonstrated by Mantovani and collogues, called M2a, M2b, M2c and M2d.

- M2a phenotype is produced *in vitrothrough* exposition to IL-4 or IL-13 that, acting through IL4Rα, increase the expression of CD206, Arg1 and TGFβ[128–130].

- M2b phenotype is produced by exposing cells to Ig-G and LPS. Unlike other M2 macrophages, this phenotype is characterized by high levels proinflammatory cytokines, high levels of IL-10 and low levels of IL-12[131].

- M2c phenotype can be acquired through treatment with IL-10 or glucocorticoids that is characterized by high levels of IL-10 and low levels of IL-12 and an increase of the expression of scavenger receptor CD163[132].Furthermore, M2c phenotype, promotes the *"switching"* from M1 to M2 phenotype. It is characterized from high levels of IL-10 and Vegfα, a factor responsible of the growth of vascular endothelium[120].

Even if M2 macrophage effects seem to be mostly favorable in protection and restoring after damage, alternative activation has been associated in the complex mechanism that lead to tumor development and growth: these peculiar macrophages are defined TAMs *(tumor associated macrophages*)[133].

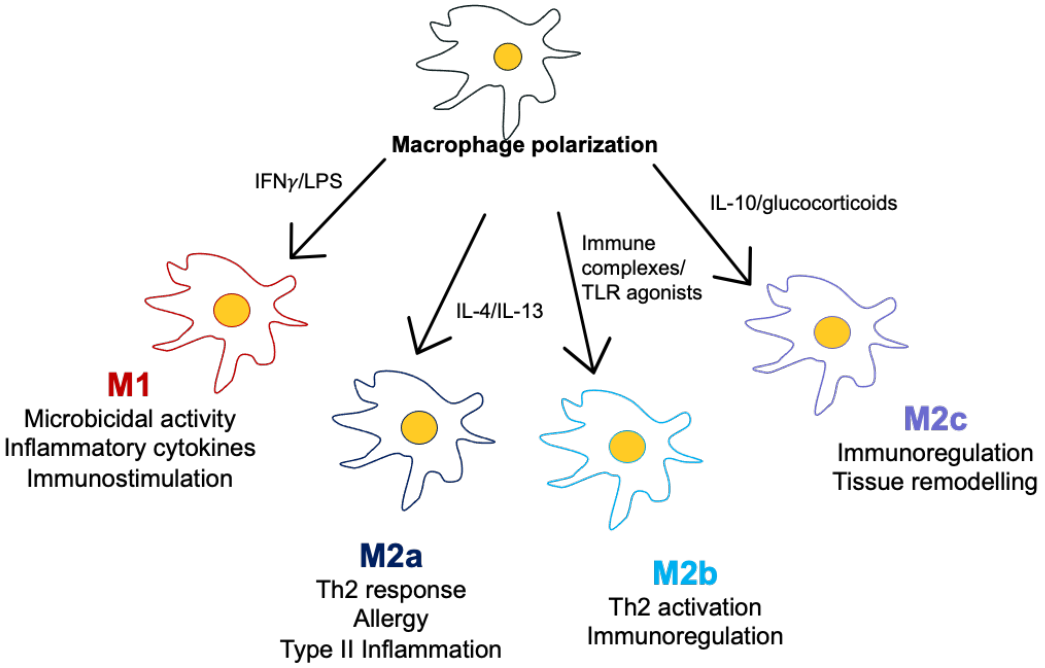


Figure 13- Macrophage polarization. Macrophage micro-environment stimuli define differential macrophage polarization via classical activation (M1) or alternative activation (M2). Pathogen-derived LPS alone or in combination with IFN-γ leads to classical activation of M1 macrophages, which improves microbicidal activity and secretion of pro-inflammatory mediators. According to the host-parasite microenvironment, alternative macrophage activation could be subdivided into three subpopulations. M2a differentiation is promoted by IL-4 or IL-3, and this subpopulation is associated with Th2 response, allergy process, internalization, and parasite killing. M2b is related to the presence of immune complexes, TLR or IL-1R agonists, and promotion of immunoregulation. Glucocorticoids and IL-10 secretion lead to differentiation into M2c, which also induces immunoregulation, tissue remodeling, and repair.

1.3.4 Immunometabolism

Intrinsic or extrinsic signals regulate metabolic pathways in order to meet cellular needs, such as growth or survival; however in the context of immunity specific alterations of metabolic pathways respond to immune effector functions, like the production of distinct set of cytokines[134].

First insight into the metabolic status of macrophages date back to more than 40 years ago, when it was demonstrated that M1 murine peritoneal macrophages show lower oxygen consumption and higher glycolytic levels when compared to resting macrophages[135]. Immunometabolism is a fast evolving field and it is now appreciated that metabolic reprogramming of immune cells is not only critical for energy metabolism but the metabolic adaptations directly influence immune cells functions by controlling transcriptional and post-transcriptional events: in fact, in order to meet energy

demand for functional specialization macrophages undergo appropriate metabolic shift[136]. There is a growing evidence that immune cells that immune cells in a specific microenvironment, like inflamed tissue or tumors, reprogram their metabolic profile in order to fulfill peculiar cellular needs like survival, growth or to perform specific tasks such as phagocytosis. So, changes in the metabolism of immune cells, particularly macrophages, could result in a modulation of their functions that can be useful in diseases with a high macrophage involvement. Therefore, macrophage metabolism could represents a new and interesting therapeutic target[137,138].

Briefly, the metabolism of M1 macrophages is characterized by enhanced glycolysis, flux through the pentose phosphate pathway (PPP), fatty acid synthesis, and a truncated TCA cycle, leading to accumulation of succinate and citrate. The metabolic profile of M2 macrophages is defined by oxidative phosphorylation, fatty acid oxidation, a decreased glycolysis, and PPP[139].

M1 macrophage metabolic signature.

Glycolysis has been shown to be involved in a large number of immune processes; M1- macrophages relies strongly on glycolysis and several works have demonstrated that macrophages treated with 2-deoxyglucose[140], that inhibits hexokinase thereby blocking the first steps of glycolysis, showed a reduced inflammatory response both in *vivo* and *in vitro*. Enhanced glycolysis occurs in LPS-activated macrophages[141], and it can be considered a hallmark metabolic change since it enables the immune cells to produce sufficient ATP to carry out effector functions, like phagocytosis and inflammatory cytokines production. Furthermore, LPS induces the activation of HIF1α, that is crucial for the production of several enzymes involved in glycolysis. A key mechanism responsible for the increase of glycolysis observed in M1-macrophages is the induction of pyruvate kinase isoenzyme M2 (PKM2), that allows direction of glycolytic intermediate to other biosynthetic pathways; moreover, PKM2 can translocate to the nucleus where it interacts with HIF1α, promoting the expression of several HIF1α,-target genes, including inflammatory factors. In

M1-macrophages another glycolytic enzyme, hexokinase-1, has been shown to activate NLRP3[142], thus enhancing the production of mature IL-1β and IL-18.

Another aspect of M1 macrophages is and enhanced PPP. The enzyme CARLK is a key control enzyme of the PPP: it limits the flux toward the PPP and it has been observed that it is highly expressed in M2-macrophages and, if CARLK is suppressed, macrophages become more M1-like, suggestion the importance of the pentose phosphate pathway in macrophage polarization[143].

In M1-macrophages TCA cycle is truncated at the level of isocitrate dehydrogenase (IDH1) and succinate dehydrogenase (SDH) leading to the accumulation of two of its intermediates, namely citrate and succinate. Excess of citrate is then utilized for the production of fatty acids, contributing to membrane biogenesis, and it serves as a precursor for the production of the macrophage-specific metabolite, namely itaconic acid, that is endowed with bactericidal effects. Accordingly, immunoresponsive gene 1 (Irg1), that codes for the conversion of aconitate, produced from citrate, to itaconic acid is one of the most upregulated gene in IFNγ/LPS-treated macrophages[144].

On the other hand, accumulation of citrate is responsible of HIF1α stabilization, by limiting prolyl hydroxylase domain activity, thus increasing the expression of IL-1β[138].

In contrast to fatty acid oxidation, it seems that fatty acid synthesis positively regulates generation and functions of pro-inflammatory macrophages. Several studies demonstrate that LPS stimulation triggers an induction of fatty acid production in macrophages[145,146].

It was found that in the differentiation from monocyte to macrophages, upon stimulation with M-CSF1, lipid synthesis increases thanks to the upregulation of sterol-regulator element- binding 1c (SREBP1c), inducing the expression of several fatty acid synthesis-related genes, like FASN. The observed increase of fatty acid synthesis in this context was crucial to the differentiation and inflammatory functions of macrophages[147]. Furthermore, fatty acid are used as precursor for prostaglandin production in macrophages stimulated with LPS, TNFα or IFNγ.

Adequate supplies of glutamine have been found to be essential for the induction of IL-1 in macrophages treated with LPS; glutamine metabolism is also important for the generation of nitric oxidethrough feeding into arginine metabolism.

Finally, macrophages use arginine in two different ways: flux of this amino acid into the nitric oxide cycle is associated with the M1 phenotype; in this way arginine is converted into nitric oxide by iNOS[148].

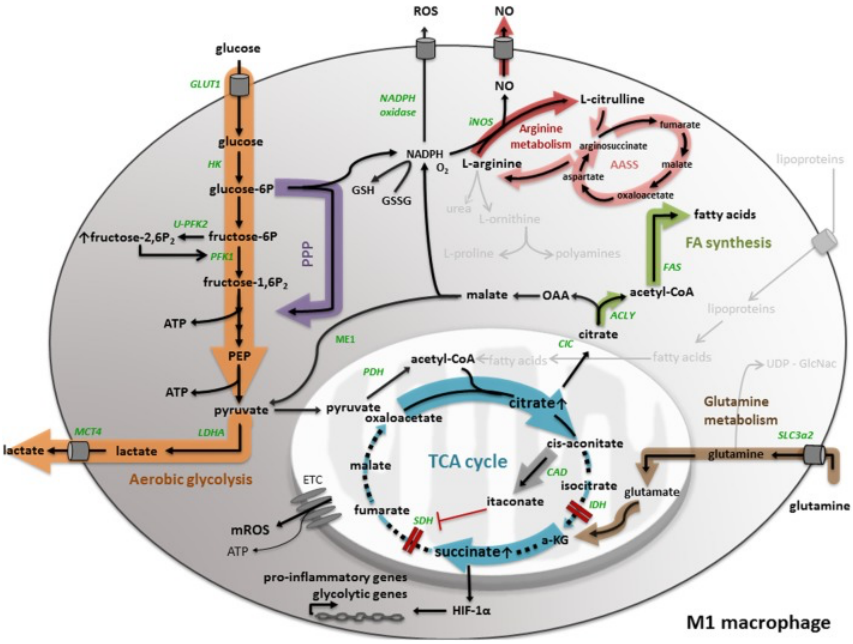


Figure 14 -M1 macrophage metabolism[138] M1 macrophage metabolism is characterized by enhanced aerobic glycolysis, converting glucose into lactate. M1 macrophages have an increased flux through the pentose phosphate pathway (PPP), generating NADPH, used for the generation of the antioxidant glutathione (GSH) and the inflammatory mediators nitric oxide (NO) and reactive oxygen species (ROS). In M1 macrophages, the tricarboxylic acid (TCA) cycle is broken in two places, leading to the accumulation of succinate and citrate. While the accumulation of succinate leads to HIF-1α stabilization and the transcription of pro- inflammatory and glycolytic genes, citrate is used for the generation of fatty acids, NO, ROS, and the synthesis of itaconate. Another aspect of M1 macrophage metabolism is the conversion of l-arginine to NO and l-citrulline.

M2 macrophage metabolic signature.

One of the major differences in the metabolism of M1 and M2 macrophages is in their energy metabolism. While M1 macrophages preferentially rely on glycolysis, M2 macrophages mainly produce ATPthrough an oxidative TCA cycle coupled wit oxidative phosphorylation. To fuel an oxidative TCA cycle IL-4 stimulated macrophage rely on FAO and glutamine metabolism.

The role of glycolysis in M2 macrophages is controversial[137]: several studies have shown that it is active in M2 cells and it can be blocked used known glycolysis inhibitor like 2-deoxyglucose resulting in an impairment of M2 polarization and functions. However, glycolysis is not required for M2 differentiation, as long as OXPHOS remains intact[149], suggesting that M2 macrophages display a more flexible metabolic profile since they can supply OXPHOS in absence of glycolysis using glutamine.

Correlated to low glycolytic rates, M2 macrophages have a limited fluxthrough the PPP: M2 macrophages show high levels of CARKL, an enzyme known to limit the flux through the pentose phosphate pathway; furthermore, overexpression of CARKL leads to a decrease in the expression of pro-inflammatory cytokines that is consistent with the M2 phenotype.

M2 macrophages are known to display an intact and functional TCA cycle, which plays a key role in providing ATP necessary to the high glycosylation levels necessary for M2 macrophage function[150].

In contrast with M1 macrophages (induced by stimulation with LPS or IFNγ), M2 macrophages rely on a program of fatty acid oxidation and mitochondrial biogenesis, promoted by signal transducer and activator of transcription 6 (STAT6) and PPARγ-co-activator 1β (PGC1β) and works to inhibit inflammatory[151,152].

Beside differences in the energy metabolism, M1 and M2 macrophages show opposite arginine metabolism and it represent the perfect example of how strict metabolic regulation can drive opposite phenotype. In contrast to arginine flux through NO synthesis pathway, M2 macrophages direct arginine into the arginase pathway that ultimately leads to collagen production and it is associated with a more tolerant immune phenotype, often linked to wound healing.

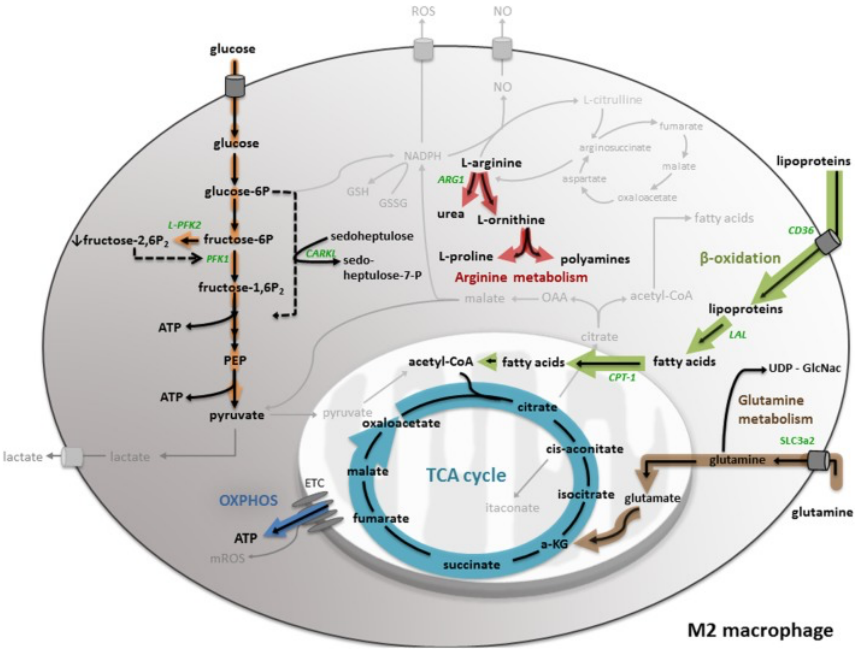


Figure 15- M2 macrophage metabolism[138]**.** M2 macrophages mainly produce ATP through an oxidative TCA cycle coupled to oxidative phosphorylation (OXPHOS). To fuel the TCA cycle, M2 macrophages rely on fatty acid oxidation (or β-oxidation) and glutamine metabolism. Furthermore, M2 macrophages show a lowered glycolysis and pentose phosphate pathway (PPP). Moreover, M2 macrophages convert l-arginine into urea and l-ornithine, which serves as precursor for l-proline production.

