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EXPLORING THE EXPRESSION OF THE NLRP3 INFLAMMASOME IN REACTIVE ASTROCYTES

VOLUME 1

Dissertação no âmbito do Mestrado em Investigação Biomédica, ramo de Neurobiologia, orientada pela Professora Doutora Cláudia Alexandra dos Santos Valente de Castro e pela Professora Doutora Ana Paula Pereira da Silva Martins apresentada à Faculdade de Medicina da Universidade de Coimbra

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Resumo

A neuroinflamação é um mecanismo essencial do sistema imunitário inato no Sistema Nervoso Central, mas, quando desregulada, está associada ao desenvolvimento e progressão de várias patologias, como as doenças neurodegenerativas. Os astrócitos e a microglia são os principais efetores imunitários do SNC e têm a capacidade, quando há disrupções na homeostasia cerebral, de se tornarem reativos. Desta forma, estas células contribuem para o desenvolvimento de um ambiente inflamatório no SNC através da secreção de citocinas pro-inflammatórias, como a IL-1β. Para além disto, os astrócitos e a microglia encontram-se em constante comunicação e podem regular a sua atividade mutuamente. Mais especificamente, a microglia reativa pode secretar fatores que induzem um fenótipo pro-inflamatório nos astrócitos, o fenótipo A1.

O principal mecanismo de produção e secreção de IL-1β é o inflamassoma NLRP3, um complexo citosólico multiproteico que deteta interferências na homeostasia celular. O inflamassoma é ativado através de dois passos: primeiro, um estímulo de *priming* (como LPS) induz um aumento na produção da proteína NLRP3 e de pro-IL-1β. Um segundo estímulo (como ATP) induz a oligomerização do inflamassoma e consequente maturação e secreção de IL-1β. Sabe-se que a microglia consegue expressar o inflamassoma NLRP3 ativo, mas a presença deste em astrócitos ainda é debatida.

Este projeto tem como objetivo caracterizar a expressão e as funções do inflamassoma NLRP3 em astrócitos A1 e determinar o papel da microglia nestes eventos. Para isto, culturas primárias de astrócitos foram isoladas de ratos Sprague-Dawley recém-nascidos. Após 19 dias *in vitro*, as culturas puras de astrócitos foram incubadas com LPS (100ng/mL), ATP (1mM) ou ambos. Para simular os sinais secretados pela microglia, os astrócitos foram estimulados com um cocktail de fatores: TNF-α (30ng/mL), IL-1α (3ng/mL) e C1q (400ng/mL).

Os nossos resultados demonstram que, sob estimulação com LPS/ATP, os astrócitos tornam-se reativos e secretam IL-1β, maioritariamente devido à atividade do inflamassoma NLRP3. Para além do mais, TNF-α, IL-1α e C1q ativam os astrócitos, atuando como um estímulo de *priming* capaz, de induzir a produção de NLRP3 e pro-IL-1β. No entanto, os astrócitos necessitam de um segundo estímulo (por exemplo, o ATP) para que ocorra oligomerização do inflamassoma e consequente maturação de citocinas.

Em resumo, este trabalho demonstra o papel preponderante dos astrócitos na neuroinflamação e reforça a importância da comunicação microglia-astrócitos para a homeostasia e patologia no SNC.

Palavras-chave: Neuroinflamação, Inflamassoma NLRP3; Astrócitos; Fenótipo A1; Microglia.

Abstract

Neuroinflammation is an essential mechanism of innate immune defense in the CNS, but when unregulated it has been associated with the onset and progression of several pathologies, as neurodegenerative diseases. Astrocytes and microglia are the main immune effectors of the CNS and can become reactive when brain homeostasis is disrupted. In this way, these cells can contribute to the development of an inflammatory environment through the secretion of pro-inflammatory cytokines, like IL-1β. Furthermore, astrocytes and microglia are in constant communication and can regulate each-other's activity. Specifically, reactive microglia can secrete factors that induce a pro-inflammatory phenotype in astrocytes, the A1 phenotype.

The main mechanism of IL-1β production and secretion it's the NLRP3 inflammasome, a multiprotein cytosolic complex that senses interferences in cell homeostasis. This inflammasome is activated through a two-step mechanism: a priming stimulus (like LPS), which induces an increased production of NLRP3 protein and pro-IL-1β, and a second stimulus (like ATP), responsible for inflammasome oligomerization and consequent maturation and secretion of IL-1β. The NLRP3 inflammasome assembly is well established in microglia, but it's presence in astrocytes remains controversial.

This project aimed to characterize NLRP3 inflammasome expression and function in A1 astrocytes and assess the role of microglia in these events. For this purpose, primary cultures of astrocytes isolated from newborn Sprague-Dawley rats were used. After 19 days *in* vitro, pure astrocytic cultures were incubated with LPS (100ng/mL), ATP (1mM) or both. To simulate microglia signaling, astrocytes were also stimulated with a cocktail of factors: TNF-α (30ng/mL), IL-1α (3ng/mL) and C1q (400ng/mL).

Our results show that under LPS/ATP stimulation, astrocytes become reactive and secrete IL-1β, mostly through NLRP3 inflammasome activity. Furthermore, TNF-α, IL-1α and C1q, besides activating astrocytes, act as a priming event capable of inducing NLRP3 and pro-IL-1β production. However, astrocytes still require a second stimuli (as ATP) for inflammasome oligomerization and subsequent cytokine maturation.

Overall, our work shows a preponderant role for astrocytes during neuroinflammation and reinforce the importance of microglia-astrocytes communication for CNS homeostasis and pathology.

Keywords: Neuroinflammation; NLRP3 inflammasome; Astrocytes; A1 phenotype; Microglia.

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Abreviation List

A

AD – Alzheimer's Disease
ANOVA - Analysis of variance
ASC - Adaptor apoptosis-associated speck-like protein
ATP - Adenosine triphosphate

B

BBB - Blood-Brain barrier
Bcl10 - B-cell CLL/lymphoma 10
BMDM - Bone marrow derived macrophage
BSA - Bovine Serum Albumin

C

C1qR - C1q Receptor
CARD - Caspase recruitment domain
CCK-8 - Cell counting kit
CNS - Central nervous system
COPs - CARD-only proteins
COX-2- cyclo-oxygenase 2

D

DAMP - Danger-associated molecular pattern
DIV - Days in vitro
DMEM - Dulbecco's modification of Eagle's media
DUB - Deubiquitinating enzyme

E

EDTA - Ethylenediamine tetraacetic acid
ELISA - Enzyme-linked immunosorbent assay
ER - Endoplasmic reticulum

F

FADD – Fas-associated protein with death domain
FasL – Fas Ligand
FBS - Fetal bovine serum
FLA - Factors, LPS and ATP
FLICA - Fluorochrome-labeled inhibitors of caspases
FRET - Fluorescence resonance energy transfer

G

GAPDH - Glyceraldehyde-3-phosphate dehydrogenase
GFAP - Glial fibrillary acidic protein
GSDMD - Gasdermin D

H

HRP - Horseradish peroxidase
Hsp - Heat-shock protein

I

IFN - Interferon
IKK - IκB kinases
IL - Interleukin
IL-1R - IL-1 receptor
iNOS- nitric oxide synthase
IRAK - Interleukin-1 receptor-associated kinase

J

JAK - Janus kinase
JNK - c-Jun N-terminal kinase

L

LCN2 - Lipocalin 2

LPS - Endotoxin lipopolysaccharide

LRR - Carboxy-terminal leucine rich repeat domain

M

MAPK - Mitogen-activated protein kinase

MAVS - Mitochondrial antiviral-signaling protein

MHC - major histocompatibility complex

MS- Multiple Sclerosis

mtDNA - Mitochondrial DNA

mtROS - Mitochondrial reactive oxygen species

MyD88 - Myeloid differentiation primary response 88

N

NACHT or NBD - Central nucleotide-binding and oligomerization

NEK7 - NIMA-related kinase 7

NFκB - nuclear factor kappa-light-chain-enhancer of activated B cells

NLR - Nod-like receptor

NLRP3 - NACHT, LRR and PYD domains-containing protein 3

NO - Nitric oxide

NP-40 - Nonyl phenoxypolyethoxylethanol

NRF2 - Nuclear factor erythroid 2-related factor

P

P2X7R - P2X purinoceptor 7

PA - Astrocyte enriched culture

PAMP - Pathogen-associated molecular pattern

PBS - Phosphate buffered saline, 1x, pH 7.4

PBS-T - PBS Tween 20

PD- Parkinson's Disease

PDL - Poly-D-Lysine

PFA - Paraformaldehyde

PKA - Protein kinase A

PMSF - Phenylmethylsulfonyl fluoride

POPs - Pyrin-only proteins

PR3 – proteinase 3

PRR - Pattern recognition receptor

PTM - Post translational modification

PVDF - Polyvinylidene fluoride

PYD - N terminal pyrin domain

PYHIN - Pyrin and HIN domain containing

R

RD - Reagent Diluent

RIPK1 – Receptor-interacting serine/threonine-protein kinase 1

ROS - Reactive oxygen species

RT - Room temperature

S

SEM - Standard error of the mean

STAT3 - Signal transducer and activator of transcription 3

T

Th1 - T helper 1 cell

TIR- Toll IL-1 receptor

TLR - Toll-like receptor

TNF - Tumor necrosis factor

TNFR - Tumor necrosis factor receptor

TRAF - Tumor necrosis factor receptor (TNFR)-associated factor

1. Introduction

1.1 Neuroinflammation

Neuroinflammation is a necessary process of response in the brain or spinal cord[1], to noxious stimuli like pathogens, danger signals or sterile (non-infectious[2]) threats.[3,4] Under regulated conditions, it promotes clearance of the invading agents, removal of damaged cells and tissue repair.[3] However, unregulated neuroinflammation becomes chronic and can lead to tissue injury, neuronal death and related pathologies, like neurodegenerative diseases.[3–7] Infections, trauma, stroke, aging, among others, can all induce neuroinflammation[1,7,8] and its severity usually varies with the type, context and duration of the challenge.[1,8]

"Normal", transient neuroinflammation is characterized by activation of glial cells and mild production of chemokines and cytokines (as Interleukin (IL)-1β, Tumour necrosis factor (TNF)-α and IL-16).[7] Cytokine exposure is short due to temporary glial cells activation.[1] There is no peripheral leukocyte infiltration nor Blood Brain Barrier (BBB) breakdown or cell death.[1,9] This type of inflammatory response, denominated acute, is thus a defensive mechanism, removing the source of the challenge and allowing later repair of damaged tissues.[7,9]

Chronic, pathological neuroinflammation, however, results from persistent stimulation.[9] Typical hallmarks include activation of astrocytes and microglia, followed by cytokines and chemokines secretion[1,6,7] and reactive oxygen species (ROS) production[1,7], increase of the BBB permeability, leukocyte infiltration and ultimately neuronal death.[1,6–8] Continuous release of inflammatory mediators (cytokines, chemokines and ROS) activates resident and infiltrating cells, which in turn also produce these molecules, perpetuating neuroinflammation and attracting more immune cells from the periphery.[7] Additionally, glial cell activation reduces their ability for engagement in reparatory pathways.[1] In this way, neuroinflammation leads to neuronal death and propagates itself in an "hard to break loop". Hence, nowadays, neuroinflammation is considered a potential cause for the development of multiple Central Nervous System (CNS) diseases, like Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's Disease and Multiple Sclerosis (MS).[5]

Anti-inflammatory mechanisms limit inflammation and prevent chronicity. So, although the initial immune response tries to eliminate the origin of the challenge and repair any damage, chronic neuroinflammation with prolonged glial cell activation contributes to disease progression and neuronal damage.[7]

Importantly, the CNS is considered an immune privileged environment, protected from insults and immune responses that occur in the periphery.[10,11] This relative isolation, which is not absolute[9], is due to the BBB, Blood Cerebrospinal Fluid Barrier and astrocytes' endfeet, that do not allow the entry of peripheral immune cells.[12,13] During chronic neuroinflammation, there's BBB breakdown and infiltration of peripheral immune cells that contribute to the inflammatory environment.[14]

Besides microglia, macrophages and astrocytes, endothelial cells of BBB and peripheral invading leukocytes also secrete cytokines and chemokines.[1,15] Peripheral insults can also lead to cytokine secretion to the circulation that then, through various mechanisms, activate CNS glial cells.[8] For example, CNS pathologies can be induced by overactivation of glial cells (and consequent overproduction of cytokines), but also through glial cell dysfunction[16], as will be further explained in section 1.3. Even though the exact mechanism that drives neuroinflammatory dysfunction is not yet fully determined, it is known that the lack of inflammation resolution leads to cytotoxicity and neuropathologies. As will be discussed next, glial cells seem to be the main effectors of neuroinflammation, being potentially relevant for the development of neuropathologies.

1.2 The Interleukin - 1 family

Pro-inflammatory cytokines attract immune cells to the site of inflammation and through paracrine and autocrine signaling, activate neighbor cells. IL-1 family of cytokines, in which IL-1α, IL-1β and IL-18 are included, are the main pro-inflammatory family of cytokines along with Tumor Necrosis Factor (TNF) family and is involved in host defense and inflammation propagation, being implicated in neuropsychiatric and neurodegenerative diseases.[3,17]

IL-1 and TNF initiate inflammatory reactions and induce inflammation propagation, but they also stimulate IL-6 production, which may then promote an anti-inflammatory pathway.[17] This illustrates CNS capacity to, under mild neuroinflammation, regulate inflammatory processes and promoting resolution and damage repair.

Importantly, IL-1 and TNF families have roles in normal CNS functioning. They are able to regulate cell growth, survival and differentiation of cells.[18] In particular, IL-1β has a key role in homeostasis and clearance of the immune system [19], besides learning[1], memory and cognition processes.[14,17]

IL-1α and IL-1β bind to the same receptor, IL-1RI. IL-1RI activation recruits Myeloid differentiation primary response 88 (MyD88) and activates IL-1R-associated kinases (IRAK), which in turn activate nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and mitogen-activated protein kinase (MAPK)-regulated transcription factors. [2,20] These transcription factors are responsible for the transcription of genes related to neuroinflammation[21], leading to its exacerbation.[22] Although through a different mechanism, IL-18 is also able to activate the NF-κB pathway.[2,4]

IL-1RII is another receptor for IL-1α and IL-1β, but it's not capable to start the signal transduction pathway, downregulating their activity.[23] Importantly, IL-1β and lipopolysaccharide (LPS) stimuli have an overlapping effect, since both of their receptors, IL-1R1 and Toll-like receptor-4 (TLR4) respectively, have a Toll IL-1 receptor (TIR) domain that triggers the same signaling cascades.[22]

Deregulated IL-1 production, mainly IL-1β, leads to inflammation propagation through NF-κB and MAPK signaling pathways and results in neuronal death.[3] IL-1 secretion is induced by pathogen associated molecular patterns (PAMPs, as LPS), cell-derived danger signals and other pro-inflammatory cytokines (TNF-α, IFN-α, IFN-β and IL-1β).[20]

Both IL-1β and IL-18 are synthetized as procytokines that need to be processed to become active, but IL-1α is produce already in its active form.[23] Another important characteristic of the IL-1 family is the absence of a classical secretion signal, which points to an alternative mechanism of release.[2] For IL-1β and IL-18, that mechanism may be pyroptosis induced by inflammasome activity.[2,3] However, proteinase 3 (PR3), cathepsin G, streptococcal pyrogenic exotoxin B and matrix metalloproteinases can process IL-1β and caspase-8, PR3 and granzyme B can process IL-18.[2] The inflammasome NLRP3, the main topic of this thesis, will be further discussed in later sections, but it's important to highlight that excessive activation of this multiprotein complex (which is the main player in IL-1β and IL-18 secretion), has been regarded as harmful for the CNS and capable of leading to the development of neurodegenerative diseases.[24]

In the CNS, various cells express receptors for IL-1β and IL-18, facilitating inflammation exarcebation.[4,14] Glial cells, neurons and endothelial cells are the main cell types that bind these cytokines.[4]

Besides the already mentioned pathways, IL-1β can induce nitric oxide synthase (iNOS), cyclo-oxygenase 2 (COX-2) and superoxide products synthesis [25], besides increasing the expression of adhesion molecules[26] and modulating BBB permeability.[3] Thus, IL-1β contributes to leukocyte infiltration in the CNS.[26] This cytokine can then activate the recently infiltrated cells, exponentially increasing the production of pro-inflammatory and neurotoxic mediators.[3]

IL-18 induces the synthesis of adhesion molecules[3], other pro-inflammatory cytokines (IL-6, IL-8, TNF-α and IL-1β) and chemokines.[3,23] It also promotes T helper 1 (Th1) and B cells activity[18] and interferon (IFN) secretion[3,20], and is associated with increase of caspase-1 production in microglial cells and Fas ligand expression. Therefore, it is linked to mechanisms of cell death.[3]

There are polymorphisms in genes related to IL-1 that increase the risk for neurodegenerative diseases like AD[27] and inhibition of IL-1 has been shown to be neuroprotective.[28] It's clear that IL-1 family of cytokines are ubiquitously expressed in the CNS, and their overproduction can lead to increased neuroinflammation and neurodegeneration.[29]

In the next sections, the importance of glial cells for neuroinflammation will be discussed. Their activation and the physiological relevance of their activity is broadly due to IL-1 signaling through the CNS.

1.3 Glial Cells

Glial cells- mainly astrocytes, microglia and oligodendrocytes' lineages[30]- are the main immune effectors in the CNS.[17] Glial cells have been extensively described as actively contributing for neuropathologies[18,31] as mediators of neuroinflammation.[7] In response to a challenge, glial cells become activated and undergo a process of gliosis, resulting in their hypertrophy, increased expression of receptors for immune molecules and secretion of cytokines, chemokines and other inflammatory mediators.[8,31]

As described above, IL-1α and IL-1β bind to IL-1RI receptor in cell membranes and activate NF-κB and MAPK pathways.[2,20] Glial cells are the main cell types expressing Patterns Recognition Receptors (PRRs, like IL-1RI), being the main effectors of pro-inflammatory pathways.[3]

For a balanced immune response followed by its resolution, coordinated interaction between glial cells is necessary.[12] So, although glial cells protect the CNS, sustained activation leads to excess of cytokines and exacerbated neuroinflammation, resulting in neuropathologies.[17,32]

The cells of the CNS may sense PAMPs or danger associated molecular patterns (DAMPs) derived from the pathogens or damaged cells, respectively. These signals are sensed through PRRs, which can be membrane bound (as TLRs) or intracellular (like NOD-like receptors, NLRs). PRRs are mainly expressed in microglia, macrophages and astrocytes[4] and the activation of these cell types through binding of pro-inflammatory mediators leads to cytokine and chemokine secretion.[1,5,15]

In the next sections, microglia and astrocytes contributions for the healthy and the damaged CNS will be explored.

