C/EBPβ and C/EBPδ in neuroinflammation

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Ph.D. Thesis

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A mio padre

"Science... never solves a problem without creating ten more."

George Bernard Shaw

"The scientific mind does not so much provide the right answers as ask the right questions"

Claude Lévi-Strauss

"Dear Hilde,

If the human brain was simple enough for us to understand,

we would still be so stupid that we couldn't understand it.

Love, Dad"

Jostein Gaarder, Sophie's World

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Probably this is the most difficult part because there is no bibliography on this topic; however I will try to do my best.

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ABSTRACT

ABSTRACT

Neuroimmunology studies the relationship between immune and nervous system. It originated in the last decade of the 19th century with Santiago Ramón y Cajal work, but only recently it has exploded as an active research topic. The increase incidence of neurological disorders, especially in high-income countries, has pointed out one of the main clinical aspects of neuroimmunology, the neuroinflammation. Abnormal endogenous and/or exogenous signals can cause neuroinflammation which represents a pleiotropic physiological response initiated by the central nervous system (CNS) innate immunity arm and sometimes mediated by the adaptive peripheral arm.

Astrocytes and especially microglia are the immune cell effectors in the CNS. In the last decades many functions of glial cells have been shown, such as adult neurogenesis reservoir or constitutive players in neuronal synaptic transmission. However, one of the main glial roles is to maintain the homeostasis in the adult nervous system by supporting neuronal activity, regulating the microenvironment and buffering any disturbances. When CNS homeostasis is compromised over a physiological threshold, astrocytes and microglia undergo enormous phenotypical changes when attempting to reestablish the original environmental state. This process is known as glial activation.

This thesis focuses on glial activation. Since glial activation is highly interconnected with several aspects of immune physiology of CNS, many features of nervous-immune system will be briefly introduced. Glial activation causes a marked change in glial cell physiology, resulting in a profound reorganization of transcriptional machinery and its fine regulation. So far, several transcription factors have been described to control glial activation during pro- and anti-inflammatory response. This thesis focus on the transcriptional regulation of pro-inflammatory glial activation and particularly the role of two members of Ccaat Enhancer Binding Protein family (C/EBPs), the transcription factors C/EBPβ and C/EBPδ.

Through bioinformatic, biochemical and cell biology analyses we have characterized transcriptional targets of C/EBPβ and C/EBPδ during glial activation *in vitro*. *In vitro* mixed glial cultures and *in vivo* models of C/EBPs deficient mice have been used to demonstrate the key role of C/EBPβ and C/EBPβ in glial activation, especially in activated microglia.

This thesis clearly demonstrates that C/EBPβ and C/EBPδ interact with key pro-inflammatory gene promoters and their absence reduces mRNA and protein levels of target genes in glial activation. In addition, it is shown that C/EBPβ and C/EBPδ control the neurotoxic potential of microglia *in vitro*. Gene expression analysis of systemically LPS-treated knockout mice has also shown an *in vivo* C/EBPs transcriptional role in brain cortex neuroinflammation.

This work demonstrates for the first time the important role triggered by C/EBPβ and C/EBPδ in glial activation, highlighting these transcription factors as potential therapeutic targets to reduce the detrimental consequences of neuroinflammation.

RESUMEN

La neuroinmunología estudia la relación entre el sistema inmune y el sistema nervioso. Sus orígenes se remontan a la última década del siglo diecinueve, pero sólo recientemente está en pleno auge. El continuo aumento de la incidencia de las enfermedades neurológicas, especialmente en los países de ingresos altos, ha resaltado la importancia de la clínica de la neuroinflamación. Señales endógenas y/o exógenas anormales pueden determinar el inicio del proceso neuroinflamatorio, el cual representa una respuesta fisiológica pleiotrópica del sistema inmune innato del sistema nervioso central (SNC) que puede ser seguida por la activación del sistema inmune periférico.