1.4 Sexual differences in immunity

Sex is a biological variable that affects immune responses both to self and foreign antigens. Sexual dimorphism in the immune response have been documented and, indeed, clinical manifestations of infection or autoimmune diseases differ between males and females[153]. Different factors may be considered responsible for the disparity observed between the two sexes, such as genetic factors, hormonal mediators and also environmental factors (like nutrition and composition of the intestinal microbiota).

Today the general hypothesis is that females develop stronger innate and adaptive immune response; in fact females respond better to various kind of vaccination and are less susceptible to a broad spectrum of infections caused by bacteria, fungi, viruses and parasites such as *Staphylococcus* spp., *Mycobacterium tuberculosis*, parainfluenza virus, respiratory syncytial virus, hepatitis B virus, *Entomoeba histolytica* and *Aspergillosis fumigatus*[154,155].

It is increasingly important to acknowledge sex- differences in immune response when we considered the differences observed in several pathologies. For

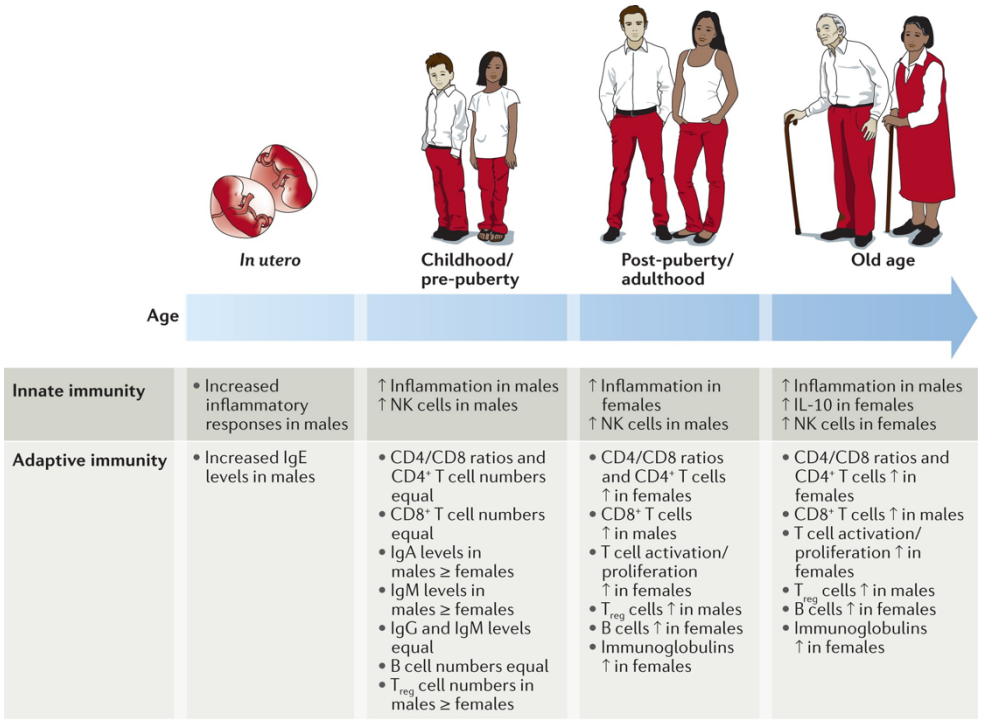
example, the incidence of sepsis is higher in man compared to women, 80% of autoimmune diseases occur in females, men show an almost two-fold higher risk of death for malignant cancer than women and females respond better to seasonal influenza vaccination[153]. The higher susceptibility to infections in men is observed from birth to adulthood, suggesting that other factors including genetic factors and hormonal mediators, beside sex hormones, play a role in the sexual disparity in immune response.

Sex-differences in immune response are not restricted to mammals, but they evolved in several species from insects to lizards and birds; in lizards for istance the phagocytic activity of macrophages in greater in females when compared to males.

There are several possible explanations for the disparity between male and female immuntity[156]. First the different composition of sex chromosomes: better innate resistance to infection is observed early in life in females, indicating that sex chromosomes have a major role in sex differences in immunity. Indeed, several gene involved in immunity are located on the X chromosome and they include genes involved in phagocytosis like CYBB, factor crucial for erythropoiesis and macrophage differentiation like IL3RA and GATA1, transcription factor like FOXP3 and GATA1 and pattern recognition receptors (PPRs) such as TLR7 and TLR8.

Second, sex hormones play a role in the transcriptional regulation of the immune system[157]; the mechanism underling immune protection in females is often reconciled with the activity of 17β-estradiol, which is known to directly influence the synthesis of multiple cytokines. However, in vivo studies on anti-inflammatory actions of estrogens have given conflicting results partly due to the multiple actions of estrogens on several cell types and to the limitation in the experimental doses of estrogens used that do not fully represent the biological differences between the sexes. Experimental models have shown that the development of innate immune cells and the immune responses to danger signals are highly influenced by estrogen, converging on an anti-inflammatory effect of this hormone in dose and context-dependent manners. In particular, estrogen can shape and modulate the activation state of macrophages, that are

an important source of cytokines and key players of innate immune response [158]. Third, stimulation related to pregnancy and breastfeeding, of course, are only limited to females; pregnancy is known to depress maternal immunity as a means to prevent rejection of the fetus[159]. Fourth, there are behavioral differences between males and females, with regard to the frequency and timing of exposure to challenges (viral, bacterial, chemical, trauma, and others), and food intake prioritization in the family[160].



Innate immunity	• Increased inflammatory responses in males	↑ Inflammation in males ↑ NK cells in males	↑ Inflammation in females ↑ NK cells in males	↑ Inflammation in males ↑ IL-10 in females ↑ NK cells in females
Adaptive immunity	• Increased IgE levels in males	• CD4/CD8 ratios and $CD4^+$ T cell numbers equal • $CD8^+$ T cell numbers equal • IgA levels in males ≥ females • IgM levels in males ≥ females • IgG and IgM levels equal • B cell numbers equal • T_{reg} cell numbers in males ≥ females	• CD4/CD8 ratios and $CD4^+$ T cells ↑ in females • $CD8^+$ T cells ↑ in males • T cell activation/ proliferation ↑ in females • T_{reg} cells ↑ in males • B cells ↑ in females • Immunoglobulins ↑ in females	• CD4/CD8 ratios and $CD4^+$ T cells ↑ in females • $CD8^+$ T cells ↑ in males • T cell activation/ proliferation ↑ in females • T_{reg} cells ↑ in males • B cells ↑ in females • Immunoglobulins ↑ in females

Figure 11- Changes in immune responses in human males and females over the life course. Multiple immunological factors vary between the sexes throughout the course of life. For certain factors (for example, pro-inflammatory responses), the sex differences change at puberty and then wane in later life suggesting hormonal effects. For other factors the sex difference remains constant from birth to old age (for example, higher numbers of CD4+ T cells andCD4/CD8 T cell ratios in females). The paucity of studies in this area is notable, particularly in utero sex differences in which results are conflicting. IL-10, interleukin-10; NK, natural killer; Treg, regulatory T.

Monocytes and macrophages

Monocytes, which accounts for about 5 to 10% of the circulating white blood cells, play a key role in the innate immune response since they are an important source of cytokines.

Cytokines released from monocytes are involved in the acute phase of the inflammatory response and they are responsible for the recruitment of other inflammatory cells.

The number and functions of innate cells have been reported to differ between males and females, both in humans and rodents, but with contrasting results[161,162].

Innate cells isolated from females generally show a more intense response to inflammatory stimuli. A higher number of pleural and peritoneal macrophages, a more efficient phagocytosis and higher level of TLR2, TLR3 and TLR4 have been observed in female than in male mice[162].

A recent study from the Immune Genome Project, a major project that assessed the variability of human cytokines response, highlighted important difference between the two sexes: the analysis of 11 immune lineage revealed that phenotypic differences between males and females existed regardless of any immune stimulation and are mostly manifested in macrophages, isolated from different tissues, namely peritoneum, spleen and central nervous system (microglia)[156].

Female sex was associated with higher circulating levels of IL1Ra and lower IL-18 binding protein[163].

Regarding myelomonocytic cells, the production of pro-inflammatory cytokines released from monocytes (IL-1β,TNFα, IL-6 and INFγ) was higher in men after stimulation with different stimuli such as LPS and although the use of oral contraceptives did not have strong effects on cytokine production capacity in vitro, women using oral contraceptives showed a further decreased IFNγ and TNFα response after stimulation with LPS. Interestingly the majority of cytokines and mediators that differed between the two sexes did not correlate with progesterone or testosterone circulating levels, excluding a potential role of this hormones in explaining gender differences.

A study has reported a differential polarization of male and female mice macrophages infected with Coxsackievirus B3, that causes severe myocarditis in male but not female mice[164]. Macrophages infiltrating the myocardium from

infected male mice expressed high levels of classically activated M1 markers and female macrophages were associated with M2 phenotype.

Adoptive-transfer experiments revealed that the excessive presence of M1 macrophages may cause damage to the host and that M2 macrophages were protected against infection-induced myocarditis, suggesting a role for macrophage polarization in defining the sex-related susceptibility to viral myocarditis. Moreover, the higher incidence of asthma observed in female mice was also associated with higher polarization of macrophages to a M2 phenotype when compared to male mice[165,166].

Sex-differences have been reported also in microglia, the immune macrophage population residing in the brain, which represent the first line of defense in the adult brain against pathological insults and play a key role in controlling tissue homeostasis. A study showed that microglia cells are sexually differentiated, as indicated by the sex-specific expression of a significant number of genes. Microglia cells maintain sex- specific expression independently by the circulating sex steroids, which was demonstrated by cell cultures of adult microglia or by their transplantation in the brain of the opposite sex. In addition, ovariectomy, thus the abolition of estrogen production, did not show very significant changes in microglia gene expression[167]. Whole-genome molecular signature analysis of transcription factors (TFs) pointed to nuclear factor kB (NF-kB) as the TF most involved in the regulation of the differentially expressed genes (DEGs) preferentially expressed in males, together with other TFs associated with inflammatory processes (RUNX1), migration (FOXM1), and negative regulation of neurogenesis (GATA2). In contrast, no association with inflammation was found in the DEGs distinctive of female microglia that associated with pathways like morphogenesis, development, or cytoskeleton organization under the control of several TFs such as NANOG and TCF3, linked to the inhibition of inflammatory response and promotion of repair mechanisms. In addition, microglia conserved their specific phenotype even after transplantation in mice of the opposite sex. Moreover, female microglia are better apt than male microglia to reduce the ischemic damage also when transplanted in males, suggesting an intrinsic sex-specific microglia phenotype

that might be independent from the hormonal environment. This is in line with the observation that in humans, in fact, males have a higher incidence of stroke and poorer outcomes afterward.

AIM

My working hypothesis is that the sexual dimorphism in PD is due to a distinct ability of macrophages of the two sexes to drive inflammatory and neuromodulatory actions, resulting in a sexually dimorphic onset and progression of PD. The aim of my research is to study the extent to which neurotoxic molecules, that are typically associated with PD pathogenesis, or neuronal signals generated in response to them, could modulate macrophage cells and induce immune, metabolic and oxidative stress responses that influence PD progression. In addition, my goal is also to assess whether these specific functions of macrophages could be different in male and female mice.

METHODS

3.1 Animals and treatment

C57BL/6 female and age-matching male mice of 4 months of age were supplied by Charles River Laboratories (Calco, Italy) and used for gene expression analyses. Animals were allowed to food and water access *ad libitum* and kept in temperature-controlled facilities on a 12-hour light and dark cycle. Animals were housed in the animal care facility of the Department of Pharmacological and Biomolecular Sciences at the University of Milan. Animal investigation has been conducted in accordance with the ethical standards and according to the Guide for the Care and Use of Laboratory Animals, as adopted and promulgated by the US National Institute of Health, and in accordance with the European Guidelines for Animal Care and Use of Experimental Animals. Animals were sacrificed by a lethal ketamine/xyilazine solution (150 and 12 mg/kg, respectively). Generation of NFκBLuc and ARE-luc2 animals has already been described[168,169]; mice were used at 4 months of age. All animal experiments were approved by the Italian Ministry of Research and University and controlled by an academic panel of experts.

3.2 Primary cultures of peritoneal macrophages

Peritoneal cells were recovered by peritoneal lavage as previously described[158]. Briefly, 5 ml of pre-chilled 0.9% NaCl were injected into the peritoneal cavity using a 21 G needle, cell suspension was recovered and centrifuged; following incubation with ACK solution (0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM EDTA; pH 7.3) for 5 minutes at 4 °C, cells were seeded at the concentration of 1×10^6 cells/mL in RPMI (Life Technology-Invitrogen, Milan, Italy) supplemented with 10% endotoxin-free FBS, 1% penicillin/streptomycin and 1% Na-pyruvate. After 45 minutes and several washes in PBS, medium was replaced with RPMI w/o phenol red supplemented with 10% dextran coated charcoal (DCC)-FBS (RPMI + 10% DCC). On the next day, cells were treated with vehicle (DMSO 20% in H_2O), MPP^+ (4mM) Tert-Butylhydroquinone tBHQ (100 μM), LPS (1 μg/mL) or the combination of the two stimuli, as specified in each experiment. Cell suspension was centrifuged at 1200xg, cell pellets were re-suspended in TRIzol reagent (Life Technology-Invitrogen), and stored at -80°C for RNA, while

supernatant were stored for ELISA analyses. tBHQ induces a strong antioxidant response through mitochondrial oxidative stress and NRF2 activation[170], without inducing cell toxicity in our experimental conditions (data not shown).

3.3 RNA preparation and expression analyses

Total RNA was purified using Direct-zol RNA Miniprep (Zymo Research, Milan, Italy), according to the manufacturer's instructions, including a step with deoxyribonuclease incubation. For real time PCR, 200 ng RNA was used for cDNA preparation using 8 U/µl of Moloney murine leukemia virus reverse transcriptase (Promega, Milan, Italy) in a final volume of 25 µl. The reaction was performed at 37 °C for 1 h, and the enzyme inactivated at 75 °C for 5 min. Control reactions without the addition of the reverse transcription enzyme were performed (data not shown). A 1:4 cDNA dilution was amplified using GoTaq®qPCR Master Mix technology (Promega) according to the manufacturer's protocol. The PCR was carried out in triplicate on a 96-well plate using QuantStudio®3 real time PCR system (Applied Biosystems) with the following thermal profile: 2 min at 95 °C; 40 cycles, 15 sec at 95 °C, 1 min at 60 °C. Primer sequences are reported in Supplementary table 1. Data were analyzed using the $2^{-\Delta\Delta Ct}$ method and normalized using 36b4 as housekeeping gene.