1.3.1 Microglia

Microglia are macrophage-like, resident immune cells of the CNS[3,32–35] with functions in homeostasis surveillance. They develop from myeloid precursors in the embryonic yolk sac[3,5,34] and migrate to the neural tube around embryonic day 10.5.[36] There is a second population of progenitors originated from blood monocytes and foetal macrophages. By post-natal day 20, microglia are maturated and distributed through the CNS.[36] Microglia turnover in adult life occurs not from myeloid cells in the bone marrow but from a unknow source in the CNS. The limited and potentially slow turnover make this cells susceptible to inflammatory damaged caused by stress, trauma, bad diet or aging.[1]

Non activated microglia help with neuronal development, synapse pruning, hemostasis maintenance and neurogenesis.[1,3,5,34,37] The resting phenotype of microglia is highly ramified and surveys the CNS for noxious factors.[7,9] Once it finds a challenge, it acquires an ameboid shape[7] and starts the innate immune response, with production of inflammatory mediators.[9] However, various reports suggest that microglia can have more than one activation state.[38]

Additionally, the relation between cell shape and activation state has been under debate because there is evidence that cell shape doesn't fully predict microglial functional state.[38,39] Microglial activation helps protect and repair the CNS, but under chronic or exacerbated activation, this inflammatory activity can lead to pathologies, neurodegeneration and neurobehavioral complications[1]. (*Figure 1*) After an activation stimulus, microglia become reactive and change their gene expression profiles, altering physiological functions of homeostasis maintenance (like ion balance).[35] Variations of stimuli and microglia location in the CNS result in different activation patterns and genetic expression.[5,37] Adding to its inflammatory profile, microglia can present antigens to lymphocytes.[18]

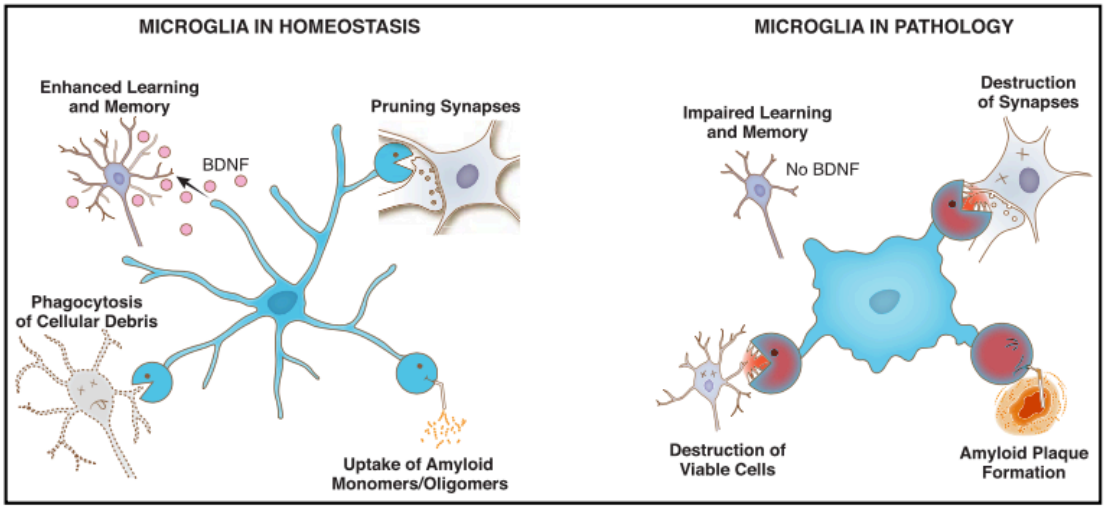

Figure 1 - Microglia are important for health but also for neuronal dysfunction. Resting microglia secretes trophic factors and prunes synapses. These highly ramified microglia also survey the environment for occasional threats. Activated, ameboid, microglia lose their support capacities and induce neuroinflammation. Adapted from: Herz *et al.*, 2017

Resting microglia are extremely ramified but when activated they acquire an ameboid shape, undergo gliosis (and thus, proliferate and migrate), increase the expression of complement receptors major histocompatibility complex molecules II (MHC-II), ROS and pro-inflammatory cytokine production.[3,5,33,37] These are hallmarks of the M1 phenotype, which also has high phagocytic activity to eliminate pathogens and cell debris.[3,5,35] ROS production seems to be one of the first steps of M1 response, through NADPH oxidase activity. It stimulates expression of pro-inflammatory cytokines (like IL-1β), chemokines[37], prostaglandin E_2 and nitric oxide (NO).[5] Additionally, M1 microglia are the main IL-1β

secreting cell type of the CNS.[32] Microglia secretion of chemokines and stimulation of expression of adhesion molecules in endothelial cells, is associated with leukocyte recruitment.[18] M2 phenotype, however, is highly ramified and has a more anti-inflammatory profile, developing at the end of the inflammatory response for its resolution and for tissue repair.[3,35] M2 microglia can be either M2a, M2b or M2c. Importantly, M2a is involved in phagocytosis and tissue repair through JAK/STAT pathway activation.[35]

As discussed before, mild reactivity of glial cells is benefic for CNS immune response and repair. However, overactivation leads to cytotoxicity and pathologies.[33] For example, in aging, is common for glial cells to become more sensitive and increase the expression of proinflammatory cytokines, while simultaneously losing neuroprotective properties.[17] In this way, microglia contributes for an increase in neuroinflammatory context in the CNS. This example illustrates that M1 microglia, as immune effectors and cytokine secretors, can contribute to neuroinflammation exacerbation and propagation, oxidative stress and cell death.[3,5,35,37]

1.3.2 Astrocytes

Astrocytes are highly heterogeneous.[3,40] Besides protoplasmic (in grey matter) and fibrous (in white matter) astrocytes, there are region-specific astrocytes, like Bergmann glia (cerebellum) or Müller cells (retina). Morphological and functional differences are also found inside the same brain region. For example, there are various subtypes of cortical astrocytes.[41]

Astrocytes are the most abundant cell type in the CNS.[32,41,42] They contiguously tile the entire CNS[43] and present varied roles in homeostasis maintenance like glutamate uptake, preservation of ionic balance, synaptogenesis, neuronal support, BBB upkeep, blood flow regulation, secretion of neurotrophic factors[3,5,41–44] and modulation of neurotransmission and synaptic homeostasis. Astrocyte mediated calcium signaling leads to release of gliotransmitters, purines and others, thus modulating synaptic transmission.[31,45] Other than calcium signaling and punctual K^+ and Na^+ inward currents, astrocytes are electrically silent.[9,16]

Along with microglia, astrocytes are immune effectors in the brain[17], undergoing a process of astrogliosis when activated.[3] Astrogliosis is a process of increased reactivity with astrocytic hypertrophy and hyperplasia[3,46] and increased expression of intermediate filaments of the cytoskeleton, namely Glial fibrillary acidic protein (GFAP) and vimentin.[5,47]

For this reason, upregulation of GFAP has been largely used as a marker for reactive astrocytes.[42,47] One important aspect of astrogliosis is scar formation, seemingly regulated by GFAP and Signal transducer and activator of transcription 3 (STAT3) related signaling[42], that restricts the damaged area and prevents inflammation spreading to the CNS.[48] Besides astrocytes, the glial scar can be formed by microglia, endothelial cells and fibroblasts.[31] Scar formation has classically been thought to inhibit axonal regrowth.[35] However, a 2016 report by Anderson and colleagues suggests that, in a severe spinal cord injury model, astrocyte scar formation actually aids axonal regeneration.[49] Liddelow and Barres thus argue that the inhibitory effect on axonal regrowth previously reported may be due to other cell types in the glial scar or even to differently activated astrocytes.[10] An example of this is the inhibition of nerve regeneration when microglia phagocytic activity is insufficient for myelin debris clearance in spinal cord injury.[50]

In line with the theory of differently activated astrocytes, Liddelow and colleagues distinguished between two phenotypes of reactive astrocytes: A1 and A2, similarly to M1 and M2 microglia phenotypes.[51] A1 astrocytes are thus pro-inflammatory and up-regulate NF-κB and complement cascade related genes, suggesting a neurotoxic role for this phenotype. A2 astrocytes are mainly anti-inflammatory, with high expression of neurotrophic factors that may aid CNS regrowth and survival.[12] Considering the increased expression of STAT3 in A2 phenotype[12], these astrocytes may be responsible for scar formation.

Reactive astrogliosis is a gradual process, with different molecular, morphological and functional alterations, but it can be divided in three main categories according with the degree of reactivity: mild, where astrocytes can reversibly change gene expression; moderate, in which astrocytes undergo hypertrophy but maintain cellular domains (*Fig. 2b*); and severe, where astrocytes, together with other cell types, proliferate in such a way that individuals domains disappear (*Fig. 2c and 2d*) leading to scar formation (*Fig. 2d*).[31,47] Mild/moderate astrogliosis can be triggered by non-penetrating trauma, CNS lesions in distant areas or mild innate immune activation. Severe astrogliosis is associated with severe focal lesions, severely infected areas or areas with active neurodegenerative signaling pathways. Scar formation occurs in penetrating trauma, neoplasms, chronic neurodegeneration or chronic inflammation.[47]

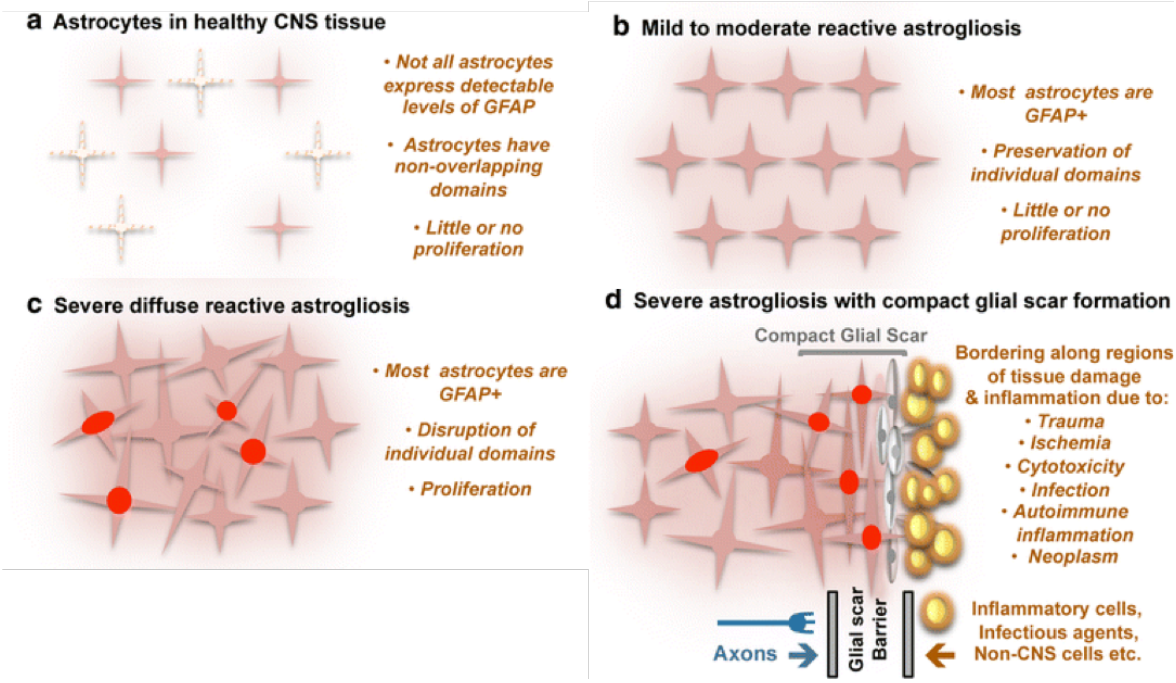

Figure 2 – Evolution of reactive astrogliosis. A. Resting astrocytes. B. Mild to moderate reactive astrogliosis. There is reversible alteration of gene expression and physiological functions and a slight hypertrophy without overlap of individual astrocyte domains. There can be inflammation resolution and return of astrocytes to their resting state. C. Severe diffuse reactive astrogliosis. Hypertrophy and proliferation of astrocytes and other cell types (represented in red), with overlap of individual domains and long-term tissue reorganization. D. Severe reactive astrogliosis. Cellular domains overlap, forming glial scars with deposition of collagenous extracellular matrix. There is proliferation of new astrocytes and other cells (in grey in the figure). Glial scars delimit the damaged zone, limiting spreading of inflammatory mediators, but also inhibiting axonal regrowth. Adapted from Sofroniew and Vinters, 2010.

Figure 2 illustrates that reactive astrogliosis is not an all-or-none response. According with the triggering insult and the context of each individual astrocyte, there is a "graded continuum of progressive alterations" that may or may not result in scar formation.[43] It is also important to consider that the same stimulus can lead to different cellular pathways and different stimuli may induce the same response.[42]

Independently of the degree of astrogliosis, reactive astrocytes increase cytokines levels[17,42] (IL-1, IL-6, IFN, TNF)[39] and chemokines and ROS secretion[3], contributing for neuroinflammation (either healthy or pathological)[17] and adaptative immune defense[42], and even altering neuronal function.[31] IL-1β then activates other astrocytes[36] and since these

glial cells express innumerous receptors for IL-1β and TNF-α in resting state[52], they are highly sensible to a pro-inflammatory context and become quickly activated.

Reactive astrocytes can protect the CNS through various mechanisms, like uptake of glutamate, ROS[43] and neurotoxins[18], adenosine release, repair of the BBB, maintenance of ion balance[43] and secretion of neurotrophins.[18] Regulated astrogliosis is thus imperative for CNS protection and normal function.[16] However, unregulated astrogliosis can lead to pathologies, either by astrocytic loss or gain of function.[43] For example, unchecked ROS production leads to oxidative stress and excess glutamate leads to excitotoxicity, both resulting in neuronal damage. Reactive astrocytes not only increase ROS production, but also lose the ability to scavenge glutamate and ROS, contributing to an increased interaction of these molecules with neurons and ultimately resulting in cytotoxicity.[53] In this way, astrogliosis contributes to pathologies through secretion of pro-inflammatory mediators, and also through the loss of regulatory astrocytic functions.[54]

Heterogeneity in astrocyte function comes not only from their diverse morphology through the CNS but also from the different reactivity states. Different stimuli trigger different signaling pathways and cellular responses. Moreover, the distance from the trigger zone can also modulate reactivity intensity.[16]

Through the described mechanisms, it is possible to conclude that astrocytes undergoing astrogliosis and communicating with other cells, can contribute or even cause inflammatory-associated pathologies in the CNS.[43,46]

1.3.3 Microglia-astrocytes interaction

No cell in the CNS is isolated and astrocytes are no exception. Astrocytes communication with other cell types, and particularly with microglia, is essential for astrocytic activity and reactivity.[12,18,47,54] A clear example is the tripartite synapse, a continuous communication between neurons and glial cells that modulates each other's activities.[31] Under neuroinflammatory conditions, damaged neurons may signal to microglia, activating them. Microglia will subsequently activate astrocytes. Reactive astrocytes then secrete neurotoxins that destroy the damaged neurons.[12] Additionally, according to some reports, microglia may increase astrocytes' proliferation but decrease GFAP expression and scar formation during neuroinflammation, while simultaneously pushing astrocytes to a pro-inflammatory phenotype.[46] Considering this, it seems that microglia input stimulates astrocytes to switch

to the A1 phenotype previously described, and increase of GFAP during astrogliosis is associated with the A2 phenotype and scar formation.

Importantly, it has recently been described that factors secreted by microglia (TNF-α, IL-1α and C1q) are sufficient and necessary for induction of the A1 phenotype in astrocytes.[51]

TNF-α is a pro-inflammatory cytokine involved in innate immune system responses and neuroinflammatory processes. It can exist in a transmembrane form or, after cleavage, as a soluble homotrimer. Both forms can bind to the TNF-α receptors (TNFR) 1 or 2. Binding of soluble TNF-α to TNFR1 leads to receptor trimerization, recruitment of adaptor proteins and downstream activation of caspase 8 and 10, which ultimately results in apoptosis. TNFR1 signaling is also associated with c-Jun N-terminal kinase (JNK), MAPK and NF-kB pathways.[55]

IL-1α is part of the IL-1 family and, similarly to IL-1β, binds to the IL-1R, promoting NF-kB translocation to the nucleous.[20,26]

C1q is a member of the classical component pathway that triggers C1 complex activation when it senses ligands. C1q can bind to the receptor C1qR and increase ROS production, which is associated with inflammasome activation.[56] Importantly, C3 is another complement member that acts downstream of C1q.[57]

Astrocytes are also able to induce microglial reactivity, mainly through connexin channels-mediated ATP release.[58] ATP binds to purinergic receptors in microglia surface, activating them, and inducing cell death.[59]

Microglia-astrocyte communication is thus essential not only for CNS homeostasis but also for glial cell reactivity regulation.[58] Glial cells can exert both pro and anti-inflammatory effects in each other, contributing for inflammation resolution or propagation.[12,43] The main mechanism involved in glial cell-mediated neuroinflammation seems to be the inflammasome, that will be discussed next.

1.4 The Inflammasomes

Inflammasomes are multi-protein cytosolic complexes that survey cell homeostasis and act as a branch of innate immunity.[21] When activated, they oligomerize and cleave pro-caspase 1 (a cysteine dependent protease), which leads to the maturation and release of pro-inflammatory cytokines.[4]

Inflammasomes are associated with various brain infections (bacterial or viral), injuries (cerebral ischemia, traumatic brain injury, stroke), metabolic disorders (atherosclerosis, type 2 diabetes, obesity) and neurodegenerative diseases (AD, MS, Amyotrophic Lateral Sclerosis, prion diseases, PD, Huntington's disease)[3,14,21,60] and, through the propagation of inflammatory mediators, can either be a cause or a consequence of these pathologies.[21]

Inflammasome expression has been described in various cell types, both from the peripheral immune system (like macrophages and monocytes[61]), and in the CNS. Microglia is considered the main expressing cell,[3,14] but astrocytes[3,21,44], neurons[14,21], perivascular CNS macrophages, oligodendrocytes and endothelial cells also contribute for inflammasome activity.[14]

A functional inflammasome is comprised by three subunits: a cytosolic PRR (that recognise PAMPs or DAMPs), an adaptor protein and caspase-1. The PRRs that are contained in the inflammasome can either be a part of the NLR family or the pyrin and HIN domain-containing (PYHIN) family. NLR proteins have a C-terminal leucine rich repeat (LRR) domain, a central nucleotide-binding and oligomerization (NACHT or NBD) domain, common to all proteins from the NLR family, and an N-terminal pyrin (PYD) domain or caspase recruitment (CARD) domain (*Fig.3*). LRR senses ligands and may act as an auto-inhibitor through binding to the NACHT domain. The NATCH domain allows ATP-dependent oligomerization of the NLR protein to other NLR proteins and the PYD domain permits homotypic interactions of NLR to the adaptor apoptosis-associated speck-like protein (ASC). ASC contains a CARD that allows binding to pro-caspase-1.