Los astrocitos y en particular la microglía son las células efectoras del sistema inmune en el SNC. En las últimas décadas se han evidenciado varias funciones de la glía, como la regulación de la trasmisión sináptica o como reservorio celular de la neurogénesis en el cerebro adulto. Aun así, una de las principales funciones de la glía es la de mantener la homeostasis del parénquima del SNC, regulando el microambiente y tamponando eventuales cambios. Cuando la homeostasis del SNC está comprometida más allá de los niveles de tolerancia fisiológica, los astrocitos y la microglía experimentan profundos cambios para restablecer las condiciones necesarias para una óptima actividad neuronal. Este proceso es conocido como activación glial.

Esta tesis doctoral se centra en la activación glial, la cual representa un aspecto fundamental de la neuroinflamación. La activación glial es un proceso complejo y altamente relacionado con varios aspectos del sistema inmunológico del SNC y para una mejor comprensión del mismo se introducirán los aspectos fundamentales que lo componen.

La activación glial es un cambio importante en la fisiología de la glía, representando profundos cambios del transcriptosoma y de su regulación fina. Hasta ahora han sido descritos diferentes factores de transcripción como reguladores de la transcripción génica durante la activación glial pro- y anti-inflamatoria. Esta tesis estudia la activación glial pro-inflamatoria y en particular el papel de dos miembros de la familia de factores de transcripción de las CCAAT Enhancer Binding Proteins (C/EBPs), los factores C/EBPβ y C/EBPδ.

A través de análisis bioinformáticos, bioquímicos y de biología celular, se han caracterizado los promotores de genes dianas de C/EBPβ y C/EBPδ durante la activación glial *in vitro*. El uso de ratones deficientes en C/EBPβ o C/EBPδ para cultivos primarios de glía mixta y para un modelo de inflamación *in vivo* ha permitido demonstrar la importancia fundamental de estos dos factores en la activación glial.

La presente tesis demuestra claramente cómo C/EBPβ y C/EBPδ interaccionan con los promotores de algunos de los principales genes pro-inflamatorios y cómo la ausencia de uno de los dos determina una reducción de la expresión de los mRNA y de las proteínas de los genes diana. Además, se demuestra que C/EBPβ y C/EBPδ regulan la neurotoxicidad de la microglía *in vitro*. Finalmente se demuestra que los mismos factores pueden regular la expresión de genes pro-inflamatorios en un modelo *in vivo* de neuroinflamación.

Esta tesis determina el importante papel de los factores de transcripción C/EBPβ y C/EBPδ en la activación glial, evidenciándolos como posibles dianas farmacológicas en el tratamiento terapéutico de la neuroinflamación para prevenir los efectos perjudiciales de la activación glial.

CONTENTS

ABBREVIATIONS

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α-MSH	**α-Melanocyte Stimulating Hormone**
aa	amino-acids
AD	Alzheimer Disease
ALS	Amyotrophic Lateral Sclerosis
Ap1s2	Adaptor-related Protein complex 1, Sigma 2 subunit
APC	Antigen Presenting Cell
BBB	Blood Brain Barrier
BCSFB	Blood Cerebro Spinal Fluid Barrier
C or Ct	Control
CD	Cluster of Differentiation
C/EBP	Ccaat Enhancer Binding Protein
CNS	Central Nervous System
CSF	Cerebro Spinal Fluid
CNTF	Ciliary NeuroTrophic Factor
CFH	Complement Factor H
COX2	CycloOXygenase 2
cPGES	cytosolic Prostaglandin E Synthase
CXCLs	Chemokine (C-X-C motif) Ligands
DAMP	Damage-Associated Molecular Products
DIV	Days In Vitro
E	Embryonic
EAE	Experimental Autoimmune Encephalomyelitis
EC	Endothelial Cells
ECM	Extra Cellular Matrix
ED	Embryonic Day
ERKs	Extracellular-signal Regulated Kinases
GABA	γ-Amino Butirric Acid
GFAP	Glial Fibrillary Acidic Protein
HAD	HIV-associated dementia
HIV	Human Immunodeficiency Virus
HPRT1	HypoxanthinePhosphoRibosylTransferase 1
ITIM	Immunoreceptor Tyrosine-based Inhibition Motif
Inpp5d	Inositol polyphosphate-5-phosphatase D
ICAM	InterCellular Adhesion Molecule
IFNγ	InterFeronN gamma
Ig	Immunoglobulin
IL	InterLeukin
IRAK-1	Interleukin-1 Receptor-Associated Kinase 1
ISF	InterStitial Fluid
Ko	Knock-out
LAP	Liver-enriched activator protein

