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Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre, realizada sob orientação científica da Doutora Andrea Patrícia Ribeiro da Cruz, Investigadora Pós-Doc no Instituto de Biologia Molecular e Celular da Universidade do Porto (IBMC) e do Doutor António Francisco Rosa Gomes Ambrósio, Investigador Principal no Instituto de Imagem Biomédica e Ciências da Vida da Faculdade de Medicina da Universidade de Coimbra (IBILI)

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“Don´t take any wooden nickels when you sell your soul”
Music by Eels

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Abstract
Microglia are the resident innate immune cells of the CNS. These cells are capable of actively scanning the environmental tissue in the search for cues that might endanger the brain. This vigilant state is known as the 'resting state' of microglia. Microglial cells are very sensitive, responding to the slightest change in brain homeostasis. They possess phagocytic ability that allows for the clearance of cellular debris and pathogens in order to maintain CNS homeostasis. In this resting state, microglia is not inactive. These cells perform several active roles to maintain CNS integrity and playing supportive actions during development. Microglia is known to control synaptic activity by directly interacting with synaptic structures; regulating neuronal survival and neurogenesis; and interacting with other components of the CNS, such as oligodendrocytes and astrocytes, in order to provide an effective functioning network between all elements of the CNS. Microglia originates from progenitor cells from the yolk sac that colonize the brain during early stages of development. At the appropriate stage of development, the blood-brain barrier is formed creating a physical separation between the CNS and the periphery. This allows for the settling of an autonomous population of microglial cells in the CNS, providing this system with specialized immune features. Upon encounter with a potentially harmful situation, microglial cells are able to engage in a spectrum of complex alterations leading to their activation. These modifications include morphological transformations and the release of several inflammatory mediators, such as cytokines and also neurotrophic factors. Although microglia activation can induce a positive outcome in CNS homeostasis, if this situation is uncontrolled or becomes chronic it can have serious detrimental effects in the brain leading to neuronal death and other consequences that may favour the progression of neurological diseases. Therefore, focusing on the processes following this activation event is crucial to develop new therapeutic targets for these devastating diseases. However, microglia deactivation is a very unstudied mechanism, contrary to microglia activation. RNA-binding proteins (RBPs) are key regulators of numerous cellular mechanisms, involving gene regulation. They play crucial roles in every aspect of mRNAs, since splicing control, post-transcription regulation, mRNA transport, stabilization and translation. Studies have shown that RBPs are involved in the biology of several cytokines, influencing activation mechanisms of macrophages. In this thesis, we aim at characterizing the microglia deactivation process and assessing the potential role of RNA-binding proteins in this process as well as in the activation of microglia. We started by establishing an in vitro protocol that allowed for the study of the deactivation process; for this, we analysed several time-points after microglial activation. We observed that these cells do not present any significant changes in cytokine expression/production 72 hours after the activation period, allowing us to define this as the 'deactivation time-point'. Deactivated microglia revealed a lower threshold for activation in the presence of a second stimulus than naive cells, analysed by inflammatory

mediators RNA expression. This suggests that microglia might become ‘primed’ after a first insult to respond stronger when facing another insult, raising the interesting question of whether these cells may retain a sort of memory from past events as an adaptive feature. We next evaluated the profile of several RBPs candidates during activation and deactivation of microglia. Our results show a change in expression of all candidates during the activation process, but only one revealed a different profile during the deactivation process comparing with controls, which was Nova1. This provided us with strong evidence that RBPs are actively involved in microglia physiology. Additionally, we analysed the intracellular localization of these RBPs and observed changes in their localization associated with the different microglia states: resting, activation and deactivation. Focusing on Nova1, we tested its expression in microglia subjected to different stimuli and concluded that Nova1 expression is only altered upon LPS stimulation. LPS is a ligand for toll-like receptor 4 (TRL4), suggesting that Nova1 is somehow involved in the signalling pathways of TRLs in microglia. Finally, we observed that deficiencies of the chosen RBPs directly impact on the ability of microglia to respond to LPS, by altering the expression/ production of several inflammatory mediators

A tight regulatory program over microglia inflammatory actions is crucial to avoid impairments in the CNS, whether by controlling translation of mRNAs encoding for inflammatory mediators or by stabilizing and degrading them, among others. Overall, this thesis provides new perspectives to further expand our understanding on the regulation of activation/deactivation of microglia.

INDEX

FIGURES

ABBREVIATIONS

RBP	RNA-binding protein
Nova1	neuro-oncological ventral antigen 1
PAPBC1	polyadenylate-binding protein, cytoplasmic 1
PABPN1	polyadenylate-binding protein, nuclear 1
YBX1	Y-box binding protein 1
A2BP1	ataxin 2-binding protein 1
PTBP1	polypirimide tract binding protein 1
UTR	untranslated region
BDNF	brain-derived neurotrophic factor
EGFP	enhanced green fluorescent protein
shRNA	small hairpin RNA
RNP	ribonucleoprotein
mRNA	messenger RNA
E	embryonic
Pre-mRNA	precursor mRNA
CNS	central nervous system
BBB	blood-brain barrier
PNS	peripheral nervous system
TGF	transforming growth factor
TNF	tumour necrosis factor
OPC	oligodendrocyte precursor cell
GABA	gamma-amino butyric acid
TNFR	TNF receptor
PET	positron emission tomography
fMRI	functional magnetic resonance imaging
PAMPS	pathogen-associated molecular patterns
TLR	toll-like receptor
AD	Alzheimer’s disease
PD	Parkinson’s disease
HD	Huntington’s disease
ALS	amyotrophic lateral sclerosis
Aβ	amyloid beta
LPS	lipopolysaccharide
NO	nitric oxide
IL	interleukin
IGF	insulin-like growth factor
MHC	major histocompatibility complex
EAE	experimental autoimmune encephalomyelitis
IFN	interferon
ECM	extracellular matrix
POMA	paraneoplastic opsoclonus-myoclonus ataxia
ER	endoplasmic reticulum
AREs	AU-rich elements
PRR	pattern recognition receptor

INTRODUCTION

1. Components of the Central Nervous System (CNS)

Several types of cells constitute the normally functioning brain tissue. Neuronal physiology is supported and preserved by glial cells, which have highly diverse and incompletely understood functions. These functions may include myelination, secretion of trophic factors, maintenance of the extracellular environment and ionic homeostasis, and clearing of molecular and cellular debris [1, 2].

Glial cells also participate in the formation and maintenance of the blood-brain barrier (BBB), a highly selective permeability barrier that separates the circulatory system from the brain and that serves as the molecular gateway to brain tissue [3].

The term neuroglia, or “nerve glue”, was devised in 1859 by Rudolph Virchow, who conceived of the glia as an inactive “connective tissue” holding neurons together in the CNS [1]. Metallic staining techniques developed by Ramón y Cajal and del Rio-Hortega allowed to distinguish three types of supporting cells in the CNS: oligodendrocytes, astrocytes and microglia. In the peripheral nervous system (PNS), Schwann cells are the major glial component.

1.2. Glia vs. Neurons

All types of glia have physiological characteristics that impact on neural connectivity, both at the structural and functional level. They respond to neurotransmission and influence these complex maps in several ways, being equally important for the neural information connectivity networks as neurons themselves [4].

Glia and neurons differ in very contrasting ways, from morphology and signalling mechanisms to spatial localization and metabolic rates. Although, glia and neurons share common neurotransmitter-based signalling systems [5, 6, 7] and thus they are part of a mutual system and glia is now righteously considered a crucial component of the connectivity pathways operating in the brain.

Furthermore, there is a dependence of neurons from glia, as glial cells offer support for metabolic functions [8], regulation of synaptic transmission [9], local regulation of blood flow to small populations of neurons [10] and glial cells also able to provide growth factors, cytokines and other neuromodulatory molecules that help regulate neuron structure, function and connectivity, for example brain-derived neurotrophic factor (BDNF), cholesterol, ephrin,

transforming growth factor beta (TGF beta) and tumour necrosis factor (TNF)[4, 7].

The components of glia impact all three scales of neuronal interactions: long-range communications, local circuits and individual synaptic connections. Astrocytes, through their highly organized distribution, allow the formation of compartments for neural connectivity, modulating volume transmission within the extracellular space [11]. They are also able to guide neurite outgrowth [12] , regulate structural dynamics of dendritic spines, stimulate synaptogenesis and remove synapses [13]. Microglia can also remove synapses in an activity-dependent manner, thereby impacting directly on neural connectivity networks [14].

Astrocytes and microglia produce growth factors that modulate neural stem cell differentiation and therefore they play a role in neurogenesis [7, 15, 16].

Oligodendrocyte progenitor cells (OPCs) continue their proliferative process even in the adult brain, indicating the existence of a re-myelinating process in the mature CNS, which consequently may provide a restructuring and optimization of long-distance networks between neurons during adulthood [17].

Glia elements connect with each other through numerous mechanisms. Astrocytes communicate with each other and other cells by gap junctions and also by neurotransmitter-based signalling that enables selective communication with other astrocytes that possess the respective membrane receptors [18]. Microglia, astrocytes and oligodendrocytes all connect with each other and also with neurons through diffusible molecules that can be produced by contact-mediated signalling pathways [4].

Taken together, these evidences demonstrate the importance of considering the architectural and functional mechanisms underlying all components of the CNS, and not focusing exclusively on neural physiology, in order to understand the complex connectivity networks of the brain.

However, and besides neural networks are quite well characterized, the physical and functional arrangements between glial cells are not well established.

2. Microglia

Pio del Rio-Hortega, a student of Santiago Ramón y Cajal, presented the concept of microglia as a cellular component of the CNS in a book chapter called “Microglia”, edited in 1932 [19]. In this document, Rio-Hortega proposed the following: 1) microglia enter the brain during early development; 2) these invading cells have amoeboid morphology and are of mesodermal origin; 3) they use vessels and white matter tracts as guiding structures for migration

and enter all brain regions; 4) they transform into a branched, ramified morphological phenotype in the more mature brain; 5) after a pathological event, these cells undergo transformation; 6) transformed cells acquire amoeboid morphology similar to the one observed early in development; 7) these cells have the capacity to migrate, proliferate and phagocytose [20].

In spite of the several decades that separate us from Rio-Hortega, his statements are still valid today and he continues to be extensively cited in the present. At that time, and for the next 50 years, definition of the different types of glial cells was not straightforward, despite the term astrocyte being introduced in 1891. However, the observation of differential morphology of some cells in pathological tissues comparing to healthy brain tissue was made by the pathologists of that time.

It was not until the late 1960s that the modern era of microglial research propelled, beneficing from the facial nerve lesion model, a preparation that allowed for the study of microglia responses to injury in tissue with an intact blood-brain barrier [19].

2.1. Origin and Development of Microglia

Currently, it is well established that microglia originates from progenitors cells with mesodermal origin that migrated to the brain from the periphery [1].

Early in development, stem cells found in the yolk sac migrate and colonize the brain tissue. Once in the CNS, these progenitors differentiate into microglia. After the migration process, the BBB starts to be constituted and effectively separates microglia from the periphery [21].

The amoeboid morphology of these immigrating cells allows for an easy visual identification, but after full invasion of the brain parenchyma, these cells suffer transformation and adopt a ramified morphology [19]. In the mature brain, infiltration of other cells, such as monocytes and macrophages is almost negligible and therefore microglia constitutes an autonomous population in the CNS. Studies have shown that ablation of 99% of total microglia, repopulation happens in a very fast and efficient manner from nestin-expressing cells spread throughout the CNS [21]. This has led to the conclusion of the existence of a microglia progenitor population that normally co-exists in the brain. However, the rate at which microglia would normally be turned over is still unclear.

In pathological conditions, after BBB damage and consequent permeabilization, a subpopulation of monocytes from the periphery is recruited to the brain and transforms into microglia [22].

2.2. Microglia in The Healthy Brain

In the healthy mature CNS, which includes the brain, spinal cord, as well as the eye and the optic nerve, microglia can be found having a ramified morphology, with fine processes extending throughout the brain parenchyma. This morphology has been associated with the 'resting' state of microglia. [19]
Microglia is able to sense the slightest disturbance in the brain tissue, such as loss of homeostasis, which may indicate a potential or real danger to the CNS. Is response, microglia suffers drastic transformations in morphology, changes in gene expression and functional behaviour, which is summarily defined as microglial 'activation'. [23]
During activation, microglial cells adopt a morphological amoeboid shape, more similar to macrophages, and can respond to chemotactic gradients that attract them to the site of injury in order to engage resolution. [24] Proliferation can also be detected in some regions as to aid the defence response of these cells to the insult.[25, 26] These phonotypical and behaviour alterations are part of the response of microglia to attempt to restore brain homeostasis. Rearrangements in expression of surface molecules and changes in intracellular enzymes, as well as the release of several mediators with pro- and anti-inflammatory functions are also part of this response. Additionally, microglia engages in alterations to potentiate its phagocytic ability in order to clear cellular and molecular debris from the extracellular space.[23]
The recruitment of other immune cells to the site of injury is made by the release of chemoattractive factors and activation of T cells is performed by the ability of microglia for presenting antigens to these cells. Therefore, microglia potentiates the engagement of the adaptive immune system for a successful resolution. The range of microglial activities also covers for neurotrophic support by the release of trophic factors and by physical association with endangered neurons.[19]
Activation confers microglia morphologic similarities with macrophages, but characteristics of the highly specialized environment in which microglia resides require special protection from potential harming consequences of the immune response. This special condition has been described as the "immune privilege" of the CNS and consequently requires for highly regulated processes of microglia activation. [27] In addition to complex and heterogeneous organization of the brain parenchyma, some studies have suggested the existence of an also heterogeneous population of microglia in the brain, providing different immune characteristics depending on the region. Therefore, the effects of an immune response may be controlled by the different biochemical microenvironments and cell subpopulations but this heterogeneity may also be the reason for different vulnerabilities of some brain regions. [28, 29]

In a recent past, resting microglia was considered as quiescent cells, with no functional activity due to the absence activation markers expression. Their ramified phenotype was considered static or with very low mobile capacity. But recent years have produced large proof that, in reality, the 'resting' state is nothing but resting. Microglia ramifications are very motile and allow the cells to actively scan the environment, searching for cues and interacting actively with other cells. [15] The 'resting' state is thus characterized as a very motile state in which microglia actively interacts with the environment and is prompt to respond to potential insults, transforming and becoming active executors.

In vivo imaging studies using mice with enhanced green fluorescent protein (EGFP)- expressing microglia demonstrated that their fine processes are in constant motion scanning their neighbourhood without disturbing the delicate neuron circuitry. [30]

The 'resting' state is thus a microglia state where these cells examine their environment and are in state of alert for potential damaging insults for the CNS. On the other hand, 'activated microglia' engage in several activities in order to deal with the potential threat. Taken together, these two states are the fundamental basis by which microglia controls of the maintenance of CNS integrity. [28]

Nevertheless, while considering the 'resting' state as an important surveillance state for brain homeostasis, several maintenance functions of microglia may be easily undetected. In the case of small vascular defect or single neuron impairment, microglia can act directly by providing protection and trophic support or inactivating synapses by the process of 'synaptic striping'. [7] Because these local and transient responses would not induce full activation of microglia, they could be overlooked. Today, the physiologic daily functions of microglia are not well characterized, but the absence of inactivity moments and the constant functional importance of microglia in the CNS are widely accepted. [30]

Moreover, the activation process provides microglia with diverse expression profiles with the ability to synthesize a broad spectrum of mediators that not always work in the same sense, ranging from neurotrophins to pro-inflammatory cytokines, therefore activation of microglia is not an 'all-or-none' process. [6]

'Resting' microglia dwells through the neuroenvironment, scanning synapses with its fine processes allowing a very dynamic surveillance. Abnormal levels of neuronal excitability may have damaging consequences for the normal function of the brain. The ability of microglia to sense synaptic activity is made through their repertoire of neurotransmitter receptors. In vivo imaging studies have shown that microglia play an active role in the control of synapses, by

establishing brief contacts with synaptic structures.[31] It has been reported that in conditions of ischemia, these contacts are more long lasting and that in some situations the synaptic structure actually disappears.[32] This process is known as “synaptic striping”. These evidences strongly support an active role of ‘resting’ microglia in the maintenance of CNS integrity; however, questions on what molecular mechanisms allow for microglia discerning on which synapses to remove still need to be addressed.
Any abnormality on synaptic strength or performance may induce a response in microglia, which although not reaching a full activation state, initiates the remodelling of synaptic assemblies.

At the same time, microglia may also be involved in the opposite phenomenon, synaptogenesis. The formation of synapses, known as synaptogenesis, is especially active during the maturation of the CNS and several studies suggest that microglia may be involved in this process in the post-natal brain in rodents.[33] Also, microglia has been reported to have a regenerative role in synaptogenesis following an insult. The regeneration of synaptic connectivity in neurons by microglia and the repair of synapses have been well documented.