The NLR protein family members that instead of a PYD contain a CARD (like NLRC4 and NLRP1) can directly bind to pro-caspase-1. Pro-caspase-1 has a catalytic domain (p20) and a catalytic subunit in the C-terminal (p10). When the NLR protein is stimulated it oligomerizes through NATCH-NATCH domains interactions and recruits ASC, forming ASC specks. ASC recruits pro-caspase-1 which undergoes autocleavage (between p20 and p10) and becomes activated. After autocleavage, Caspase-1 cleaves pro-IL-1β and pro-IL-18 into their activated forms, which are then secreted from the cells.[3,4,14,17,21,32,56,62,63] Caspase-1 remains attached to ASC until CARD-p20 processing allows the release of p20-p10, ending the proteolytic activity.[56] Caspase-1 activation can also, through Gasdermin D (GSDMD), lead to pyroptosis[4,14,21,56], a form of inflammatory programmed cell death[56] that will be later addressed in the context of the NLRP3 inflammasome.

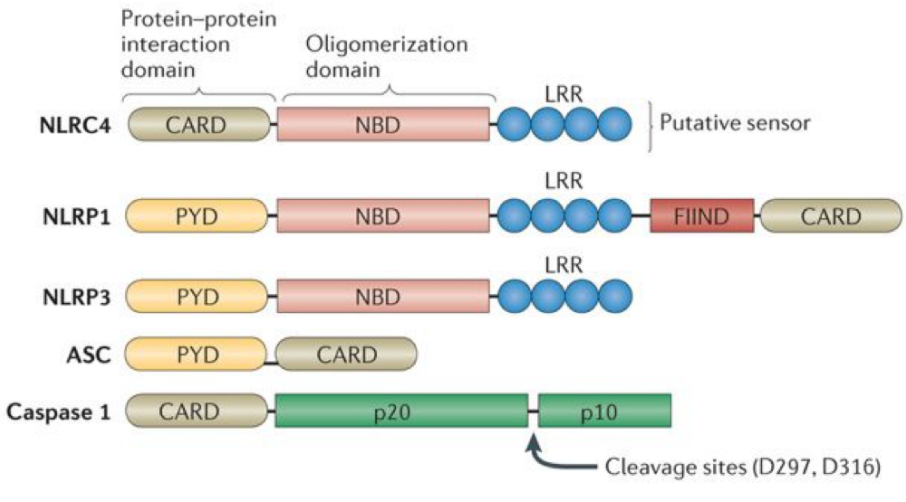

Figure 3- Inflammasomes structure. In NLRS, the sensory domain is the LRR. NBD which mediates oligomerization. PYD domains interacts with the PYD domain of ASC. ASC also contains a CARD domain that interacts with pro-caspase-1. NLRC4 and NLRP1 can interact directly with pro-caspse-1 with their CARD domains. Adapted from Walsh *et al*., 2014

ASC is normally found in the nucleus of cells and it relocates to the cytosol when the inflammasome is activated, forming speck-like aggregates, usually close to the nucleus.[3,21] These specks can be released upon cell death (like pyroptosis) and cleave pro-IL-1β in the extracellular matrix, as well as activate pro-caspase-1 when endocytosed by other cells. In this way, ASC helps to spread inflammation.[21,64] ASC redistribution and alternative splicing may act as regulators of inflammasome activity.[65]

The NLR family has three subfamilies: NODs, NLRPs/NALPs and IPAF (NLRC4 and NAIP).[62]

Different inflammasomes are able to respond to different PAMPs and DAMPs. NLRP3 is the only PRR capable of distinguishing various unrelated stimuli (including viral RNA)[56], NLRP1b recognizes a toxin from *Bacillus anthracis*, NLRC4 senses bacterial flagellin and AIM2 is activated by dsDNA.[14]

In this thesis, the focus is on the NLRP3 inflammasome, the most vastly expressed inflammasome, capable of sensing a vast array of stimuli.[3,21,56] It is the only inflammasome that requires a two-step activation mechanism.[14]

1.4.1 The NLRP3 inflammasome

From all known inflammasomes, the NLRP3 inflammasome is the most studied and extensively characterized one, being believed to be the main pathway for IL-1β production in the CNS.[3,4] It's presence in microglia has been well established,[3,66] but debate still arises about NLRP3 inflammasome presence and activity in astrocytes.[3,67] NLRP3 inflammasome activity in neurons and CNS macrophages has also been reported.[4] This inflammasome has been associated with various immune cryopyrin-associated periodic syndromes[4,68], neurological diseases[4,19,21], and aging and metabolic disorders.[6,64]

NLRP3 inflammasome acts as a sensor for misfolded proteins[4], viral and bacterial infections[2,19,22,56] and for overall environmental and intracellular stress.[4,6,56,66]

It's PRR is a NLR family pyrin domain containing 3 (NLRP3) and binds to an ASC adaptor for pro-caspase 1 recruitment (*Fig.3*).[3,56,66] By leading to the release of IL-1β and other inflammatory mediators (through pyroptosis), is associated with acute and chronic neuroinflammation, possibly leading to pathologies.[4]

In the next sections, NLRP3 inflammasome priming, activation and regulation will be addressed. Hereafter, the NLRP3 inflammasome will be referred simply as "inflammasome" and "NLRP3" will be used to designate the PRR protein domain.

1.4.2 Mechanisms of activation

1.4.2.1 Canonical pathway

For inflammasome activation and oligomerization two steps are required: a priming stimuli that induces NLRP3 and pro-IL-1β production through the NF-κB pathway, and an activation step that allows the inflammasome oligomerization (Fig. 5).[3,4,56,62,63] TLR and NLR ligands, like IL-1β, IL-1α , TNF, PAMPs and DAMPs can all induce both priming and oligomerization of the inflammasome.[3,56,62] Activation signals can be delivered through DAMPs like ATP, particulates, monosodium urate and cholesterol crystals[56] and also aggregated or misfolded proteins.[4]

LPS binds to TLR in cell membranes, acting as a priming event.[4,62] TLR are membrane bound PRRs.[14] Inflammasome activation and IL-1β production without a priming stimuli is possible, but for a significant pro-inflammatory cytokine production priming is needed, since it activates NF-κB, a transcription factor associated with inflammatory mechanisms.[4,56]

Binding of LPS to TLR4 recruits MyD88, that through IRAK activates B-cell CLL/lymphoma 10 (Bcl10) (*Fig.4*). Bcl10 activates the IκB kinases (IKK) family, which phosphorylate IκB and signals it for ubiquitination and degradation. NFκB, no longer inhibited by IkB, translocates to the nucleus and acts as a transcription factor for pro-inflammatory genes.[29,35,69–71]Additionally, LPS increases the production of ROS, decreasing Heat shock protein 27(Hsp27) phosphorylation and leading to NF-κB activation.[69]

Besides the MyD88 dependent TLR pathway, there is also a MyD88 independent pathway, where LPS induces Interferon (IFN)-β production.[70]

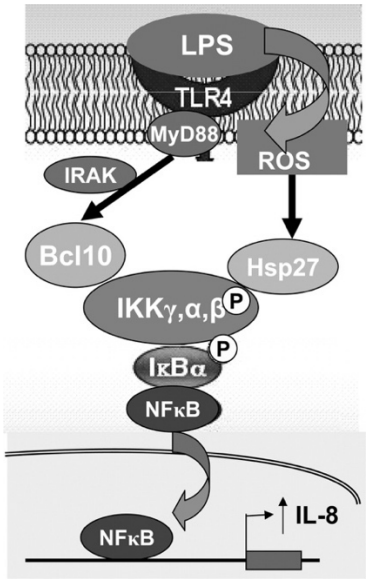

Figure 4- LPS mediated NF-κB activation. LPS binding to TLR4 recruits MyD88, activates IRAK and leads to IKK-mediated IκB phosphorylation, releasing IkB from NF-kB. NF-κB migrates to the nucleus where it acts as a transcription factor. Another pathway for IKK activation is mediated by increase of ROS production due to LPS stimulation. Source: Bhattacharyya *et al.*, 2008

Priming, besides increasing the levels of NLRP3 in the stimulated cells, has a non-transcriptional role by inducing post-translational modifications (PTMs) in this NLR protein, allowing it to quickly respond to stimuli.[56] For example, in bone marrow-derived macrophages (BMDMs), it induces NLRP3 deubiquitination, which allows its activation[21,60] and leads to NLRP3 phosphorylation at S194, a critical step for NLRP3 oligomerization with ASC.[64] Importantly, this phosphorylation is mediated by TLR-JNK1 axis.[64]

Priming non-transcriptional mechanisms seem to be partially regulated by mitochondrial reactive oxygen species (mtROS).[60,72]

The second step in the inflammasome canonical pathway is denominated activation and it leads to NLRP3, ASC and caspase-1 oligomerization into the functional inflammasome complex, which results in IL-1β maturation and pyroptosis (Fig.5).[4,56]

ATP is a DAMP that acts has an activation stimulus by binding to ionotropic purinergic receptors P2X7 (P2X7R). Sustained activation of this receptor leads to the formation of a membrane pore permeable to molecules up to 900 Da.[25,33,73] P2X7R activation is followed by increase of intracellular Ca^{2+} [33], K^+ efflux [62,74–76] and Na^+ influx.[75] P2X7R normally acts as a selective cationic channel, but after ATP-mediated activation, it leads to the secretion of larger molecules, through the formation of the membrane pore.[23] The main candidate for pore formation is pannexin 1.[62,73,74] The formation of the pore may allow the entry of agonists that directly activate NLRP3.[62] Also, K^+ efflux may trigger inflammasome assembly.[74] However, conflicting reports exist about pannexin-1 involvement in P2X7R-dependent pore formation. Qu and colleagues, later supported by Alberto and co-workers., defended that pannexin-1 was not responsible for the pore formation, and that P2X7R and pannexin-1 are two different signaling pathways.[77,78]

ATP can also trigger apoptosis through mtROS production[6], as will be explained in the "Mitochondrial dysfunction" section.

It is widely established that NLRP3 can recognize and induce cellular stress, but how this recognition happens and what pathways are activated upstream of NLRP3 activation that allow the engagement of this PRR are still debated.[56] Table 1 summarizes most of the NLRP3 inflammasome activation stimuli known.

Table 1- Known NLRP3 activators. Source: Swanson *et al*., 2019

Activator	Source	Examples	Refs
DAMP	Self-derived	ATP, cholesterol crystals, monosodium urate crystals, calcium pyrophosphate dihydrate crystals, calcium oxalate crystals, soluble uric acid, neutrophil extracellular traps, cathelicidin, α-synuclein, amyloid-β, serum amyloid A, prion protein, biglycan, hyaluronan, islet amyloid polypeptide, hydroxyapatite, haeme, oxidized mitochondrial DNA, membrane attack complex, cyclic GMP–AMP, lysophosphatidylcholine, ceramides, oxidized phospholipid 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine and sphingosine	20,25,26,57,99,111,156–163
	Foreign-derived	Alum, silica, aluminium hydroxide, nanoparticles, carbon nanotubes, chitosan, palmitate (also self-derived), UVB, imiquimod (R837)/CL097 and resiquimod (R848)	28,40,164
PAMP	Bacterial	Lipopolysaccharide, peptidoglycan, muramyl dipeptide, trehalose-6,6′-dibehenate, c-di-GMP–c-di-AMP, bacterial RNA and RNA–DNA hybrid	31,165–169
		Toxins: nigericin (*Streptomyces hygroscopicus*), gramicidin (*Brevibacillus brevis*), valinomycin (*Streptomyces fulvissimus* and *Streptomyces tsusimaensis*), β-haemolysin (*Streptococcus* sp. 'group B'), α-haemolysin (*Staphylococcus aureus*), M protein (*Streptococcus* sp. 'group A'), leucocidin (*Staphylococcus aureus*), tetanolysin O (*Clostridium tetani*), pneumolysin (*Streptococcus pneumoniae*), listeriolysin O (*Listeria monocytogenes*), aerolysin (*Aeromonas hydrophila*), streptolysin O (*Streptococcus pyogenes*), enterohaemolysin (*Escherichia coli* O157:H7), haemolysin BL (*Bacillus cereus*), adenylate cyclase toxin (*Bordetella pertussis*), M protein (*Streptococcus* sp. 'group A') and maitotoxin (*Marina* spp. dinoflagellates)	170–173
	Viral	Double-stranded RNA and single-stranded RNA	62,66,67
	Fungal	β-Glucans, hyphae, mannan and zymosan	174,175

DAMP, damage-associated molecular pattern; NLRP3, NOD-, LRR- and pyrin domain-containing protein 3; PAMP, pathogen-associated molecular pattern; UVB, ultraviolet B.

Since NLRP3 answers to a multiple array of DAMPs and PAMPs, it is possible that instead of being able to bind to all of these stimuli, NLRP3 senses a disruption of cell homeostasis that results from them.[2,4,21,63] The activation stimuli activates upstream signaling pathways like K^+ efflux, Ca^+ flux, release of mtROS, DNA and cardiolipin, lysosomal disruption, metabolic changes, trans-Golgi disassembly and Cl^-efflux.[21,56,63] Interestingly, cell swelling has been proposed as a "conserved homeostatic mechanism" linked to NLRP3 activation, since most of the signaling pathways described are affected during this phenomena and, *in* vivo, neuronal cell swelling is linked to caspase-1 activity. In this way, NLRP3 could sense membrane integrity.[79]

K^+ efflux

K^+ efflux has been described as a necessary and sufficient step for NLRP3 activation and oligomerization.[4,19,56,63,67] However, recent reports contradict this dogma and question if there is a downstream pathway necessary for inflammasome activation.[80] Nonetheless, K^+ efflux is induced by almost all NLRP3 activating stimuli.[4,19,56,63,67] The lowering of intracellular K^+ concentration is sufficient to activate the inflammasome and its hypothesized that it leads to conformational changes in NLRP3, which allows its interaction with ASC and regulatory proteins, as the NIMA Related Kinase 7 (NEK7).[63,79] K^+ efflux is also necessary for inflammasome activation in the non-canonical pathway.[63]

ATP activates P2X7R that allows Ca^{2+} and Na^{+} influx and mediates K^{+} efflux. Low extracellular concentrations of K^{+} activate the inflammasome and high extracellular concentrations inhibit it.[79]

Ca^{2+} flux

Ca^{2+} flux seems important particularly for the activation of the NLRP3 inflammasome, but not as much for other inflammasomes.[63] Nevertheless, conflicting reports exist stating that Ca^{2+} signaling is not necessary for inflammasome activation.[56,63] Ca^{2+} can come from either the extracellular medium, entering the cell through the opening of channels in the plasma membrane, or from intracellular stores in the endoplasmic reticulum (ER). Both pathways are usually linked: Ca^{2+} may enter the cell through P2X7R, which simultaneously promotes K^{+} efflux that induces Ca^{2+} release from the ER.[56]

Changes in Ca^{2+} signaling may lead to mitochondrial dysfunction with increased mtROS production and inflammasome activation.[63]

Cl^{-} efflux

ATP-induced IL-1β secretion is enhanced by low extracellular concentrations of Cl^{-} and high levels of this ion in the extracellular milieu inhibit the inflammasome. Cl^{-} efflux is necessary for ASC polymerization and may be dependent on mtROS, since they induce the formation of the Cl^{-} channels.[63]

Lysosomal rupture

Endocytosis of uric acid, cholesterol crystals, amyloid-β, alum, silica, asbestos, viral and bacterial toxins leads to lysosomal rupture and release of cathepsin B (a cysteine protease) to the cytosol, which can activate the inflammasome.[4,32,56,62,63]

Mitochondrial dysfunction

ROS production is one of the most conserved mechanisms of innate immunity [62] and most of inflammasome activators, like ATP and particulate matter, induce ROS production.[56,62] Mitochondria normally produces ROS as a consequence of oxidative phosphorylation, but under cellular stress this production increases into potentially damaging levels. The increase of mtROS levels in the cytosol is thought to activate the inflammasome[4,32,56], as well as the

release of oxidized mitochondrial DNA (mtDNA).[4,56,63] The switching of cardiolipin (a mitochondrial phospholipid) from the inner membrane of mitochondria to the outer layer can also bind and activate NLRP3.[56,63] Interestingly, inhibition of ROS only blocks the priming step of the inflammasome, but not the activation phase [56,63] and mtDNA can be released downstream of the activation of the inflammasome.[63] Priming with TLR agonists activates Interferon type I (IFN-1), which increases mtDNA synthesis.[56] These results, together with a report that describes mitochondrial dysfunction as unnecessary for inflammasome activation[68], show how mitochondrial role in inflammasome activation still needs to be clarified.

Nonetheless, if mitochondrial dysfunction does indeed affect NLRP3 activation, mitophagy (the process by which damaged mitochondria are eliminated) may help control the activation of the inflammasome.[4,56] This suggests a connection between inflammasome activity, mitochondrial dysfunction and apoptosis. Interestingly, ATP, besides activating NLRP3, can induce apoptosis through mtROS production and consequent caspase-3 activation.[6]

Different mtROS-mediated activation mechanisms of NLRP3 have been proposed. A report suggests that after priming, mtROS activates DUB enzymes that deubiquitinate NLRP3, allowing its activation after the second stimuli. However, different DUBs may be activated, some which are not mtROS dependent (for example, LPS activates a mtROS dependent-DUB and ATP activates a DUB independent from mtROS).[60,64] Other reports suggested that NLRP3 may bind to mitochondrial membranes, where it forms the inflammasome. [81,82]

The pathways described may act independently of each other or may converge and influence one another, culminating in inflammasome activation. For example, K^+ efflux and mitochondrial dysfunction may converge in Golgi disassembly. The resultant trans-Golgi network exposes phosphatidyl-linositol-4-phosphate, that recruits and activates NLRP3, leading to inflammasome assembly.[56] Additionally, lysosomal damage induces K^+ efflux[4,56] and Ca^{2+} influx.[56] Also, mtROS and Ca^{2+} flux lead to increase of mitochondrial permeability and formation of transition pores that allow mtROS and mtDNA secretion.[56] Due to the difficulty in disentangling each individual pathway, the exact mechanisms of inflammasome activation by mtROS remain to be elucidated.