Gene	Forward sequence	Reverse sequence
36b4	5'-GGCGACCTGGAAGTCCAACT-3'	5'-CCATCAGCACCACAGCCTTC-3'
Hmox1	5'-CCTGGTGCAAGATACTGCCC-3'	5'-TGGGGGCCAGTATTGCATTT-3'
Idh1	5'-CCAGTCGCTGTTACCGTATG-3'	5'-AATAACCCTCTTCACTCTGGAC-3'
IL-6	5'-CTGGATATAATCAGGAAATTTGCCT-3'	5'-TGGGGTAGGAAGGACTATTTTATGT-3'
Il1β	5'-TGCCACCTTTTGACAGTGATG-3'	5'-GCTGCGAGATTTGAAGCTGG-3'
Irg1	5'-ACCAAAGAGATTCCACCCTCCCTCT-3'	5'-GCTATGGGTGCCCCTGCGTG-3'
Ki67	5'-AGAGCTAACTTGCGCTGACT-3'	5'-TCAATACTCCTTCCAAACAGGCA-3'
Nqo1	5'-GGCCGATTCAGAGTGGCAT-3'	5'-CCAGACGGTTTCCAGACGTT-3'
Ppat	5'-CAGTAGCGCGAGCCTCTTG-3'	5'-TCCTGACCTCGGTGCTGTAG-3'
Taldo1	5'-CCACAGAAGTTGATGCAAGGC-3'	5'- GTTCCTCCAGCTCCTTTCCAG- 3'
Tkt	5'-ACAAAGCCAGCTACCGAGTC-3'	5'-GCGCTTCTGGTAGATGTCCA-3'
TNFα	5'-GCCCACGTCGTAGCAAACC-3'	5'-GTGAGGAGCACGTAGTCGG-3'
Vegfα	5'-AGCAGAAGTCCCATGAAGTGA -3'	5'-ATGTCCACCAGGGTCTCAAT -3'

Table 2- Primer sequences.

3.4 Cell culture

SKNBE neuroblastoma cell line was purchased from the American Type Culture Collection (ATCC) and grown in RPMI 1640 medium (Life Technologies) supplemented with 10% Fetal Bovine Serum (FBS) (Sigma-Aldrich) and streptomycin-penicillin (50,000 IU plus 50 mg/l) (Gibco, Life Technologies).

3.5 Luciferase enzymatic assay

Cells were lysed with Luciferase Cell Culture Lysis Reagent (Promega) and the luciferase assay carried out in luciferase assay buffer (470 μm luciferin, 20mm Tricine, 0.1 mm EDTA, 1.07 mm $(MgCO_3)4{\cdot}Mg\ (OH)_2 \times 5H_2O$; 2.67 mm $MgSO_4 \times 7H_2O in H_2O$, pH 7.8, with 33.3 mm DTT and 530 μm ATP), using 20 μL of cell lysate and 100 μL of luciferase assay buffer. Luminescence emission was measured with a Veritas luminometer (Promega). Protein concentration was determined by the Bradford assay and used to normalize luciferase units/μg protein, obtaining the relative luminescence units (RLU).

3.6 ELISA assay

Culture medium from peritoneal macrophages treated with different combinations of tBHQ and LPS were assayed for IL-1β, IL-6 and TNFα protein levels by enzyme immunoassay (murine ELISA kits from Bio-Techne, Milano, Italy). Dilutions of 1:10 were made for IL-6 and TNFα assays.

3.7 Bioinformatic analysis

BaseCall files were converted to FastQ files using Casava 1.8.2. Sequencing reads were aligned to the mouse genome (mm10) using TopHat v.2.0.9. Transcripts were reconstructed and quantified using Cufflinks v2.1.1 and differential expression analysis was performed using CuffDiff [309]. CuffDiff uses the test statistics T = E[log(y)]/Var[log(y)], where y is the ratio of the normalized counts between two conditions. A t-test is used to calculate the P value for Differential Expression [310]. A threshold of 0.05 was applied to False Discovery Rate (FDR) adjusted p values in order to select the differentially

expressed genes (DEGs) to use in downstream analysis. Lists of DEGs upregulated in male or female were analysed with DAVID database for functional annotation and with Enrichr database for transcription factor enrichment analysis. Biological processes and transcription factors were investigated focusing on enriched terms with a Benjamini adjusted p-value less than 0.05.

3.8 Statistical analyses

All data are presented as mean ± SEM of $n=3$ observations. Unless otherwise indicated, results were analyzed by one-way ANOVA followed by Bonferroni's multiple comparison test. A statistical level of significance of $p<0.05$ was accepted. Statistical analysis was performed using GraphPad Prism software, version 8.

RESULTS

The aim of the present study was to investigate the macrophage response to neurotoxic and pro-oxidant stimuli under physiological and inflammatory conditions, in order to characterize the inflammatory process associated with neurodegeneration. To reach this aim, I performed some experiments in which I assayed the reactivity of macrophages in response to signals derived from degenerating dopaminergic neurons. The neuroblastoma cell line, namely SKNBE, was used to perform initial studies, since it shows a dopaminergic phenotype and has all the advantages of cell lines compared to primary neuronal cell cultures. To induce neurodegeneration, cells were treated with MPP^+, the active metabolite of MPTP selectively up taken by dopaminergic cells, through the dopamine transporter, where it interferes with complex I of electron transport chain leading to cell death. Thus, MPTP is widely used to replicate the pathological features of PD that specifically target dopaminergic neurons. On the other side, I used primary cultures of macrophages isolated from two transgenic mouse lines, each carrying a reporter gene of inflammatory and oxidative events, respectively. This experimental setting is intended to follow the inflammatory and oxidative responses of macrophages, induced by neuronal demise. The genome of these transgenic mice contains the luciferase gene under the transcriptional control of inflammatory and oxidative transcriptional factors, NFκB and NRF2; the assessment of bioluminescence emission triggered by the luciferase enzyme is a reliable and quantitative measurement of NFκB and NRF2 activity.

Before starting with my experiments of co-culturing of reporter cells I performed an experiment on SKNBE cells alone, using different doses of MPP^+, at different time points. Morphological analysis (shrinkage, blebbings, nuclear fragmentation and, finally, disappearance of the cells from the microscope field) and survival percentage were used to select the best dose (4mM) and time point (12-24h) in which the neuronal damage is maximum and consequently the conditioned medium enriched with factors secreted by suffering neurons that possibly are able to activate macrophages.

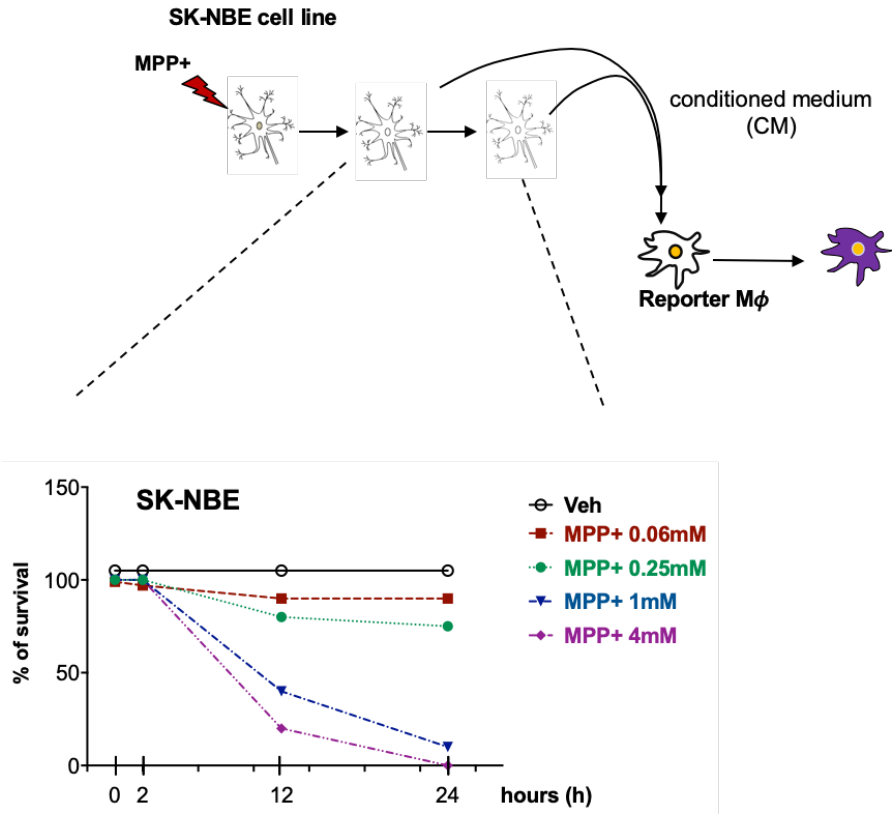


Figure 17- Time- course experiment of SKNBE degeneration following MPP^+ treatment

4.1 Imaging of NFκB activation in macrophages in response to degenerating neurons.

Neuroinflammation is a key factor in neurodegenerative diseases; in fact, neurotoxic insults such as deposition of alpha-synuclein or beta amyloid aggregates trigger neuronal demise as well as microglia activation; in turn, the production of neuroinflammatory mediators by microglia, like cytokines and oxidative species, further contributes to neuronal death creating a self-amplifying circle. It is however still unknown whether MPP^+ activatea NfkB in macrophages.

First, I studied the activation of the inflammatory response in macrophage isolated from NFκB reporter mice, developed in the laboratory of Adriana Maggi[169]. As alluded to, the luciferase enzyme (responsible of bioluminescence) is placed under the control of NFκB, a transcription factor known as a master regulator of the inflammatory response in macrophage. Primary culture of peritoneal macrophages was obtained from reporter mice and treated with the

conditioned medium collected from SKNBE cells treated for 12 or 24h with 4mM MPP^+. Protein extracts were prepared and tested to measure bioluminescence emission: in this experimental condition activation of NFκB pathway in macrophage following 12h treatment with conditioned medium collected from SKNBE doesn't occur. (Figure 18).

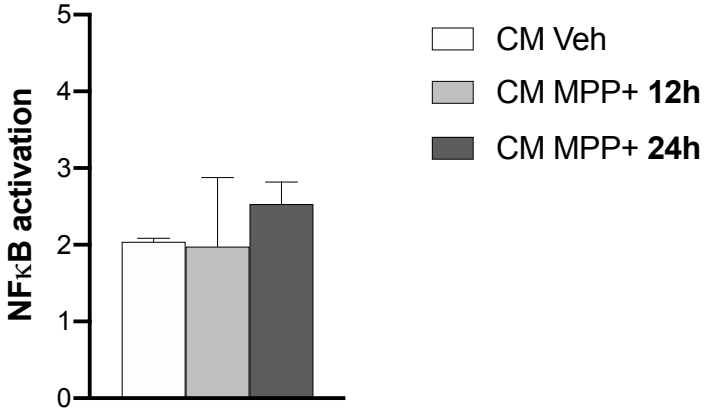


Figure 18- Imaging of NFκB activation in macrophage in response to signals secreted by dying neurons. Peritoneal macrophages were treated with conditioned medium collected from SKNBE treated with MPP^+ 4mM for 12h (light grey bar), 24h (dark grey bar) or veh (open bar) and luminescence measured after 12h. Data are presented as mean values ± SEM (n=3) of a single experiment representative of at least two other independent experiments.

To test if the problem was the responsiveness of the system, I used LPS, a component of the wall of Gram-negative bacteria and a known activator of NFκB pathway (Figure 19). Peritoneal macrophages isolated from animals were treated with 100 ng/mL of LPS for 3 or 6h. As expected, treatment with LPS results in the increase of luciferase activity in a time-dependent manner, reflecting the effect of NFκB-driven transcription.

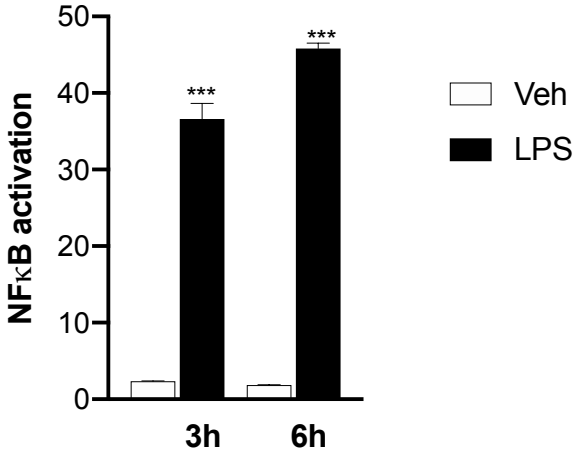


Figure 19- Imaging of NFκB activation in macrophage following LPS treatment. Peritoneal macrophages isolated from NFκB reporter mice were treated with vehicle (white bar) or LPS (black bar) and luminescence measured after 3 or 6h. Data are presented as mean values ± SEM (n=3) of a single experiment representative of at least two other independent experiments. Results were analyzed by one-way ANOVA followed by Bonferroni's multiple comparisons test. (***) $p < 0.001$ *versus* own veh.

Thus, on the bases of the results of this first experiment, it was possible to conclude that neurodegenerative signals secreted by dying neurons do not induce NFκB in macrophages, suggesting that at least under these experimental conditions NFκB-driven inflammation is not involved in macrophage-neuron communication under MPTP stimulation.

4.2 Imaging of NRF2 activation in macrophages in response to degenerating neurons.

Macrophages were isolated from NRF2 reporter animals and used in similar experimental conditions as those previously described. In these mice, luciferase expression and thus bioluminescence emission is controlled by NRF2, a transcription factor activated by oxidative stress, mitochondrial dysfunctions and neuroinflammation to regulate the expression of protective proteins that buffer cells against damage; a growing body of evidence demonstrated that NRF2 is a key regulator of defense pathways protecting brain from several insults, including those coupled with oxidative stress, mitochondrial dysfunctions, autophagy and neuroinflammation, all of which are associated with neurodegenerative processes, including PD[171,172]. This system has recently been generated in the lab and tested for the response to oxidative stress both in vivo and in vitro[168]. Using the conditioned medium from SKNBE treated for 12h

with MPP^+, a strong induction of NRF2 activity can be observed in peritoneal macrophages cells extracts, suggesting that dying neurons produced signals able to trigger the induction of NRF2 in peritoneal macrophages (Figure 20). In order to confirm if NRF2 activation observed in peritoneal macrophages was caused by signals secreted by dying neurons rather than by the direct action of the neurotoxicant, SKNBE cells weres extensively washed with PBS after 1h of treatment with MPP^+ in order to eliminate the neurotoxin from the culture medium; this MPP^+ - free medium did not induce NRF-2 activity in macrophages (Figure 22), suggesting that NRF2 activation in peritoneal macrophages is caused by the direct action of MPP^+, rather than by signals secreted by dying neurons.