The influence of microglia in the plasticity of the CNS may also include the support in neurogenesis.[34] Many modulating factors produced by microglia may play an important role in neuronal formation, affecting the growth and differentiation of neurons, and also neuronal connections.[35] ATP stimulation of microglia triggers the release of BDNF, which in turn affects the calcium (Ca^{2+}) equilibrium in neurons, turning the gamma-aminobutyric acid (GABA)- and glycine- mediated postsynaptic responses from inhibitory to excitatory.[19]

The release of neurotrophins may occur during development or as a response to damaged neurons and confer microglia a trophic and supportive role, which is or particular importance during this stage of the nervous system.[36]
During development, the CNS relies on a sensible equilibrium between neurogenesis and neuronal death and, as described above, microglia plays a dual role in these processes. The mechanisms underlying the detrimental vs. supportive effects of microglia are still not well characterized, although it is believed that it might be influenced by the activation status of microglia in a particular spatial-temporal environment, therefore balancing the production of cytotoxic vs. supportive factors.[22]
The role of microglia in the removal of neurons during development has been reported in several brain regions.[34] For example, in the embryonic (E12-E13) spinal cord, invading microglia control the apoptotic death of motor neurons

by secretin of TNF; coincidentally, motor neurons have been described as expressing TNF receptor 1 (TNFR1) precisely at the same stage. [19]

In conclusion, microglia have the potential to participate in several regulation processes, such as development, and architecture and function of neural networks. They constantly and actively scan the neural environment, receiving information from neurons and directly controlling their connectivity, thus participating in an active remodelling of the neural physiology.
Multiple activation states can be found in microglial cells and the existence of an active 'resting' state or simply active compartments in the 'resting' microglia may contribute to the support and maintenance of the healthy brain homeostasis and provide additional plastic capabilities. [19] The overlapping environmental orientations that induce responses in microglia still need to be further addressed. It will be interesting to identify the sources of these instructing signals as they guide the functional orientations engaged by microglia during the activation process.
Likewise, very little is known about heterogeneity of microglia in the brain. It would be important to understand the functional differences between individual subpopulations of microglia within the CNS.
Finally, in pathological conditions with periphery-derived monocytes and macrophages infiltrating the brain, overlapping and complementing functions between these cells and microglia may produce both detrimental and beneficial consequences. It is important to understand the physiology and pathophysiology of microglia and its interactions with other cells in pathological conditions to evaluate the potential of correcting its deregulation.

2.3. Microglia in Pathologic Conditions

Cumulating evidence indicates that deregulation of microglia physiology can exacerbate pathogenesis in multiple diseases of the nervous system. While activation of microglia can have a supportive role, when uncontrolled may engage in exacerbated reactivate patterns, producing abnormal amounts of pro-inflammatory and toxic factors. Summing this uncontrolled reaction to the presence of other potential damaging signals can result in harmful effects in the CNS. The inflammatory response of microglia has been widely suggested to play a detrimental role in several pathologies of the nervous systems, such as Alzheimer's disease (AD), Huntington's disease (HD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), ischemic stroke, Multiple Sclerosis (MS) and psychiatric disorders. [22, 37, 38]

A variety of signals, involved or not in pathologies, was shown to trigger a response in microglia, such as ATP and neurotransmitters produced by neurons; products from necrotic cells, such as RNA and DNA; and abnormally folded proteins, such as amyloid-β (Aβ) and α-synuclein.[22]

The CNS, due to its specific characteristics, benefits from a specialized immune system comparing to other tissue and organ systems. The endothelium of the BBB regulates the interconnection between the brain and the periphery with a selective permeability to cells and molecules, providing a sterile environment. This immune specialization within the CNS provides protection by strictly regulating inflammatory responses and avoiding damaging consequences to neurons, which have limited regenerative abilities.[25]
In a scenario of neurological disease, microglia can either have a beneficial neural protective role or a negative impact by exacerbating and accelerating the disease process, depending on their activation profile.[22] Thus, microglia has a strong impact in CNS disease progression.

In parallel with several other neurological diseases, neuropathology in ALS and its animal models co-occurs with strong microglial activation, and whether microglial activation is a cause or a consequence of the disease is still not known. Substantial experimental evidence with the ALS animal model has identified microglia activation as a contributing factor for ALS progression.[39] Experimental animals stimulated with bacterial endotoxin lipopolysaccharide (LPS) showed microglia producing much higher amounts of pro-inflammatory mediators, such as TNF, nitric oxide (NO), superoxide, interleukin (IL)-6, and lower levels of insulin-like growth factor 1 (IGF-1), when compared to microglia of wild-type control animals. Interestingly, microglia from the experimental model revealed different phenotypes along disease progression, adopting a neurotrophic phenotype at disease onset and changing to a neurotoxic phenotype at disease end-stage.[25]
Therefore, microglia are triggered to respond to different signals along the disease progression, balancing from the production of neurotrophic factors versus toxic factors. Leaning this balance towards a neurotrophic role may be a promising site for intervention.

In Alzheimer's disease, the inflammatory component that is characteristic of this pathology also still remains to be characterized as a potenciator of disease progression or as a protective element. In brain tissue samples from patients with this disease, microglia can be observed to accumulate around Aβ deposits and to constitute a cellular component of the senile plaques.

Upon activation, microglial cells migrate to the senile plaques and multiple receptors expressed by microglia have been shown to interact with Aβ.[37] Activation of microglia with Aβ has been shown by in vitro studies to produce toxic factors that induce neuronal death, when co-cultured. Contrary, isolated neuronal cultures stimulated with Aβ show lower levels of neuronal death, indicating that microglia is the main player in this process.[25] While several studies support this idea of a neurotoxic effect of microglia, others have been suggesting the opposite idea.

Overexpression of IL-1β in AD animal models has been shown to increase microglia activation but resulting in a decreased Aβ deposition in the hippocampus.[40]

In the past few years, the concept of microglia senescence has gained attention and has been intensively associated with the development and progression of AD.[41] With increasing age, microglia reveals morphologic and functional changes. The number of microglial cells increases in certain areas of the mouse brain, their spatial distribution becomes uneven and they adopt a less ramified and branched morphology. In the human aged brain, microglia morphology resembles that of the activated state and some cells appear dystrophic. On the functional side, aged microglia is known to express high levels of pro-inflammatory cytokines, such as TNF and IL-1beta, and decreased expression of anti-inflammatory IL-10.[42]

Taken together, these facts suggest that with ageing, microglia adopts baseline activation, being more prone to engage in a response to environment cues than its younger counterparts. This would indicate a more efficient response at clearing Aβ deposits. However, in vitro experiments show that despite the increased activation state of microglia, Aβ membrane receptors and intracellular Aβ-degrading enzymes are decreased.[43]

Therefore, a model for the role of microglia in the progression of AD has been proposed, in which, during an initial stage of the disease, microglia plays a protective role by clearing accumulating Aβ; as age and disease progress, and despite the increased number of microglial cells, these become impaired and unable to efficiently clear Aβ.[25] In addition, microglia increases the expression of pro-inflammatory mediators that emphasize inflammation progression, with higher levels of neuronal death and also increased activity of γ- and α-secretases, which enhances Aβ production and the formation of senile plaques.[44] Potentially, all these events create a self-perpetuating cycle of inflammation and Aβ accumulation.

Also in Multiple Sclerosis, a demyelinating disease, active lesions are associated with activated microglia or macrophages.[45] The belief that microglia may be involved in MS has been emerging, although whether these cells promote damaging or relieving effects is not known. On the other hand,

since the pathology is characterized by intercalated symptomatic and non-symptomatic periods, a possible dual role of microglia is also debated.

Reports of microglia activation in the early stages of the disease might suggest that these cells play an initial triggering role for disease progression.[25] While they may not be the main agent for the high inflammatory profile of MS, microglial cells may allow for the entry of peripheral harmful inflammatory monocytes due to damages inflicted to the BBB. Characteristic to microglial activation is the potential damage produced to the BBB; disruption of this barrier allows free passageways for circulating cells, molecules and parasites.[46]

Another hypothesis for the role of microglia in MS is the antigen-presenting ability of these cells. In MS, the number of self-antigens that are presented to T cells increases during disease progression, and this dissemination of epitopes is thought to play a role in enhancing demyelination.[47] 'Resting' microglia does not normally express major histocompatibility complexes (MHC I and MHC II), which are responsible for presenting antigens to T cells. Although, when activated, microglia can express both MHC I and MHC II and in vitro studies have shown that microglia is able to efficiently present myelin peptides to T cells.[48]

On the contrary, in the experimental autoimmune encephalomyelitis (EAE) model, microglia has been correlated with beneficial effects in disease progression by phagocytizing myelin debris, which impair re-myelination. Production of trophic factors by activated microglia also plays a positive role by stimulating oligodendrocyte precursors cells.[25]

Evidence that microglia plays multiple roles in human disease is well established. Impairment of several functions of microglia, such as clearance of debris and production of neurotoxic factors, has been shown to contribute to the establishment of multiple pathogenesis. Therefore, focusing on microglial cells role in several pathologies is though to be a promising therapeutic approach. However, the limited knowledge on the molecular mechanisms that regulate these cells has been an obstacle to identifying strategies to modulate microglia in vivo.

Currently, it seams that the most successful approaches are based on a transplantation of bone-derived myelomonocytic cells that are able to colonize microglial niches within the CNS.

These cells can be engineered to produce factors that directly impact on disease progression, such as specific enzymes missing in lysossomal diseases, therefore serving as long-term delivery factories for the treatment of such neurological diseases. In the context of cell therapy, restoration of normal microglia functions would also be an assertive target to explore.[25]

In all these applications, microglia replacement or transplantation could play a promising role in several CNS diseases that currently remain in the obscurity of therapeutic approach.

2.4. Microglia Activation-Deactivation

As mentioned above, microglia is able to switch from 'resting' default, ramified state to a more amoeboid-like and 'activated' form. Such switch between different morphological phenotypes after CNS injury is also associated to changes in gene expression and function.
After activation, microglia can be polarized into classical, M1, or alternative, M2, activation states. M1 is a phagocytic state induced by T helper 1 (Th1) cytokines, interferon gamma (IFN gamma), TNF, and LPS; associated with the production of pro-inflammatory cytokines (ILs and TNF), and cytotoxic substances (oxygen-free radicals and NO). M2 is a phagocytic phenotype induced by Th2 cytokines (IL-4 and IL-10), that triggers anti-inflammatory responses (IL-10, TGF beta), and promotes tissue repair (extracellular matrix components (ECM), arginase1).[49] Importantly, the M2 category has been further divided into: alternative activation-wound healing or acquired deactivation-regulatory. After the resolution of the injury, microglia 'deactivates', and reverts to a more resting phenotype.[28]

In order for effective restoration of tissue homeostasis, magnitude, duration and quality of the activation response by microglia have to be tightly regulated. In the context of neurodegenerative diseases, the persistence of a pathological stimulus may induce a chronic form of microglia activation and subsequent exacerbated neurotoxicity. Therefore, unravelling the mechanisms that positively and negatively regulate microglia may offer new therapeutic targets for several neuroinflammatory diseases.
Although neuroprotective functions of microglia, by releasing anti-inflammatory mediators and neurotrophic molecules, have been well characterized, they are part of the activation response of microglia. These restoring and protective functions result from a later-phase in the activation process. The question remaining to be answered is what happens in the regulatory machinery of microglia after the full resolution of an immune insult.

Emerging studies have begun to focus on the deactivation stage of microglia, but still there are very few conclusions that can be withdrawn.
Evidence has showed that several molecules induce downregulation of microglia, such as IL-10 and TGF beta [28, 39] and several ligands expressed by

neurons, astrocytes and oligodendrocytes.[6]. TGF beta and IL-10 both inhibit immune-stimulated IL-1beta, IL-6 and TNF production, decrease chemokine levels and decrease the expression of MHC II. These molecules have growth factor properties and promote survival of neurons and increase tight junctions at the blood-brain barrier.[28] This downregulation of microglia is, in most cases, defined as 'acquired deactivation'. However, some studies have shown that longer periods after several types of brain injury reveal low levels of these mediators (as well as the pro-inflammatory mediators) compared to control.[50, 51] Therefore, this may be the starting of microglia deactivation process. Microglia deactivation, however, is a very complex and multifaceted concept due to the vast array of microglia phenotypes that can emerge from different types of stimulus, duration and potency of the insult, and even from different brain regions subjected to insult.

Another concept that has been emerging possibly relating to microglia deactivation is microglia 'priming'.[6] A 'primed' profile can be defined by increased basal expression of inflammatory mediators, lower thresholds for engaging in activation and exaggerated reactivity upon facing a stimulus. These functional characteristics are accompanied by morphological changes.[52]
Microglia 'priming' has been associated with contexts of ageing, neurodegenerative diseases and traumatic brain injury, but there has not yet been identified a single source for the causing of priming.[6, 51, 53]
Changes in morphology, upregulation of cell surface antigens and the proliferation of microglial cells are all associated with microglia priming.
Environmental cues to which the healthy CNS is presented can activate microglia but also prime these cells. For example, a transient peripheral infection or chronic exposure to low levels of infectious pathogens, characterized by being below those necessary to cause clinical signs, can induce these phenotypes in microglia. Prolonged exposure to very low levels of LPS can induce priming rather tolerance to LPS in macrophages, which can be of particular importance in cases of PD patients, which are very prone to suffer from constipation. In these cases, LPS may escape from the gut to the systemic circulation and this increased systemic exposure may result in disease progression through induction of microglial priming.[6]

Several studies have been focused on the duality of macrophage priming and tolerance, two mechanisms that may suggest the existence of a primitive innate immune memory.
When cells are exposed to LPS they can become hyporesponsive to a second LPS insult in terms of pro-inflammatory cytokines and other mediators, a mechanism defined as endotoxin tolerance, which serves to

avoid collateral inflammatory damage. In contrast, low levels of LPS can cause the opposite effect by priming cells to respond more robustly to a secondary LPS challenge.[54] The molecular mechanisms underlying these two phenomena are still not well understood, but evidence has shown that low doses of LPS can induce changes at the molecular level by removing transcriptional suppressors from the promoters of inflammatory genes, despite not being able to induce a fully functional activation in these cells.[54]
Studies with rodent animal models have shown that systemic inflammatory challenges in the presence of a primed microglia can produce fever and highly expressed symptoms of sickness such as prostration and anorexia. These behaviours are accompanied with high expression of cytokines in the brain, such as IL-1, TNF and IL-6, as well as NO.[51]
The increased expression of these cytokines can have damaging effects in neuronal survival, but in addition to this, primed microglia can also respond to stimuli by modifying its surface receptors, which can have important consequences. Functional magnetic resonance imaging (fMRI) studies of healthy human volunteers have shown that vaccination with low levels of Salmonella *typhi* endotoxin can trigger an increase in systemic levels of IL-6.[55] Results of a positron emission transmission (PET) imaging study with nonhuman primates demonstrated that intravenous administration of low doses of LPS induced the increase of IL-1beta and IL-6 levels in serum on these animals, correlated with high levels of microglia activation.[56]
According to this hypothesis, the treatment of systemic inflammations could potentially enforce the delay of the progression of chronic neurodegeneration.

The processes that result in nonspecific innate immune memory have been suggested to be mediated through epigenetic mechanisms. Changes in chromatin accessibility were described to be differential in situations of tolerance and priming of monocytes. The authors conclude that in induced priming in vitro, monocytes are able to implement a long-term epigenetic program in contrast to LPS-induced tolerance.[57]

In the context of microglia, further studies on the priming mechanism still need to be made as several questions arise from this issue: does microglia ever reach a deactivated state parallel to the resting state? Or does microglia retain a permanent molecular mark (innate memory) upon a first activation? Is it possible to revert this molecular mark? Is it possible to revert microglia priming?
Identification and characterization of the core mechanisms underlying these functional states is thus important, as it allows better understanding of the full capabilities of these innate immune cells.

3. RNA-Binding Proteins

Microglia recognition is mediated by pathogen-associated molecular patterns (PAMPs), such as the Toll-like receptors (TLRs). After stimulation a genetic program is initiated, and different genes are differently modulated. Part of this regulation is performed by the action of RNA-binding proteins (RBPs).

RBPs play crucial roles in post-transcriptional processing of RNAs, which can occur at every aspect of the life of mRNAs, from pre-mRNA splicing to mRNA localization, turnover, polyadenylation, translational control, nuclear export and editing.[58]

Post-transcriptional regulations are part of the evolutionary machinery that allowed increasing complexity in the control of gene expression throughout evolution. Cells are therefore able to adjust their protein composition in order to respond to developmental cues and other stimuli, providing post-transcriptional regulation with an important role in multiple cellular processes.