Due to the amount of activation stimuli, and considering the various upstream pathways described, it is plausible to hypothesize that different stimuli have different outcomes.[2] For example, in a report by Gordon and colleagues, caspase-1-dependent pyroptosis was induced

by nigericin treatment but not by α-synuclein in microglia.[66] Other authors report that NLRP3 may be activated through mitochondrial antiviral-signaling protein (MAVS) interaction only when stimulated with soluble stimuli, being MAVS irrelevant when NLRP3 is stimulated with particulate matter.[63]

After activation, the inflammasome oligomerizes and activates caspase-1, that ultimately produces IL-1β and IL-18 (*Fig.5*). Additionaly, the inflammasome can also lead to pyroptosis, a form of inflammatory cell death.[3,4,21,62,63,66] GSDMD is cleaved by caspase-1, releasing its C-domain, and binding to phospholypids in the inner cell membrane (mainly phospatidylinositol and phosphatidylserine), forming membrane pores. The pores allow water influx, cellular swelling and osmotic lysis, leading to the secretion of IL-1β and other pro-inflammatory contents (like IL-1α, HMGB1 and ASC).[3,4,14,56,62] Pyroptosis allows ASC specks to be secreted and fill the extracellular space, where they can maturate caspase-1, and consequently activate IL-1β and IL-18.[66] In these ways, pyroptosis results in the spreading of inflammation and neuronal death. Pyroptosis is thought to be a response to viral and bacterial infections and is mechanistically different from apoptosis and autophagy.[4,56,62] For example, DNA cleavage (distint from DNA laddering of apoptosis) occurs.[62] Reports hint that once caspase-1 is activated, the cells become irreversibly commited to pyroptosis.[19]

Alternative pathways for IL-1β secretion exist, including direct release by transporters, multivesicular bodies with exosomes, microvesicles' shedding and secretory lysosomes.[23,26]

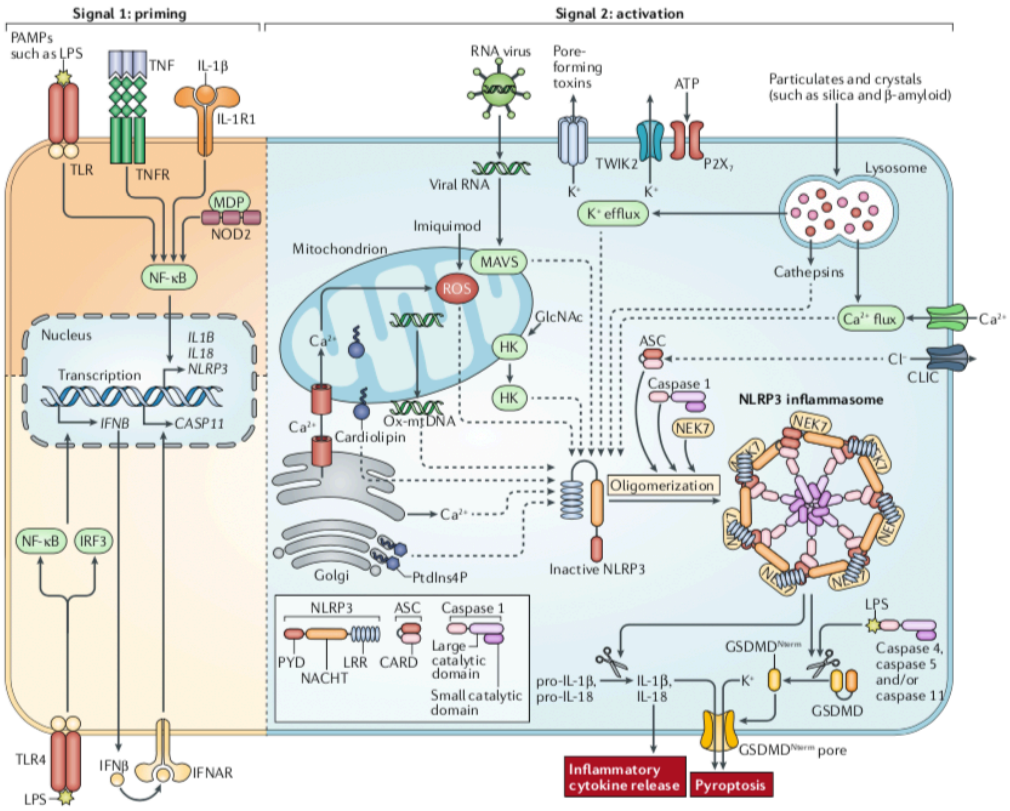

Figure 5- NLRP3 inflammasome activation pathways and regulatory mechanisms. Priming induces NF-κB translocation to the nucleus, increasing the expression of NLRP3, IL-18 and IL-1β. Priming also induces post-translational modifications that allow inflammasome assembly. The second stimulus induces the formation of the inflammasome. Activation may be due to relocation of NLRP3 close to the mitochondria, sensing of mtROS, mtDNA and cardiolipin, sensing of K^+ efflux or lysosomal disruption. NLRP3 then oligomerizes with ASC and pro-caspase-1. Pro-caspase-1 CARD domain is cleaved and the effector p10 heterodimer is formed. Pro-IL-1β and GSMD are cleaved, pyroptosis is induced and the cytokine is released. Source: Swanson *et al.*,2019

1.4.2.2 Regulatory mechanisms

Before activation, the inflammasome is maintained in an inactive state by the binding to chaperones as ubiquitin ligase-associated protein SGT1 and Hsp90.[83]

Also, PTMs regulate NLRP3 activation and stimuli-induced response through all inflammasome activation stages (before stimuli and during priming, activation and resolution) (*Fig.6*). These PTMs, mainly ubiquitination, phosphorylation and sumoylation,

allow NLRP3 to be inactive but ready to rapidly respond to challenges. Additionally, PTMs can be interconnected (for example, phosphorylation of S194 of NLRP3 is needed for further deubiquitination) and may be either inhibitory or activating.[56,64] Ubiquitination signals proteins for degradation in the proteasome, phosphorylation allows self-association, activation, deubiquitination and also inhibition, and sumoylation suppresses NLRP3 activity. Of notice, not only NLRP3 undergoes PTMs.[56] For example, ASC needs to be ubiquitinated to be active.[21,56]

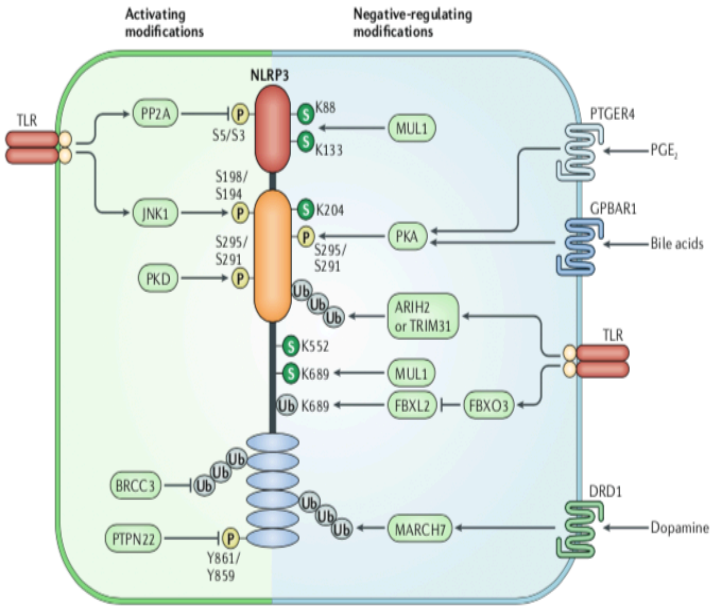

Figure 6 -Post transcriptional modifications in NLRP3 protein. On the left are activating modifications and, on the right, the inhibitory ones. For phosphorylation, human and mouse amino acid residues are mentioned, but for ubiquitylation and sumoylation only mouse protein residues are listed. P-phosphorylation; Ub-ubiquitylation; S-sumoylation;

ARIH2- E3 ubiquitin-protein ligase ARIH2; BRCC3- BRCA1/BRCA2-containing complex subunit 3; DRD1- D1A dopamine receptor; FBXL2- F-box/LRR-repeat protein 2; FBXO- F-box only protein 3; GPBAR1- G protein-coupled bile acid receptor 1; JNK1- JUN N-terminal kinase 1; MARCH7- membrane-associated RING finger protein 7; MUL1- E3 SUMO protein ligase MUL1; PGE2- prostaglandin E2; PKA- protein kinase A; PKD- protein kinase D; PP2A- protein phosphatase 2A; PTGER4- prostaglandin E2 receptor EP4 subtype; PTPN22- protein tyrosine phosphatase non-receptor type 22; TLR- Toll-like receptor; TRIM31- tripartite motif-containing protein 31. Source: Swanson *et al.,*2019

Besides the regulatory mechanisms already described, like PTMs, a series of proteins control NLRP3 activation. MicroRNAs, NO, Pyrin-only proteins (POPs) and CARD-only proteins (COPs) and Nuclear factor erythroid 2-related factor (NRF2), mainly act as negative regulators. NEK7 seems to positively regulate NLRP3 activation.[3,56,62] Syk/JNK axis allows inflammasome oligomerization.[84]

NRF2 controls antioxidant genes expression, limiting ROS levels and inhibiting NLRP3 activation. Through NF-κB activity regulation, NRF2 also transcriptionally controls NLRP3, pro-IL-1β and pro-IL-18 levels.[56]

POPs and **COPs** are decoy proteins that bind to inflammasome components through homotypic interactions. Increase of NF-κB activity, and also of IL-1β, upregulates POP1 and POP2, inhibiting NLRP3 activation and NLRP3-ASC interactions. COPs bind to pro-caspase-1, avoiding its autoactivation.[56,62] Besides COP, other CARD-containing proteins (like caspase 12) act as decoy for caspase-1.[62]

The kinases **Syk** and **JNK**, through phosphorylation, allow ASC speck formation and oligomerization with NLRP3.[84]

NEK7 regulates inflammasome assembly in what seems an inflammasome-specific interaction. Its binding to the LRR of NLRP3 is necessary for ASC speck formation and caspase-1 activation, downstream of K^+ efflux and ROS production.[21,22,56,63,64]

Another form of inflammasome regulation may be autophagy. This process of elimination of damaged cell components or even pathogens is upregulated during cell stress. Autophagy seems to inhibit inflammasome activity, either by directly targeting the complex or by inhibiting ROS production. Additionally, autophagy may inhibit pyroptosis.[62]

ASC alternative splicing can also regulate inflammasome activation since a report showed that different isoforms of ASC can inhibit or potentiate caspase-1 maturation and influence pyroptosis.[65]

1.4.2.3 Non-canonical and alternative pathways

The inflammasome can be activated by alternative pathways to the caspase-1-mediated IL-1β production (*Fig. 7*).

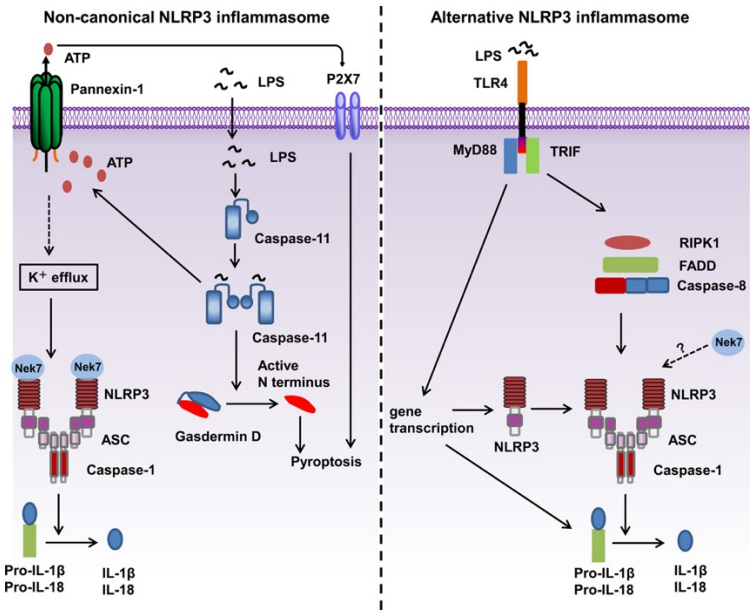

Figure 7- Non-canonical and alternative pathways of NLRP3 inflammasome activation. On the non-canonical NLRP3 inflammasome activation, LPS enters the cell by transfection or infection and binds to caspase-11, activating pannexin-1 pore and K^+ efflux. NLRP3 is activated and IL-1β is produced. Caspase-11 also mediates GSDMD-mediated pyroptosis and the resulting release of ATP activates P2X7 and further stimulates cell death. In the alternative inflammasome pathway, LPS binding to the TLR4 activates caspase-8 upstream of NLRP3 activation. There is a gradual IL-1β secretion, independent of pyroptosis. Source: He *et al*., 2016

In the non-canonical inflammasome activation, murine Caspase-11 (or caspases 4 and 5 in humans) can become activated upon LPS sensing in the cytosol, cleaving GSDMD and inducing pyroptosis. The release of pro-inflammatory mediators is thus independent of the inflammasome. This pathway is normally activated in response to Gram negative bacteria.[14,21,56,63] Also, caspase-11 opens the pannexin-1 channel, allowing K^+ efflux and NLRP3 activation[56,63] besides ATP secretion, that will activate P2X7R. These caspases are only able to cleave GSDMD and not IL-1β. It's the inflammasome activation through K^+ efflux and ATP binding to P2X7R that induces IL-1β processing.[22]

The last pathway for inflammasome activation it's the **alternative pathway**, so-called since activation of the inflammasome it's not accompanied by the usual hallmarks (ASC specks- even though ASC is required- K^+ efflux and pyroptosis).[19,56,63] This pathway it's human and porcine-specific and it's characterized by TLR4-TRIF-RIPK1-FADD-caspase 8 axis, upstream of NLRP3 activation.[19,22] LPS binds to TLR4 and induces caspase 8 activity and NLRP3 activation independently of a second stimuli.[19,56,63] It also challenges the concept that inflammasome activation is an all-or-nothing response, since a gradual release of IL-1β, without the occurrence of pyroptosis, is observed.[19] Caspase-8 is also implicated in canonical and non-canonical activation of the inflammasome and caspase-1 functions.[21] Elliot and Sutterwala hypothesize that caspase-8 may be involved in loss of mitochondrial integrity and consequent NLRP3 activation.[80]

2. Aims

This work was based in four assumptions:

i. Exacerbated neuroinflammation promotes CNS disease genesis and progression;

ii. Microglia and astrocytes are the main effectors of CNS immunity and thus responsible for the secretion of pro-inflammatory mediators (as IL-1β);

iii. Microglia is known to activate astrocytes through the secretion of soluble factors;

iv. The NLRP3 inflammasome is a crucial mechanism of IL-1β secretion and its expression in reactive astrocytes is still controversial.

Taking this into consideration, this work aimed to: i) characterize NLRP3 inflammasome expression and assembly in reactive isolated A1 astrocytes; ii) evaluate the role of NLRP3 inflammasome in IL-1β secretion from reactive A1 astrocytes; iii) assess if microglia-derived factors can promote NLRP3 inflammasome expression and assembly in A1 astrocytes.

3. Methods

3.1 Animals

Cell cultures were prepared using Sprague-Dawley rats from Charles River Laboratories (Barcelona, Spain). The European Union Guidelines (2010/63/EU) and the Portuguese law regarding the protection of animals for scientific purposes were followed throughout all experiments. The methods for this thesis were approved by the Ethical Committee of the Faculdade de Medicina da Universidade de Lisboa. All measures were taken to reduce the number of animals and its suffering.

3.2 Reagents and Drugs

Unless stated otherwise, all basic reagents were purchased from Sigma (St. Louis, MO, USA).

Table 2- Working concentration and supplier of drugs administered to astrocytes

Drug	Supplier	Working concentration
Endotoxin lipopolysaccharide **LPS**	Sigma	100 ng/mL
Adenosine 5′-triphosphate disodium salt hydrate **ATP**	Sigma	1mM
Interleukin-1α **IL-1α**	Sigma	3 ng/mL
Tumor necrosis factor-α **TNF-α**	Peprotech (London, UK)	30 ng/mL
C1q	bioNova científica, s.l. (Madrid, Spain)	400 ng/mL
MCC950	AdipoGen Life Sciences (San Diego, USA)	10 µM

3.3 Cell Cultures

Newborn Sprague-Dawley pups, with 1-3 postnatal days, were sacrificed and their brains removed in phosphate buffer saline solution (PBS, 137 mM NaCL, 2.7 mM KCL, 8mM Na_2HPO_4, $2H_2O$, 1.5 mM KH_2PO_4, pH 7.4). Under a dissection microscope, the meninges were removed, and the hemispheres separated. The cortexes were collected and mechanically dissociated in complete Dulbecco's modification of Eagle's media (DMEM, Gibco, Paisley, UK) composed of 10% fetal bovine serum (FBS, Gibco), 1% L-glutamine (Gibco) and 1% antibiotic-antimycotic solution, and then strained through a 200µm cell strainer. The resulting cell suspension was centrifuged for 10 minutes at room temperature (21º C, RT) and 1200 rpm. The pellet was resuspended in DMEM and filtered again, now through a cell strainer with 70µm. This cell suspension was centrifuged, in the same conditions as before, and the pellet was resuspended in DMEM. Cells were plated in Poly-D-Lysine (PDL) coated plates. For immunocytochemistry assays, cells (diluted 1:3 in DMEM) were seeded in glass coverslips (Corning-Costar, Corning, USA) placed into 24-well plates and coated with 25µg/mL PDL. For western blot assays, cells (diluted 1:2 in DMEM) were seeded in 60mm Petri dishes coated with 10µg/mL PDL. All cultures were incubated at 37ºC and 5% CO_2 in a humidified atmosphere. The growth medium was changed twice a week.

3.3.1 Astrocytic-enriched cultures

The protocol for astrocyte enriched cultures was executed as previously described[85–87] with slight modifications with slight modifications.

To obtain cultures enriched in astrocytes (PA), at 6-7 days in vitro (DIV) mixed glial cell cultures were shaken overnight, at 300 rpm and 37ºC, in an orbital shaker. By the end of the shaking period, the medium containing loosened microglia was changed to fresh DMEM. At 13-14 DIV, the remaining microglia was removed using clodronate encapsulated in liposomes (Liposoma BV, Netherlands), according to the protocol outlined by Kumamaru and co-workers.[85] Cells were incubated overnight with 100µg/mL of clodronate and the medium was changed the next morning.