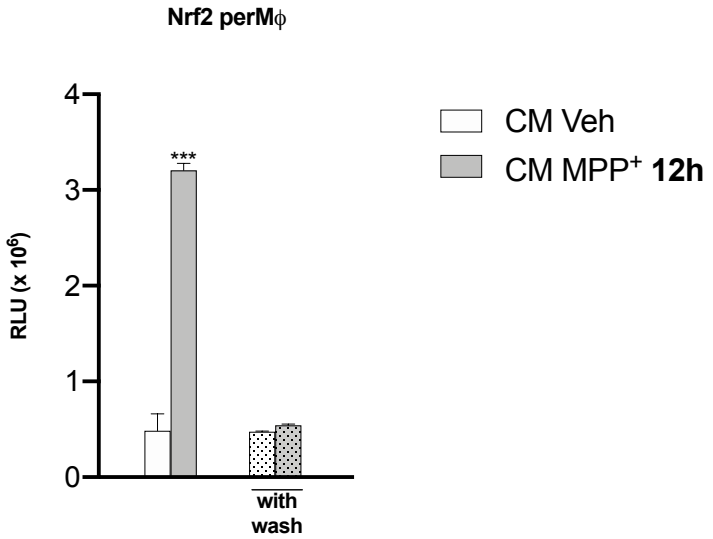


Figure 20- Imaging of NRF2 activation in macrophage induced macrophage induced by degenerating neurons. Peritoneal macrophages were treated with conditioned medium collected from SKNBE treated with MPP^+ 4mM for 12h (light grey bar), or veh (open bar); as a control to test the direct effect of MPP^+ on macrophages conditioned medium collected from SKNBE treated for 12h with veh (dotted open bar) or MPP^+(dotted light grey bar); in order to eliminate MPP^+, after 1h plate were extensively washed with PBS. In both cases, luminescence was measured after 12h. Data are presented as mean values ± SEM (n=3) of a single experiment representative of at least two other independent experiments. Results were analyzed by one-way ANOVA followed by Bonferroni's multiple comparisons test. (***) $p < 0.001$ *versus* own veh.

This hypothesis was confirmed by the direct effects of MPP^+ on macrophages, which provide a 4-fold induction of NRF2 transcriptional activity (Figure 21): surprisingly, NRF2 resulted higher in females suggesting that NRF2 pathway is more activated in females; these results suggest that the higher activation of NRF2 may be responsible of higher antioxidant defense in females thus

potentially explaining the reduced risk of PD observed in women as compared to men.

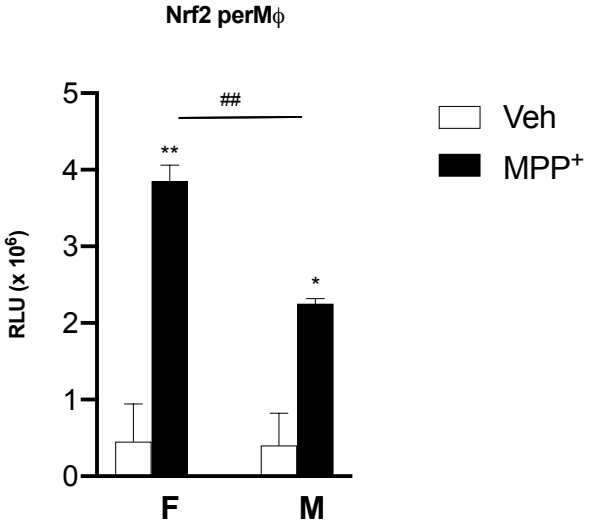


Figure 21- Sexual dimorphism in NRF2 activation following MPP^+ treatment. Peritoneal macrophages were treated with MPP^+ 4mM for 12h (black bar), or veh (open bar); luminescence was measured after 12h. Data are presented as mean values ± SEM (n=3) of a single experiment representative of at least two other independent experiments. Results were analyzed by one-way ANOVA followed by Bonferroni's multiple comparisons test. (**) $p < 0.01$ *versus* own veh.; (*) $p < 0.05$ *versus* own veh.*;* (##) $p< 0.01$ *versus* other sex.

Taken together, these experiments lead us to conclude that signals secreted by degenerating neurons do not activate NFκB or NRF2 in macrophages, at least under our experimental conditions. Future experiments are planned to assay neuroblasts-macrophages direct communication in co-culture systems.

4.3 Validation of NRF2 target genes in macrophages

The ability of MPP^+ to directly activate NRF2 in macrophages led us to further investigate the signaling of this transcription factor in macrophagic cells. As said before, NRF2, coupling an antioxidant activity with a shift in energetic metabolism, is a key system in cell protection against oxidative stress. So, we asked if the activation of NRF2 in peritoneal macrophages treated with MPP^+ may correlate with the induction of a positive immune phenotype in macrophages. In fact, recent studies, using animal models of dysregulated inflammation which also carry genetic manipulation of the KEAP1-NRF2 complex, demonstrated that NRF2 in also necessary for mounting an appropriate innate immune response, and its activity has been associated with a reduction in the production of pro-inflammatory cytokines during the inflammatory response[2–4,22,173].

Considering the role of NRF2 in metabolism we envisioned that this change in the immune phenotype may be achieved through changes in macrophages immunometabolism.

Diverse NRF2-target genes have been identified, depending on the cell type analyzed and specific function required by NRF2 activation. In order to study the endogenous transcriptional activity of NRF2 in peritoneal macrophages we performed a preliminary experiment, analyzing the expression of two classical NRF2 target genes, namely *Hmox1*, heme oxygenase 1, the key enzyme in heme metabolism and *Nqo1,* NAD(P)H:quinone reductase 1, an enzyme involved in detoxification, in peritoneal macrophages treated with MPP^+ or tBHQ, a molecule that causes a strong antioxidant responsethrough mitochondrial oxidative stress, thus activating NRF2.

Short-term treatment with tBHQ results in a strong, significant induction of both genes analyzed; even treatment with MPP^+ is able to induce expression of Hmox1 and Nqo1, although with a much weaker effect (Figure 22).

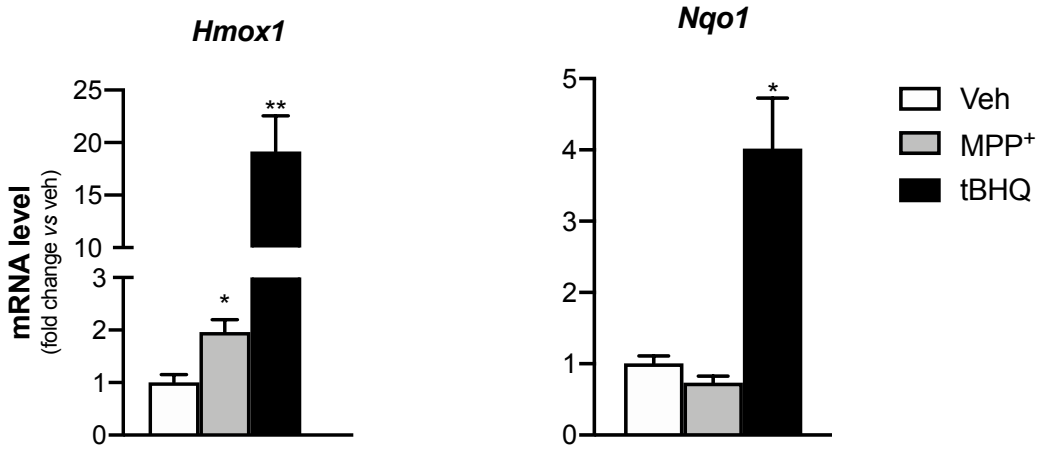


Figure 22- Expression of NRF2 target genes in peritoneal macrophages, in response to NRF2 activating signals. The expression of candidate NRF2 target genes was measured in peritoneal macrophages treated with vehicle (open bar), MPP+ (4mM, grey bar) or tBHQ (100μM, black bar) for 6h. Real time PCR was used to analyze the mRNA levels coding for HMOX1 and NQO1. Data sets for each gene were calculated using the $2^{-\Delta\Delta Ct}$ method and expressed in relation to veh samples. Data are presented as mean values ± SEM (n=3) of a single experiment representative of at least two other independent experiments. Results were analyzed by one-way ANOVA followed by Bonferroni's multiple comparisons test. (*) $p < 0.05$; (**) $p < 0.01$ *versus* veh.

Neurotoxic administration is able to induce NRF2 activation and the parallel increase in the expression of NRF2-target genes, HMOX1 and NQO1, suggesting that NRF2 can represent a mediator of the macrophage response to the neurodegenerative stimulation.

Considering MPP^+ lower efficacy in inducing NRF2- target genes, we decided to use tBHQ as a pharmacological activator of NRF2 signaling pathways in our subsequent studies.

We first performed dose and time-dependent experiments with tBHQ and assessed the mRNA abundance of candidate target genes. We started analyzing the expression of *Hmox1* and *Nqo1.* As shown in Figure 23, the mRNA levels coding the antioxidant protein HMOX1 are readily increased by high concentrations of tBHQ with stronger effects detected at later time points; also, *Nqo1* mRNA levels are induced in a time and dose-dependent manner, although with a lower efficacy.

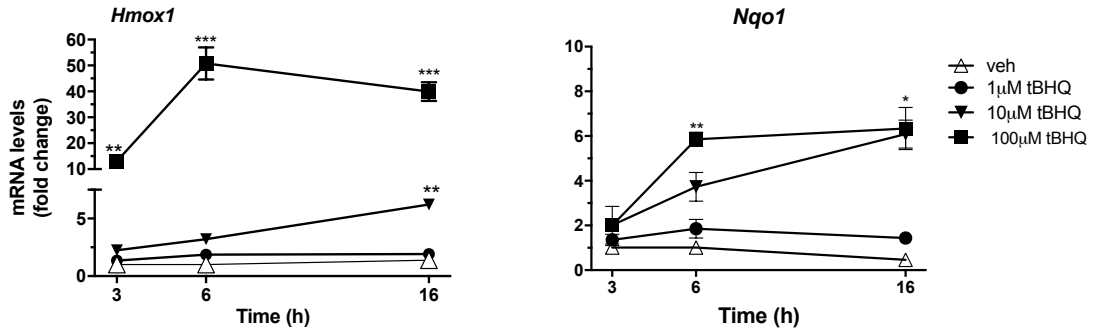


Figure 23- Time and dose-dependent effect of the NRF2 activator tBHQ on NRF2 target genes in peritoneal macrophages. The expression of candidate NRF2 target genes were measured in peritoneal macrophages treated with vehicle (open triangles) or increasing concentrations of tBHQ (1μM, filled circles; 10μM, filled triangles; 100μM, filled squares) for 3, 6 and 16 h, as indicated. Real time PCR was used to analyze the mRNA levels coding for HMOX1 and NQO1. Data sets for each gene were calculated using the $2^{-\Delta\Delta Ct}$ method and expressed in relation to 3h veh samples. Data are presented as mean values ± SEM (n=3) of a single experiment representative of at least two other independent experiments. Results were analyzed by two-way ANOVA followed by Dunnett's multiple comparisons test. (*) $p < 0.05$; (**) $p < 0.01$ (***) $p < 0.001$ *versus* veh.

In parallel, we analyzed the expression of NRF2-target metabolic enzymes, namely Transaldolase-1 (TALDO1) and Transketolase (TKT), that sustain the pentose phosphate pathway (PPP) associated with macrophage polarization, as well as phosphoribosyl pyrophosphate amidotransferase (PPAT), a rate-limiting enzyme in purine biosynthesis which has been reported to be increased by NRF2 activity in proliferating cancer cells[3,174], Figure 24 shows that *Taldo1* mRNA levels increase in a dose and time-dependent manner following NRF2 chemical activation, while later time points and higher concentrations of tBHQ are necessary to increase *Tkt* expression. Unexpectedly, the mRNA levels encoding PPAT are not modified by tBHQ at any concentration used. Altogether, these experiments provide the dynamics of red-ox and metabolic genes expression that is induced by the pharmacological activation of NRF2 in peritoneal macrophages.

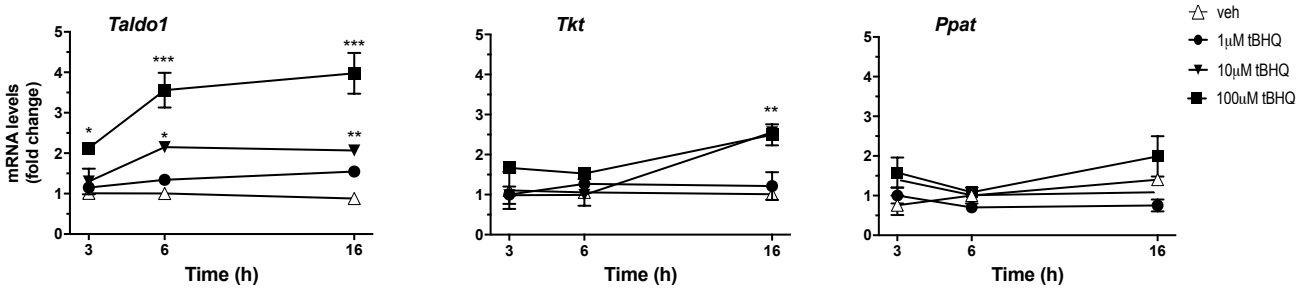


Figure 24- Time and dose-dependent effect of the NRF2 activator tBHQ on NRF2 target genes in peritoneal macrophages. The expression of candidate NRF2 target genes was measured in peritoneal macrophages treated with vehicle (open triangles) or increasing concentrations of tBHQ (1μM, filled circles; 10μM, filled triangles; 100μM, filled squares) for 3, 6 and 16 h, as indicated. Real time PCR was used to analyze the mRNA levels coding for TALDO1, TKT and PPAT. Data sets for each gene were calculated using the $2^{-\Delta\Delta Ct}$ method and expressed in relation to 3h veh samples. Data are presented as mean values ± SEM (n=3) of a single experiment representative of at least two other independent experiments. Results were analyzed by two-way ANOVA followed by Dunnett's multiple comparisons test. (*) $p < 0.05$; (**) $p < 0.01$ (***) $p < 0.001$ *versus* veh.