RBPs interact with specific cis-regulatory elements present in the mRNA to form ribonucleoprotein (RNP) complexes, thereby controlling expression and function of their target RNAs.[59] Several RBPs have been described to be ubiquitously expressed in several cells and tissues, although many examples have been reported of RBPs being specifically expressed in different cell types or tissues.[58]

Additionally, there are macromolecular complexes that contain more than one RBP and these specific arrangements can also be cell- and tissue-specific. This specificity of RBPs expression patterns adds genetic diversity also contributes for the cell- and tissue-specific manifestations in cases where RBPs are deficient or mutated. Another characteristic of RBPs is the fact that they may possess multiple target mRNAs, regulating the expression and function of several target mRNAs. Consequently, affecting the function of a single RBPs may produce manifestations in the post-transcription of numerous target RNA transcripts, a phenomenon that has gained relevance in several neurodegenerative disease researches.[60]

Despite the lack of information on the precise mechanisms underlying the ability of RBPs to achieve their specificity in the regulation of their target genes, several studies have correlated a large number of mutations in RBPs with a wide range of human diseases, taking to the conclusion that RBPs are important in human biology and pathophysiology.[59]

Nova1

Nova1 was first identified in an autoimmune syndrome called paraneoplasic opsoclonus myoclonus ataxia (POMA), a form of paraneoplastic

degeneration. In this disorder, the immune system recognizes Nova1 as a non-self antigen and initiates an autoimmune response consequently producing auto-antibodies for Nova1.[61]
Nova1 has been described as being highly expressed in the brain and possessing several functions, such as alternative splicing (including of its own pre-mRNA), neuronal cell survival in the post-natal stage, inhibitory synaptic transmission, synaptogenesis and neuronal migration.[62] Nova1 knockout mice do not survive the second week of life, indicating a crucial role for this RBP in development.[63]

YBX-1

Y box binding protein 1 (YBX-1) is a major component of cytoplasmic mRNPs.[64] It has been referred to be involved in mRNA splicing, translation, packaging and stabilization due to its ability to shuttle between cytoplasm and the nucleus.[65] This RBP can also be secreted to fulfil extracellular functions.[64]
YBX-1 is known for its association with cancer, being considered as a molecular marker in some types of transformed tissues.[66] Several evidences have demonstrated its overexpression in some types of cancer and suggested that this might be a result of its roles in regulating cell proliferation and apoptosis, although the mechanisms underlying these functions are unknown. YBX-1 has also been associated with cellular stress responses, promoting the decrease of cell sensitivity to some types of insults.[64]
YBX-1 has been identified in dendrites of neurons, appearing as puncta related to mRNPs, suggesting their role in some neuronal functions.[67] Additionally, a role in neuronal development is also documented; this RBP has been positively correlated with neural stem cell markers, promoting cell growth and inhibiting differentiation.[68]

PABPC1

In most eukaryotic cells, the poly(A) tail of mRNAs is bound to two important proteins, poly(A)-binding protein cytoplasmic 1 (PABPC1) and poly(A)-binding protein nuclear 1 (PABPN1). These are RBPs of the same family, being that PABPC1 is mainly localized in the cytoplasm and PABPN1 in the nucleus, although they may shuttle between the two compartments.[69] PABPC1 is known to be one of the main constituents of RNPs and is able to bind to the majority of mRNAs. This RBP is involved in mRNA stability, degradation, transport and translation. PABPC1 may either bind directly to the poly(A) and other (A)-rich sequences in the 3'UTR of mRNAs, or associate with other

protein partners and bind to other kind of sequences. PABPC1 has been demonstrated to bind to the 3'UTR of the mRNA of Ybx1 and promote translation. Although in other cases, binding to the 3'UTR may result in translation inhibition.[70]
Reduced levels of PABPC1 in the brain have been related to cerebellar vulnerability in Spinocerebellar Ataxia.[71]

PTBP1

Polypirimidine tract-binding protein (PTBP1) is a RBP involved in cell differentiation.[62] In the case of neurons, PTBP1 has been described as being responsible for the switch between splicing programs that lead to neuronal and non-neuronal cell differentiation.[59]
Being characterized as a splicing factor and suppressor, PTBP1 as been associated to deregulation of the glucose metabolism and consequently have a negative impact on breast tumorigenesis, promoting the maintenance of transformed cells.[72] In the neurodegenerative disease context, this RBP seems to present increased levels in patients with Parkinson's disease, suggesting a potential biomarker for this disease.[73]

A2BP1

Ataxin 2-binding protein 1 (A2BP1) is documented to influence transcription, splicing factors and synaptic proteins implicated in neuronal development and differentiation.[62] Mutations in A2BP1 are associated with retardation, autism and epilepsy, suggesting an important role for RBP in the electrophysiology homeostasis of the CNS.[60] In the brain, interactions between A2BP1 and ataxin-2 may be underlying the pathophysiology of Spinocerebellar Ataxia.[74]

3.1. RNA-Binding Proteins and Gene Regulation

The mRNAs are produced in the cellular nucleus, when genes are transcribed by RNA polymerase II to generate their pre-mRNA transcript(s).[75] 5'-end capping and 3'-end polyadenylation of pre-mRNAs can occur in the same time frame of transcription. During transcription by RNA polymerase complex, several nucleotide sequences are removed from the pre-mRNA transcript by the multi-component splicing machinery called the spliceosome, a mechanism that characterizes the "pre-mRNA splicing" step.[60]

These coordinated mechanisms of 5'capping, 3'polyadenylation and splicing all result in the production of a mature mRNA ready to be transported to the cytoplasm and be translated.[75] Once in the cytoplasm, the mRNA is associated to the protein synthesis machinery, which may be localized in the endoplasmic reticulum (ER) or at several sites far away from the nucleus. An example of local protein synthesis is seen in neurons where the translation of proteins in made locally at specific regions of axons and dendrites.[76] This mechanism allows the cells to control their protein synthesis according to specific needs and is generally called 'local translation'. During mRNA transportation, the mRNA is protected and translation is repressed.[77]

Throughout all these processes of the life of mRNAs, these are associated with RBPs, many of which influence more than one aspect of post-transcriptional regulation, as described before. The close relation between mRNAs and RBPs allows for the complex temporal and spatial regulation of eukaryotic gene expression.[60]

Recent evidence has shown that post-transcriptional regulation plays crucial roles in the most complex system, the brain. Local translation mRNAs in neurons is required for neuronal functions, such as learning and memory; therefore defects in RBPs present in neurons lead to deficiencies in the post-transcriptional regulation of these genes, resulting in neurological diseases.[78]

Research on neurodegenerative diseases, with the support of new technologies, such as bioinformatics analysis, genetic mouse models and biochemical approaches, has increasingly sought to understand RBPs biology since they strongly suggest being involved in many human neurological diseases. Still, the mechanisms that regulate RBPs expression that leads to defects in neuronal functions are quite unclear. One single mutation may produce vast and distinct clinical manifestations in different individuals, thus it would be important to have access to specific information about what defects in RNA processing can result in disease outcome. Also, the mechanisms by which the cell interprets information from the multitude of RBPs and integrates them in its cellular functions still need to be elucidated.

The association of RBPs to neurological disease has been established and the biological functions of RBPs and pathogenic mechanisms have been intensively studied, although many questions remain unanswered. Knockout models are some times used in this field, but because mutations in RBPs related to diseases not always result in loss of gene expression, attention must be taken when using these approaches. Animal models in combination with cellular and molecular approaches have been starting to focus on pathogenic mechanisms, although much more work needs to be done to identify the early events and biomarkers of neural damage and for the development of new effective therapeutic strategies to address these devastating diseases.

3.2. RNA-Binding Proteins and The Immune System

Accurate control and regulation of gene expression is a key feature of the immune system. Transcripts and their related proteins need to be under very tight control so that their concentrations are adequate for specific locations within the cell and over time. Curiously, as organism complexity evolves so does the lengthening of the 3' untranslated region (UTR) of mRNAs, revealing the importance of this region. In some cases such as with ILs, the length of the UTRs can exceed that of the coding region.[79]

After leaving the nucleus, RNA is wrapped in proteins that control transcript stability, translation and localization, being able to respond to signalling pathways.[80] Short-living RNAs are more sensitive to changes in transcription and thus, RNA decay works in concert with transcription to ensure accurate dynamics of expression. Noteworthy, unstable transcripts are often the products of genes that encode regulators of transcription or signal transduction, indicating that these mechanisms are highly susceptible to changes in transcription and post-transcriptional regulation.[81]

The majority of transcripts that encode for cytokines contain in their 3'UTR, one or more AU-rich elements (AREs), which regulate mRNA stability. ARE motifs are known to interact with many RBPs, which can influence the stability of the mRNA in a positive or negative manner.[82]

Additionally, each sequence of the mRNAs, including coding and non-coding, can interact with different RBPs with complementing or opposing functions. Large-scale studies focused at identifying sequences bound by RBPs brought up evidences that these recognition elements lack high levels of complexity.[83]

Innate immune cells have evolved to recognize harmful elements and threatening agents, clear cellular debris and infectious organisms, provide support to the adaptive immune system and help tissue regeneration. In order to fulfil these functions, cells of the innate immune system undergo an active and continuous regulation of RNPs configuration and post-transcriptional programs in response to the inflammatory microenvironment.

Pathogens, infected cells and damaged or transformed tissues are detected by immune cells through the germline-encoded pattern recognition receptors (PRRs), such as TRLs. PRRs use multiple adaptor complexes to initiate active intracellular signalling cascades that induce cells to engage in pro- or anti-inflammatory biosynthesis and cellular execution.[80]

RBPs can influence PRRs signalling thresholds in many ways. For example, prolonged TRL activation signalling via the adaptor MyD88 induces nuclear proteins to promote skipping of an exon in Myd88 pre-mRNA.[84]

Studies of macrophages and microglia in hypoxic conditions have shown that some RBPs are transported to the cell surface and interact with TRL4 co-receptors promoting their pro-inflammatory activity.[85]
Innate cells deactivation seems to be accompanied by degradation of pro-inflammatory mRNAs, which seems to be an efficient way to avoid the accumulation of pro-inflammatory mediators that can result in tissue and cell destruction.[80] Regulatory endonucleases are the key players in restricting basal transcription of genes encoding for inflammatory molecules. In macrophages, the endonuclease regnase-1 targets the 3'UTR of IL-6 mRNA, inhibiting it. When in resting conditions, regnase-1 in macrophages is active; however, upon TRL signalling, phosphorylation of regnase-1 is promoted and this endonuclease is degraded, allowing the increase in IL-6 levels. When environmental cues are lost, the activity of regnase-1 is restored and pro-inflammatory IL-6 is inhibited.[86] This evidence of a time-controlled regulation is crucial for the functions of the innate immune system.
Some RBPs may also protect mRNAs by preventing proteasomal degradation, allowing the control of concentrations of inflammatory mediators and chemokines by macrophages and microglia. Contrary, in other situations, the signals received by the cell may promote the gain of access of specific RBPs to their targets and induce mRNA degradation. Anti-inflammatory cytokines, such as IL-4, IL-10 and TGFβ may promote even further the expression of these RBPs, creating a safety mechanism against chronic inflammation.[80]
Multiple RNA elements may be engaged to induce mRNA degradation to ensure the deactivation of innate immune responses. The anti-inflammatory effects of negative regulators are associated with their ability to bind to their targets during the time frame of deactivation.

Taken together, the functions of RBPs in cells of the innate immune system clearly demonstrate their importance and how remodelling of RNPs can influence the activation states of these cells via post-transcriptional regulation. Influence of RBPs over all aspects of the life of mRNAs is a key strategic mechanism that allows cells from the innate immune system to determine their functions and phenotypes according to the specific environmental cues.

AIMS

AIMS

In this thesis we addressed three main objectives:

- To give insight into the molecular and cellular mechanism of Microglia Deactivation.

- To investigate the mechanism of ‘Microglia Priming’.

- To ascertain the role of RBPs in Microglia Activation and Deactivation processes.

MATERIALS AND METHODS

IN SILICO ANALYSIS

Nucleotide sequences for the several mRNAs analysed were obtained from the NCBI (http://www.ncbi.nlm.nih.gov) and Ensemble (http://www.ensembl.org) databases. Study of conservation of nucleotide sequences among species was performed using the Geneious v4.7 software.

MICROGLIA PRIMARY CULTURES

Microglia cultures were prepared as described before.[87] Briefly, wild type Wistar rat pups with two days of age (P2) were quickly euthanized by decapitation with scissors. The heads of the animals were dissected by cutting the skin and skull, exposing the brain. After dissecting the brains, meninges were removed and cortices isolated. These were placed in Hank's balanced salt solution (Gibco, Life Technologies) on ice to preserve living tissues. The dissected cortices were homogenized, strained and plated on Poly-D-lysine (PDL) coated T75 flasks in complete Dulbecco's Modified Eagle Medium (Gibco, Life Technologies). Mixed glial cell cultures were maintained 10 days in culture.

After, microglial cells were mechanically isolated from the mixed glial cultures by shaking (200 rpm, 2 hours), pelleted by centrifugation at 1200g for 5 min, and plated at a density of 3 x 10^5 cells/cm^2 in DMEM/F-12 medium (Gibco, Life Technologies) supplemented with 10 % Fetal Bovine Serum (FBS), 1 % penicillin/streptomycin (PS) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (1 ng/ml, Sigma-Aldrich). Microglial cells were left 48 hours for recovery and adhesion.

MICROGLIA CULTURE TREATMENTS

When appropriated and as described in the text, microglia cultures were treated with 100ng, 10 ng, 2 ng, 0,2 ng, 0,02 ng, 0,002 ng and 0,002 ng of LPS (from Escherichia coli, Sigma-Aldrich), or 10 ng of recombinant IL-4 (PrepoTech).

For the hypoxia conditions, cells received fresh medium 30 min before treatment. They were then subjected to a gas mixture of 5 % O_2 and 95 % of N_2 in a hyperbaric chamber. The cells were incubated for 2 hours in these conditions, at 37°C.

RNA EXTRACTION

Total RNA was extracted with TRIzol reagent (Invitrogen, Life Technologies) following the standard protocol. Cells were incubated with 500 µl of TRIzol for 5min at RT and then overnight at -80°C. To separate the RNA fraction, chloroform was added to the lysates, these were mixed and centrifuged for 15 min at 16.000g, at 4°C. The aqueous phase was transferred to a fresh tube, where 1 µl of GlycoBlue (15 mg/mL, Ambion, Life Technologies) and the same volume as the aqueous phase of isopropanol was added and mixed. Samples were frozen overnight at -80°C and after thawing, centrifuged twice, for 20 min and 10 min at 16.000g, at 4°C, with a washing step of 75% cold ethanol between centrifugations. The pellet was air dried and resuspended in Nuclease-free water (Thermo Scientific). RNA quantification was performed in a NanoDrop™ 1000 Spectrophotometer (Thermo Scientific) and RNA was then stored at -80°C for future analysis.

REVERSE TRANSCRIPTION

cDNA was synthesized using SuperScript® III Reverse Transcriptase enzyme Kit (Invitrogen, Life Technologies). 500 ng of RNA were used following manufacturer's instructions. Briefly, RNA was denatured for 5 min at 65°C with dNTPs (10mM), random hexamers (50 mM) and Nuclease-free water (Thermo Scientific), followed by a 5 min cycle at 4°C. The mixture for reverse transcription was prepared with cDNA synthesis buffer (5X), DTT (0,1M), RiboLock RNase inhibitor (Thermo scientific) and SuperScript III reverse transcriptase enzyme (200 units/ µl), then added to the denatured RNA samples and gently mixed. Samples were then incubated for 10 min at 2°C, 60 min at 50°C and 10 min at 70°C to inactivate the enzyme.

REAL-TIME qPCR

mRNA expression was analysed by real-time qPCR (RT-qPCR) analysis using the StepOne Real-Time PCR System (Applied Biosystems, Life Technologies) thermocycler.
Each primer pair was first optimized for reaction efficiency. Briefly, a standard curve was performed using serialized dilutions of cDNA (undiluted; 1:10 and 1:100 dilutions) added to each primer pair (10 mM), SYBR Select Master Mix (Applied Biosystems, Life Technologies) and Nuclease-free water (Thermo Scientific) to a final volume of 10 µl. Conditions of amplification used were recommended by the company: 2 min at 50°C, 2 min at 95°C and 40 cycles of 15 sec at 95°C and 1 min at 60°C or 58ºC. For an ideal efficiency, the

slope of the standard curve generated with 10-fold dilutions would be -3,32. The slope obtained is used to calculate the primer pair efficiency using the formula $E = 10^{(-1/slope)}$ followed by calculation of the correspondent percentage of efficiency. Primer pairs were considered efficient with percentages between 90% and 110%. To quantify the relative mRNA expression levels, specifications listed above were used with minor alterations. The $2^{-(2\Delta\Delta Ct)}$ (Pfaffl) method was used to calculate differences (fold changes) between the expression of the gene of interest (GOI), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (endogenous control) and experimental controls (reference genes).

Target Gene	Primer Pairs	Sequences 5' ➔ 3'
GAPDH	GAPDH F	TGGAGTCTACTGGCGTCTT
	GAPDH R	TGTCATATTTCTCGTGGTTCA
TNF	TNF F	ATCTACCTGGGAGGCGTCTT
	TNF R	GAGTGGCACAAGGAACTGGT
IFNβ1	IFNβ1 F	GAAGCTCCAGTTCCGACAAA
	IFNβ1 R	AGCTGAGGTTGAGCCTTCCA
IL-6	IL-6 F	ACTCATCTTGAAAGCACTTG
	IL-6 R	GTCCAACAAACTGATATGCCTTAG
IL-10	IL-10 F	ATCCGGGGTGACAATAACTG
	IL-10 R	TGTCCAGCTGGTCCTTCTTT
PABPC1	PABPC1 F	AACCGTGCTGCATACTATCCT
	PABPC1 R	GCATATTCTGGAATGGATGAGGTC
YBX1	YBX1 F	GAAGGAGAAAAGGGTGCGGA
	YBX1 R	TGGTAATTGCGTGGAGGACC
Nova1	Nova1 F	TTCCATCTCAACTTCCGCCC
	Nova1 R	TCAGAGAGAGGCTGGACGAA
PTBP1	PTBP1 F	CGTCCCAGACATAGCAGTCG
	PTBP1 R	ATGATGAAGGGGCCGTTGCT
A2BP1	A2BP1 F	CGGCCTATGGCGGTGTTG
	A2BP1 R	GTAGGGGTCGGCAGCATAAA

ELISA

Supernatants were collected from microglia cultures at several time-points and stored at -80°C. Quantification of released TNF protein levels was performed using the BioLegend ELISA MAX™ Standard Set. Protocol was performed following manufacturer's instructions. Briefly, the ELISA plate was incubated overnight at 4°C with diluted Capture Antibody in Coating Buffer. After washing the plate four times with Washing Buffer, blocking of non-specific binding was performed by incubating the plate with Assay Solution for 1 hour at RT, with mechanical shaking. Standard curve was prepared from stock solution (500 pg/ml) and six two-fold serial dilutions in Assay Solution (Assay Solution alone was used as the zero standard, 0 pg/mL). After a four-time washing step, the plate with samples and standard curve was incubated for two hours at RT, with shaking. The plate was then washed four times and further incubated with the Detection Antibody for one hour at RT, with shaking. After another four-time washing step, plate was incubated with diluted Avidin-HRP in Assay Solution and left for reacting for 30 min, at RT with shaking. The final washing step is crucial for minimizing background and thus a one-minute five-step washing was performed. To visualize the signal, o-Phenylenediamine dihydrochloride (OPD) substrate was added to the plate and left incubating in the dark for 15 min (or until desired colour signal developed). The reaction was stopped with Stop Solution (2N H_2SO_4) and absorbance was read at 450 nm. TNF concentrations were calculated by extrapolation of the standard curve values.