3.4 Pharmacological approaches

To characterize NLRP3 expression and assembly, PA were subjected to different conditions: LPS stimulated, ATP stimulated, LPS/ATP stimulated, IL-1α/TNF-α/C1q stimulated (hereafter designed as "Factors (F)") and F/LPS/ATP stimulated. To assess the effect of NLRP3 inhibition on IL-1β, LPS/ATP stimulation was used together with MCC950, the selective NLRP3 inhibitor. The timeline of culture maintenance and pharmacological administration is schematized in Figure 8.

After 20 DIV, cells were either fixed or frozen at -80ºC, according to the molecular assay to be performed. For immunofluorescence assays, cells were fixed with Paraformaldehyde (PFA, Sigma) at 4% for 15 minutes at RT, after a brief washing with PBS. Fixed cells were maintained in PBS at 4ºC until further use. For western blots, cells were scrapped and stored in lysis buffer (Tris 50 mM, ethylenediamine tetraacetic acid (EDTA) 5 mM, NaCl 150 mM, 1% nonyl phenoxypolyethoxylethanol (NP-40), 10% Glycerol, pH 8) supplemented with phenylmethylsulfonyl fluoride 1mM (PMSF) and proteases inhibitors (Mini-EDTA-free, Roche, Germany) at -80ºC until further use. Supernatants were also collected and kept at -80ºC until further use.

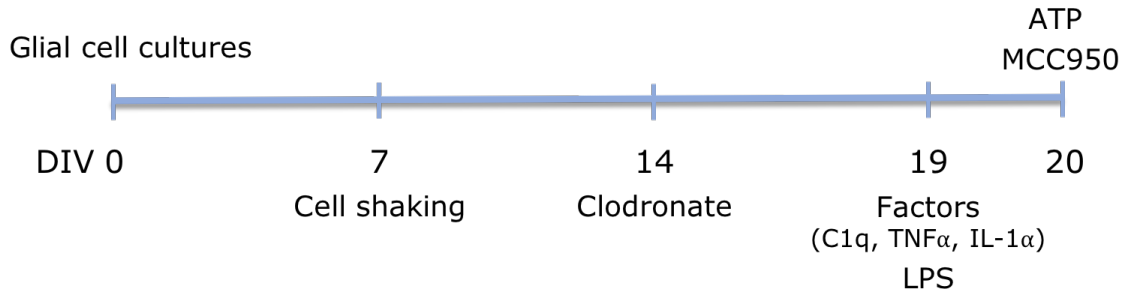

Figure 8- Astrocyte culture timeline

3.4.1 Inflammasome activation

The formation of a functional NLRP3 inflammasome requires two-steps: 1) a priming event and, 2) an activation stimulus. LPS as the priming step, and ATP as the activation challenge are well characterized in the literature and were hence chosen as stimuli for NLRP3 inflammasome induction.[56]

At 19 DIV, LPS 100 ng/mL was added to the PA for 24h (according to previous reports).[33,86,87] ATP 1mM was administered at DIV 20, 6h before the end of LPS incubation.

3.4.2 A1 phenotype induction

For induction of the reactive A1 phenotype, a cocktail of IL-1α (3 ng/mL), TNFα (30 ng/mL) and C1q (400 ng/mL), previously described by Liddelow and co-workers[51] as sufficient and necessary microglia-secreted factors for astrocytes activation, were added to PA for 24h at DIV 19.

3.4.3 NLRP3 Inflammasome inhibition

NLRP3 inflammasome inhibition was carried out with MCC950, a small molecule inhibitor of this complex.[88] MCC950 (10μM) was added to cells at 20 DIV for 6 hours.

3.5 Cell viability assay

According to previous reports[23], more than 15 to 30 minutes of ATP stimulation can lead to cell death and release of cellular content to the extracellular medium. To establish the best duration for the ATP stimulus, a CCK-8 metabolic viability assay was performed (Cell Counting kit-8, Dojindo Molecular Technologies; Rockville, Maryland).

PA cultures were prepared as described in section 3.1, but were shaken at DIV 11 and replated at DIV 13-14 in 96 multi-wells plates. For replating, cells were incubated with 0,05% Trypsin-EDTA (Gibco) for 2 minutes. DMEM was added to stop the trypsinization reaction. The cells were then diluted in DMEM and plated in 96-wells plates, coated with PDL 10μg/mL, for 48h to ensure complete adherence to the wells. ATP 1mM was then added for 15min, 30min, 1h, 3h or 6h, to cells incubated, or not, with LPS 100ng/mL (for 24h). 10μl of CCK-8 were added to the cells for 2h, the medium was changed to a clean 96 multi-wells plates and the absorbance was measured at 450 nm in a microplate reader (TECAN Infinite M200, TECAN Trading AG, Switzerland).

3.6 Immunocytochemistry

This assay was performed to evaluate the activation of astrocytes and the expression and localization of NLRP3. After cell fixation with 4% PFA, cells were incubated for 10 minutes with 0,1M Glycine (NZYTech, Lisbon). Next, cells were permeabilized with Triton X-100 0.1% in PBS for 10 min and blocked with FBS 10% for 1h at RT. Primary antibodies were incubated overnight at 4ºC and the cells were then washed with PBS supplemented with 0.05% Tween 20 (PBS-T). Cells were incubated with the fluorescent-labeled secondary antibodies for 1h at RT. The nuclear marker (Hoechst 33342, 20µM, Sigma) was added for 5min and the coverslips were mounted in Mowiol mounting medium over a microscope slide and allowed to dry.

The coverslips were observed under inverted widefield fluorescence microscope Zeiss Axiovert 200 (Carl Zeiss Inc, Germany) with a 40x Plan-Apochromat (Zeiss, Germany) with a frame size of 1240x1240 pixels. Fluorescent images were acquired using AxioVision 4 (Zeiss). 4 to 5 fields per coverslip per condition were obtained. Images were analyzed with the software ImageJ 1.44b (NIH).

Table 3- Primary antibodies utilized in immunoblot and immunocytochemistry

Primary antibodies	host	Application and dilution	supplier
NLRP3	mouse	ICC (1:500)	Adipogen
NLRP3	rabbit	WB (1:300); ICC(1:600)	Abcam
pSTAT3^{Y705}	rabbit	WB(1:500); ICC (1:600)	Abcam
GFAP	mouse	ICC (1:400)	Millipore
GFAP	rabbit	WB (1:5000)	Sigma

Table 4 – Secondary antibodies utilized

Secondary antibodies	Application and dilution	supplier
Donkey Anti-mouse Alexa Fluor® 568	ICC (1:250)	Invitrogen
Donkey anti-mouse Alexa Fluor® 488	ICC (1:250)	Invitrogen
Donkey anti-rabbit Alexa Fluor® 568	ICC (1:250)	Invitrogen
Donkey anti-rabbit Alexa Fluor® 488	ICC (1:250)	Invitrogen
Goat anti-rabbit IgG-HRP	WB (1:10000)	Biorad

3.7 ELISA

The enzyme linked immunosorbent assay (ELISA) was conducted to quantify IL-1β production and secretion. DuoSet ELISA Development kit (R&D Systems, Abingdon, United Kingdom), which detects both pro-IL-1β and mature IL-1β, was used following the manufacturer's protocol.

To quantify IL-1β in media, IL-1β standard curve was prepared in DMEM and no dilution was required for the samples. The IL-1β standard curve ranged from 1.5 to 1000 pg/mL. The IL-1β capture antibody (goat anti-rat IL-1β in PBS, 0.8 µg/mL) was added to the wells overnight at RT. The blocking step was performed with reagent diluent (RD, 1% bovine serum albumin, BSA, in PBS) for 1h at RT. The standard and samples were then added to the wells for 2h at RT, followed by incubation with the detection antibody (goat anti-rat IL-1β in PBS, 100 ng/mL). After 2h at RT, the streptavidin-HRP solution was added for 20min and then the substrate solution incubated for another 20min. Between each step, the wells were washed three times with PBST (Wash Buffer). To end the reaction, STOP solution (2N H_2SO_4) was added and the optical density was measured at 450nm, with a reference absorbance at 570 nm, using the Microplate Reader TECAN Infinite M200.

3.8 Western Blot

3.8.1 Protein extraction

For the western blot protocol, the protein content was extracted from the cells. Cells previously stored at -80ºC in lysis buffer were lysed using a 25G syringe (Terumo Europe, Leuven, Belgium) through up-and-down movements and agitated at 4ºC for 15 min in an orbital shaker. Cell lysates were centrifuged at 11696,1 rpm for 10 min at 4ºC, the supernatant was collected and stored at -20ºC for future quantification.

3.8.2 Protein extraction

For protein quantification, Bradford protein assay (Bio-Rad DC Protein Assay kit, Bio-Rad, Hercules, CA, USA) was utilized. Samples were diluted 1:10 and the manufacturer protocol was followed. A protein standard curve, ranging from 0 to 1 mg/mL of bovine serum albumin (BSA, NZYTech), was prepared. Absorbance was measured at 750nm using the microplate reader TECAN Infinite M200.

3.8.3 SDS-Page

To quantify the total expression of NLRP3, STAT3 and GFAP, Western Blot was performed. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the loading control.

Molecular weight markers (NZYColour Protein Marker II, NZYTech) and cell lysates, containing 40µg of total protein and sample buffer (36% glycerol, 12% sodiumdodecylsulphate (SDS), 0.015% bromophenol blue, 420 nM Tris, pH6.8), were boiled for 10 min at 100ºC and were run on a 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) at 120V. After proteins were separated by size, they were transferred to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad) using a current of 300A for 90 min. After electrotransference, the membranes were blocked for 1h using BSA 3% in TBS-T (20 mM Tris-base pH 7.6, 137 mM NaCl, 0,1% Tween 20, pH 7.6) and incubated overnight at 4ºC, with the primary antibodies also diluted in BSA 3%. Next, the membranes were washed with TBS-T and incubated for 1h a RT with the secondary antibodies, coupled to horseradish peroxidase (HRP). For development of the signal, ECL Plus Western Blotting Detection System (Amersham-ECL Western Blotting Detection Reagents, GE Healthcare, Buckinghamshire, UK) was used. The intensity of the signal was

measured in Chemidoc XRS+ System (Bio-Rad). Densitometry analysis was performed using ImageJ Software.

3.9 Stastical Analysis

For statistical analysis GraphPad Prim software (San Diego, CA, USA) was utilized. One-way Analysis of Variance (ANOVA) followed by Bonferroni's Comparison Test was used to compare multiple conditions. Data are represented as mean ± standard error of the mean (SEM).

4. Results

Incubating astrocytes with ATP for 6 hours doesn't lead to an increase in cell damage

For inflammasome activation, a two-step stimulation is needed.[56,92] To induce assembly and activation of the inflammasome in astrocytes, a combined stimuli of LPS (100 ng/mL) and ATP (1mM) was chosen according to previous reports.[33,37,89] However, ATP stimulation has also been associated with membrane damage and consequent cell death[75] and with release of non-maturated IL-1β.[23] Although these observations are concomitant with more recent theories that pyroptosis is the main mechanism of IL-1β release[92], it's still not fully understood how this mature cytokine is secreted from cells. In previous projects from the group, ATP incubation times were higher than the 15 min mark reported by Ferrari and colleagues.[23] Since higher incubation times may induce higher secretion of IL-1β due to cell damage, the first step in this project was to assess the cell viability of astrocytes exposed to different incubation periods of ATP, using the metabolic cytotoxicity test CCK-8 (*Fig.9*). The viability of astrocytes is presented as the percentage of cell viability in the absence of ATP. No statistical differences were found between the five different periods of ATP incubation tested (15min, 30min, 1h, 3h and 6h), either with or without LPS. Based on ISO 10993-5:2009 "Biological evaluation of medical devices-Part5: Tests for in vitro cytotoxicity", only a reduction of cell viability by more than 30% is considered a cytotoxic effect, which did not occur in this experiment. Therefore, an ATP incubation period of 6h was chosen in order to keep continuity with previous data.

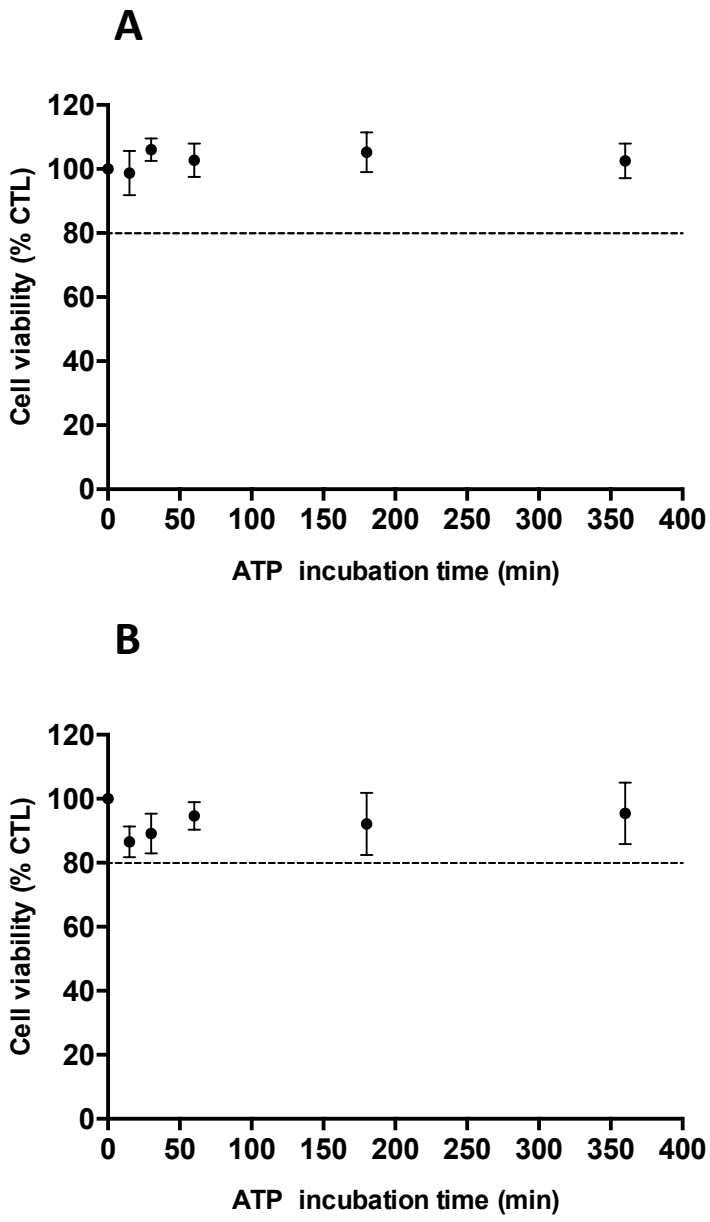

Figure 9- Viability of astrocytes when incubated with ATP. Astrocytes were incubated with ATP 1mM for 15 min, 30 min, 1 h, 3 h or 6 h in the presence **(A)** and absence **(B)** of LPS 100 ng/mL. LPS was maintained in cell medium through 24h. Viability of astrocytes is presented as the percentage of cell viability in the absence of ATP. Values are mean ± SEM from 3-4 independent cultures. Statistical tests were performed with one-way ANOVA followed by Bonferroni's test.

A combined stimulus of LPS and ATP activates astrocytes

After confirming that 24 hours of LPS (100 ng/mL) combined with 6 hours of ATP (1mM) didn't affect cell viability, the activation state of astrocytes in response to these stimuli was verified. GFAP and pSTAT3^{Y705} were used as activation markers. In pathological or inflammatory conditions, as the ones mimicked by LPS/ATP[56], astrocytes become reactive, displaying cellular hypertrophy and upregulating the expression of GFAP under a process denominated reactive astrogliosis.[43,93] As described in section 1.3.3, two distinct reactive phenotypes were proposed for astrocytes, a pro-inflammatory neurotoxic phenotype (A1) and an anti-inflammatory neuroprotective phenotype (A2), similarly to M1 and M2 subtypes used for microglia.[51]

STAT3, a signal transducer molecule from the JAK-STAT pathway, was also associated with reactive astrocytes, more specifically with the anti-inflammatory process of astrogliosis[12,43,93]. Activated STAT3 is phosphorylated in the tyrosine residue 705 (pSTAT3^{Y705}) and migrates to the nucleus, where it acts as a transcription factor[93]. Furthermore, STAT3 seems to be negatively correlated with the NF-kB pathway and consequently with NLRP3 transcription, being considered a potential marker for the A2 phenotype of astrocytes.[12,42]

Considering this the levels of GFAP and pSTAT3^{Y705} were assessed by Western Blotting, and double detection of GFAP and pSTAT3^{Y705} by immunocytochemistry was used to characterize the activation state of astrocytes in response to LPS, ATP or both.

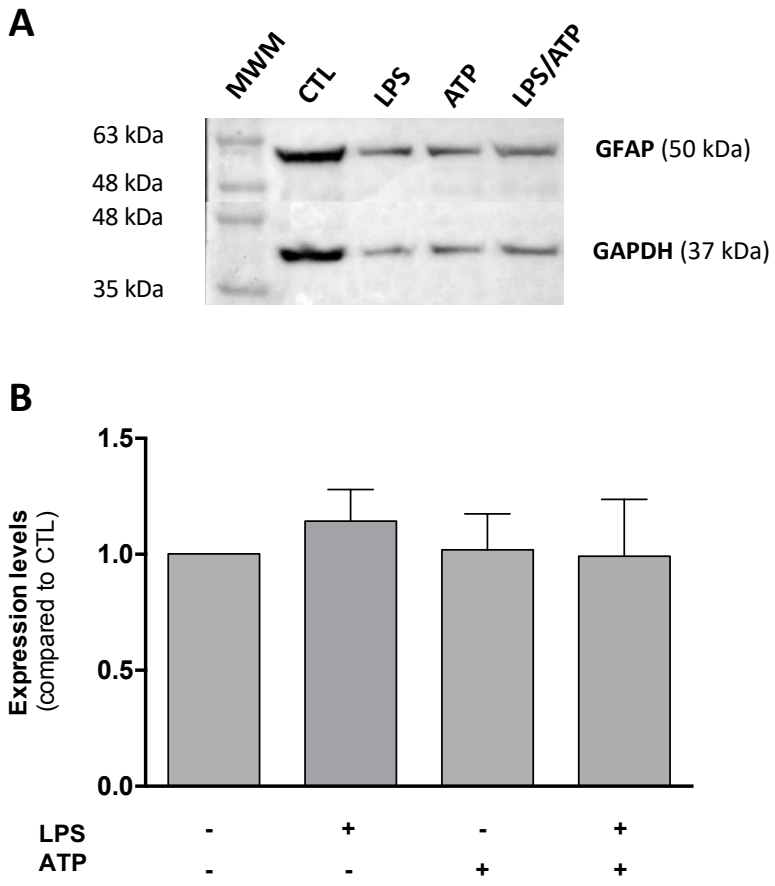

Figure 10- GFAP quantification through Western Blot in Blot in primary cultures of pure astrocytes under no stimulation (CTL) or stimulated with LPS, ATP or both. A. Representative immunoblot for GFAP and GAPDH. **B**. GFAP quantification, compared to control. All values are mean ± SEM from 5 independent cultures. Statistical analysis was performed with a One-way ANOVA followed by Bonferroni's comparison test. No statistical differences between conditions were found.