LIP	Liver-enriched Inhibitory Protein
LPS	LipoPolySaccharide
MAP2	Microtubule-Associated Protein 2
MMP	Metallo Proteinase
MeCP2	Methyl CpG binding Protein 2
MAPK	Mitogen Activated Protein Kinase
MN	Motor neuron
mPGES	microsomal Prostaglandin E Synthase
MS	Multiple Sclerosis
mSOD1	mutant SuperOxide Dismutase-1
n.a.	not available
n.d.	not determined
NeuN	Neuronal Nuclei
NGF	Nerve Growth Factor
NCAM	Neural Cell Adhesion Molecule
NOS2	Nitric Oxide Synthase 2
NE	NorEpinephrine
P	Post-natal
PAMP	Pathogen-Associated Molecular Patterns
PD	Parkinson Disease
PEM	Perivascular Extracellular Matrix
PGE_2	ProstaGlandin E2
PI3K	Phosphatidyl-Inositol 3-Kinases
PND	Post-Natal Day
PTGES	ProsTaGlandin E Synthase
PTGS1	ProsTaGlandin-endoperoxide Synthase 1
qChIP	quantitative Chromatin ImmunePrecipitation
REST	RE1-Silencing Transcription factor
rfu	relative fluorescence unit
Rn18s	18s Ribosal RNA
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SOD	Super Oxide Dismutase
sORF	short out-of-frame Open Reading Frame
TAD	TransActivation Domain
TBAXS1	ThromBoXane Synthase 1
TNF	Tumor Necrosis Factor
VIP	Vasoactive Intestinal Peptide
Wt	Wild-type

1. INTRODUCTION

1.1 The immune system in the central nervous system

In the last decade, the classical concept of the central nervous system (CNS) as an immune privileged organ which is isolated and passive with respect to the peripheral immune system has radically changed. Now several studies have revealed how the CNS is a site of high regulation of immune system responses[1,2].

In this thesis, the term immune privileged is used referring to the highly specialized and regulatory inter-communication of resident CNS cells (glia and neurons) with the immune system. Among all CNS regions, the only immune privileged region is the CNS parenchyma. Ventricles, meninges, cerebrospinal fluid, circumventricular organs and sub-arachnoid space are immune-competent structures where robust immune innate response has been observed.

The CNS regulation of the immune native response is due to four main features:

- The presence of CNS barriers
- The absence of a proper lymphatic system
- The immune-regulatory environment established by neurons
- The resident immune effector cells, astrocytes and microglia

1.2 The blood-brain barrier

Neuronal communication is performed through chemical and electrical signals, this implies that the CNS microenvironment has to be highly regulated in order to maintain the solute homeostasis.

Mammalian CNS has developed three main interfaces to strictly control the interchanges between blood and CNS: the blood-brain barrier (BBB), the blood-cerebrospinal fluid-barrier and the avascular arachnoid epithelium (figure 1). Among them, the BBB is the most important exchange organ, because it is the main responsible for brain parenchyma immune privilege. In adult human brain, it has between 12-18 m^2 of total exchange area, representing the major interchange surface of the brain[3].