CLONING INTO PSICOR VECTOR

The pSicoR vector was previously linearized by digestion with the appropriate restriction enzymes, followed by gel extraction.
The shRNAs were designed using the PSICOOLIGOMAKER program. The oligo sequences are listed below:

shPABPC1
sense
TGTAACATCCTTTCATGTAATTCAAGAGATTACATGAAAGGATGTTACTTTTTTG
antisense
TCGACAAAAAAGTAACATCCTTTCATGTAATCTCTTGAATTACATGAAAGGATGTTACA

shYBX-1

sense

TGAGAACCCTAAACCACAAGATTTCAAGAGAATCTTGTGGTTTAGGGTTCTCTTTTTTC

antisense

TCGAGAAAAAAGAGAACCCTAAACCACAAGATTCTCTTGAAATCTTGTGGTTTAGGGTTC
TCA

shNova1

sense

TGGATGTAGTTGAAATAGCATTCAAGAGATGCTATTTCAACTACATCCTTTTTTC

antisense

TCGAGAAAAAAGGATGTAGTTGAAATAGCATCTCTTGAATGCTATTTCAACTACATCCA

Annealing step: in a single tube, 1 µl of each oligo (100 mM) was mixed with 25 µl of annealing buffer and 23 µl of Nuclease-free water (Thermo Scientific), to a final volume of 50 µl. The mix was incubated for 4 min at 95°C, 10 min at 70°C and then slowly cooled down to 4°C (1,5°C per minute). The resulting annealed oligo stock was stored at -20°C until the ligation step.
Ligation step: The annealed oligos were pre-diluted (1:20) and 1 µl was added to 2 µl of 10X ligase buffer, 1 µl of the digested vector (50-100 ng), 0,5 µl of T4 ligase (1U, Roche) and Nuclease-free water (Thermo Scientific) was added up to a 20 µl of final volume.

TRANSFORMATION OF COMPETENT BACTERIA

For transformation, 1 µl of the ligation (pSicoR vector + annealed oligos) was added to 25 µl of Novablue competent cells (Novagen, Merck Millipore). Cells were incubated for 15 min on ice following a heat shock at 42°C for 30 sec and put back 2 min on ice. 125 µl of SocMedium was added and then LB medium up to 1000 µl. The cells were incubated at 37°C for one hour, in orbital shake (250 rpm). Transformed cells were then centrifuged and the resuspended pellet was plated in LB plates with ampicillin (1:100) and left growing overnight at 37°C.

COLONY PCR

Colonies grown in LB and ampicillin plates were submitted to colony PCR. Reaction mix was prepared containing GoTaq DNA Polymerase (Promega), Green GoTaq Reaction Buffer (Promega), MgCl2 (25mM), dNTPs mix (10mM), pSicoR Multiple Cloning Site (MCS) forward and reverse primers and ddH2O up to 10 µl. With a sterile tip, each colony was individually dipped into

the PCR tubes containing the reaction mix. For storage and future use, simultaneously each of the colonies were inoculated in a new LB with ampicillin (1:100) plate and incubated overnight at 37°C. After running the agarose gel, positive colonies were selected from the stored plates and inoculated in liquid LB medium. Plasmid DNA was extracted using PureLink Quick Plasmid Miniprep Kit (Invitrogen, Life technologies), following manufacturer's instructions. Samples were then prepared for sequencing outside the lab.

LENTIVIRUS PRODUCTION

Lentiviral particles were produced using jetPRIME® (Polyplus Transfection). HEK293T cells were thawed and plated in T75 flasks in DMEM (glutamax, high glucose, piruvate, Gibco, Life Technologies) supplemented with 10 % FBS and 1 % PS. Cells were left for 3 days in culture to achieve the correct confluence (~80%). On the evening of transfection, HEK293T cells were split into 3 PDL-coated plates (10 cm). On the day of transfection, medium was replaced with fresh medium 1 hour before transfection. The transfection mix was prepared with 10 µg of total DNA (2,7 µg VSVG, 4,2 µg psPAX2, 3,1 µg shRNA plasmid, ratio 1:1:1) and 500 µl of jetPRIME buffer in a sterile tube. After vortexing, 20 µl of jetPRIME reagent was added and mixed for 10 sec. After a brief spin down, the mix was incubated for 10 min. 500 µl of the mix were then added to each plate, drop wise. The plates were gently rocked for even distribution of the transfection complexes. After 4 hours of incubation, the medium was replaced with fresh medium cells were left incubating for 48 hours. After this period, supernatant was collected and centrifuged for 15 min at 3000 rpm, at 4°C. The supernatant was immediately transferred to clean tubes and stored at -80°C. Titration of lentivirus and calculation of the biological titter was performed according to protocol in BD FACSCalibur™ (BD Biosciences).

CELL TRANSDUCTION

Microglia cells were transduced with shRNAs at the multiplicity of infection of 1. Transduced cells were visualized by the presence of the green fluorescent particle (GFP). On the day of plating, microglial cells were incubated with the shRNAs and left incubating for 48 hours. At the end of this period, cells received fresh medium and were left incubating for 24 hours.

IMMUNOFLUORESCENCE (IF)

For the IF assays, cells were plated over coverslips treated with PDL at the density of $3x10^5$ cells/cm^2. Fixation was then performed for 10 min with 4% paraformaldehyde (PFA). After three washes with PBS (1X), cells were stored at 4°C. Permeabilization was performed with 0,1% Triton X-100 for 15 min and cells were washed three times for 5 min before the blocking step, which was performed by incubating the cells in 3% BSA (Bovine Serum Albumin) in PBS (1X) for one hour at RT. Primary antibodies were incubated in blocking solution overnight at 4°C. After a three-time washing step, cells were incubated with secondary antibodies in blocking solution for one hour. 4',6-Diamino-2-Phenylindole Dihydrochloride (DAPI, 1:20000) (Molecular Probes) was used to stain nuclei (DNA staining). Coverslips were then mounted in Immu-Mount™ medium and analysed with the Leica DMI6000 B inverted microscope.

IF ANTIBODIES (ABs)

Target	Primary AB	Secondary AB
PABPC1	Rabbit 1:500	αRabbit 568 1:1000
YBX-1	Goat 1:100	αGoat 488 1:1000
Nova1	Rabbit 1:200	αRabbit 1:1000
Actin	Phalloidin 647 1:50 (conjugated)	
Nucleus (DNA)	DAPI 1:20 000	

RESULTS AND DISCUSSION

1. Microglia Deactivation

1.1. Definition of the Microglia Deactivation Time-Point

Microglia activation is a well-defined process, characterized in terms of cytokine and inflammatory mediators release, morphologic changes and phagocytic ability.[19] After resolution of the injury, microglia deactivates and reverts to a more resting phenotype.[28] Nonetheless, the process of microglia deactivation remains unclear, and so far, available studies focussed mainly on morphological and cytokine alterations [50], and failed to provide satisfactory mechanistic insight into the regulation of microglia deactivation.

To elucidate the mechanisms that underlie deactivation of microglia, and since there are no deactivation protocols described, we started by implementing an in vitro protocol in the lab (Figure 1). We use primary rat cortical microglial cell cultures prepared as previously described.[88]

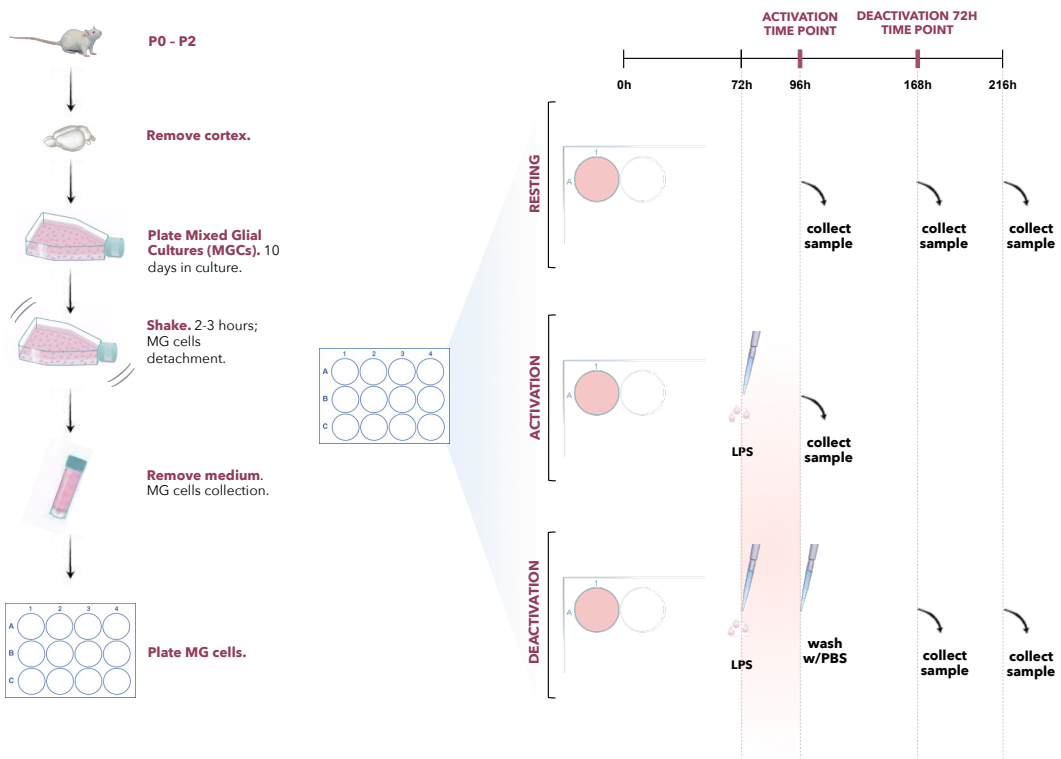


Figure 1. Schematic representation of the experimental activation-deactivation

Therefore, 72 hours after plating, a period defined by us to allow recovery of the cells from the extensive manipulation subjected during the plating process, we induced an M1 activation of microglia by addition of LPS. Upon a 24h-period of stimulation, the media was removed and replaced with new culture medium without LPS. Next, we analysed the mRNA expression of several cytokines by RT-qPCR at different time points after removal of LPS. We tested five time points of deactivation: 24h, 72h, 96h, 120h and 240h.

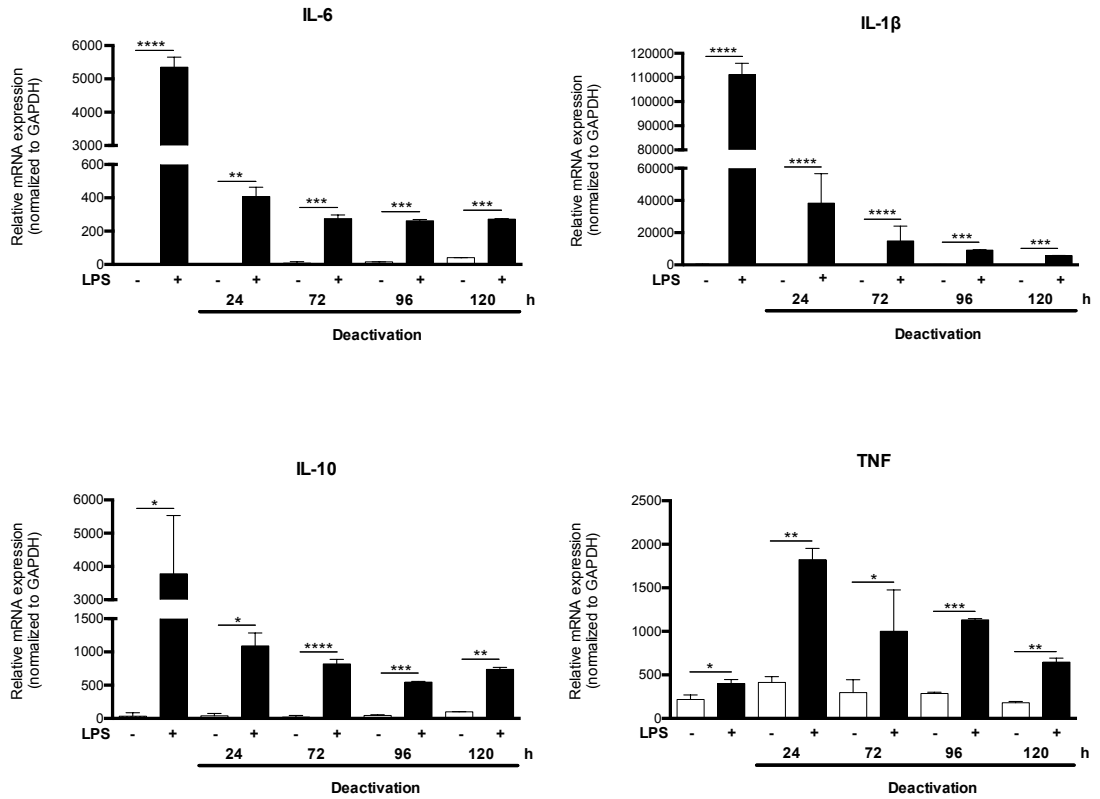


Figure 2. Cytokine mRNA expression in microglia during activation and deactivation. mRNA expression from *Il-6, Il1beta*, *Il-10* and *Tnf* was quantified by RT-qPCR. Error bars show the standard deviation (SD) for at least three independent experiments. *p<0,05, **p<0,01, ***p<0,001. n=4

As expected, the mRNA of the several cytokines increases upon PLS stimulation and starts to decreases upon LPS removal (black bars) (Figure 2). However, the mRNA levels never reach levels as low as the resting levels (white bars) (Figure 2), suggesting that microglial cells retain a pool of mRNAs after facing an LPS insult. We additionally tested a 240h-period of deactivation in one experiment and the results demonstrated the same trend (data not shown). Altogether, we show that after 72 hours of deactivation there are no significant changes in mRNA expression between deactivation time-points, indicating that the cell, despite the time left in culture, never

lowers its mRNA levels. Taking these results into account, and since *in vivo* studies based in morphological criteria suggest that microglia initiates deactivation three days after an insult [26], we selected the 72h deactivation time-point to continue our work.

To further assess the response of microglial cells during deactivation, we decided to study if *TNF* mRNA, which remains upon deactivation was being translated into protein. For that, we quantified the TNF protein production in samples collected from all the deactivation points (Figure 3). We observed that during deactivation, microglial cells stop releasing TNF, although keeping its mRNA (Figure 2 and 3).

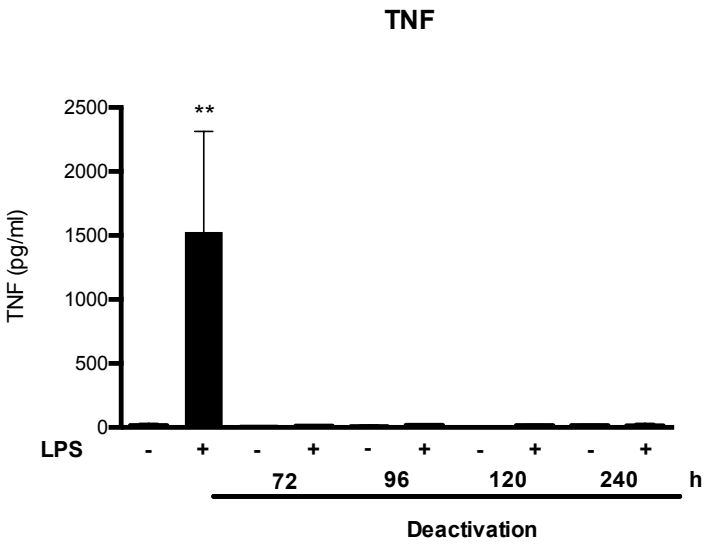


Figure 3. TNF production in activated/deactivated microglia. Supernatants from microglial cultures were harvested at different time points and the production of TNF assessed by immunoassay. Error bars show the SD for at least three independent experiments. **p<0,01. n=3

These results may suggest that the cell maintains the mRNA silenced until further stimulations. In addition, these results may also be responsible for the observed exacerbation of inflammatory reaction described in secondary responses (further discussed in chapter 1.2). In the same way, another possible scenario is that during deactivation, RBPs maintain the correspondent mRNAs silent, inhibiting their translation into protein (discussed in more detail in the chapter 3).

1.2. Microglia Deactivation Suggests a Priming State

The fact that microglial cells showed no TNF protein production during deactivation (Figure 3) but retained a pool of TNF mRNA (Figure 2) led us to question whether these cells had a mechanism of becoming primed and therefore be able to prepare a stronger and faster response to a second stimulus. The mechanism of priming has been studied in macrophages [54], raising the interesting question on whether cells from the innate immune system share some characteristics with cells from the acquired immune system, since priming would suggest some sort of 'memory-like' phenotype.
In line with these evidences, we decided to evaluate this "priming" capacity in our model. For this, we designed an experiment that would allow assessing potential differences in the response to a second stimulus, comparing microglial cells that were pre-stimulated with LPS and naive cells (Figure 4). Our goal was to define an LPS dose that would produce stronger responses in the pre-stimulated microglia. For that, we tested the ability of microglia to produce TNF, upon a second stimulation with several LPS doses (Figure 5).