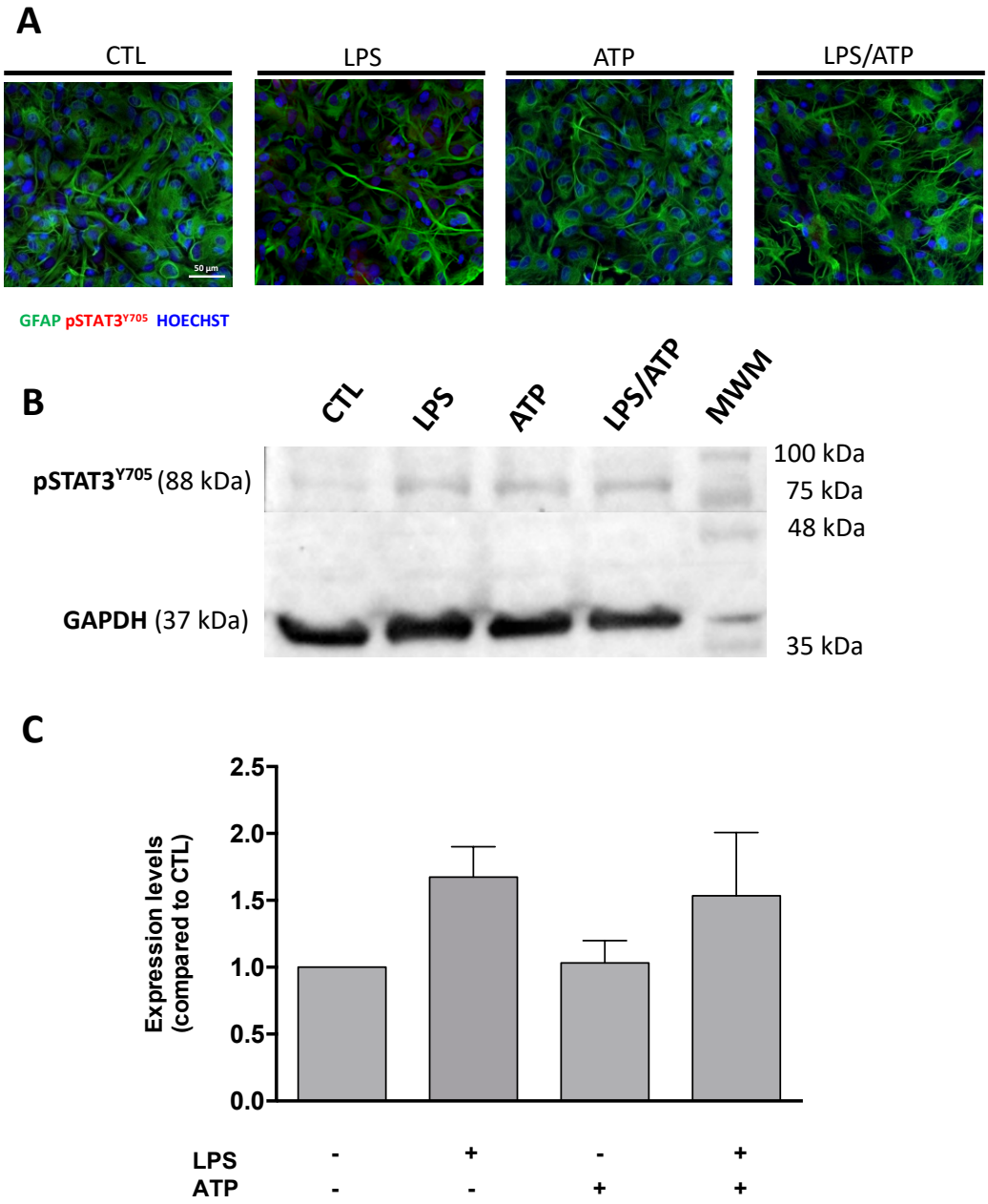

Figure 11- Detection of pSTAT3^{Y705} in primary cultures of pure astrocytes under no stimulation (CTL) or stimulated with LPS, ATP or both. A. pSTAT3^{Y705} (red) was not detected in GFAP-positive astrocytes (green). Nuclei were stained with Hoechst 33423 (blue). Images were acquired with a 40x objective in a fluorescence microscope. Scale bar, 50µm. **B.** Representative immunoblot for pSTAT3^{Y705} and GAPDH**. C.** pSTAT3^{Y705}quantification, compared to control. GAPDH was used as the loading control. All values are mean ± SEM from 4-5 independent cultures. Statistical analysis was performed with a One-way ANOVA followed by Bonferroni's comparison test. No statistical differences between conditions were found.

Regarding the expression of GFAP, no significant differences were obtained between conditions (*Fig.10*), unlike what would be expected, since reactive astrocytes become hypertrophic and are described to upregulate GFAP.[47]

Immunocytochemistry images show that astrocytes incubated with LPS, ATP and LPS/ATP depict very low expression of pSTAT3^{Y705} in the nucleus *(Fig. 11A*). Likewise, the western blot results *(Fig. 11B, C*) do not show significant differences in pSTAT3^{Y705} expression between conditions. It is worth to mention that western blots were performed with cell lysates, which contain only a very small portion of the nuclear fraction. Thus, pSTAT3^{Y705} expression in cell lysates was not expected to be significant.
Overall, and since no pSTAT3^{Y705} was found in astrocytes nucleus (*Fig.11*), we can hypothesize activation of A1 astrocytes.

Activated astrocytes express NLRP3

Next, NLRP3 protein levels were evaluated. The literature has contradictory reports regarding NLRP3 expression in astrocytes. Some authors describe NLRP3 presence in this cell type[6,44,94], but others believe that astrocytes by themselves are not able to express neither the protein nor the functional inflammasome[32]. LPS stimulus is regarded as a priming event of the canonical NLRP3 inflammasome pathway, through activation of NF-kB pathway upon binding of LPS to TLR4.[37,56,72] Thus, it was expected that under LPS and LPS/ATP stimulus, NLRP3 inflammasome expression in astrocytes was increased.
Through immunocytochemistry, a significant increase in NLRP3 expression in GFAP-positive cells was observed when the cells were stimulated with LPS and LPS/ATP(*Fig.12*). It is possible to observe a strong NLRP3 staining in some cells, indicative of increased NLRP3 expression, as a response to LPS stimulation (*Fig.12A*), which was corroborated by cell counting (*Fig.12B*).
As expected, ATP does not affect NLRP3 expression, since it is established that it acts in the oligomerization step of inflammasome assembly.

Western blot analysis didn't show significant differences between the conditions evaluated (*Fig. 13*), only a tendency for NLRP3 increased expression in LPS/ATP condition.
Overall, and considering the need for increasing sample size for western blot analysis, astrocytes respond to LPS and LPS/ATP by increasing the expression of NLRP3, probably through activation of NF-kB pathway.

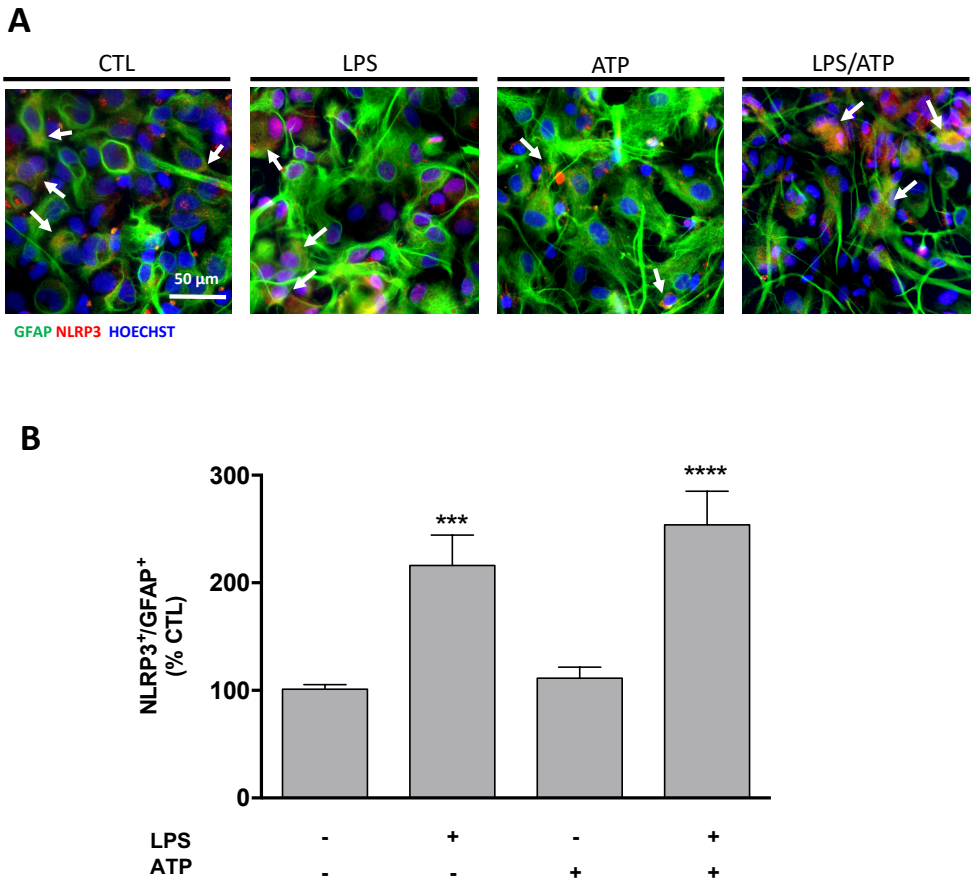

Figure 12- NLRP3 expression in primary cultures of pure astrocytes under no stimulation (CTL) or stimulated with LPS, ATP or both. A. NLRP3 (red, arrows) was detected in the cytoplasm of GFAP-positive astrocytes (green). Nuclei were stained with Hoechst 33423 (blue). Images were obtained with a 40x objective in a fluorescence microscope. Scale bar, 50µm **B.** Quantification of NLRP3-expressing GFAP-positive cells. Values were calculated as percentage of non-stimulated astrocytes (CTL). All values are mean ± SEM in 4-5 random fields per slide, from 7 independent cultures. ***p<0.001, ****p<0.0001. Statistical tests were performed with a One-way ANOVA followed by Bonferroni's comparison test.

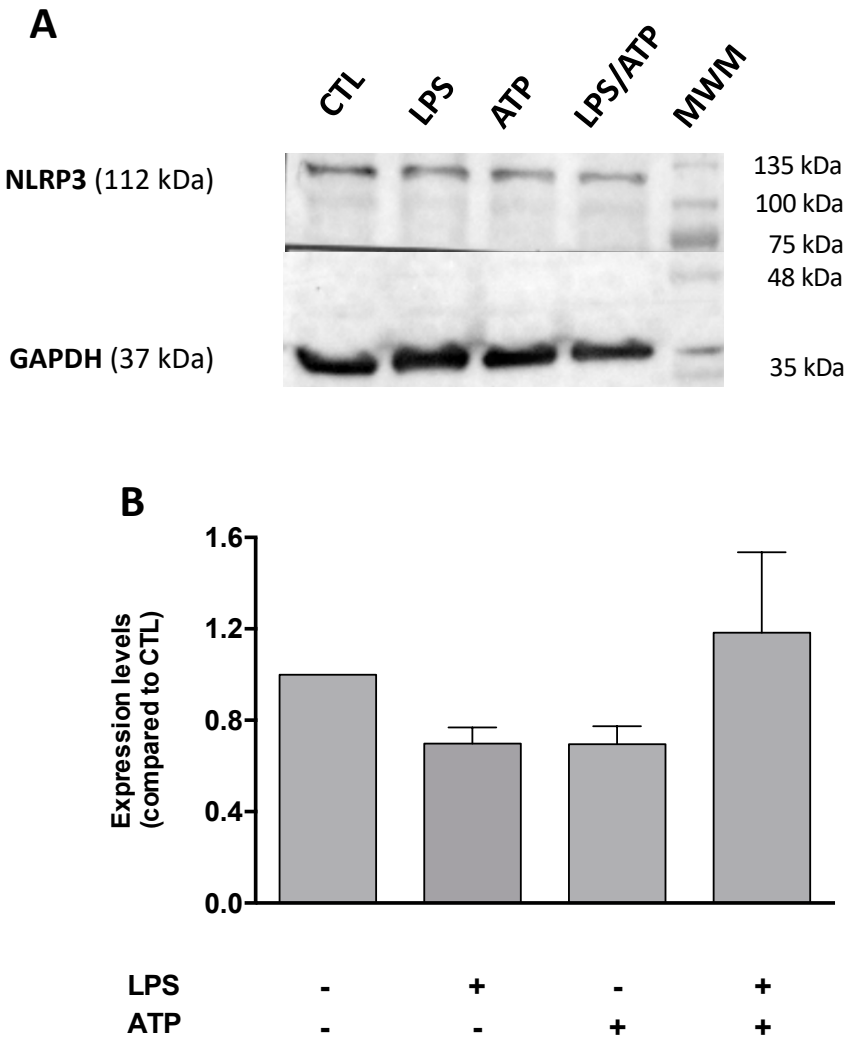

Figure 13- NLRP3 quantification through Western Blot in pure cultures of astrocytes under no stimulation (CTL) or stimulated with LPS, ATP or both. A. Representative immunoblot for NLRP3 and GAPDH. **B**. NLRP3 quantification in astrocytic cultures, compared to control. All values are mean ± SEM from 3-4 independent cultures. Statistical analysis was performed with a One-way ANOVA followed by Bonferroni's comparison test. No statistical differences between conditions were found.

Activated astrocytes secrete mature IL-1β

LPS activates the NF-kB pathway and leads to increased expression of NLRP3 domain and pro-IL-1β. ATP binds to P2X7R causing K^+ efflux from the cell and triggering inflammasome oligomerization, which activates caspase-1 and induces IL-1β maturation and secretion.[56]

Previous results in the group showed an increased IL-1β secretion in co-cultures of astrocytes and microglia comparatively to pure microglia cultures (*Supplementary data 1*). Therefore, it was hypothesized that astrocytes were a player in IL-1β secretion. To assess if astrocytes, in response to an inflammatory stimulus, were indeed capable of assembling a functional NLRP3 inflammasome, the levels of mature IL-1β secreted to the medium in PA cultures were measured through ELISA (*Fig.14)*. As expected, there is a significant increase in the secretion of this cytokine when astrocytes are incubated with LPS/ATP. LPS alone increased levels of pro-IL-1β, as observed in Supplementary data 1, but not IL-1β secretion. Only a tendency for increased IL-1β secretion was obtained under LPS insult, possibly as a result of cytokine maturation due to inflammasome activation by other cell DAMPs, or by alternative pathways of IL-1β secretion[95,96].

These results additionally hint that the activation of the inflammasome is the main pathway for IL-1β maturation, since after LPS stimulation, there isn't a marked increase of the cytokine maturation, unless the ATP activation stimulus is applied. An isolated ATP stimulus doesn't lead to an increase in IL-1β secretion, since astrocytes don't have significant basal levels of NLRP3.

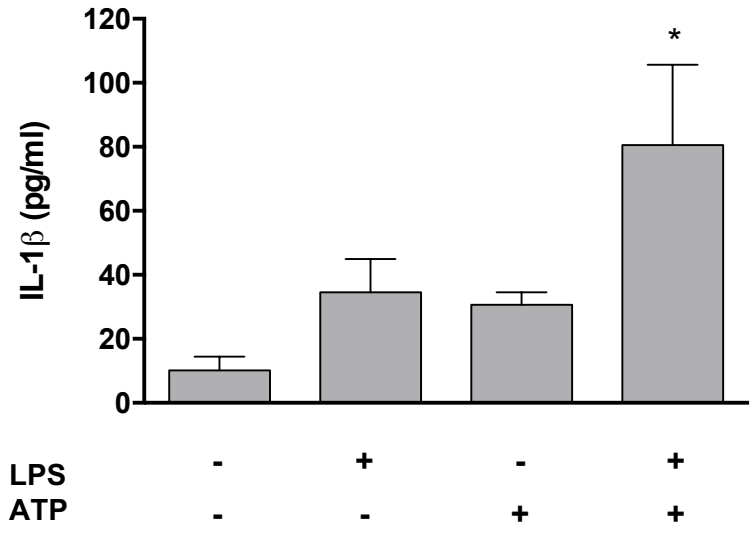

Figure 14- Secretion of IL-1β from pure cultures of astrocytes non-stimulated or stimulated with LPS, ATP or both. Results are represented as mean ± SEM from 3 independent cultures. *p<0.05. Statistical analysis was performed with a one-way ANOVA followed by Bonferroni's test.

IL-1β is produced by the NLRP3 inflammasome

Due to ELISA kit limitations in assessment of mature IL-1β levels and considering that this cytokine can be synthetized through alternative processes,[95,96] a small molecule that selectively inhibits the NLRP3 inflammasome was administered to PA after LPS/ATP insult. MCC950 is a specific inhibitor of NLRP3, described to block the canonical and non-canonical pathways.[91]

Expression of NLRP3 doesn't necessarily mean that the protein oligomerizes with ASC and activates caspase-1. Also, other inflammasomes may be at play (for example, NLRP1 which can also be activated by LPS[97]). If NLRP3 inflammasome is indeed crucial for the processing of pro-IL-1β and consequent increase in IL-1β secretion, it's inhibition should abolish IL-1β levels in the extracellular medium. Indeed, preliminary results indicate that, in pure cultures of astrocytes, NLRP3 inflammasome is the main player in IL-1β secretion, since its inhibition leads to a considerable abolishment of IL-1β release (*Fig.15)*.