4.4 Reciprocal interference between NRF2 and NFκB transcriptional activity in macrophages.

These preliminary results lead us to predict that NRF2 is working in peripheral macrophages, inducing a peculiar metabolic program slightly different to what is reported in literature. At this point we asked what happens to this activation when macrophage is activate by a classical inflammatory signal, such as LPS. Although cell metabolism and immune functions are strictly interconnected in macrophage physiology, the effects of the concomitant activation of inflammatory and NRF2-activating signals in the immunometabolic adaptation of resident macrophages is still poorly defined. We first took advantage of the ARE-luc2 reporter mice, a transgenic strain engineered to express the enzyme luciferase under the control of AREs-containing promoter[168], to readily obtain biological evidence of the influence of inflammatory signals on NRF2 activity. Primary cultures of peritoneal macrophages were obtained from ARE-luc2 mice, treated with tBHQ and LPS, either alone or in combination, and protein extracts were prepared after 6 and 16h to measure bioluminescence emission. As expected, treatment with tBHQ results in the increase of luciferase activity that is proportion with the time of incubation, reflecting the induction of NRF2-driven transcription (see Figure 25A). Also, LPS induces similar although much weaker effects as compared with tBHQ, supporting the notion that inflammatory signals induce NRF2 transcriptional activity in macrophages. Importantly, the

tBHQ+LPS treatment potentiates both the short and long-term effects of tBHQ alone on NRF2 transcriptional activity, suggesting that inflammatory conditions are able to influence the pharmacologically induced activity of NRF2 in macrophages.

We then asked whether the pharmacological activation of NRF2 causes the metabolic and immune reprogramming of M1 macrophages. To this aim, we used the NFκB-luc2 reporter mice, engineered to express the luciferase enzyme under the control of a promoter containing NFκB responsive elements[169]. NFκB-luc2 peritoneal macrophages were treated as reported above. As expected, LPS induces luciferase activity in a time-dependent manner, with 10 and 3-fold inductions observed after 6 and 16h, respectively (See Figure 25B). Both short and prolonged NFκB-mediated transcriptional effects are reduced with the tBHQ+LPS treatment; interestingly, tBHQ alone also provides inhibitory effects at the later time point analyzed. Thus, these results suggest that the pharmacological activation of NRF2 modifies the inflammatory response of macrophages to LPS by triggering significant and persisting inhibitory effects on NFκB transcriptional activity.

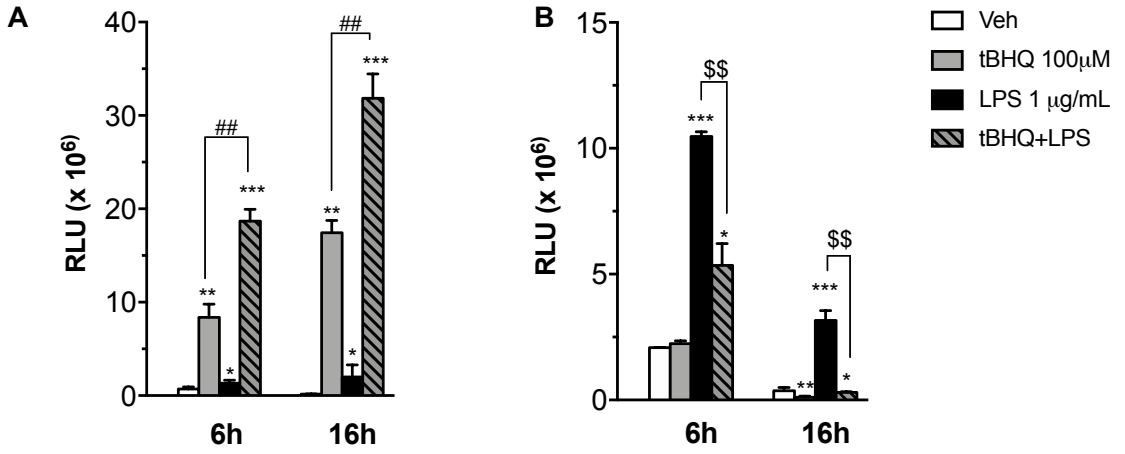


Figure 25- Regulation of luciferase reporter activity by oxidative and inflammatory stimuli in peritoneal macrophages from ARE-*luc2* and NFκB -*luc2* mice. Peritoneal macrophages isolated from ARE-*luc2* (A) and NFκB -*luc2* (B) reporter animals were used to assay NRF2 and NFκB transcriptional activity. Cells were treated with vehicle (veh, open boxes), tBHQ (grey boxes), LPS (filled boxes) or the combination of the two stimuli (dashed grey boxes) and luminescence measured after 6 or 16 h, as indicated. Luciferase activity is represented as relative luciferase units (RLU) per μg protein and expressed in relation with 6h veh-treated samples. Data are presented as mean values ± SEM (n=3) of a single experiment representative of at least two other independent experiments. Results were analyzed by one-way ANOVA followed by Bonferroni's multiple comparisons test. (***) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$ *versus* 6h veh*;* (##) $p< 0.001$ *versus* tBHQ; ($$*)* $p < 0.001$ *versus* LPS.

4.5 Modulation of metabolic, cell cycle and inflammation-related genes by tBHQ and LPS in macrophages

Metabolic adaptation and immune activation are interlinked and concomitant events in macrophage immune response. The results obtained using the two strains of reporter mice lead us to predict that inflammatory conditions and NRF2 activation reprogram the transcriptional effects induced by each signaling pathway, causing a reciprocal interference on macrophage immunometabolism. Separated or combined treatments with tBHQ and LPS were used in time-course experiments to assess NRF2 and LPS-target genes expression. In line with the positive effects of LPS on NRF2 activity shown in Figure 25A, we observed that the increase in *Hmox1* mRNA induced by short-term tBHQ treatment is potentiates by the inflammatory signal (see Figure 26A). On the contrary, the combined tBHQ+LPS treatment causes a reduction in the effects of tBHQ alone on the expression of the NRF2-target metabolic enzymes TALDO1 and TKT (see Figure 26B). Unexpectedly, we also observed that the treatment with LPS alone significantly reduces *Taldo1* and *Tkt* mRNA levels,

while it increases the expression of PPAT, an enzyme involved in *de novo* purine biosynthesis, suggesting that LPS may reduce energy fueling through the PPP, as already reported[175], and support cell proliferation, which still needs biological confirmation. Thus, these results show that LPS regulates gene expression in a peritoneal macrophages-specific manner and that it is able to modify the transcriptional effects of NRF2 associated with cell redox and metabolic adaptation.

To analyze the NRF2-mediated interference with the metabolic effects of LPS, we took advantage of the fact that the metabolic signature of M1 macrophages is characterized, among others, by a truncated TCA cycle[137]. We thus assayed the expression of isocitrate dehydrogenase (IDH1) and immune-responsive gene 1 (IRG1*),* two key enzymes in macrophage TCA cycle that display opposite activities on citrate metabolism[176,177]. Accordingly with published data[138], LPS downregulates the mRNA levels of *Idh1* and strongly up-regulates those coding *Irg1* in peritoneal macrophages, as shown in Figure 26C. More importantly, these effects are significantly different following the combined treatment with LPS+tBHQ. In fact, this association reduces the inhibitory effects of LPS on *Idh1* and potentiates its long-term effects on *Irg1* mRNA levels.

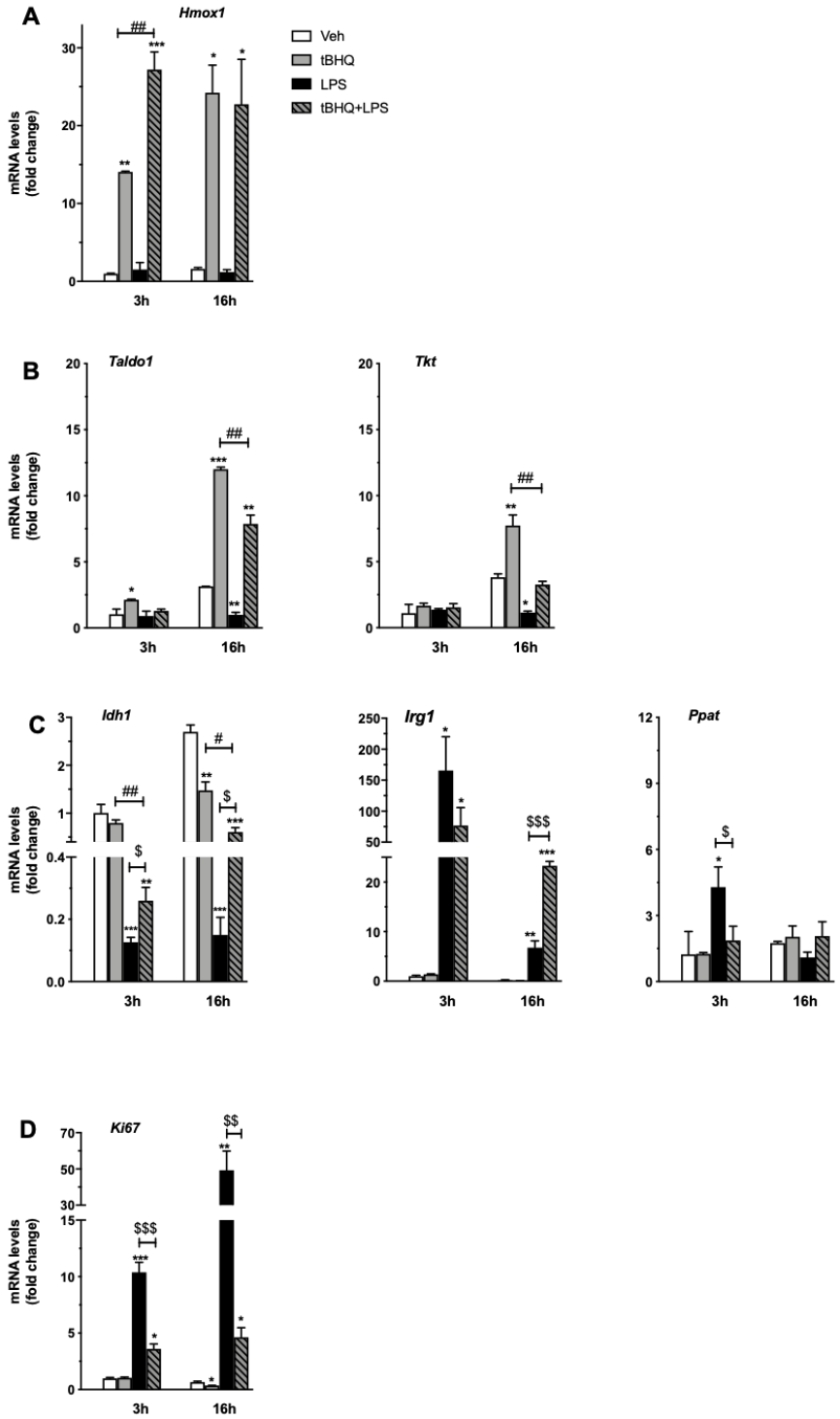


Figure 26- Expression of NRF2 target genes in peritoneal macrophages in response to NRF2 and LPS activation. The expression of NRF2 and LPS-target genes was evaluated in peritoneal macrophages treated with vehicle (veh, white bars), tBHQ (grey bars), LPS (black bars) and the combination of the two stimuli (dashed grey bars) for 3 or 16 h, as indicated. Real time PCR was used to analyze the mRNA levels coding (A) HMOX1 (B,C) TALDO1, TKT IDH1, IRG1 and PPAT and (D) KI67. Data sets for each gene were calculated using the $2^{-\Delta\Delta Ct}$ method and expressed in relation to 3h veh samples. Data are presented as mean values ± SEM (n=3) of a single experiment representative of at least two other independent experiments. Results were analyzed by one-way ANOVA followed by Bonferroni's multiple comparisons test. (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$ *versus* 6h veh; (###) $p < 0.001$ *versus* tBHQ; (*$$$*) $p < 0.001$ *versus* LPS.

Since metabolic adaptation to LPS is supposed to correlate with cell proliferation, we assessed the expression of KI67, a marker of proliferating cells. The results are reported in Figure 26D and show that LPS induces an immediate and persistent increase in *Ki67* expression and that the co-administration of tBHQ significantly reduces this effect, in agreement with what shown in Figure 26C on the expression of PPAT, the enzyme involved in *de novo* purine biosynthesis. These results suggest that inflammatory stimuli sustain the proliferation of peritoneal macrophages, a response that is significantly reduced by the simultaneous activation of NRF2.

Considering that metabolic adaptation is strongly connected to immune activation, we extended our analyses to inflammatory genes expression. As expected, treatment of peritoneal macrophages with LPS increases the mRNA levels coding IL-1β, IL-6 and TNFα. Interestingly, we observed a significant reduction of *Il-1β* and *Il-6* mRNA levels following tBHQ+LPS as compared to LPS alone, with a more persistent inhibition *of IL-1β*. We also analyzed TNFα since its expression is differentially regulated by inflammatory stimuli [178]. Indeed, we observed that the combined tBHQ+LPS treatment only slightly reduces the early effect of LPS, without reaching statistical significance, on *TNFα* mRNA levels; conversely, it increases the response to the endotoxin at the later time point (see Figure 27A). Notably, when added alone, tBHQ also induces a delayed reduction of *Il-1β* and *Il-6* mRNA levels, as compared to controls. In order to obtain a biological evidence for the immune regulatory effects of NRF2 on macrophage immune activation, we assessed the cytokine protein levels that were present in the culture medium of macrophages treated as above. The results reported in Figure 27B confirm the data on gene expression and show that the combined treatment with tBHQ+LPS significantly reduces the levels of IL-1β and IL-6. Interestingly, also TNFα levels are reduced at the early time point of tBHQ and endotoxin treatment, indicating an early transcriptional interference on this gene, which is counteracted at later time points only at the mRNA level. In addition, we observed a consistent reduction, beneath measurable levels, of IL-1β, IL-6 and TNFα when tBHQ was added

alone to the cells. Thus, NRF2 activation during the inflammatory response induces anti-inflammatory effects through gene-specific dynamic and effects.