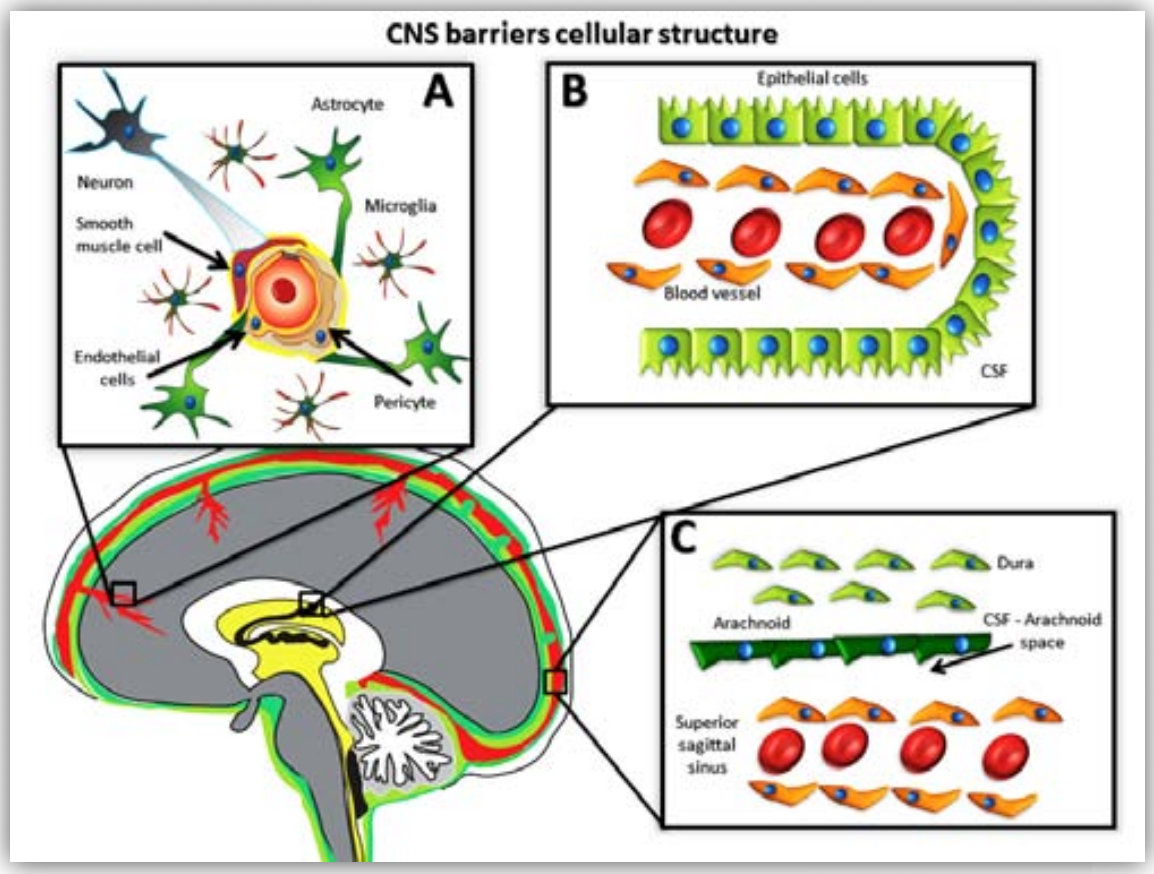

Figure 1: CNS barriers cellular structure. There are three principal barrier sites between the lumen of cerebral blood vessels and brain parenchyma. (A) In the BBB, cerebral endothelial cells form tight junctions at their margins, preventing paracellular diffusion. Pericytes are distributed discontinuously along the capillaries and partially surround the endothelium. Endothelial cells and pericytes are enclosed by perivascular extracellular matrix (grey area) different from extracellular matrix (yellow area) of the astrocytic endfeet network in CNS parenchyma. (B) At the choroid plexuses in the lateral, 3rd and 4th ventricles, the blood-cerebrospinal fluid-barrier is present. The choroid plexus blood vessels are fenestrated and form a non- restrictive barrier but tight junctions are present at the apical part of endothelial cells. (C) The arachnoid barrier. The brain is enveloped by the arachnoid membrane lying under the dura. The arachnoid is avascular but lies close to the superior sagittal sinus. It is a multi-layered epithelium with tight junctions in the inner endothelial cells layer. Arachnoid villi project into the sagittal sinus through the dura and cerebrospinal fluid drains into blood stream through the valve-like villi which permit only efflux of cerebrospinal fluid[3,4].