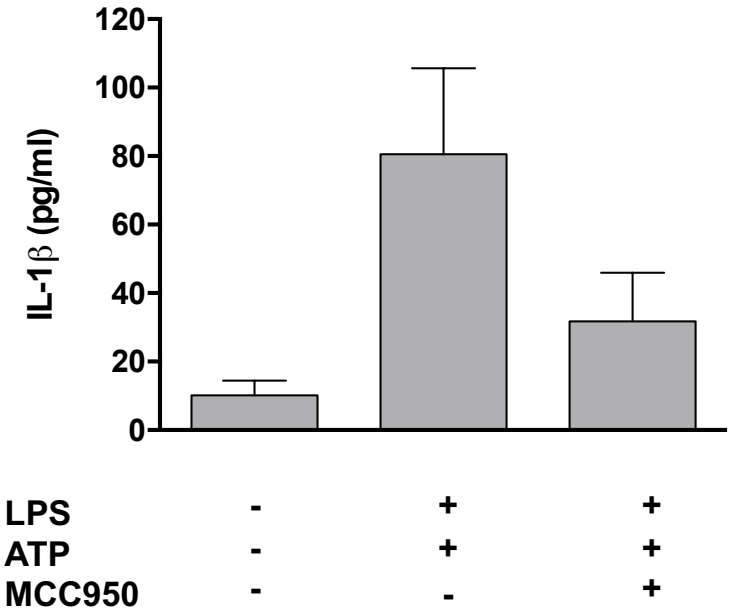

Figure 15- IL-1β levels in cell medium of astrocytic cultures non-stimulated or stimulated with LPS/ATP either with or without the NLRP3 inhibitor MCC950. Results are represented as mean ± SEM in 3-4 independent cultures. No statistical differences were found through ANOVA.

Overall, it's possible to conclude that pure astrocytes are able to respond to a pro-inflammatory stimulus by expressing a functional NLRP3 inflammasome.

Combined administration of TNF-α, IL-1α and C1q activates astrocyte

In co-cultures of microglia and astrocytes, immunocytochemistry assays with staining for the microglial marker Iba1 and NLRP3, show NLRP3 expression in Iba1-negative cells (*Supplementary data 2A, arrows)*. Furthermore, NLRP3 expression was observed in GFAP-positive cells (*Supplementary data 2B, arrowheads)*. This led to the hypothesis that the NLRP3 inflammasome could be expressed by astrocytes in response to microglia-derived signals. To further study this relation, the microglia input was simulated using a cocktail of microglia-secreted factors described by Liddelow and colleagues as sufficient and necessary for the induction of the A1 phenotype of astrocytes.[51] The cocktail was composed of three factors (F): TNF-α (30ng/mL), IL-1α (3ng/mL) and C1q (400ng/mL). Astrocytes were stimulated with either the Factors alone (F condition) or with the Factors together with LPS and ATP (hereafter called the FLA condition).

Western blot results show an increase of GFAP expression in F-stimulated astrocytes *(Fig.16)*, indicating an ability for inducing astrocytic reactivity. However, we cannot say through our results if astrocytes are indeed turning to the A1 phenotype due to the lack of an A1 marker. Nonetheless, the absence of pSTAT3^{Y705} in the nucleus of F and FLA-stimulated astrocytes (*Fig. 17A*) supports Liddelow's reports.

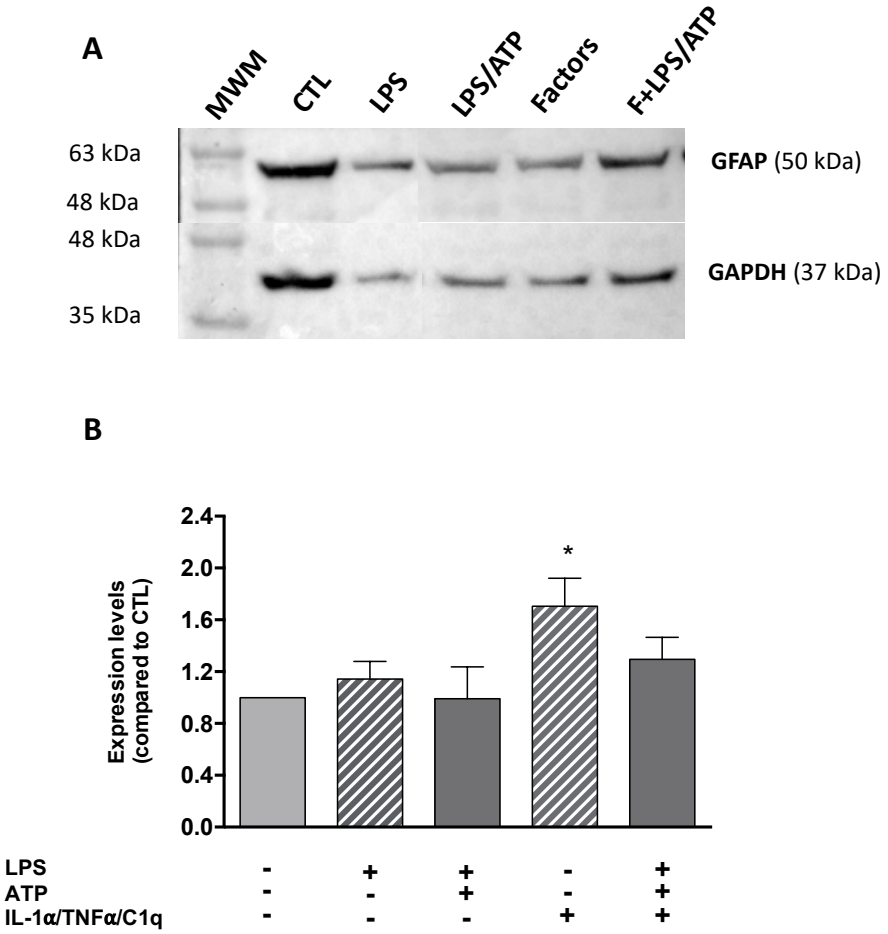

Figure 16- GFAP quantification through Western Blot in pure cultures of astrocytes under no stimulation (CTL) or stimulated with LPS, LPS/ATP, F and FLA. A. Representative immunoblot of GFAP and GAPDH. **B.** GFAP quantification in astrocytic cultures, compared to control. GAPDH was used as loading control. All values are mean ± SEM from 4-5 independent cultures. *p<0.05. Statistical analysis was performed with a one-way ANOVA followed by Bonferroni's test.

LPS/ATP, Factors and FLA didn't increase pSTAT3^{Y705} in the nucleus of astrocytes *(Fig.17A)*. Likewise, the expression of pSTAT3^{Y705} evaluated by western blot did not show any differences *(Fig.17B, C)*.

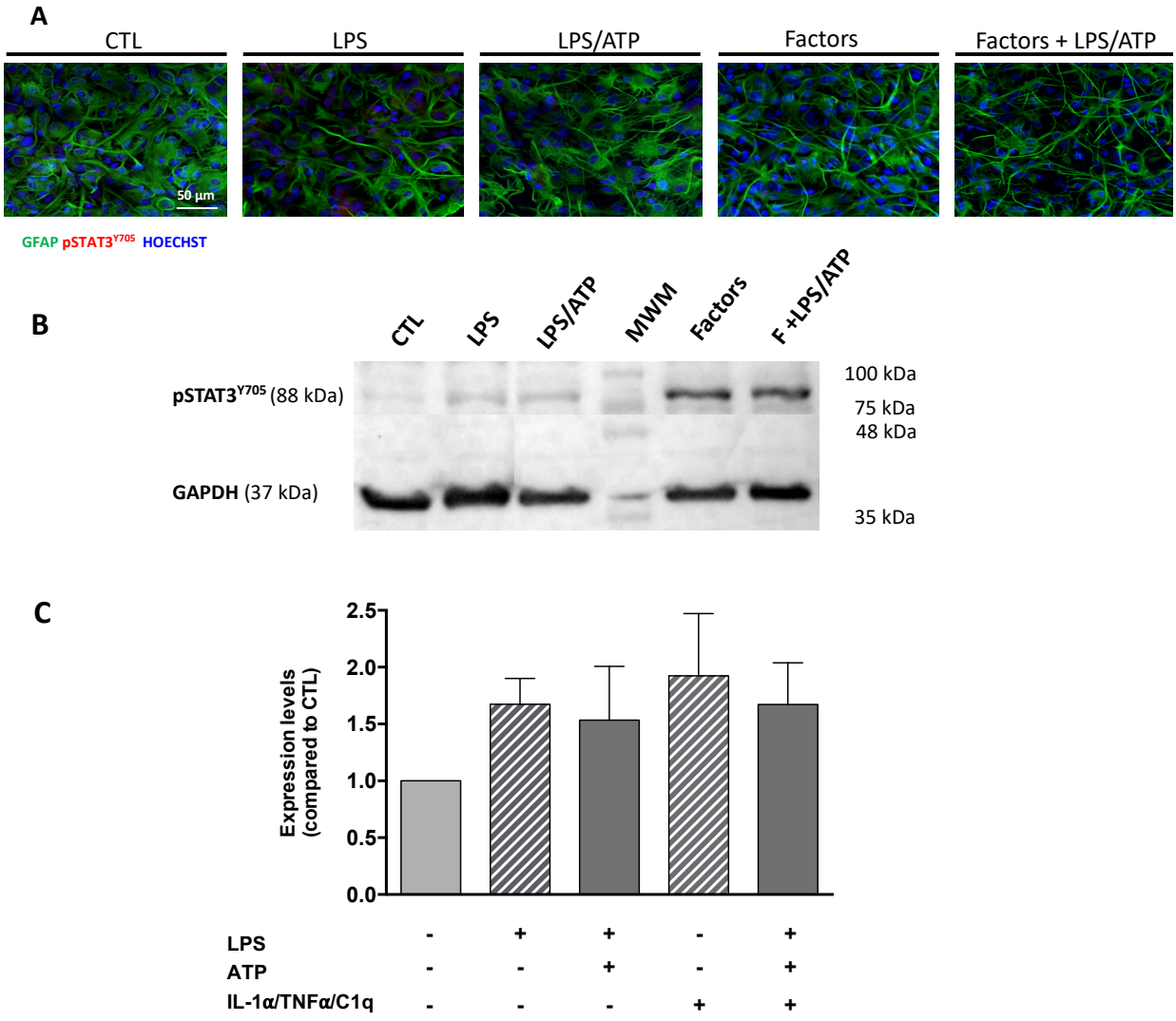

Figure 17- STAT3 expression in primary cultures of astrocytes under no stimulation (CTL), or stimulated with LPS, LPS and ATP, Factors or Factors, LPS and ATP. A. pSTAT3^{Y705} (red) was not detected in GFAP-positive astrocytes (green). Nuclei were stained with Hoechst 33423 (blue). Images were acquired with a 40x objective in a fluorescence microscope. Scale bar, 50µm. **B.** Representative immunoblot for pSTAT3^{Y705} and GAPDH. **C.** pSTAT3^{Y705} quantification compared to control. GAPDH was used as the loading control. All values are mean ± SEM from 3-5 independent cultures. Statistical analysis was performed with a One-way ANOVA followed by Bonferroni's comparison test. No statistical differences between conditions were found

Overall, our findings suggest that stimulation of astrocytes with TNF-α, IL-1α and C1q increases their reactivity.

Reactive astrocytes depict increased NLRP3 expression

Staining for NLRP3 showed an increased NLRP3 expression in astrocytes under F- and FLA-stimulation (*Fig.18)*. FLA lead to the highest increase of NLRP3 protein levels, followed by the F condition. TNF-α and IL-1α can both similarly activate the NF-kB pathway[26,55], so the factors can act as a priming event that leads to NF-κB activation and NLRP3 expression, in the same way as LPS does.

However, Western Blot of cell lysates didn't show any significant differences in NLRP3 expression between conditions (*Fig.19)*.

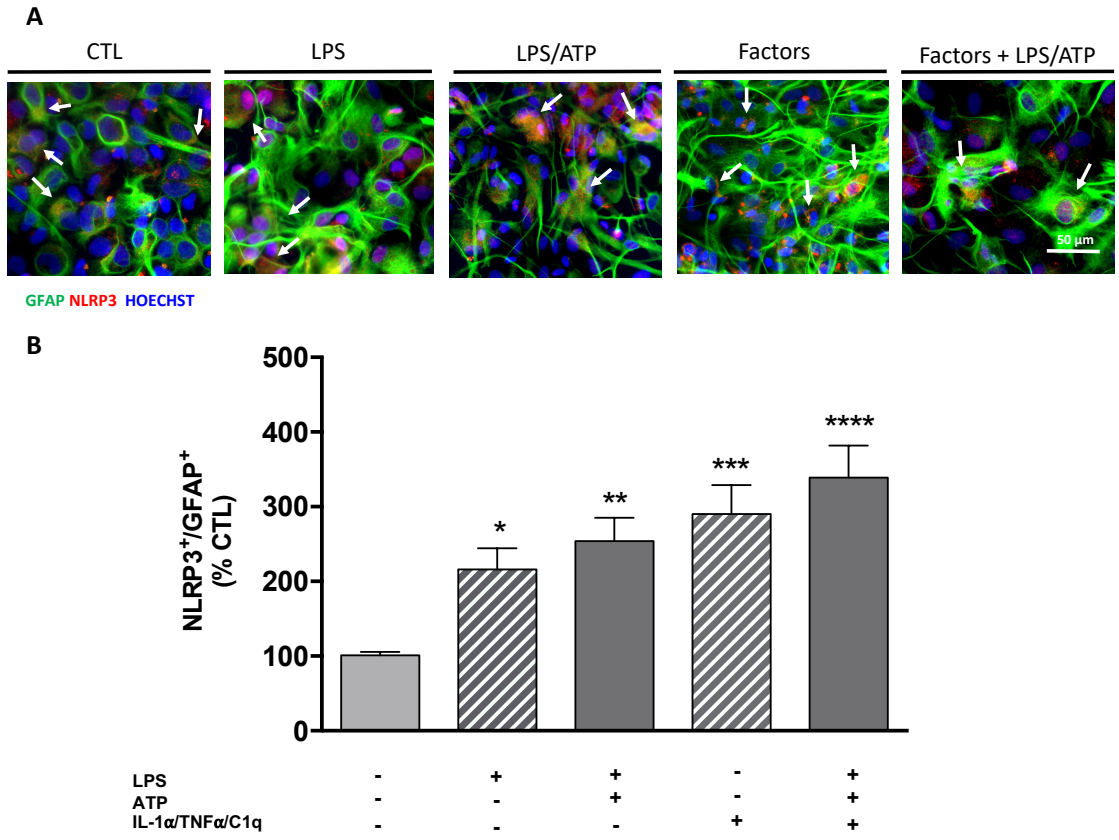

Figure 18- NLRP3 expression in primary cultures of pure astrocytes under no stimulation (CTL) or stimulated with LPS, LPS/ATP, F or FLA. A. NLRP3 (red, arrows) was detected in the cytoplasm of GFAP-positive astrocytes (green). Nuclei were stained with Hoechst 33423 (blue). Images were obtained with a 40x objective in a fluorescence microscope. Scale bar, 50µm **B** Quantification of NLRP3-expressing GFAP-positive cells. Values were calculated as percentage of non-stimulated astrocytes (CTL). All values are mean ± SEM in 4-5 random fields per slide, from 7 independent cultures. *p<0.05, **p<0.01, ***p<0.001. Statistical tests were performed with a One-way ANOVA followed by Bonferroni's comparison test.

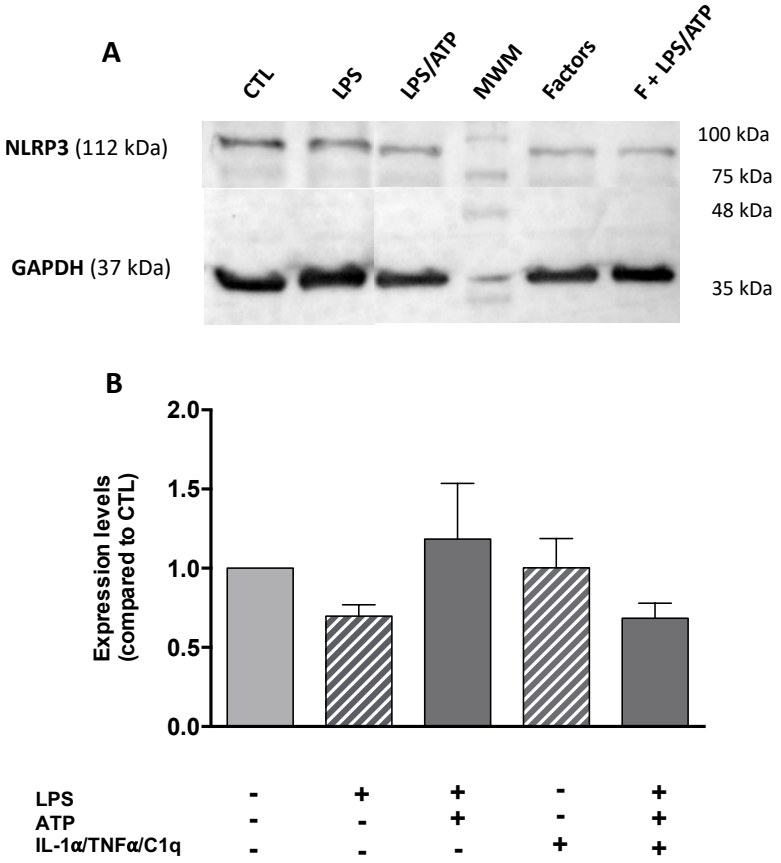

Figure 19- NLRP3 quantification through Western Blot in pure cultures of astrocytes under no stimulation (CTL) or stimulated with LPS, LPS/ATP, F and FLA. A. Representative immunoblot of NLRP3 and GAPDH. **B.** NLRP3 quantification in astrocytic cultures, compared to control. GAPDH was used as loading control. All values are mean ± SEM from 3-4 independent cultures. No statistical differences were found through ANOVA.

Microglia-induced reactive astrocytes express a functional NLRP3 inflammasome

To test if priming was followed by NLRP3 activation and IL-1β secretion, ELISA was performed.

ELISA analysis didn't show differences in IL-1β secretion between conditions (*Fig.20)*. However, there is a tendency for increased secretion in LPS/ATP and FLA conditions. This supports the hypothesis that microglia-derived factors act as a priming stimulus that can lead to inflammasome assembly when an activation signal is later delivered to the cells.