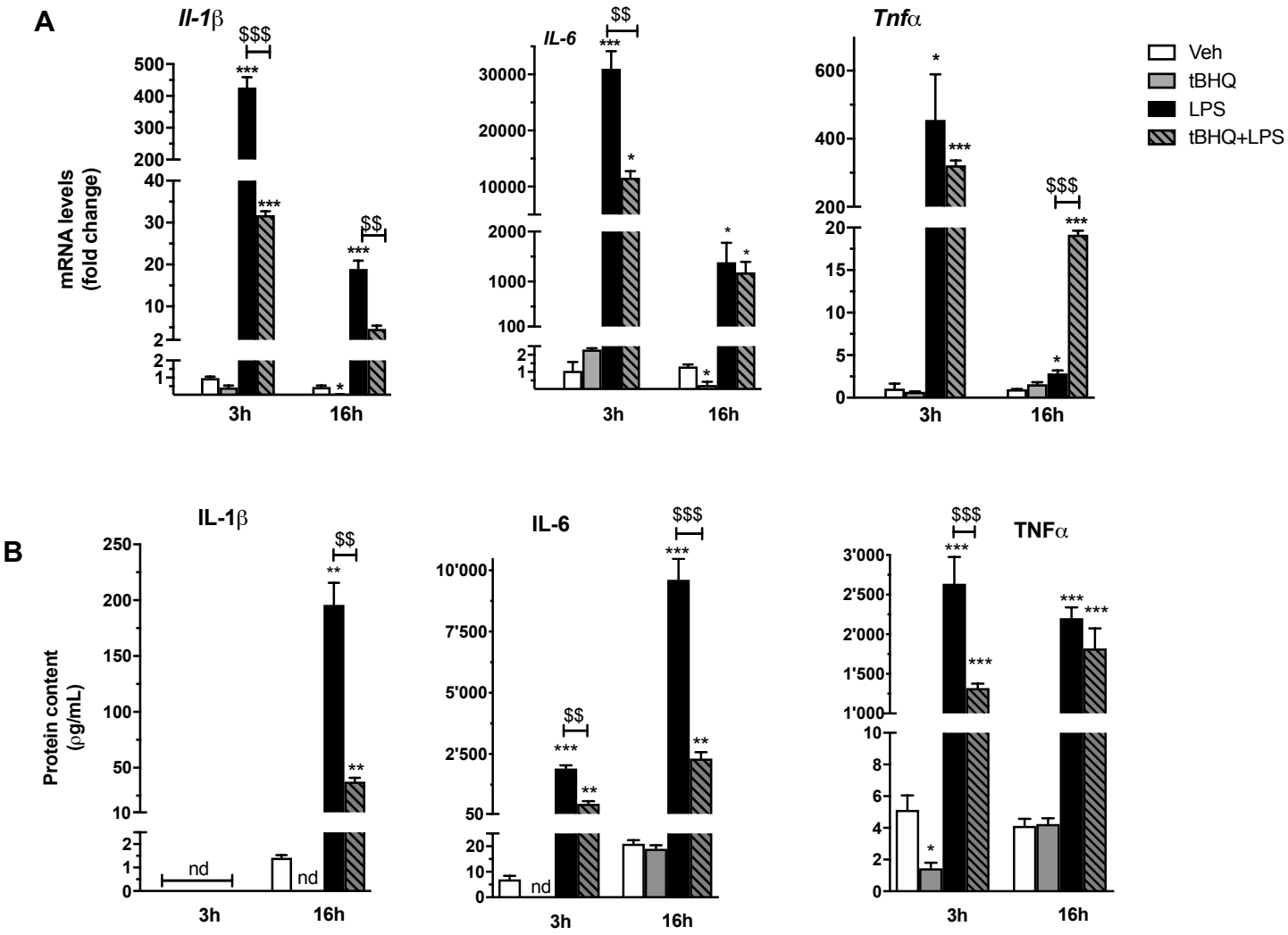


Figure 27- NRF2-mediated interference with inflammatory genes expression induced by LPS in peritoneal macrophages. The expression of inflammatory genes was evaluated in peritoneal macrophages treated with vehicle (veh, white bars), tBHQ (grey bars), LPS (black bars) and the combination of the two stimuli (dashed grey bars) for 3 or 16 h, as indicated. (A) Real time PCR was used to analyze the mRNA levels coding IL-1β, IL-6 and TNFα. Data sets for each gene were calculated using the $2^{-\Delta\Delta Ct}$ method and expressed in relation to 3h veh samples. (B) IL-1β, IL-6 and TNFα proteins were analyzed by ELISA immunoassay in the culture medium of peritoneal macrophages treated as in (A). Data are presented as mean values ± SEM (n=3) of a single experiment representative of at least two other independent experiments. Results were analyzed by one-way ANOVA followed by Bonferroni's multiple comparisons test. (*) $p < 0.05$; (**) $p < 0.01$; (***) $p < 0.001$ *versus* veh; ($$) $p < 0.01$; ($$$) $p < 0.001$ *versus* LPS, by one-way ANOVA followed by Bonferroni multiple comparisons test.

Altogether, these results show that the NRF2 and LPS pathways, when analyzed separately, induce a macrophage-specific subset of target genes and that the inflammatory response of resident macrophages can be regulated by

the pharmacological activation of NRF2 by reprogramming the expression of metabolic, proliferative and inflammatory genes.

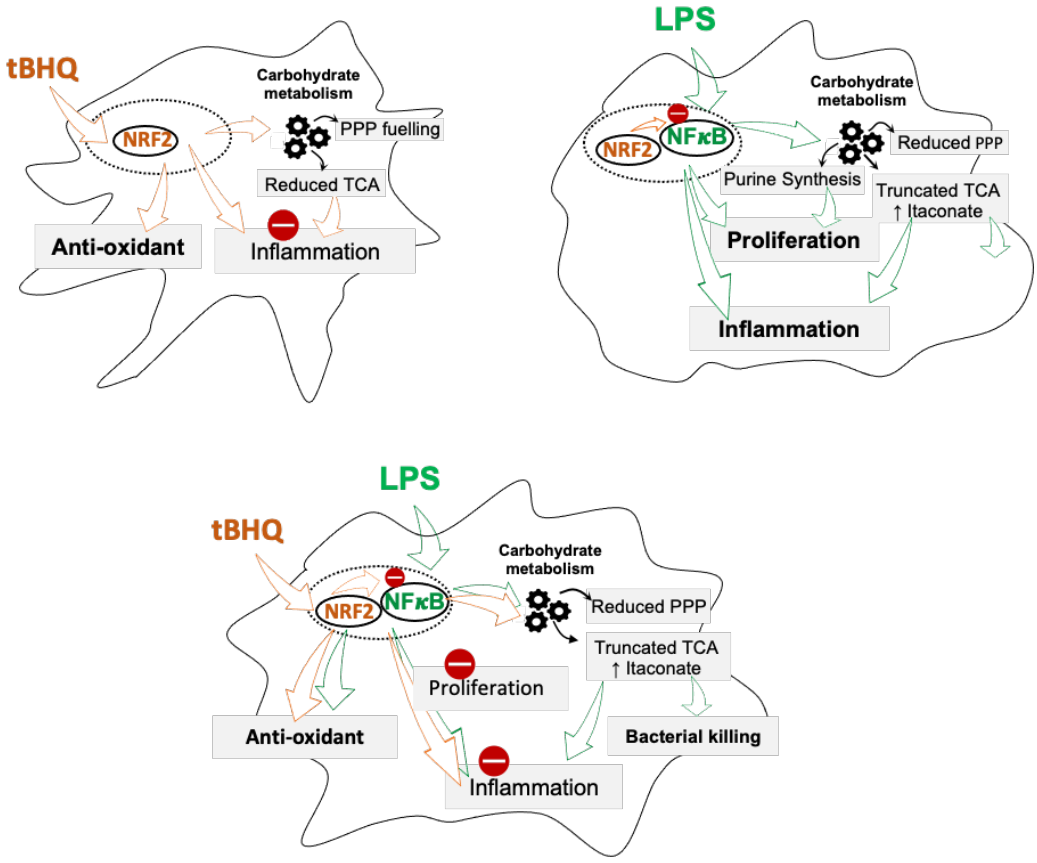


Figure 28- Schematic representation of NRF2 and LPS interference in peritoneal macrophages[179]**.** NRF2 and LPS signaling pathways activate specific metabolic and immune responses in peritoneal macrophages. Together with the antioxidant response occurring through the increased expression of Nqo1 and Hmox1, tBHQ administration regulates macrophage energy request by increasing Taldo1 and TkT and reducing Idh1 mRNA levels, suggesting that NRF2 activation increases energy fueling through the PPP with reduced TCA and, together with the transcriptional inhibition of inflammatory mediators, such as IL1b and IL6, results in an anti-inflammatory phenotype. Treatment with LPS alters the PPP, as indicated by the reduction in the expression of Taldo1 and Tkt, and truncates the TCA, through the inhibition of Idh1 and strong increase of Irg1 expression; this metabolic adaptation associates with increased proliferation, as suggested by increased expression of the cell-cycle marker, ki67, and the purine synthesis enzyme, Ppat, and pro-inflammatory activation, as indicated by the burst in pro-inflammatory cytokine expression.
Administration of tBHQ with LPS increases the effect of NRF2 on Hmox1 and the induction by LPS of Irg1 expression, suggesting that antioxidant and bactericidal responses are potentiated during concomitant activation of these two pathways, while they reciprocally reduce the metabolic, phenotypic and proliferative effects activated by each individual stimulus.

4.6 Sexual differences in macrophage gene expression.

So, pharmacological activation of NRF2 in peritoneal macrophages interferes with macrophages response to LPS not only by reducing inflammatory gene expression but also altering the expression of metabolic enzymes involved in cell phenotypic activation, thus changing macrophages immune metabolism. Since our previous results highlighted sexual differences in response to MPP^+, although in limited experimental conditions tested, we decide to further investigate NRF2 activation and its consequences on metabolism and inflammation comparing macrophages isolated from animal of both sexes.

First, I started with a genome-wide transcriptomic analysis performed in our lab in order to compare macrophages gene expression in male and female (under different estrogen conditions) mice. The analysis shows a strong difference in gene expression levels, with around 2000 genes being differentially expressed between males and females (DEGs) (figure 29, panel A); DEGs belonged to pathway linked to cell cycle or metabolism in males (figure 29, panel B) while in females they were related to antioxidant activity, chemotaxis (figure 29, panel C). These first indications suggest that peritoneal macrophages of both sexes at basal, unstimulated, conditions are imprinted to perform different tasks in the two sexes.

In order to gain a deeper insight in the molecular mechanism responsible for the sexual dimorphism in gene transcription we performed bioinformatic analysis to identify the main transcriptional factors (TFs) that are known to be involved in the regulation of DEGs expression. Transcription factors involved in up-regulated genes in males (figure 29, panel D) include transcription factors related to cell cycle, DNA replication and mitosis (FOXM1), interferon-related genes (IRF3) and regulator of cell metabolism, particularly related to cellular growth and nuclear genes required for respiration, heme biosynthesis, and mitochondrial DNA transcription and replication (NRF1). On the other hand, transcription factors involved to females up-regulated genes comprehend factors linked to immune response and cellular differentiation, such as stem cell maintenance (RELA), hematopoietic development (GATA2), embryonic

development and, of course, estrogen receptor 1 (ESR1) (figure 29, panel E). Notably, NRF2 is significantly involved in females up-regulated genes.

Interestingly, since the expression of NRF2 and other transcription factors is equal in both sexes (figure 29, panel F), a different reactivity of this TFs, rather than differences in the amount or levels of expression, may underlie the sexual difference observed in gene expression.

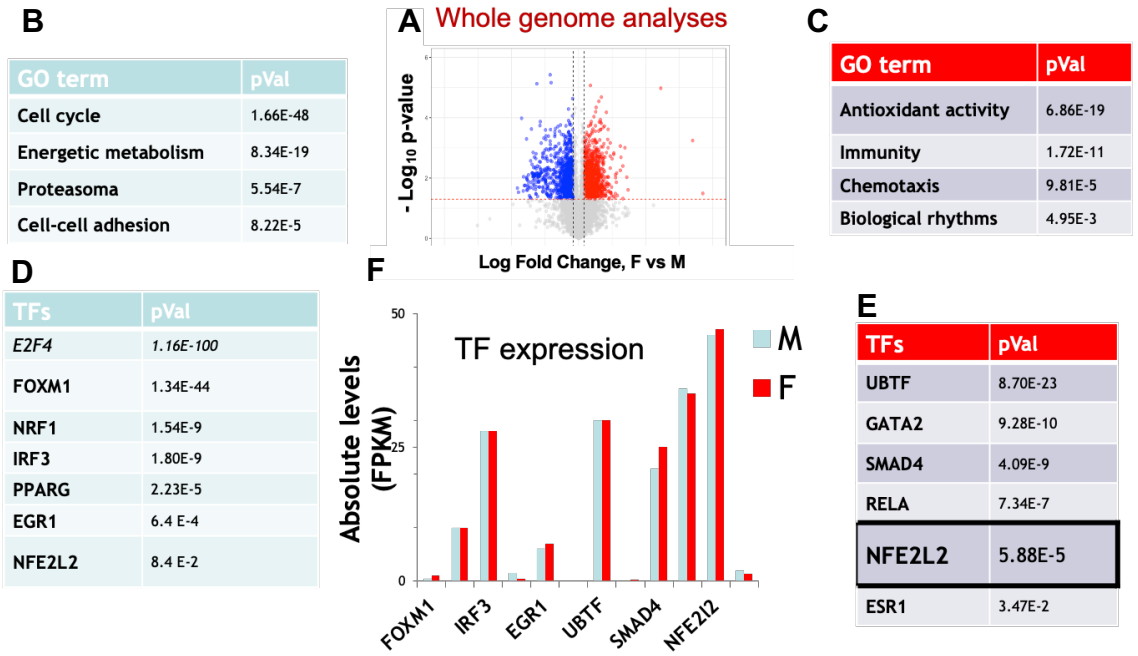


Figure 29- Sexual differences in macrophage gene expression (*unpublished data*).
A) Volcano ploto of RNA-seq data. Blue dots represent differentially expressed gene (DEGs) significantly higher in males than in females (1041 genes; $p<0.05$ by Benjamini-Hochberg correction). Red dots represent DEGs significantly higher in females than in males (945 genes; $p<0.05$). B-E) Gene Ontology (B-C) and ChIP enrichment analysis (ChEA) transcription factors (TFs) (D-E) of DEGs with higher expression in male (B-D) or females (C-E). Tables show the most significantly modulated GO terms and TFs. F) Graph represents levels of expression (FPKM) in male (blue bar) and female (red bar) of some relevant transcription factors.

4.7 NRF2 transcriptional activity presents sex-related differences.

In order to confirm the results obtained from genome-wide analysis, that showed the involvement of NRF2 in up-regulated genes in female mice, peritoneal macrophages of both sexes were isolated and treated with tBHQ, a known activator of NRF2, for different time points. In order to analyze sex-related differences in NRF2 transcriptional activity we took advantage of ARE-luc2 reporter mice.

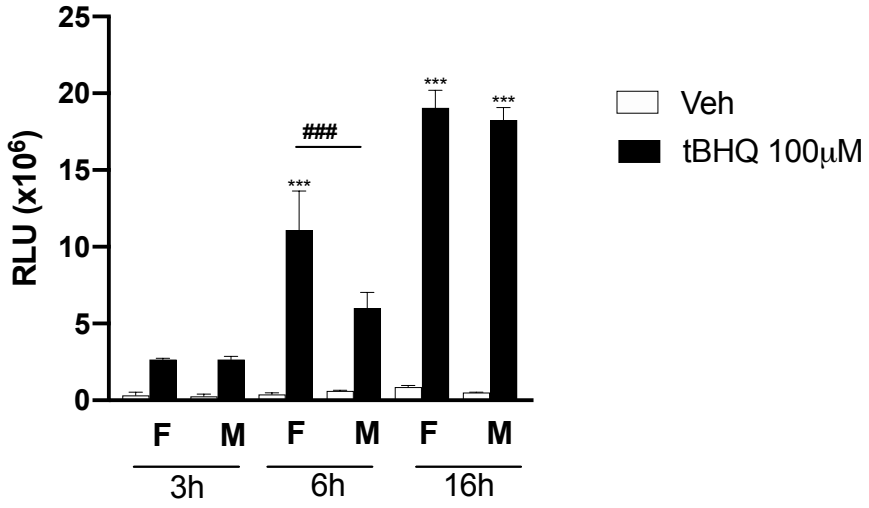


Figure 30- Sexual differences in NRF2 trascriptional activity. Peritoneal macrophages isolated from ARE-*luc2* reporter animals of both sexes were used to assay sex-related differences in NRF2 transcriptional activity. Cells were treated with vehicle tBHQ and luminescence measured after 3,6 or 16 h, as indicated. Luciferase activity is represented as relative luciferase units (RLU) per μg protein and expressed in relation with veh-treated samples. Data are presented as mean values ± SEM (n=3) of a single experiment representative of at least two other independent experiments. Results were analyzed by two-way ANOVA followed by Dunnett's multiple comparisons test. (***) $p < 0.001$ *versus* T0.