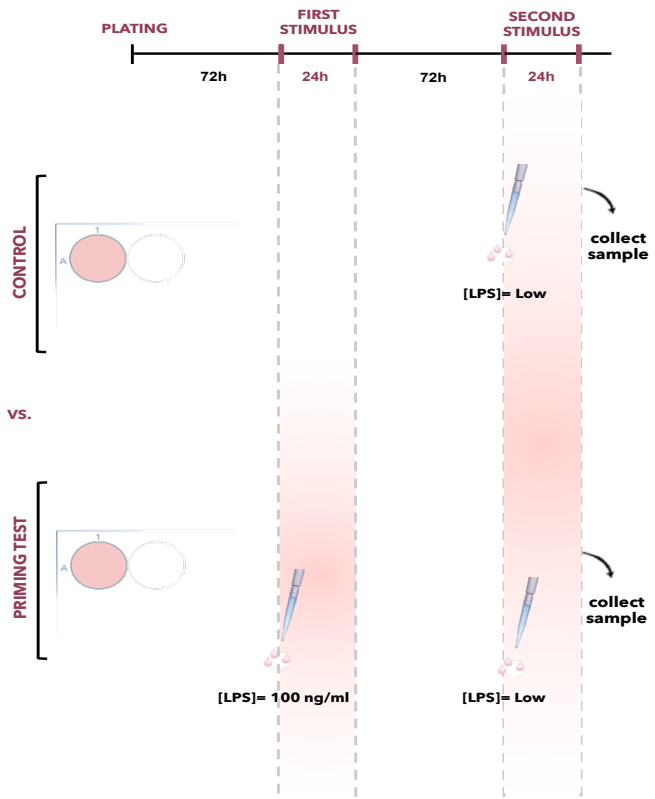


Figure 4. Schematic representation of the experimental 'priming' protocol

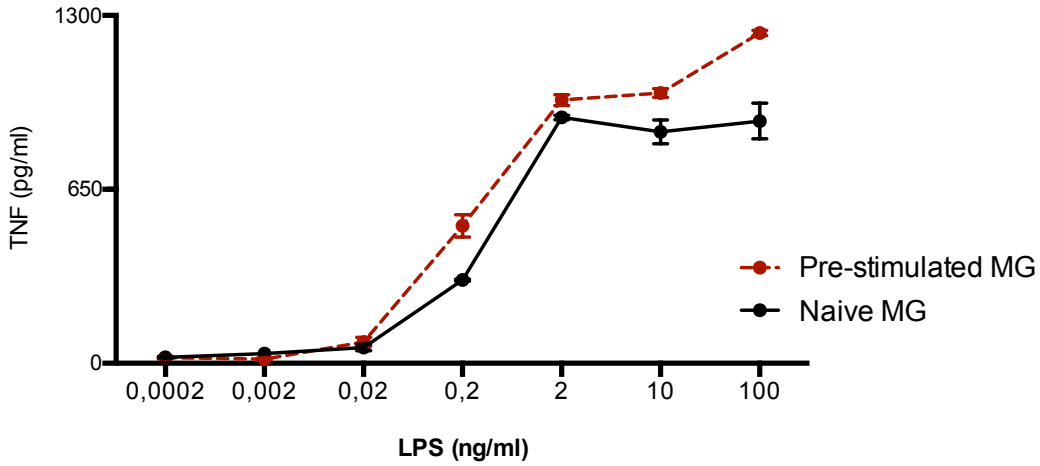


Figure 4. TNF production in primed microglia. Supernatants from microglial cultures stimulated with different doses of LPS were harvested at 24h post stimulation and the production of TNF assessed by immunoassay. Error bars show the SD. n=1

According to our results, microglial cells that potentially became 'primed' facing an LPS pre-stimulation (red line) engage in stronger production of TNF when facing lower concentrations of LPS in a second event, comparing to naive cells (black line), which did not face any prior LPS stimulation (Figure 5). Moreover, the same pattern was observed with higher LPS doses.

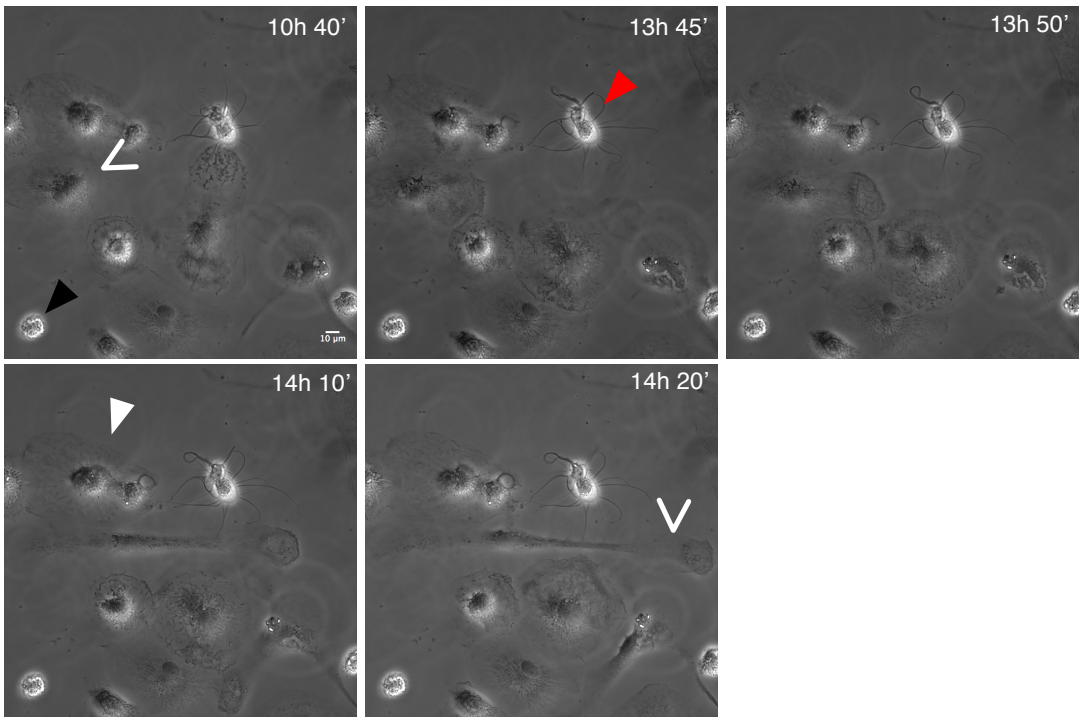
Taken in consideration all the data, these results suggest that microglial cells may be capable of becoming primed, maintaining a pool of mRNAs after a primary insult (Figure 2) that allows them to be more sensitive and more potent in their response to a second stimulus (Figure 5).
In the future, it will be important to address if this 'priming' state is exacerbated in subsequent LPS stimulations, or on the other hand, become less efficient. To address that, we will perform several cycles of microglia activation/deactivation, with multiple LPS doses. Additionally, it is also relevant to dissect whether this 'primed' state is also antigen specific, as in T cells. To assess this issue, we will perform LPS stimulations in combination with different other stimuli, e.g. other TLR ligands and the myelin basic protein (MBP) peptide. With this approach, we shall be able to understand if microglia acquires a memory-like phenotype, similar to T cells.

1.3. Microglia Does Not Reveal Visual Dynamic Changes Between Resting and Deactivation

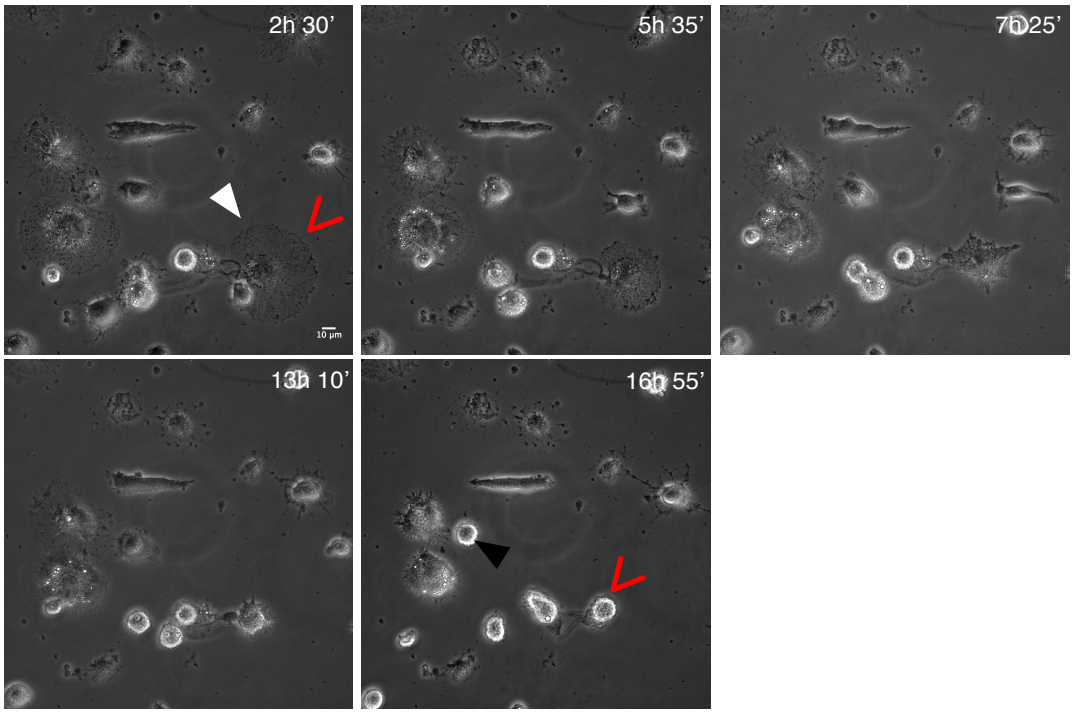
Microglial cells are highly dynamic structures. Along their spectrum of phenotypes, these cells are able to engage in multiple cytoskeleton rearrangements in order to perform specific actions, such as phagocytic clearance of cellular debris and active scanning of the environmental brain tissue.[30] Based on these evidences, we decided to evaluate the potential dynamic alterations in our model, comparing live microglial cells in resting, activation and deactivation conditions using time-lapse live imaging microscopy. Since we found strong evidence that these cells have a specific and particular molecular behaviour during deactivation, we hypothesized that during this process these highly plastic cells could reveal changes in their dynamics.

To assess this, we plated isolated microglial cells in specific microscopy plates and photographed them during long periods of time (up to 24 hours) in each of the states: resting (R), activated (A) and deactivated (D). After putting all of the frames together to create a time-lapse film, we were able to visualize the behaviour of our cultured cells during the different states (Figure 6 and supplemental video).

R.



A.



D.

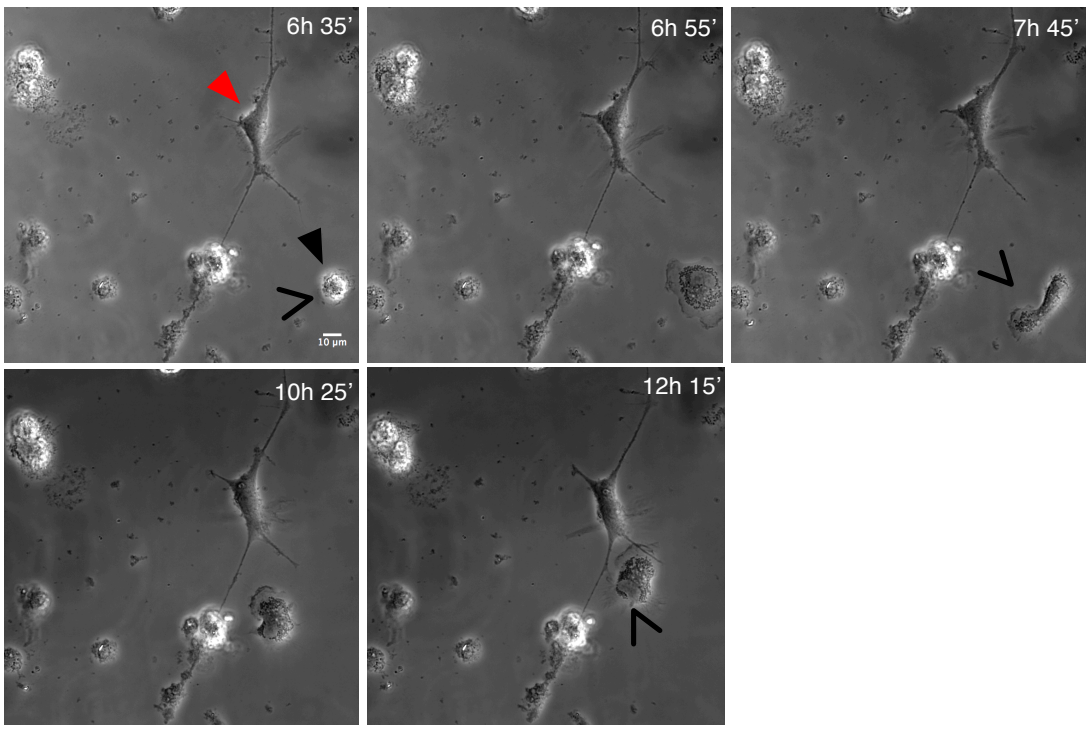


Figure 6. Time lapse images from microglia in culture. Different morphologies are evident (full arrow heads). In activation conditions, cells are seen changing their morphology to a round-shaped cell (**A**). In deactivation conditions, cells are seen reversing to a more relaxed morphology (**D**). 40x.

Three main morphologies were observed, amoeboid cells with large soma (full white arrow head), ramified cells with small soma (red arrow head) and small round-shaped cells (black arrow head) (Figure 6). Previous studies have shown that microglia under *in vitro* conditions present several different morphologies [42] and this has been related to the absence of other glial cells, such as astrocytes that provide mechanical support, but also to the possible co-existence of different sub-populations of microglia belonging to different brain regions.[22] This fact was visible in our experiments.

After analysis of the films generated, we were able to conclude that our microglial cells were healthy and highly motile, extending and retracting their processes in the ramified phenotype, and constantly remodelling their large soma in the amoeboid-shaped phenotype (Figure 6.D, empty white arrow head). We also have observed the well-characterized process of activation, defined by changes in cell morphology from the different aspects of the resting state to small round-shaped cells (Figure 6.A, empty red arrow head). Interestingly, but expected, when LPS is removed and cells are left to deactivate, the round-shaped cells characteristic of the activation state seem to restore their more relaxed and motile behaviour (Figure 6.D, empty black arrow head).

We focused in comparing the deactivation state and the resting state, since our aim is to assess whether microglia, when deactivated, returns to a resting state or to a different new phenotype. However, we did not observe significant changes in cell dynamic behaviour between the resting and the deactivation states (supplemental video). Microglial cells present the same type of phenotypes in both conditions and perform the same dynamic patterns. Despite the lack of visible changes in the deactivation state, this experiment allowed the visualization of microglial cells during a deactivation process for the first time, since published data on visual representations of microglia deactivation *in vitro* are not available.
To better evaluate these results, a more detailed analysis is needed. Morphological dynamic parameters have to be defined in order to quantitatively assess the potential changes in the deactivation state.

2. *In Silico* Analysis of Genomic Conservation of 3'UTRs of Inflammatory Genes and Putative RBPs Binding-Sites

The 3' UTR of mRNAs is a region that allows regulation of RNAs biology, such as promotion or inhibition of translation, mRNA stability or polyadenylation, by the specific binding of RNA-binding proteins and other regulators.[58] Microglia can be polarized into M1 or M2 phenotype, with expression of specific genes.[23] For this reason, previous work done in the lab has focused on the analysis of RBPs putative binding sites on the 3'UTR of the genes commonly known to be involved in microglia polarization in M1 and M2 phenotypes. This analysis was made with the 'PBS-Finder', a bioinformatics tool created in the lab, that allows for faster searches in several gene and RBPs databases. The results of the bioinformatics analysis are shown in Table 1.

Table 1. *In silico* analysis of the putative binding sites for several RBPs in the 3'UTR of genes characteristic from M1 and M2 microglia profile.

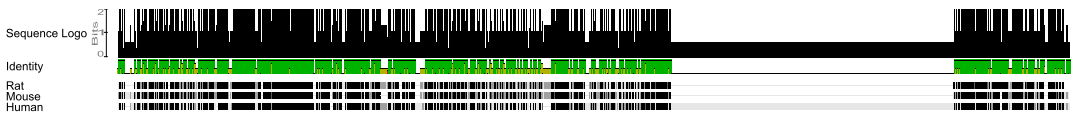
	gene	EIF4B	PABPC1	PUM2	MBNL1	RBMX	SFRS1	KHDRBS3	SFRS13A	ELAVL1	FUS	YTHDC1	NONO	RBMY1A1	YBX2-A	SFRS9	KHSRP	VTS1
M1 phenotype	*Nos2*	x	x	x	x	x	x	x	x	x	x	x		x		x		
	Ifnb1	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
	Tnf	x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	
	Il6	x	x	x	x	x	x	x	x	x				x				
	Il6ra	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
	Smad2	x	x	x	x	x	x	x	x	x	x	x		x		x	x	x
	Socs1	x	x	x	x	x	x	x	x	x	x		x	x		x	x	x
	Cebpb	x	x	x	x	x	x	x	x	x	x	x						x
	Nfkb1	x	x	x	x	x	x	x	x	x	x	x				x	x	x
	il1b	x	x	x	x	x	x	x	x	x	x	x		x	x	x	x	x
M2 phenotype	*Jun*	x	x	x	x	x	x	x	x	x	x	x	x	x		x	x	x
	Tgfb1	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
	Arg1	x	x	x	x	x	x	x	x	x	x	x						
	Retnla (FIZZ)	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	
	Il10	x		x	x	x	x	x	x	x	x	x	x		x	x	x	x

	gene	VTS1	SAP-49	ACO1	ZFP36	ELAVL2	QKI	RBM4	HNRNPA1	A2BP1	NCL	PUM	PTBP1	SNRPA	ZRANB2	SFRS2	Nova	YBX1
M1 phenotype	*Nos2*					x										x	x	
	Ifnb1	x				x	x		x						x			
	Tnf			x	x	x								x				
	Il6		x		x	x											x	
	Il6ra	x	x	x		x	x	x						x				x
	Smad2	x	x				x							x	x			
	Socs1	x				x								x	x			
	Cebpb	x				x		x										x
	Nfkb1	x		x		x						x	x					
	il1b	x			x	x	x		x								x	x
M2 phenotype	*Jun*	x		x		x	x	x	x	x	x							
	Tgfb1	x	x	x														
	Arg1																	
	Retnla (FIZZ)				x	x	x		x	x								
	Il10	x	x	x	x		x											

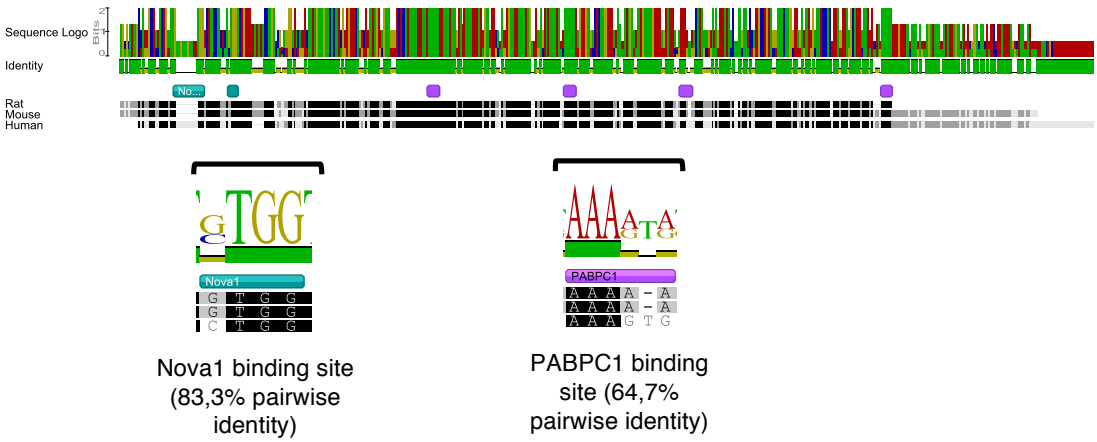
Most of the RBPs present in the table are expressed in microglia [89], but there is still not enough work done in order to understand their specific roles in these immune cells. We observed that some RBPs are more specific for M1 polarization than others: Poly(A) Binding Protein, Cytoplasmic 1 (PABPC1) has putative binding sites in almost all genes involved in microglia polarization. This RBP has the particularity of binding to the poly (A)-tails of all mRNAs, although it can also bind to the 3'UTR independent of poly(A) tails as observed in the table.[70] As for neuro-oncological ventral antigen 1 (Nova1) and Y box binding protein 1 (YBX1), the results show a specific binding in genes characteristic of the M1 phenotype; and ataxin 2-binding protein 1 (A2BP1) and nucleolin (NCL) seem to bind to target genes associated with the M2 phenotype (Table 1).
These results suggest that different RBPs are involved in different pathways of microglia physiology and therefore may control their function.
Thus we intended to investigate whether RBPs play a role in microglia activation/deactivation, by modulating the expression of M1 genes. For that, we selected 3 candidates RBPs (from Table1) that putatively only regulate M1 but not M2 target genes, which are PTBP1, Nova1 and YBX1. Additionally, as a control, we selected the RBP PABPC1 that binds to genes of both M1 and M2 phenotypes, and A2BP1 that binds to M2 genes.