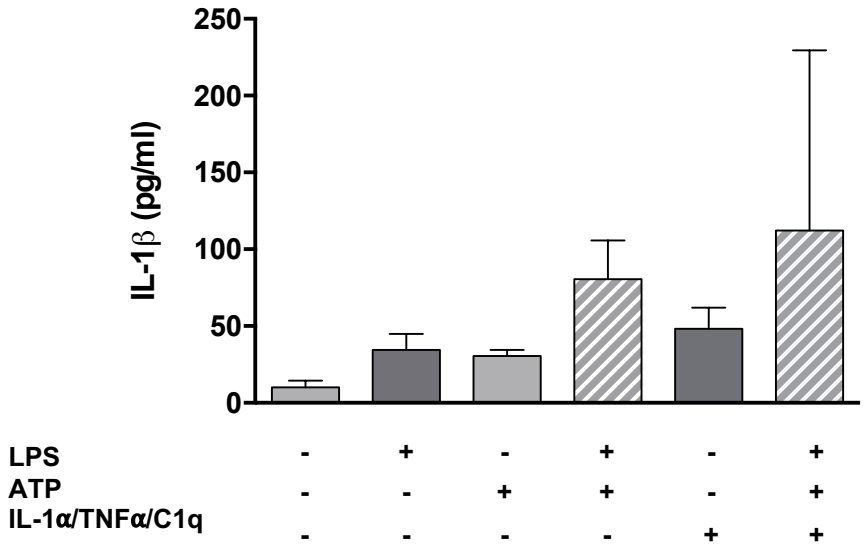

Figure 20- IL-1β levels in cell medium of pure cultures of astrocytes non-stimulated or stimulated with LPS, ATP, LPS/ATP, F and FLA. Results are represented as mean ± SEM in 3 independent cultures. No statistical differences were found through ANOVA.

5. Discussion

ATP effect

To access if ATP stimulation of astrocytes was inducing cell death, a metabolic assay for cytotoxicity was utilized. No statistical differences between the various ATP incubation periods were observed, in presence or absence of LPS. According to the results obtained, astrocytes don't become damaged under ATP stimulation. This is important for the ELISA analysis carried out throughout this thesis, since the capture antibody used in this assay doesn't distinguish between the immature 31 kDa and the mature 17 kDa forms of IL-1β. If the cells were undergoing cell death, with the consequent release of its content to the outer space, the levels measured by ELISA would be indicative of both pro-IL-1β and IL-1β. This is not ideal, since only the activated form of the cytokine is physiologically active. [20,22] CCK-8 results allow us to assert the absence of ATP effect upon cell viability and thus confirm that the levels of IL-1β reported are indeed due to the selective secretion of the mature form of this cytokine.

However, the literature states that ATP induces pyroptosis through caspase-1 mediated GSDMD cleavage and insertion in the plasma membrane.[98] Furthermore, a continuous stimuli of high concentrations of ATP, that far surpasses the physiological concentrations normally found in cells (studies suggest a physiological interval of 20 to 200μM of ATP at the plasma membrane[23]) should lead to cell pyroptosis. Previous reports showed that pyroptosis can occur after 6 hours of stimulation.[99] Also, it has been shown that ASC specks can cleave pro-IL-1β into the mature form in the extracellular matrix.[100] Finally, pyroptosis has been considered as the main pathway for IL-1β secretion.[101] Considering all this, CCK-8 results should've reflected an increase of cell death, particularly in astrocytes incubated with both ATP and LPS, as a reflection of inflammasome activation. The lack of cell death in our assay supports a previous report that states that, against what was previously hypothesized, IL-1β secretion is not an "all-or nothing"[19] response and so, there may be another mechanism regulating the secretion of this cytokine. Indeed, a vesicle-mediated mechanism for IL-1β secretion has been proposed.[102]

STAT3 as a marker of reactivity

STAT3 has been established as a marker for astrogliosis and scar formation after CNS insults.[12,103] Later, it has been proposed as a marker for anti-inflammatory, A2 astrocytes.[12,42,43] The JAK/STAT3 pathways is activated through the binding of cytokines, hormones or growth factors to receptors with associated JAKs. The JAKs phosphorylate and recruit STAT3. STAT3 is phosphorylated in Tyrosine residue 705, dimerizes and translocates to the nucleus where it acts as a transcription factor. This pathway is considered to interact with the NF-kB pathway, inter-regulating each other, but with opposing dynamics.[42,93] Considering this, NF-kB pathway activation, and consequently NLRP3 expression, should be negatively correlated with STAT3 translocation to the nucleus.[42,93] Indeed, in the conditions evaluated there wasn't $pSTAT3^{Y705}$ staining in the nucleus of GFAP positive cells through immunocytochemistry.

Regarding the cytokine cocktail, TNF-α and IL-1α can bind to JAK-associated receptors and start JAK/STAT3 pathway.[93] This may explain the tendency for a slight increase in $pSTAT3^{Y705}$ expression observed in F-stimulated astrocytes.
LPS is a known inducer of STAT3 phosphorylation in the Serine residue 727, through MAPK[93,104] and phosphorylation in this residue can inhibit transcriptional responses to STAT3 and inhibit tyrosine phosphorylation[104], but this was not assessed in this work. The lack of replication, in the FLA condition, of the tendency observed for the F stimulated-astrocytes may be due to STAT3 inhibition through Ser 727 phosphorylation instigated by LPS.

Overall, the lack of nuclear $pSTAT3^{Y705}$ supports the induction of the A1-like neurotoxic astrocytes by LPS and by the cytokine cocktail, corroborating the literature.[105]

However, to truly asses if TNFα, IL-1α and C1q promote the A1-like astrocytes, and considering the complicated relation between JAK/STAT3 and NF-kB pathways, a more straightforward marker that positively correlates with the A1 phenotype should be chosen hereafter. Lipocalin 2 (LCN2) has been described has a promoter of the pro-inflammatory phenotype of reactive astrocytes[106,107], being a possible candidate for astrocytes A1. However, LCN2 production is associated with STAT3 activity[108], making it less attractive. A second, better candidate is C3, a central protein in the complement cascade, that is released downstream of NF-kB activation.[57] C3 has previously been suggested as a marker for the

neurotoxic A1 phenotype[109,110], turning it into the best hypothesis for A1 phenotype marker in future analysis.

Besides immunocytochemistry, performing quantitative PCR in a subset of genes related to A1 astrocytes (like lcn2 and serpina3n) could also be used to access the phenotype.[51,107]

GFAP in astrocytes' reactivity

STAT3 acts as a transcription factor, regulating expression of GFAP and vimentin, the intermediate filaments known to be increased in astrogliosis.[42,93,109] However, microglia-induced reactivity in astrocytes has been shown to decrease their GFAP levels.[46] Together, these reports suggest that A2 astrocytes show increased STAT3 expression and consequently GFAP levels, while microglia-induced reactive astrocytes (potentially A1) have a decrease in GFAP levels. For this reason, astrocytes exposed to LPS and Factors (either isolated or in combination) should show decreased GFAP expression. However, GFAP levels quantified through Western Blot were generally not significantly changed between conditions, suggesting that an increased number of cultures must be processed in order to achieve statistical significance.

It is important to consider that primary cultures do not recapitulate *in vivo* conditions, and the gene expression in cultured astrocytes is profoundly altered comparatively to the physiological conditions.[111] Any differences in gene expression and protein production observed in this study doesn't necessarily translates to what occurs in the CNS under inflammatory conditions. NLRP3 expression and inflammasome oligomerization should be analyzed in more *in vivo*-like models, like organotypic cultures, and later in mice/rat models.

Microglia-derived factors effect on astrocytes

In the FLA condition, astrocytes have an increased NLRP3 expression and IL-1β secretion comparable to the changes verified in astrocytes stimulated with LPS/ATP only. Since these expression and secretion profiles in LPS/ATP-stimulated astrocytes seems to be due to inflammasome activity, it was hypothesized that the changes verified in FLA-stimulated astrocytes is also due to the inflammasome activity.

The results suggest that the factors by themselves are able to act as a priming stimulus for the inflammasome, since NLRP3 expression increases either in the presence or the absence

of the LPS/ATP stimuli, when the factors are applied. Indeed, TNF-α and IL-1α both activate the NF-kB pathway, leading to an increase of NRLP3 and pro-IL-1β. They also activate MAPK which could inhibit STAT3. [20,26,55] However, as previously explained, they can also bind to JAK-associated receptors and activate the JAK/STAT pathway.[93] Regarding C1q, is the first recognition subcomponent of the complement classical pathway. The classical pathway is initiated by antigen antibody complexes binding to C1q.[57] C1q is linked to increase in mtROS production, an inducer of the inflammasome.[56] In the peripheral immune system, it is known that C1q binds monocyte-derived immature dendritic cells, causing the NF-κB nuclear translocation, which induces expression of many genes, as NLRP3.[112]

Concomitant with the priming effect of the Factors, through ELISA, there was a tendency for increased IL-1β secretion only when, besides the factors, LPS/ATP was added. The absence of statistical differences may be resolved by increasing the number of cultures, since it is hard to normalize the plating and handling in independent cultures. In this way, differences in cell density can lead to variations in the results that affect the statistical analysis.

Taking this into account, one can state that the Factors act as a priming event that transcriptionally increase NLRP3 protein and pro-IL-1β levels in astrocytes, but require ATP as the activation stimuli for cytokine maturation. If this is the case, it is important to evaluate the patterns of NLRP3 expression and IL-1β secretion in F/ATP stimulated astrocytes.

It is also important to point that there are other secretor pathways for IL-1β that may be contributing for the ELISA results. PR3, cathepsin G and matrix metalloproteinases can all process the cytokine. [2] For this reason, the NLRP3 inflammasome inhibitor approach used in the LPS/ATP condition should also be replicated in the FLA condition.

5.1 Future perspectives

To further verify if the Factors act as a priming stimuli for the NLRP3 inflammasome and ATP can act as an activation signal in pure astrocytes, two more conditions must be studied: 1) PA stimulated with Factors/LPS, and 2) PA stimulated with Factors/ATP. All analysis mentioned in this discussion should therefore be applied to these conditions.

The number of cultures incubated with LPS/ATP/MCC950 should be increased in order to reach statistical significance in ELISA analysis. Also, an evaluation of NLRP3 complex assembly, through immunoprecipitation of NLRP3 and subsequent assessment of caspase-1 and ASC by western blot, would complement these results. Indeed, immunoprecipitation assays were attempted but the protocol still requires further optimization. Additionally, complex formation could be observed with colocalization studies, through an immunocytochemistry with primary antibodies against NLRP3 and ASC and secondary antibodies coupled, for example to ALEXA-FLUOR 488 (green emission) and ALEXA-FLUOR 568 (red emission). NLRP3 complex will be visualized as yellow dots in the cytoplasm of the cells.

To further complement this study, caspase-8, caspase-11 and caspase-1 presence should be assessed, to rule out the interference of the alternative and non-canonical inflammasome pathways from the effects observed. Caspase-1 quantification was attempted in this work, but the lack of antibody sensitivity and the high amounts of pro-caspase in cells impaired caspase-1 quantification. A flow cytometry approach through FLICAs may help increase sensitivity.

There are a number of PTMs that can change NRLP3 an even ASC activity, leading to different activation states of these proteins.[56,60] mRNA levels of NLRP3, ASC, caspase-1 and pro-IL-1β should be accessed to confirm the transcriptional role of the priming stimuli (since other factors, like PTMs could be interfering with the activity of the proteins).
Interestingly, ASC alternative splicing can regulate the activity of the inflammasome.[65,113] It would be interesting to see the effects of the microglia secreted factors in ASC isoforms and caspase-1 activation.

It should also be considered that different activation stimuli lead to different outcomes in the cell response. Binding to different receptors leads to different signaling pathways activation

and different transcriptional and regulatory effects. [56,93] The response observed in astrocytes to microglial factors should be assessed in the future with stimulation from other known activators of the inflammasome (like nigericin, particulate matter and HMGB1).[56,114,115]

6. Conclusions

Overall, it is possible to conclude that cultured pure astrocytes possess a functional NLRP3 inflammasome. Furthermore, astrocytes respond to microglia-derived stimuli by acquiring a reactive phenotype (likely A1) and increasing the production of the NLRP3 subunit of the inflammasome, preparing the response to a possible danger signal. Thus, the factors secreted by microglia can license astrocytes to respond to, for example, danger signals secreted by neighbor cells, activating the NLRP3 inflammasome. The consequent secretion of pro-inflammatory mediators, such as IL-1β, by activated NLRP3 in astrocytes may, in a more complex setting, contribute to neuroinflammation and disease progression in the CNS.

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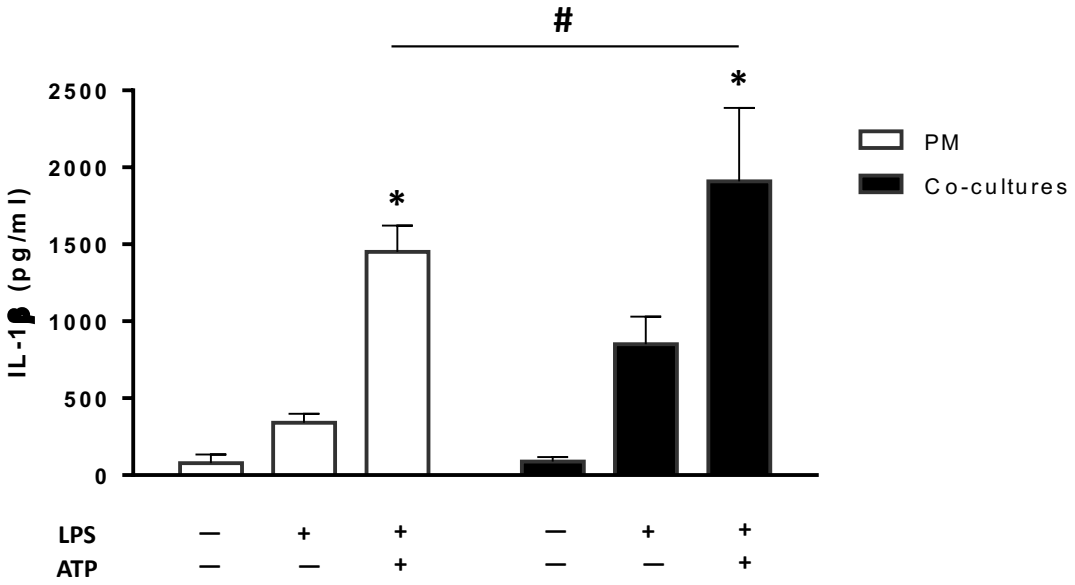
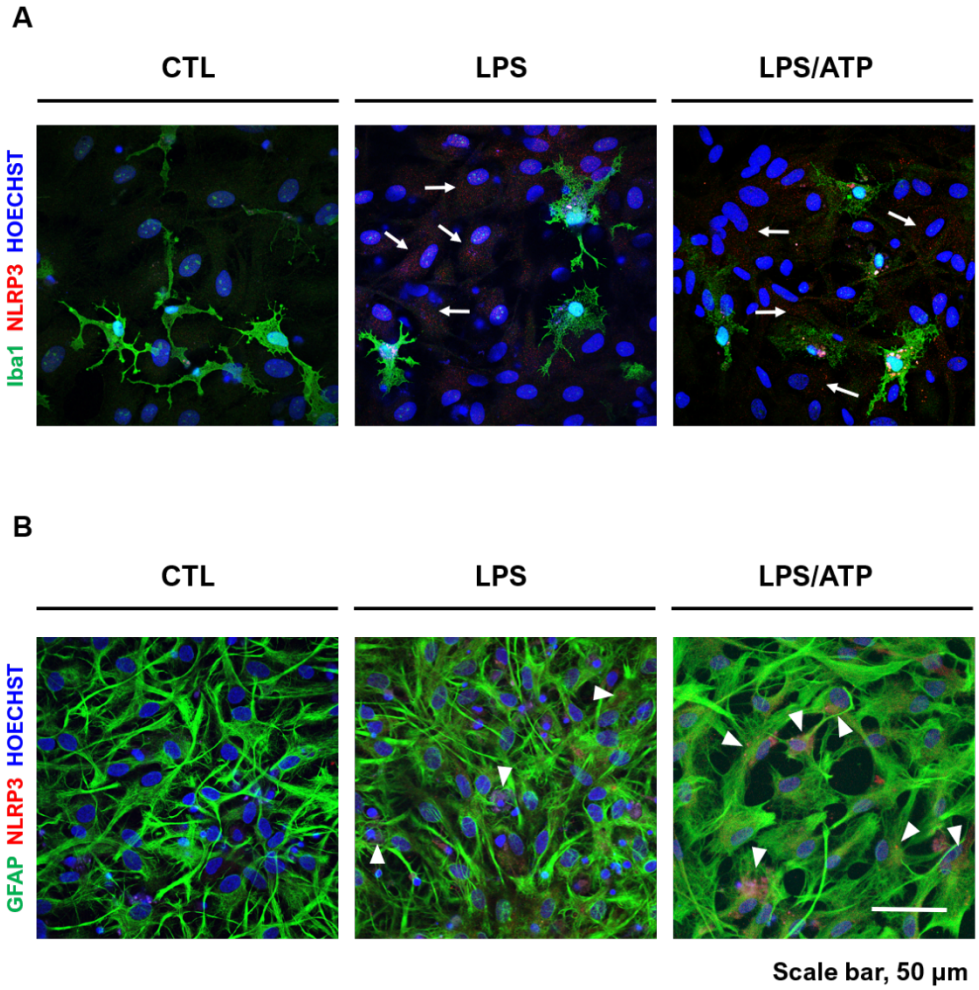
Supplementary Data



Supplementary Data 1- IL-1β released from pure microglial cultures (PM) and co-cultures of astrocytes and microglia (CoC) non-stimulated or stimulated with LPS and LPS/ATP. Results are represented as mean ± SEM in 3 independent cultures. Statistical analysis was performed with a one-way ANOVA followed by Bonferroni's test. *$p<0.05$ vs CTL, within the same culture, #$p<0.05$ between type of cultures.



Supplementary data 2 - NLRP3 expression in Co-cultures non-stimulated or stimulated with LPS and LPS/ATP. A. Immunocytochemistry of microglia labeled with Iba1 (green), NLRP3 (red) and Hoechst 33423 (blue). **B.** Immunocytochemistry of astrocytes labeled with GFAP (green), NLRP3 (red) and Hoechst 33423 (blue). Images were obtained with a 40x objective in a confocal microscope. Scale bar, 50µm. Arrows point to NLRP3 expression in Iba1-negative cells, and arrowheads indicate NLRP3-expressing GFAP-positive astrocytes.