As expected, treatment with tBHQ results in an increase of luciferase activity that is in proportion with the time of incubation, reflecting NRF2-driven transcription. Interestingly, luciferase increases faster in females compared to males, suggesting a higher reactivity of NRF2 in females' cells and thus confirming previous data obtained by genome-wide analysis.

4.8 Sex- differences in NRF2-target genes expression.

Having identified NRF2- target genes in peritoneal macrophages with my previous experiments, I then assessed sex-related differences in the expression of this genes in a time course experiments, using tBHQ as an activator of NRF2 pathways.

As shown in Figure 31, mRNA levels coding for the antioxidant protein HMOX1 are readily increased by tBHQ treatment and, notably, peritoneal macrophages isolated from female mice shows a stronger induction of this gene, particularly at intermediate time-point with where the difference between males and females is significant. Notably, basal levels of HMOX1 are equivalent in both sexes, suggesting that the observed difference is caused by a dissimilar reactivity following NRF2 activation. Also, *Nqo1* levels, even if this gene is induced with a lower efficacy, presented differences between males and females starting from

3h and persisting at all time points analyzed. Again, basal levels of the two genes are overlapping and they follow the same pattern of expression, slightly decreasing throughout time.

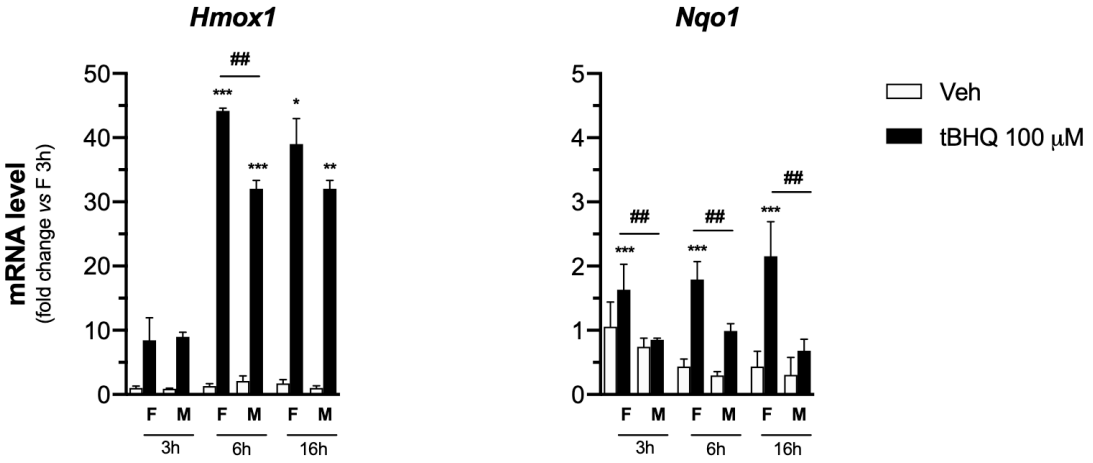


Figure 31- Time-dependent effect of NRF2 activation on NRF2 antioxidant target genes in peritoneal macrophages. The expression of NRF2 target genes was measured in peritoneal macrophages isolated from females or males mice treated with vehicle (open bar) or tBHQ (100μM, black bar) for 3, 6 and 16 h, as indicated. Real time PCR was used to analyze the mRNA levels coding for HMOX1 and NQO1. Data sets for each gene were calculated using the $2^{-\Delta\Delta Ct}$ method and expressed in relation to 3h females veh samples. Data are presented as mean values ± SEM (n=3) of a single experiment representative of at least two other independent experiments. Results were analyzed by two-way ANOVA followed by Dunnett's multiple comparisons test. (*) $p < 0.05$; (**) $p < 0.01$ (***) $p < 0.001$ *versus* veh, (#) $p < 0.05$; (##) $p < 0.01$ (###) $p < 0.001$ *versus* other sex.

Taken altogether this result confirms the dynamics of redox response induced by NRF2 pharmacological activation in peritoneal macrophages, highlighting also important sexual differences that suggest that female sex is endowed with better capacity to counteract an oxidative stress.

In parallel we analyzed the expression of two NRF2-target genes involved in the pentose phosphate pathway (PPP), namely TALDO1, a transaldolase, and TKT, a transketolase. In both sexes, Taldo1 levels increases in a time-dependent manner, with males expressing higher levels of this gene. On the other hand, mRNA levels coding for *Tkt* increases faster in males with significant differences when compared to females, especially at intermediate time-point, then *Tkt* levels of expression decreases and are comparable to females. Again, basal level of expression of these two genes are overlapping and their changesthrough time are comparable, suggesting that a different reactivity of this cells may be responsible of the sex-related differences observed.

Importantly, TALDO1 and TKT relates with the pentose phosphate pathway, that is a key metabolic feature of M1 macrophage polarization, that use PPP to support ROS and nucleotide production (Figure 32, panel A); furthermore, M1 polarization is also more correlated to males' macrophages. Accordingly, Vegfα expression, correlated with the acquisition of an alternative, M2, polarization, is upregulated in females (Figure 32, panel B).

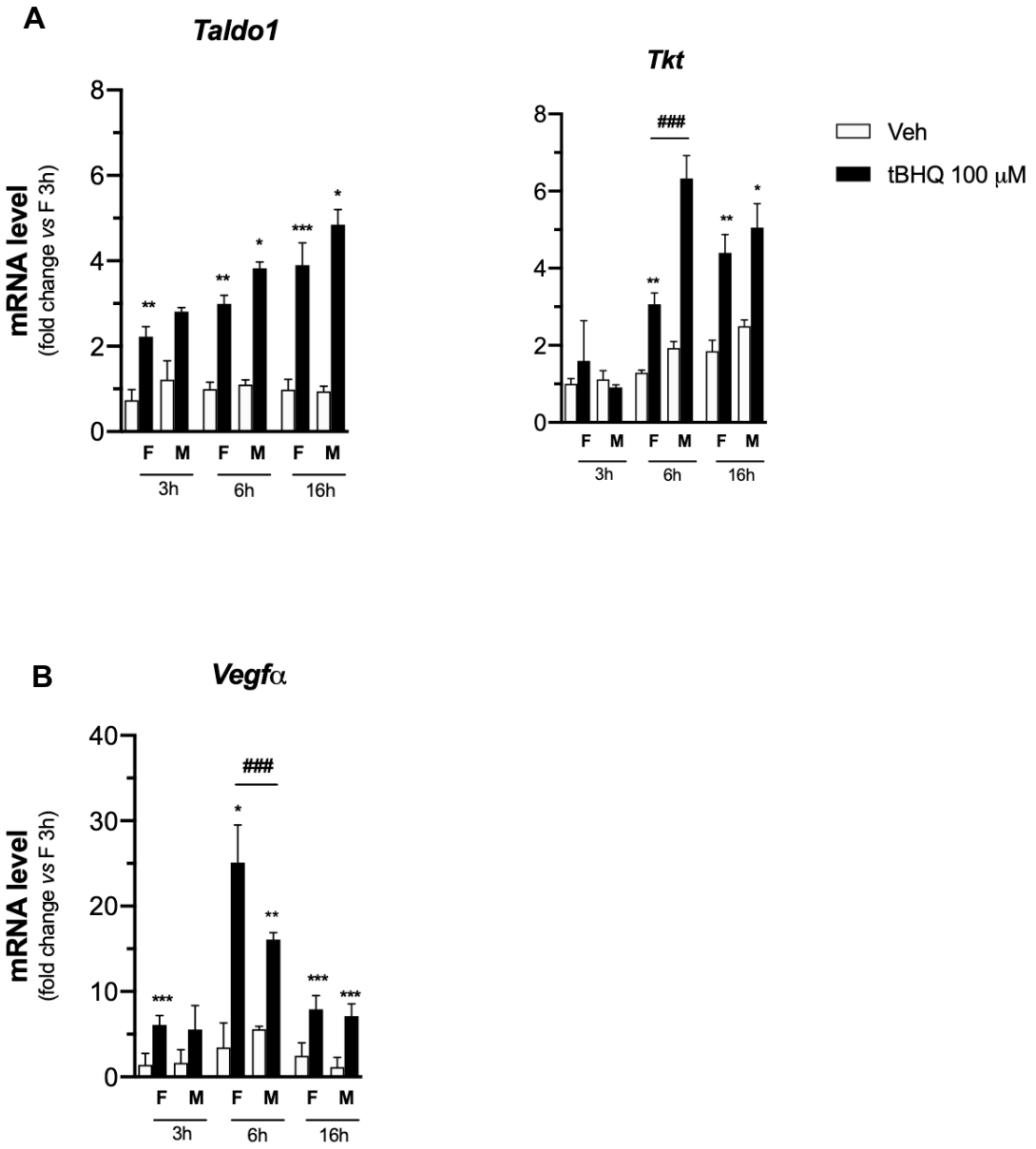


Figure 32- Sex differences in time-dependent effect of NRF2 activation on NRF2 metabolic target genes in peritoneal macrophages. The expression of NRF2 target genes was measured in peritoneal macrophages isolated from females or males' mice treated with vehicle (open bar) or tBHQ (100μM, black bar) for 3, 6 and 16 h, as indicated. Real time PCR was used to analyze the mRNA levels coding for TALDO1, TKT and VEGFα. Data sets for each gene were calculated using the $2^{-\Delta\Delta Ct}$ method and expressed in relation to 3h females veh samples. Data are presented as mean values ± SEM (n=3) of a single experiment representative of at least two other independent experiments. Results were analyzed by two-way ANOVA followed by Dunnett's multiple comparisons test. (*) $p < 0.05$; (**) $p < 0.01$ (***) $p < 0.001$ *veh;* $p < 0.01$ (###) $p < 0.001$ *versus* other sex.

Since my previous results demonstrated that pharmacological activation of NRF2 interferes with macrophage inflammatory response, even when activated

alone, reducing the expression of pro-inflammatory cytokines such as *Ilβ-1* and *Il-6* and since we have some evidence of sex-related differences in NRF2 activation, next we investigate if the NRF2-mediated effect on the inflammatory response may be sexually dimorphic.

4.9 Sex differences in LPS metabolic response.

As discussed in Introduction, immunity displays sex-related differences that involve several aspects such as immune cells number, cytokines production, response to pathogens and macrophages polarization. So, before moving on with evaluation of eventual sex-differences in the interference between NRF2 and NFκB, we assessed sex differences in LPS response analyzing the expression of its target genes related to metabolism and inflammatory response. First, we evaluate the expression of LPS-target genes involved in metabolism, focusing our analysis on the time point in which their induction was higher. According with published data LPS downregulates levels of Idh1 and strongly upregulates levels of Irg1; interestingly all three genes analyzed are more induced in males compared to females, suggesting a higher reactivity in response to LPS in peritoneal macrophages isolated from male mice.

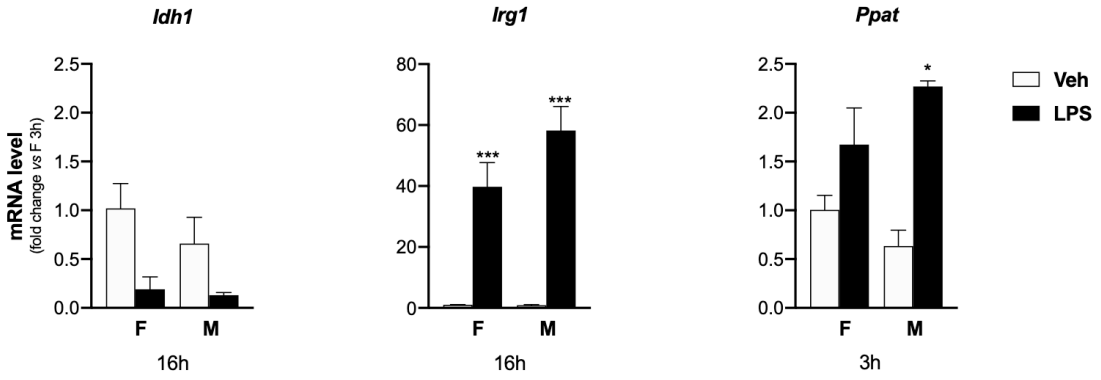


Figure 33- Sex differences in the macrophage response to LPS. The expression of LPS-metabolic target genes was measured in peritoneal macrophages isolated from females or males mice treated with vehicle (open bars) or LPS (1µg/mL, black bars) for 3 or 16 h, as indicated. Real time PCR was used to analyze the mRNA levels coding for Idh1 Irg1 and Ppat. Data sets for each gene were calculated using the $2^{-\Delta\Delta Ct}$ method and expressed in relation to 3h females veh samples. Data are presented as mean values ± SEM (n=3) of a single experiment representative of at least two other independent experiments. Results were analyzed by one-way ANOVA followed by Bonferroni's multiple comparisons test. (*) $p < 0.05$; (***) $p < 0.001$ *versus veh*.

4.10 Sex differences in NRF2-NFκB reciprocal interference.

Finally, we extended our observations evaluating sex-related difference in the expression of pro-inflammatory genes when the two pathways, NRF2-NFκB, are activated alone or in combination.

As expected, LPS treatment of peritoneal macrophages results in a strong increase of mRNA levels coding for IL-1β, IL-6 and TNFα. Interestingly, and in accordance with data reported in literature, stimulation with LPS presents sex-related differences, that reach statistical significance; these differences are present at early time points and persist through time, in particular, the production of pro-inflammatory cytokines released from macrophages (IL-1β, IL-6, TNFα) is higher in male macrophages after stimulation with LPS; suggesting a higher reactivity of these cells when triggered by an inflammatory stimulus.