To better characterize the putative RBPs binding sites, the genome conservation of the 3'UTR of the selected M1 genes and putative RBPs binding sites was evaluated. To that purpose, *TNF*, *IL-1beta*, *IL-6*, *IFN beta* and *IL-10* 3' UTR sequences from rat, mouse and human were gathered from the NCBI nucleotide database (ncbi.nlm.nih.gov/nuccore/) and Ensemble genome database (http://www.ensembl.org/), and further aligned using Geneious v4.7 software.

A. *IL-10* 3'UTR (59,5% pairwise identity)



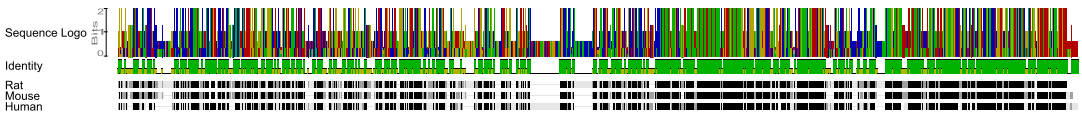
B. *IL-6* 3'UTR (77,8% pairwise identity)



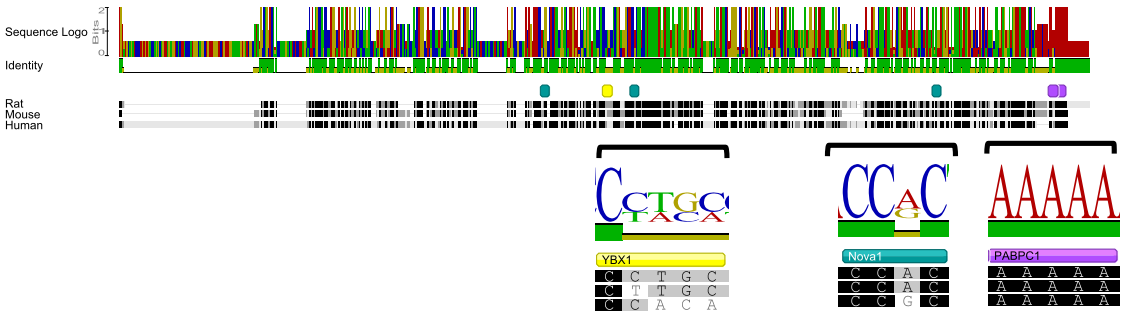
Nova1 binding site (83,3% pairwise identity)

PABPC1 binding site (64,7% pairwise identity)

C. *TNF* 3'UTR (69,0% pairwise identity)



D. *IL-1beta* 3'UTR (57,0% pairwise identity)



YBX1 binding site (46,7% pairwise identity)

Nova1 binding site (83,3% pairwise identity)

PABPC1 binding site (100,0% pairwise identity)

E. *IFN beta* 3'UTR (72,7% pairwise identity)

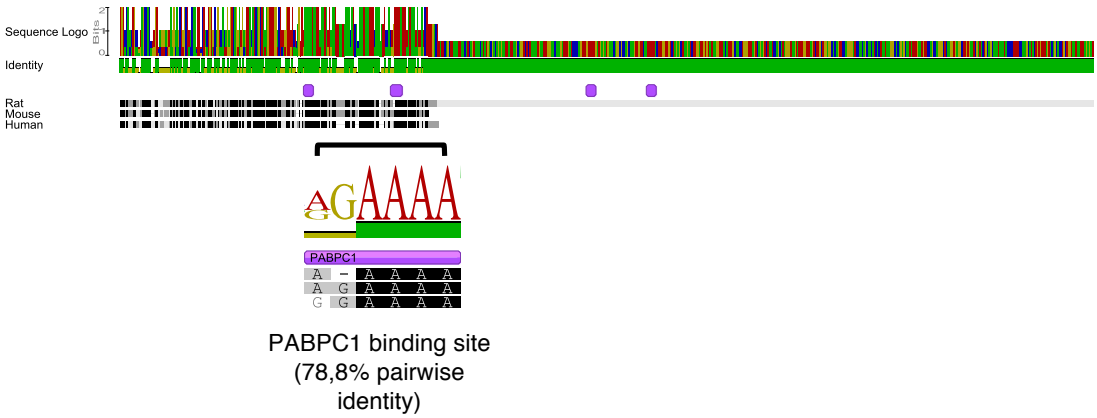


Figure 7. Genomic alignment of M1 (*IL-6*, *TNF*, *IL-1beta*, *IFN beta*) and M2 (IL-10) genes 3'UTR sequences (rat, mouse and human). 3'UTRs showing the high conservation throughout all sequences (showed by green horizontal Identity bars), with close-up on RBPs putative binding sites.

The 3'UTRs of all the genes studied revealed a pairwise identity ranging from 50% to 80%, with genes as *IL-6* sharing a 77,8% of conservation of their 3'UTR between the three mammalian species.

The putative binding sites for the several RBPs were also analysed in the corresponding target mRNAs. For example, the binding site for Nova1 in the 3'UTR of *IL-6* shares 83,3% of pairwise identity between all three species.

Although this analysis was performed in only three species, the high level of conservation reveals that these sites were maintained and persisted throughout evolution despite speciation, and it is an indicative of their importance as regulatory regions in these organisms.

3. RBPs expression in Microglia

3.1. RBPs show different expression profiles in Microglia

RBPs have been linked to stabilization of cytokines mRNA in macrophages[80] and therefore indicated to have a role in the activation of macrophages. Since microglial cells are the resident immune cells in the CNS, we sought to study the role of RBPs in the activation and deactivation processes of these cells.
For this, we studied mRNA expression of the selected RBPs (PABPC1, PTBP1, YBX1 and Nova1) by RT-qPCR in our model.

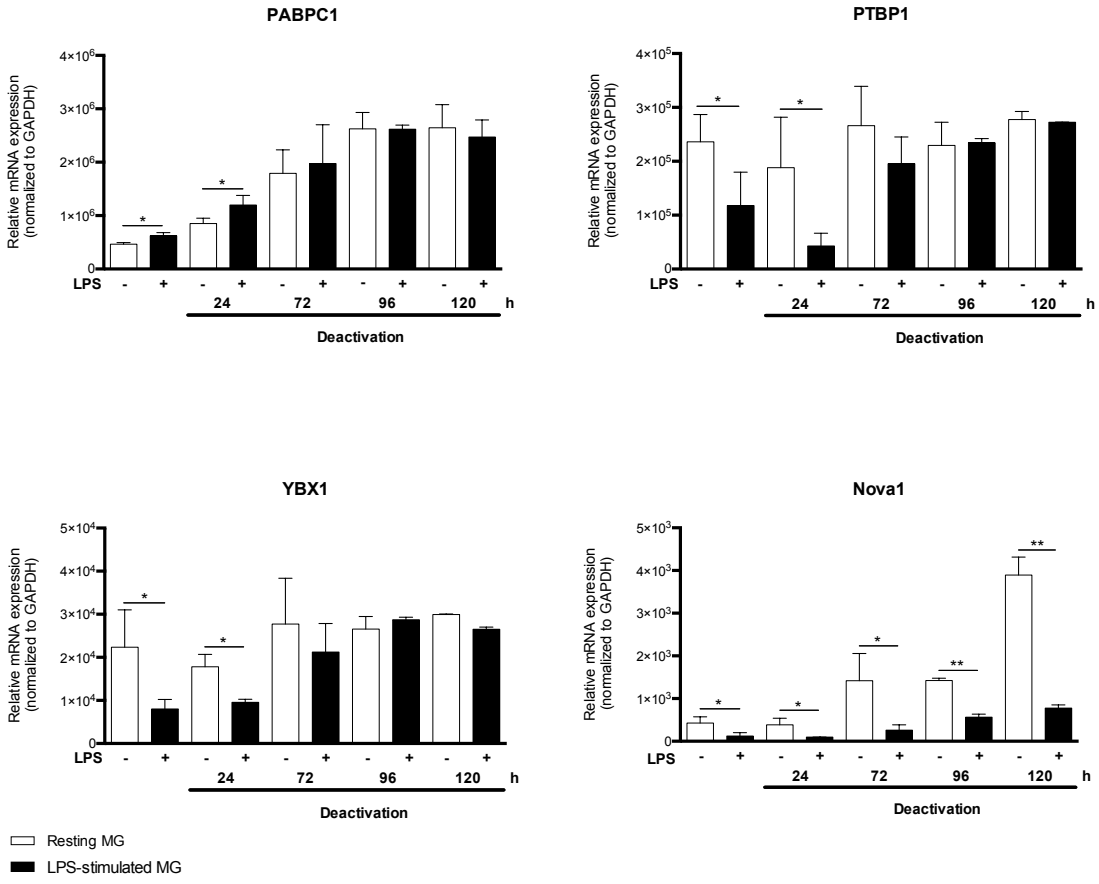


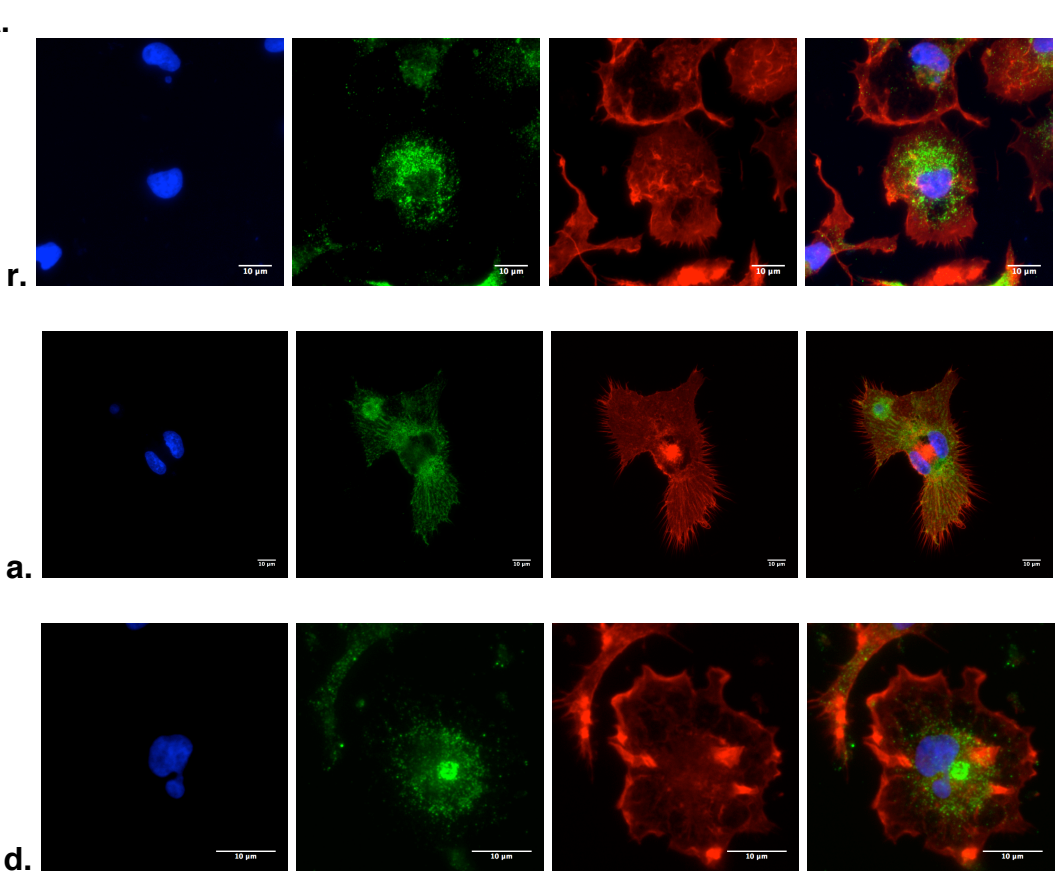
Figure 8. RBPs mRNA expression in microglia during activation and deactivation. mRNA expression from *Pabpc1*, *Ptbp1*, *Ybx1* and *Nova1* was quantified by RT-qPCR. Error bars show the SD for at least three independent experiments. *p<0,05, **p<0,01. n=4

As shown in Figure 8, all RBPs analysed were modulated during microglia activation/deactivation. *Nova1*, *Ybx1* and *Ptbp1* mRNA levels decrease during the activation of microglia; on the contrary, *Pabpc1* mRNA levels increase

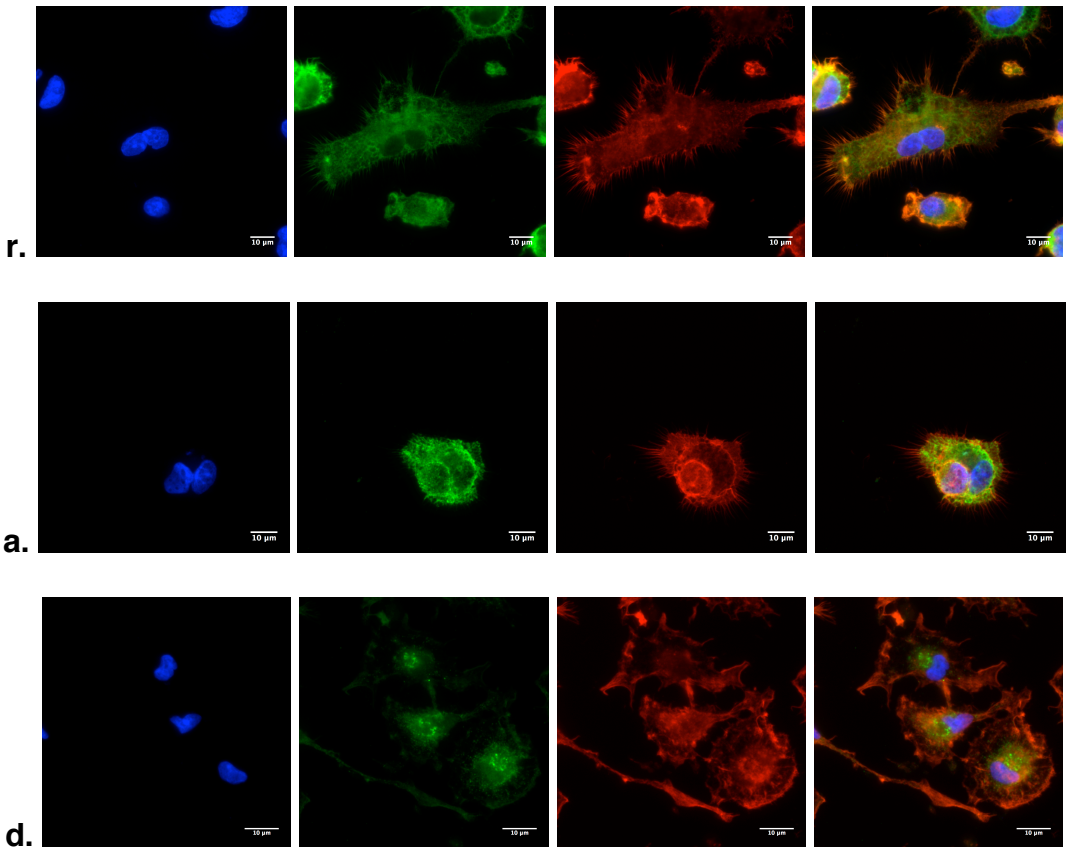
during this process. However, during the deactivation process and with the exception of *Nova1*, all the analysed RBPs return to levels identical to the resting levels, revealing no significant changes between the two conditions (cells exposed to LPS vs. naive cells) upon 72 hours (Figure 8).
Interestingly, the *Nova1* profile was different and it is clear that even after LPS removal the levels of this RBP remain low (black bars) when comparing to resting cells (white bars) (Figure 8). Also, we observed that cells in resting conditions increase their *Nova1* mRNA levels over time (white bars). This led us to the possibility that Nova1 may be necessary for maintain microglia in resting conditions.
Nova1 has been related to alternative splicing in neurons but no work has been done in microglia yet. Potentially, the fact that Nova1 levels do not return to the ones seen in resting conditions could be used as a biomarker for microglia 'priming'.

3.2. RBPs intracellular localization

Since RBPs function is intrinsically related with their cellular localization[69], we performed immunocytochemistry assays to identify the localization patterns of our candidates during the processes of resting, activation and deactivation.



B.



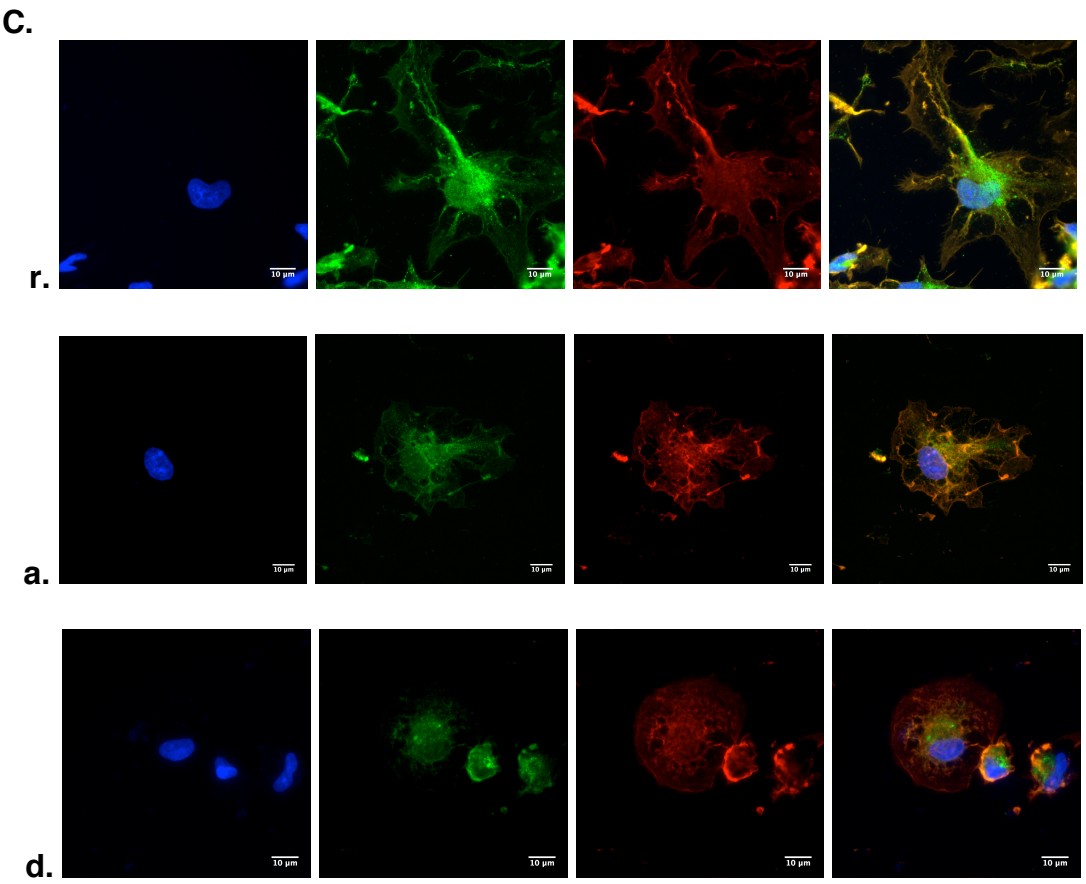


Figure 9. YBX1 (**A**), PABPC1 (**B**) and NOVA1 (**C**) intracellular localization in microglial cells in resting (**r**), activated (**a**) and deactivated (**d**) conditions. RBPs are marked in green, actin in red and the nucleus in blue. 63x.

Our results demonstrate that YBX1 localization during resting conditions is restricted to the perinuclear region (Figure 9A.r). After activation of microglia by LPS, this RBP appears spread throughout the cytoplasm (Figure 9A.a). After 72 hours of deactivation, YBX1 relocates to the perinuclar area, showing some nuclear localization also (Figure 9.A.d).

PABPC1 is known to be located mainly in the cytoplasm and this was observed in all conditions, although no relevant changes in localization of this RBP were observed (Figure 9.B)

Nova1 is described to be localized in the nucleus and also in the dendrites of neurons.[90] In resting microglia, Nova1 is localized in the cytoplasm and in the nucleus (Figure 9.C.r). Once activated, microglial cells presented Nova1 localized more spread in the cytoplasm (Figure 9.C.a). After 72 hours of deactivation, Nova1 appears to be less expressed and is localized more near the nucleus (Figure 9.C.d).

Further studies are necessary to understand the biological functions of RBPs during microglia activation/deactivation, however this data suggests that the

studied RBPs change localization during the process of activation, which may indicate that they are important for translation of genes important for this process.

3.3. Nova1 expression profile seems to be specific to LPS stimulus

In order to further explore the interesting profile of Nova1 in microglia activation (Figure 8) and assess if this is a general effect or, on the other hand, is a specific response to LPS, we tested other types of microglia activation stimulus. We subjected microglia cells to IL-4, a known anti-inflammatory cytokine that induces an M2-like polarization of microglia.

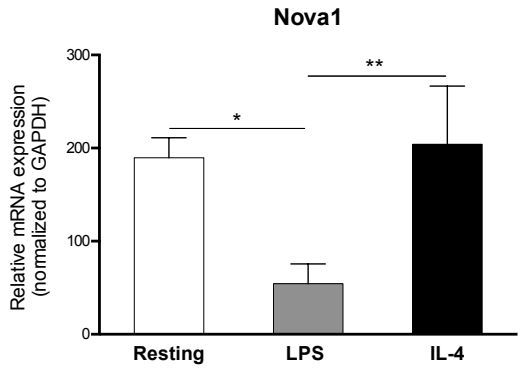


Figure 10. *Nova1* mRNA expression in microglia exposed to LPS and IL-4. mRNA expression from *Nova1* was quantified by RT-qPCR. Error bars show the SD for at least three independent experiments. *p<0,05, **p<0,01. n=3

We observed that *Nova1* mRNA levels are not altered during M2 polarization, comparing to control (Figure 10). This led us to the possibility that modulation of Nova1 could be dependent on a pro-inflammatory stimulus. We then tested another pro-inflammatory stimulus different from LPS, hypoxia.

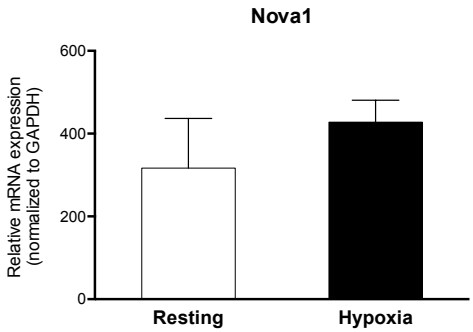


Figure 11. *Nova1* mRNA expression in microglia under hypoxic conditions. mRNA expression from *Nova1* was quantified by RT-qPCR. Error bars show the SD for at least three independent experiments. *p<0,05, **p<0,01. n=3

Interestingly, *Nova1* does not respond to hypoxia in the same way as in the presence of LPS (Figure 11), suggesting that this RBP may respond to a specific molecular pathway involved in LPS cellular recognition. LPS is a ligand to TRL4 and once this receptor is activated it engages a complex pathway cascade culminating in the production of several types of cytokines.[28] Further research is necessary to understand the molecular pathways involved in modulation of *Nova1* mRNA, namely testing other TLR ligands and other type of microglia stimulation.

3.4. RBPs modulation in Microglia

After observing that our selected RBPs were modulated during the process of activation/deactivation (Figure 8), we aimed at assessing the biological function of these RBPs in microglia.

For that, we designed shRNAs for *Ybx1*, *Nova1* and *Pabpc1* and transduced microglia cells by lentiviral vector approach. After transduction, the cells were left in culture without any stimulus for 48 hours to allow the lentivirus to silence the expression of the respective genes. After the 48 hour-period, we analysed the efficiency of the knockdown (KD) by RT-qPCR of the target genes, and we observed knockdowns of about 80% for each gene (Figure 12).

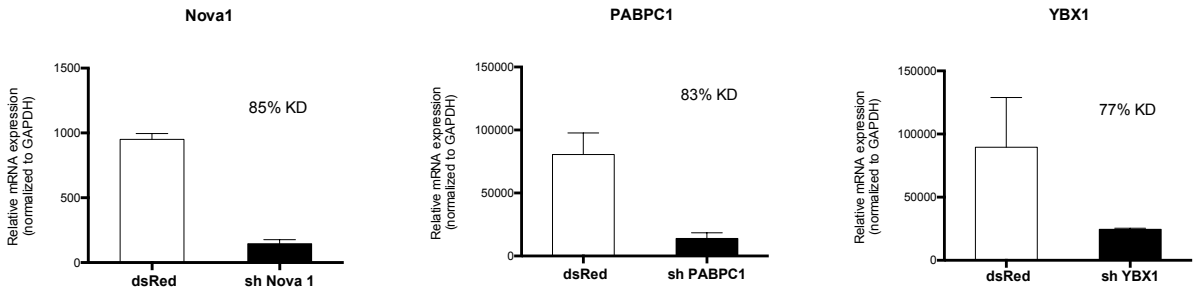
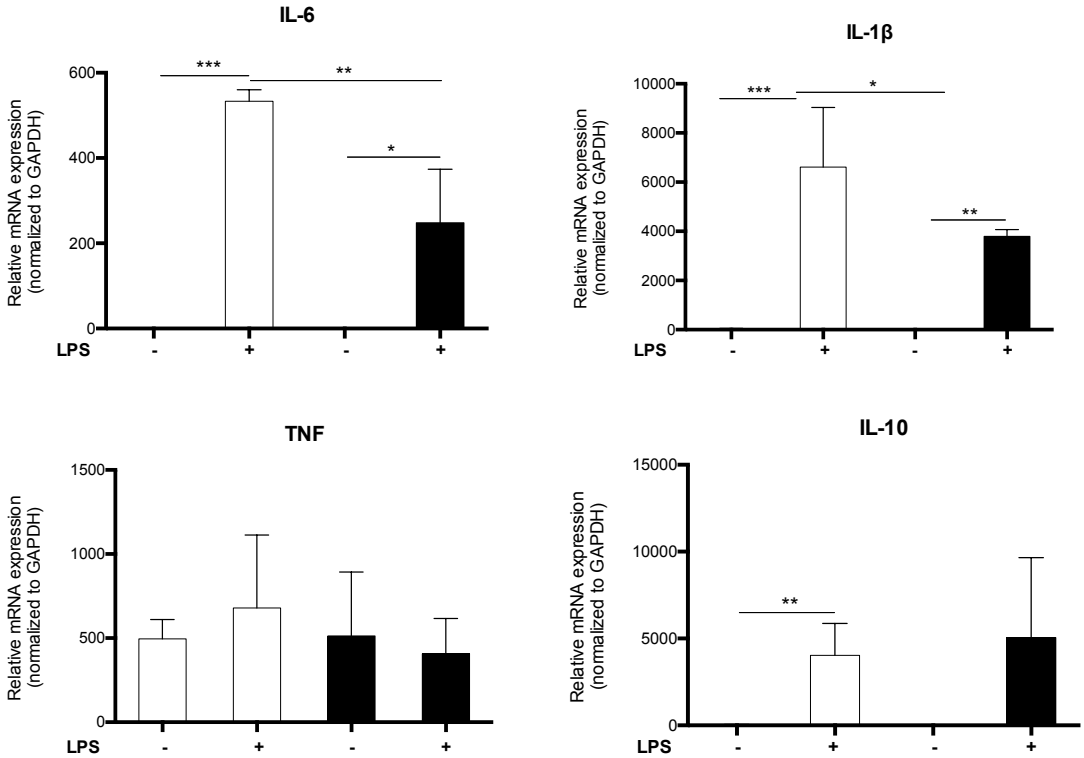


Figure 5. RBPs mRNA expression in microglia upon KD of RBPs. KD efficiency of the shRNAs for *Nova1*, *Pabpc1* and *Ybx1* was quantified by RT-qPCR. Error bars show the SD for at least three independent experiments. n=3

3.4.1. Knockdown of *Pabpc1*

After knocking down *Pabpc1*, we analysed the capacity of microglia to respond to LPS activation, by analysing the mRNA levels of *Il-6*, *Il-10*, *Il-1beta*, *Tnf* and *Ifn beta*.



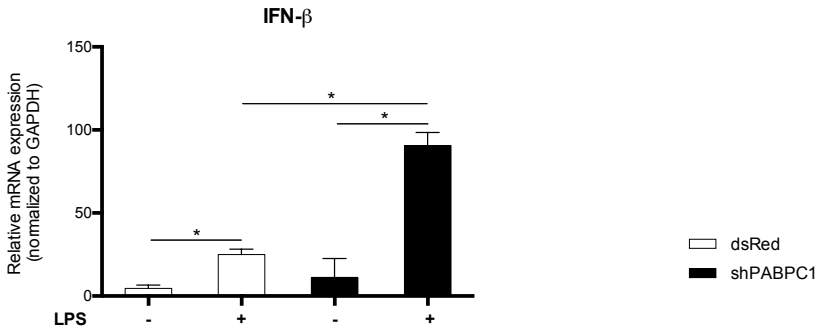


Figure 13. Cytokine mRNA expression in microglia upon shPABPC1. mRNA expression from *Il-6, Il-10, Il1beta, Tnf,* and *Ifn beta* was quantified by RT-qPCR. Error bars show the standard deviation (SD) for at least three independent experiments. *p<0,05, **p<0,01, ***p<0,001. n=4

Deficiency of PAPBC1 in microglia impairs *Il-6* and *Il-1beta* mRNA expression upon activation (Figure 13), suggesting that PABPC1 may be necessary to regulate the transcription or stability of these mRNAs. This fact is also supported by our previous results showing that *Pabpc1* increases upon microglia activation (Figure 8), evidencing a potential relationship between this RBP and microglial activation.

Il-6 has several putative binding sites for PABPC1 in its 3'UTR (Table1 and Figure 7.B), suggesting that maybe this is a regulatory region crucial for the physiologic ability of microglia to express IL-6. PABPC1 has a translation activator activity[70], meaning that it can be involved in ribosome-mediated translation of mRNAs into proteins; therefore PABPC1 may be involved in the normal processing of these mRNAs through this mechanism. In its absence, since mRNAs cannot follow the normal pathways to translation, they could undergo downregulation.

However, regarding *Il-10* and *Tnf* mRNA expression (Figure 13), *Pabpc1* KD does not induce any effects. This may be related to the fact that neither of these mRNAs have putative binding sites for this RBP in their 3´UTR (Table1).

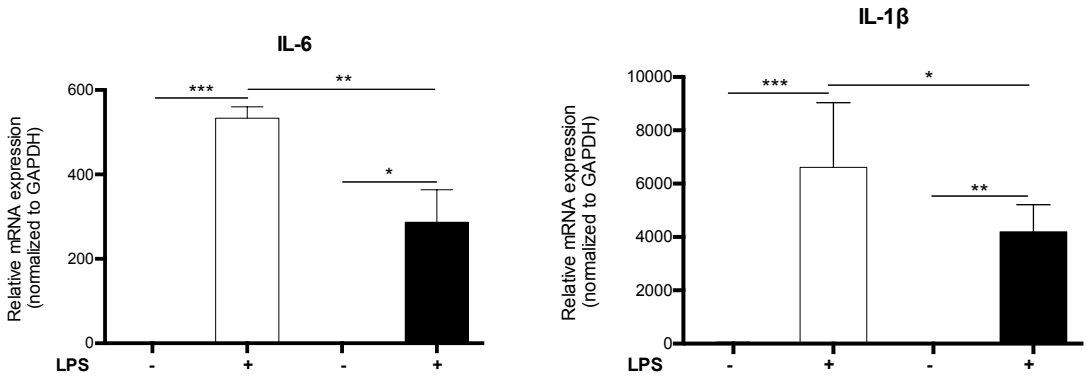
Interestingly, in the case of *Tnf* mRNA expression, we observed that there are no differences between resting and activated conditions, suggesting that microglia is not able to properly activate when exposed to LPS.

This phenomenon could be explained by the fact that microglial cells are very sensitive cells and any disturbance can be seen as an insult; therefore, when these cells are transduced with lentivirus they can already start an activation process. Additionally, TNF is one of the fastest cytokines to be produced by immune cells. So, the expression levels of *Tnf* observed in resting conditions

could result from a fast expression of *Tnf* by microglia caused by the lentiviral transduction that was interpreted as a first insult. Later, when we subject the cells to LPS, since they have already started the activation process of *Tnf* expression, and taking into account our previous data (Figure 2 and 3), the cells may be keeping the mRNA to be translated later. In this case, they might not feel the need to express more *Tnf*, but only translating it into protein, explaining the low levels of expression seen after LPS stimulation.
Considering *Ifn beta* mRNA expression (Figure 13), we observed that the absence of PABPC1 seems to potentiate the response of microglia to LPS. Analysis of Table 1 reveals putative binding sites for PABPC1 in the 3'UTR of *Ifn beta*, leading to the suggestion that this RBP could be involved in the physiology of this cytokine. PABPC1 might be involved in the silencing of the mRNA of *Ifn beta* in normal microglia activation; when it is in lower amounts, it can allow for this mRNA to be maintained.
Our results from the knockdown of *Pabpc1* indicate an intrinsic role for this RBP in microglia activation.

3.4.2. Knockdown of *Ybx1*

Similar to the KD of *Pabpc1*, knocking down *Ybx1* decreases *Il-6* and *Il-1beta* mRNA expression upon microglia activation by LPS (Figure 14).