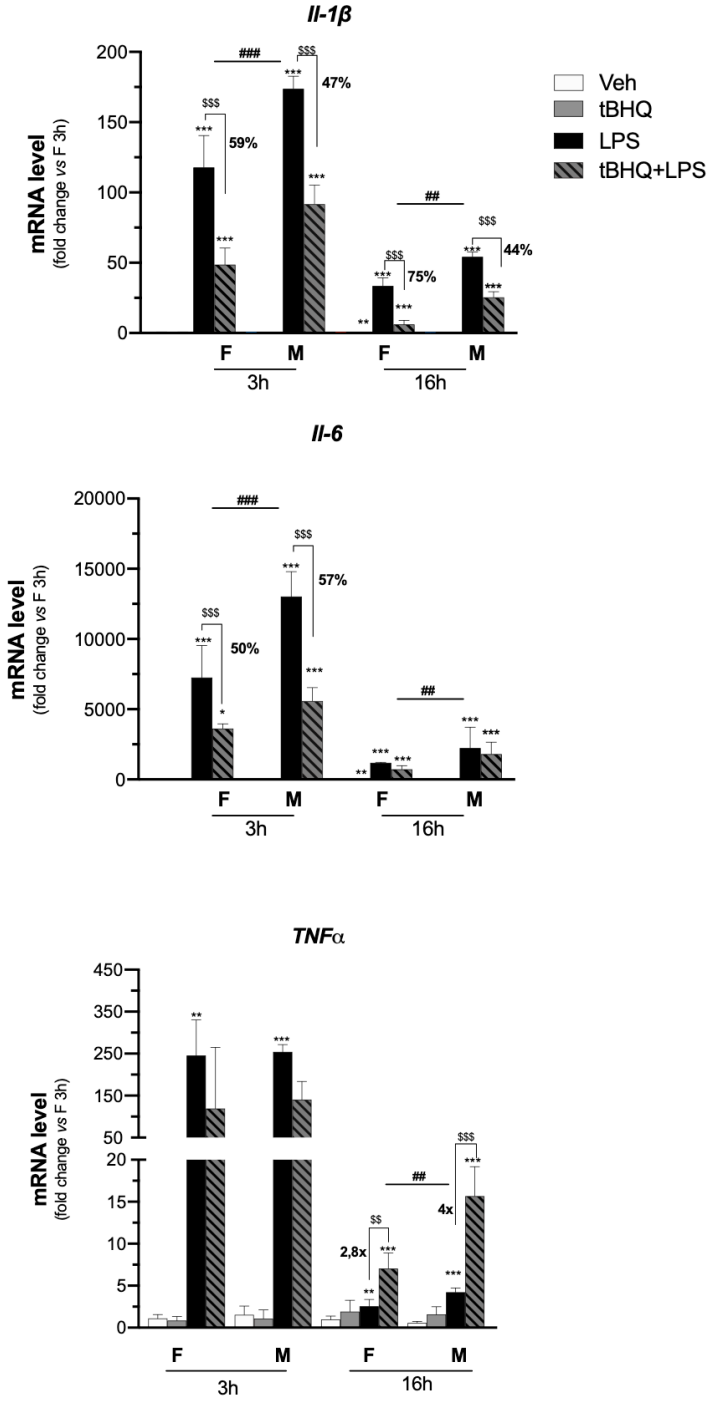


Figure 34- NRF2-mediated interference with inflammatory genes expression induced by LPS is sexually dimorphic. The expression of inflammatory genes was evaluated in peritoneal macrophages treated with vehicle (veh, open bars), tBHQ (grey bars), LPS (black bars) and the combination of the two stimuli (dashed bars) for 3 or 16 h, as indicated. Real time PCR was used to analyze the mRNA levels coding IL-1β, IL-6 and TNFα.Data are presented as mean values ± SEM (n=3) of a single experiment representative of at least two other independent experiments. Results were analyzed by one-way ANOVA followed by Bonferroni's multiple comparisons test.
(*) $p < 0.05$; (**) $p < 0.01$; (***) $p < 0.001$ *versus* veh; ($$) $p < 0.01$; ($$$) $p < 0.001$ *versus* LPS; (##) $p < 0.01$; (###) $p < 0.001$ *versus* other sex by one-way ANOVA followed by Bonferroni's multiple comparisons test.

As already demonstrated, we observed a significant reduction of the expression pro-inflammatory cytokines following tBHQ+LPS treatment as compared to LPS alone, with a more persisting effects on IL-1β; the potency of inhibition between the two sexes differs in accordance with the cytokine analyzed: for IL-1β the reduction is higher in females compared to males at both time points analyzed, while for IL-6, the ability of NRF2 to reduce the inflammatory response is equal for both sexes. Importantly, despite the potency of inhibition, levels of proinflammatory cytokines remains higher in males compared to females at both time points analyzed.

Notably, when added alone, tBHQ also induces a delayed reduction of IL-1β and IL-6 as compared to controls, that is stronger in macrophages isolated from females' mice; in accordance with our hypothesis that NRF2 is more reactive in females.

The combined treatment slightly reduces TNFα expression levels; in accordance with previous data; in contrast, levels of TNFα are increased by the combined treatment at later time points, that is higher in male compared to female macrophages, in accordance with the higher induction caused by LPS treatment alone.

Further studies on other metabolic genes are in progress to better understand sexual differences in the interference between NRF2 and NFκB pathways.

DISCUSSION

The involvement of immune cells in the neurodegenerative processes is receiving growing attention given their involvement in the onset and progression of neurodegenerative diseases, including PD. This view also extends to enteric macrophages, which are also assumed to participate in the gut-brain development of PD pathogenesis, thanks to their ability to contact directly, regulate and phagocytose enteric neurons, as well as to peritoneal macrophages, which appear to mediate the systemic transmission of the disease from dysfunctional gut to other tissues, such as metabolic organs, or the immune system. However, the reactivity of immune cells to neurotoxic molecules, which are proved to sustain pathogenic mechanisms, is still vastly unexplored. Only few publications reported that MPP^+ is able to induce the activity of antioxidant enzymes in microglia and macrophage-like cells[180,181], while the antioxidant functions of NRF2 and its protective effects in PD are well-known[32].

Based on this background, I first analyzed whether the antioxidant activity induced by MPP^+ in macrophages could be ascribed to NRF2 activation. Then, I asked to which extent NRF2 activity could be associated with an immunometabolic reprogramming of macrophages, which allows these cells to adapt their cellular metabolism in order to sustain the need for a specific immune phenotype under pathologic or inflammatory conditions. Finally, I evaluated whether NRF2 activity in cell homeostasis and immune polarization could be different among female and male macrophages.

The results of my research project clearly demonstrate that MPP+ is able to induce NRF2 transcriptional activity in peripheral macrophages. To my knowledge, this is the first evidence that identifies NRF2 as a direct mediator of the cellular response to MPP^+-induced oxidative damage. Considering the key role of oxidant injury in PD, it is important to corroborate the involvement of NRF2 protective redox and immune responses of macrophage against MPP^+-induced toxicity[180]. Interestingly, despite the increased production of ROS, the loss of mitochondrial membrane potential, the alteration of Ca^{++} homeostasis or reduction in ATP levels, MPP^+ treatment did not induce NFκB transcriptional

activity in macrophages. This finding, which could be in part ascribed to the experimental conditions and models used in my study, needs further investigation to be better explained.

My results also show that NRF2 activation correlates with the induction of a protective immune-metabolic phenotype sustained by an anti-proliferative and anti-inflammatory shift. NRF2 is considered an attractive target for immune-regulatory therapies: it has been associated with a limited induction of inflammation in *in vivo* LPS-treated animal models or *in vitro* differentiated macrophages[182,183]. Our results extend this knowledge to peripheral macrophages and show that the pharmacological activation of NRF2 interferes with the macrophage response to LPS, not only by reducing inflammatory gene expression but also by altering the expression of metabolic enzymes involved in cell phenotypic activation. Notably, it is still not known whether these effects are protective or detrimental. Nevertheless, healthy naïve macrophages are believed to sense noxious stimuli and adapt their immune-metabolic phenotype to counteract any dangerous consequences and thus provide protective effects through antioxidant and immunoregulatory mechanisms. On the other hand, the accumulation of neurotoxicants over time results in a sustained and chronic pathologic signal that possibly leads to impaired, maladaptive immune responses.

Moreover, my study shows that NRF2 activation impairs LPS response. The anti-inflammatory activity of NRF2 has been ascribed to the upregulation of antioxidant enzymes, which are able to eliminate ROS. More recently, NRF2 has been shown to inhibit the activity of the inflammatory transcription factor, NFκB[4]. The use of reporter mice in the present study allowed us to confirm this hypothesis, as we observed a persistent reduction of NFκB transcriptional activity in response to NRF2 activation by tBHQ. Moreover, I herein show that the inhibitory effects of the combined tBHQ+LPS treatment, as compared with those induced by the two individual signals, occur on both antioxidant and inflammatory genes shortly after their administration to macrophages, suggesting that the downstream biochemical processes activated by this treatment proceed in parallel and, probably, independently from each other. The

molecular mechanisms underlying the interference between NRF2 and LPS-induced inflammatory mediators, such as NFκB, involve the physical interaction with Pol II that reduces its binding to inflammatory gene promoters, or the interaction with anti-inflammatory transcription factors, such as the estrogen and peroxisome-proliferator activated gamma receptors, thus potentiating their transcriptional activity[4,184,185]. Further studies are needed to understand the molecular mechanism of NRF2 activity in macrophages.

It has been shown that selected metabolic molecules produced by NRF2 activation in response to inflammatory signals are able to switch macrophages towards an anti-inflammatory phenotype[182]. With my study I further corroborate this hypothesis and demonstrate that also the expression of metabolic genes regulated by NRF2 or LPS is reciprocally influenced by the co-occurrence of NRF2 and inflammatory-activating signals. This finding suggests that the shift in energy metabolism and consumption allows NRF2 to induce an integrated cellular response that redirects macrophage polarization towards an anti-inflammatory phenotype. Interestingly, the combined treatment (LPS+tBHQ) further potentiates the NRF2 transcriptional activity, as suggested by the increased expression of antioxidant genes. On the other hand, the NRF2-dependent regulation of metabolic genes is instead reduced by LPS, further supporting a competitive mechanism between the two transcription factors, which also needs further elucidations.

Our results demonstrate a novel activity of NRF2 in inflammatory macrophages that provides an anti-proliferative response. The underlying mechanism is still not clear; however, the metabolic effects observed following NRF2 activation are consistent with the absence of DNA replication. My data suggest that NRF2 activation in resident macrophages promotes glucose metabolism through the PPP that is not followed by purine synthesis and cell proliferation. These data are apparently in contrast with previous studies which showed a positive involvement of NRF2 in anabolic processes leading to nucleotides and amino acids synthesis. Although the reasons for this discrepancy are unknown, we believe that the use of primary cultures of non-elicited peritoneal macrophages, as those used in my experiments, allows appreciation of the reactivity and

specialized functions of this subpopulation of resident immune cells, including self-renewal, highlighting distinctions from macrophage-like cells derived from *in vitro* differentiation of monocytes or bone marrow precursor cells, or transformation into immortalized cell lines.

Finally, with my research project I have provided new knowledge on the sexual dimorphism of the immune response, showing that NRF2 activation is more rapid and potent in female as compared to male macrophages. This finding suggests that the sex differences in NRF2 activity might contribute to a sex-specific regulation of the immune response, allowing female macrophages to better adapt their antioxidant and immune-metabolic potential, thus counteracting the negative consequences derived from the chronic accumulation of neurotoxic molecules.

In summary my study demonstrates that the activation of NRF2 by chemical agents modifies immune and metabolic genes expression, reducing inflammatory and proliferative responses in peritoneal macrophages in a sex-dependent way. These effects are likely ascribable to a competition between NRF2 and NFκB, resulting in a sex-specific modulation of their target genes. This study strongly supports the hypothesis that the pharmacological regulation of NRF2 changes macrophage phenotypic activation in a sex-dependent manner and suggest that NRF2 might be a useful target for inflammatory-related diseases, such as neurodegenerative diseases.

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PhD ACTIVITIES

Publications

- Brunialti E., Villa A., Mekhaeil M., **Mornata F.,** Vegeto E., Maggi A., Di Monte D., Ciana P. 2021- "Inhibition of microglial GBA hampers the microglia-mediated anti-oxidant and protective response in neurons"- bioRxiv doi: 10.1101/2021.01.20.427380
- **Mornata F**., Pepe G., Sfogliarini C., Brunialti E., Rovati GE., Locati M., Maggi A., Vegeto E.: "Reciprocal interference between the NRF2 and LPS signaling pathways on the immune- metabolic phenotype of peritoneal macrophages." - Pharmacol Res Perspect. 2020; e00638. https://doi.org/10.1002/prp2.638
- Pepe G., Locati M., **Mornata F**., Cignarella A., Maggi A., Vegeto E.: "The estrogen-macrophage interplay in the homeostasis of the female reproductive tract" - Human reproduction Update, 24(6):652-672. https://doi: 10.1093/humupd/dmy026

Oral presentation of scientific data

- "Estrogen-macrophages interplay as a possible therapeutic target in endometriosis"- F. Mornata, G. Pepe, GC Cermisoni, A. Maggi, P. Viganò, M. Locati, E. Vegeto - 19th World Congress on Gynecological Endocrinology. Firenze, Italy, March 2020 (*rescheduled* to December 2020) (*poster presentation*)
- "Study of macrophage reactivity in the early phases of Parkinson's disease"- Spring School of the Doctorate of Experimental and Clinical Pharmacological Sciences- IV Edition, Chiesa in Valmanenco (SO), June 2020
- "Molecular imaging of the inflammatory response in the early stage of Parkinson's disease: role of macrophages and gender" - Mornata F, Pepe G, Brunialti E, Ciana P, Maggi A, Vegeto E.- NEXT STEP 10, La Giovane ricerca avanza, Università degli Studi di Milano, September 2019
- "Sexual differentiation of microglia and neurodegenerative disease" Vegeto E, Villa A., Mornata F**,** Pepe G., Maggi A.- XIV European Meeting on Glial Cells in Health and disease, Porto July 2019 (*poster presentation*)
- "Molecular imaging of the inflammatory response in the early stage of Parkinson's disease: role of macrophages and gender" - Spring School of the Doctorate of Experimental and Clinical Pharmacological Sciences - III Edition, Chiesa in Valmanenco (SO), April 2019
- "Molecular imaging of the inflammatory response in the early stage of Parkinson's disease: role of macrophages and gender" - Mornata F, Pepe G, Brunialti E, Ciana P, Maggi A, Vegeto E.- NEXT STEP 9, La Giovane ricerca avanza, Università degli Studi di Milano, July 2018
- "Molecular imaging of the inflammatory response in the early stage of Parkinson's disease: role of macrophages and gender" - Spring School of the Doctorate of Experimental and Clinical Pharmacological Sciences- II Edition, Chiesa in Valmanenco (SO), April 2018

Teaching and tutoring activities

- Tutoring activity for the course Biotecnologie farmacologiche avanzate (u.d. Laboratorio Biotecnologie Farmacologiche), held by Professor Elisabetta Vegeto- a.a 2020/2021.
- Tutoring activity for the course Biotecnologie farmacologiche avanzate (u.d. Laboratorio Biotecnologie Farmacologiche), held by Professor Adriana Maggi- a.a. 2018/2019.
- Mentoring activities during experimental thesis period for Pharmacy master's degree students (Pharmaceutical Chemistry and Technology, Pharmacy and Pharmaceutical Biotechnology).

Third-mission activities

- Supervision of one student from the project "Alternanza Scuola-Lavoro" (A.A. 2018-2019).
- Supervision of three international Medicine Students (A.A. 2018-2019, 2019-2020).