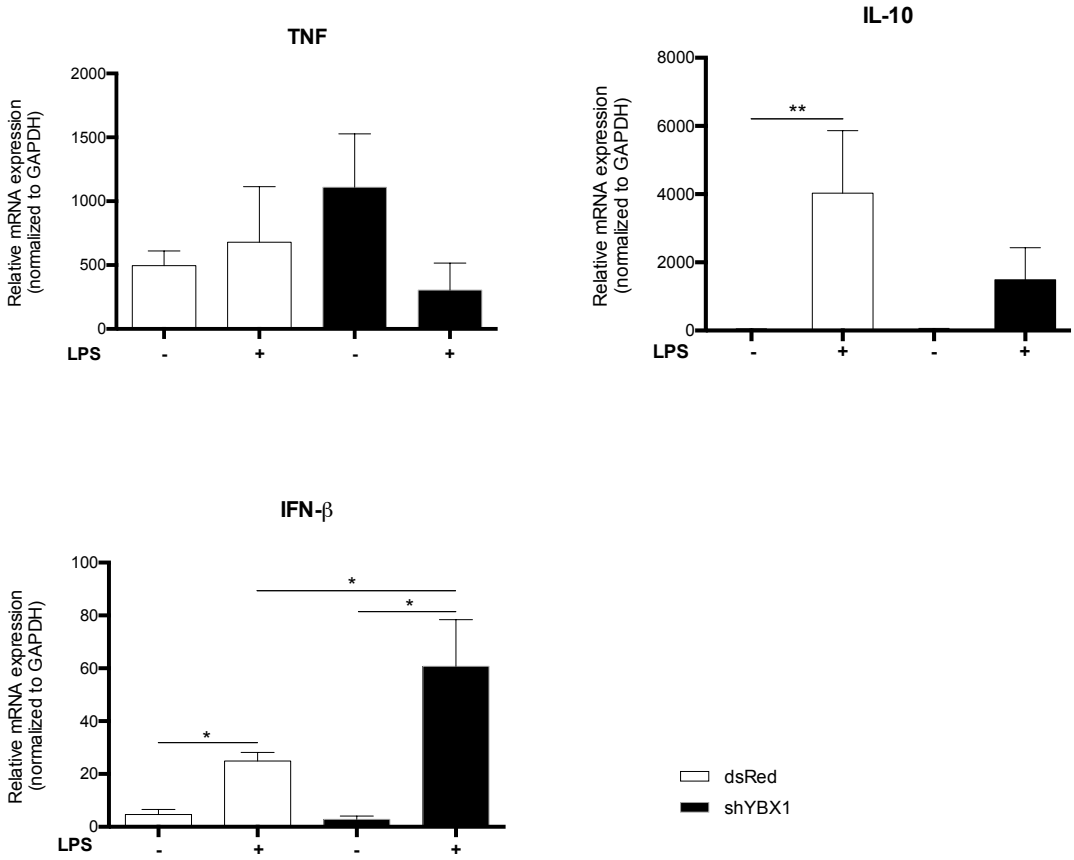


Figure 14. Cytokine mRNA expression in microglia upon shYBX1. mRNA expression from *Tnf, Il-1beta, Il-6, Ifn beta* and *Il-10* was quantified by RT-qPCR. Error bars show the SD for at least three independent experiments. *p<0,05, **p<0,01, ***p<0,001. n=4

YBX1 has been reported to be involved in transcription regulation of several mRNAs[64], suggesting that despite not having putative binding sites in the 3'UTR of *Il-6* (Table 1) it could be involved in the transcription or stability of this mRNA by an indirect pathway.

Also in the case of *Tnf*, the levels of expression in resting conditions do not change upon LPS stimulation, indicating that YBX1 is important for microglia activation. Additionally the expression of *Il-10*, is also not affected by the deficiency of YBX1 (Figure 14).

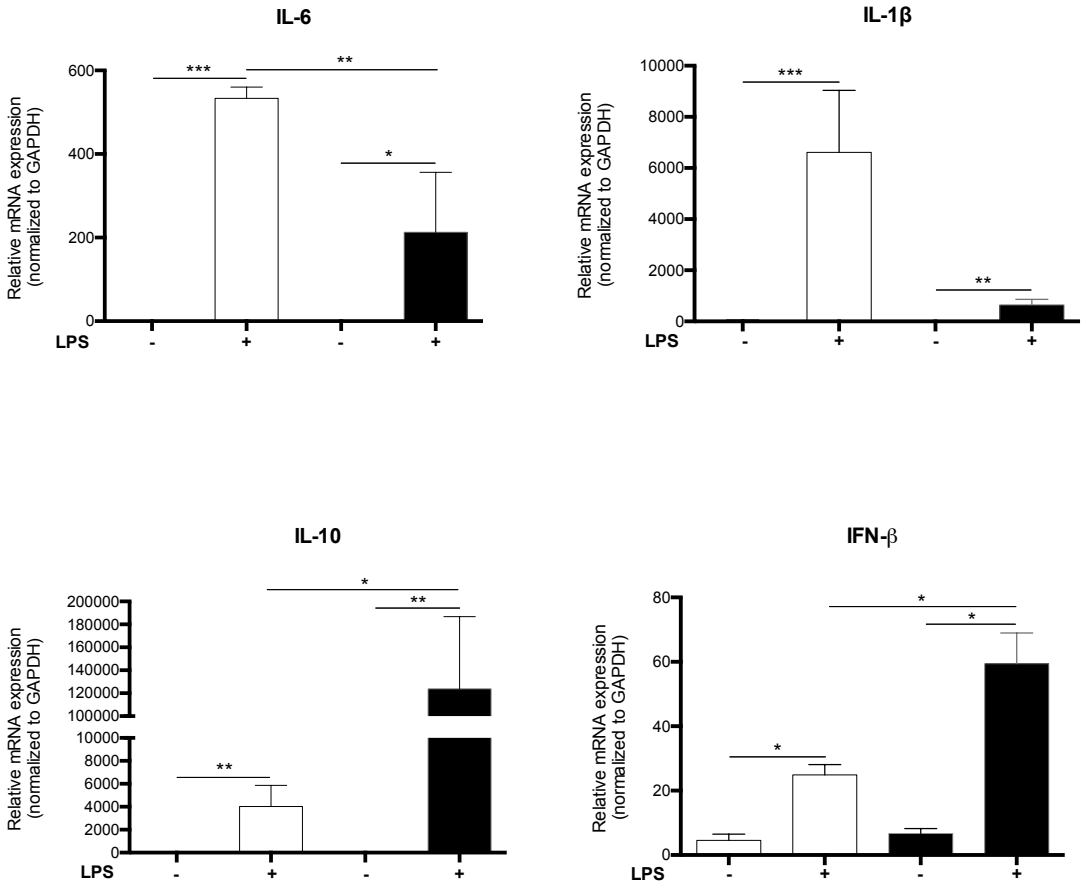
Ifn beta expression is increased upon microglia activation in the absence of YBX1 (Figure 14) similarly to the absence of PABPC1 (Figure 13). YBX1 does not have putative binding sites for the 3'UTR of *Ifn beta*, but it could, as suggested before, perform regulation of this mRNA by indirect pathways, or by indirect binding with other RBPs.

Taken together, our results of the KD of *Ybx1* suggest that the RBP encoded by this gene is also important for microglia activation.

3.4.3. Knockdown of *Nova1*

NOVA1 is a key brain-specific regulator of alternative splicing, a mechanism underlying gene expression.[63] Although this RBP has been recently identified in other types of cells outside the brain tissue, its most important role has been related to neurons.[62] Here, we intend to assess the potential biological function of this protein in microglia activation, since our results have shown an interesting pattern of mRNA expression of this gene during the processes of activation and deactivation of microglia (Figure 8).

Deficiency of NOVA1 in microglial cells impairs their ability to express *Il-6* and *Il-1beta* upon LPS-mediated activation (Figure 15). As seen in Table 1, *Il-6* and *Il-1beta* have putative binding sites for NOVA1 in their 3'UTRs, suggesting a possible direct regulation of these mRNAs by NOVA1.



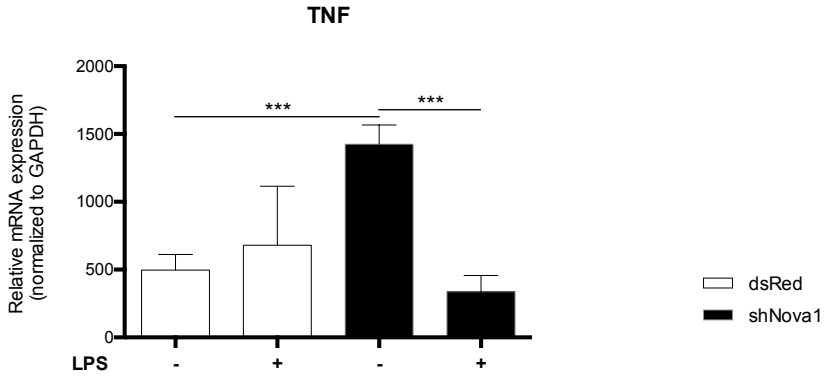


Figure 15. Cytokine mRNA expression in microglia upon shNOVA1. mRNA expression from Tnf, IL1beta, Il-6, Ifn beta and Il-10 was quantified by RT-qPCR. Error bars show the SD for at least three independent experiments. *p<0,05, **p<0,01, ***p<0,001. n=4

As also shown previously, *Nova1* increases during microglia resting conditions (Figure 8), leading to the hypothesis that the RBP encoded by this gene is necessary for maintaining microglial cells in a resting state. Likewise, when microglia becomes activated there is a decrease in the expression levels of *Nova1* (Figure 8), supporting the possibility that, for microglia to respond to LPS, NOVA1 is necessary at lower levels. If this was a direct regulation, we might expect for *Il-6* levels to become increased when knocking down *Nova1*, although this is not observed, suggesting a possible indirect mechanism of regulation.

On the other hand, the mRNA of the anti-inflammatory cytokine IL-10 shows to be increased during microglia activation (Figure 15), in the absence of NOVA1. IL-10 is known to play roles in balancing inflammatory responses, by downregulating some pro-inflammatory cytokines, such as IL-6 and TNF[91]. According to this, one possible effect of the increase of *Il-10* could be the counterbalance of *Il-6* expression, not allowing a more prominent expression of *Il-6*, as shown in Figure 15.

Interestingly, and similar to other RBPs KD, the mRNA levels of *Ifn beta* increase upon *Nova1* KD (Figure 15), although not putative binding sites were predicted in the 3'UTR of this mRNA (Table1). One possible explanation for the difference in the expression of *Ifn beta* in contrast to *Il-6* or *Il-1beta* may be related with LPS-TLR4 signalling. TLR4 activation by LPS results in intracellular signals mediated by both MYD88 and TRIF. As a result of the TRIF-pathway activation, a signalling complex involving IKK and TBK-1 is formed, which catalyse the phosphorylation of IRF3 and induce its nuclear translocation, leading to the transcription of *Ifn beta*.[80] Thus, this may indicate that the TRIF pathway is only activated in the absence of the RBPs tested, or

that since the MYD88 pathway is downregulated during KD, the TRIF pathway is upregulated. Also, this would explain why *Nova1* expression is only modulated by the activation of the TLR4 pathway (Figure 10-11). Further studies need to be performed to address these points.

Interestingly, *Tnf* expression increases in resting knocked down cells (3rd bar) comparing to control (1st bar) (Figure 15). In contrast to the other tested RBPs, this increase is significantly higher than in control cells (1st bar), suggesting that it is the specific absence of NOVA1 that is responsible for this increase. This is in accordance with our hypothesis that NOVA1 may be necessary for cells to maintain a resting state, since its absence is sufficient to induce microglia activation and *Tnf* expression. When cells are subjected to LPS in the absence of NOVA1, the results show that microglia fails to respond in terms of expression of this cytokine (4th bar) (Figure 15). This fact can be due to several reasons, one being the counterbalancing effect of *Il-10*, which is increased in these conditions (Figure 15). Another possible reason is that the cells were no longer able to produce an effective response due to the fact that removal of NOVA1 was already interpreted as a strong insult. Additionally, this could also be explained by the fact that *Tnf* mRNA has already been produced, and microglial cells are at this point engaging in protein translation and secretion.

To further assess this issue, we analysed the protein levels of TNF being produced during this process in the absence of NOVA1 (Figure 16).

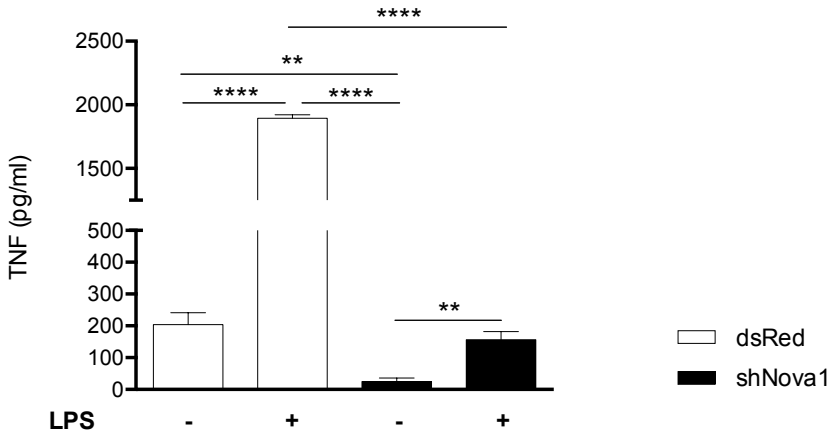


Figure 16. TNF production in microglia during KD of *Nova1*. Supernatants from microglial cultures were collected 24 hours upon LPS stimulation and the production of TNF assessed by immunoassay. Error bars show the SD. n=3

As shown, in resting conditions without NOVA1 (black bars), TNF protein levels were barely detected in the supernatant. In contrast, when microglia is subjected to LPS in the absence of NOVA1, TNF protein levels increase comparing to resting. However, they fail to reach higher levels as in control activation conditions (white bars).
The discrepancy between mRNA expression and protein production in resting conditions was intriguing but may be due to limitations in our experimental protocol. TNF is one the fastest cytokines to be produced by immune cells; studies show that this cytokine may be detectable within the first two hours of macrophage activation.[92] The fact that we collected microglia cells 72 hours after lentiviral exposure, and because microglia react promptly to this event, we could be missing the peak of TNF production. In the future, we will perform a kinetic assay in order to understand if microglia is producing TNF in earlier time-points. Another possible reason for the low levels of TNF protein might be the fact that the mRNA is not being translated due to some regulatory pathway in which NOVA1 is involved.

The role of these RBPs in microglia has never been studied before, but our results demonstrate that there is an important biological function for these proteins in microglial cells.
Although, further insight is necessary to understand the full potential of these biological effects in these innate immune cells.

CONCLUSIONS

Microglia ability to activate after identifying an insult and to deactivate upon resolution of the insult is crucial for the homeostasis of the brain.

In this study, we had to initially develop an *in vitro* system that allowed us to study the deactivation process. Additionally, we have demonstrated that microglia deactivation is characterized by the maintenance of several mRNAs that encode for inflammatory mediators involved in the microglia response to insult. The fact that, in the case of *Tnf*, these maintained low levels of mRNA are not being translated into protein, gives rise to the possibility of a state of provision awaiting further insults. Microglial deactivation has not been well characterized at the molecular level, despite the increased number of studies on microglia published in the last years. Here, we demonstrate that this deactivation process may be accompanied by a primed state, raising the possibility that microglial cells are able to retain a sort of memory from past inflammatory events, similar to adaptive immune T cells and to what has been recently suggested in macrophages. Although, questions on the possible influence of this process in the exacerbation of several neurological diseases need to be further addressed. It is of increasing importance to unravel potential ways to reverse this process and properly deactivate these cells, and even to understand if this is observed in all subpopulations of microglia in the brain and in *in vivo* conditions. Studies involving other elements of the CNS, which actively interact with microglia, are crucial and may bring new insights into these questions.

In this thesis, we obtained evidences that RBPs have an active role in the activation and deactivation processes of microglia. Accompanying the activation process are several differential changes in the expression of the RBPs studied, including a modulation in the expression of these regulators that should be further studied to fully understand their role in the microglia activation process. The potential outcomes of this regulation performed by RBPs are vast, as new targets for modulating microglial inflammatory responses are fundamental for future therapeutic approaches.

Between the RBPs studied in this work, the specificity of *Nova1* expression profile during microglial deactivation suggests that during this process, regulation is specifically performed by some elements.

In prolonged periods of culture, *Nova1* is progressively increased in microglial cells, and during the activation process, *Nova1* is decreased. This interesting profile led us to suggest that this RBP might be necessary for microglia to remain in resting conditions and further, needs to be decreased, for microglia to become activated. We studied different microglial insults and concluded that among the three tested, *Nova1* profile during activation of microglia is only responsive to LPS. These results suggest that NOVA1 is involved in some way in the signaling pathways engaged by TRLs. Further research on

these mechanisms is necessary to unravel the specific targets in these pathways that promote Nova1 alterations. Although, the revelation of a close relation between NOVA1 and microglia physiology, as demonstrated in our work, is by itself groundbreaking, since this RBP has never been associated with microglia, let alone have such strong impacts on these cells.
In the knockdown experiments, we demonstrated that RBPs are relevant elements in modulating microglia mediator-expression phenotypes. RBPs are key regulators in key cellular processes, but the multitude of interactions between them and several mRNAs provides an increased difficulty in studying them. However, after multiple approaches (bioinformatics and molecular) we have demonstrated strong and specific impacts of these RBPs in the activation process of microglia and that their deficiency can alone induce differential phenotypes in the expression of inflammatory mediators by microglia.

To conclude, future work is necessary for the understanding of these specific mechanisms, since microglial cells are key players in the CNS pathophysiology. Identification of different approaches capable of modulating their performance is a promising target for future therapeutic development. Together, our work has provided new insights on specific players that could be used in the regulation of these innate immune cells.

FUTURE PERSPECTIVES

Microglia undergoes several changes during deactivation. This work we have developed an *in vitro* model that can unravel the molecular mechanisms behind microglia activation/deactivation. Therefore, in the future, several important questions should be answered:

- What are the molecular mechanisms behind microglia priming?
- Is microglia priming antigen specific?
- What is the biological importance of RBPs in microglia activation/deactivation?
- Is NOVA1 important to keep microglia in resting conditions?
- Is modulation of NOVA1 TLR-dependent?

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