Nevertheless, the BBB could be thought as a series of functional features with physical basis, which is relatively constant in the CNS capillary system, changing in pre- and post-capillary vessels. The BBB exerts bi-directional control over the passage of several substances, such as regulatory proteins, nutrients and electrolytes. But it also exerts a defense control discarding neurotoxic substances into the blood stream, blocking the entrance of potential toxics from the blood and controlling the lymphocyte infiltration into the CNS [5,6]. Therefore, the BBB contributes to prevent alterations of CNS homeostasis that could trigger a direct insult or a native immune system response, as glial activation.

1.2.1 The neurovascular unit

The BBB is a complex system and its proper function relies on the sustained interaction with the adjacent extracellular matrix components and cellular elements. The fully-differentiated BBB has been referred to as the neurovascular unit. Within the neurovascular unit, brain vessel wall consists of the highly specialized endothelial cells are the basis of the BBB, which is supported by an underlying endothelial basement membrane embedding a high number of pericytes[7]. Pericytes are in intimate contact with endothelial cells in capillaries as well as in arterioles and venules. In pre- and post-capillaries, smooth muscle cells are present around the endothelial cells, constituting the "media". Outer vascular matrices cover pericytes and the media[8].

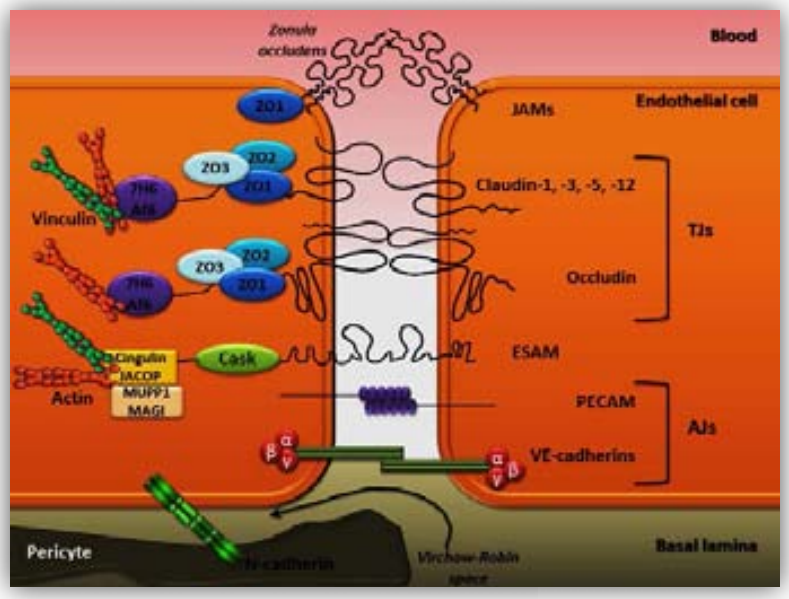

Figure 2: The molecular structure of endothelial cell junctions in the blood-brain barrier. Tight junctions (TJs) consist of proteins spanning the intercellular cleft (occludin, claudins and junctional adhesion molecules, JAMs) which are linked to cytoplasmic scaffolding and regulatory proteins (ZOs, 7H6, etc.). In adherent junctions (AJs), cadherin proteins span the intercellular cleft and are linked in the cell cytoplasm by the scaffolding proteins α-, β-, γ-catenin[3–5,10,14]. Pericytes are connected to endothelial cells through integrins, gap junctions and adherent junctions, as N-cadherin[16].

The cerebral endothelial cells are the fundaments of the capillaries architecture in the brain and the spinal cord, but in order to constitute the BBB they need to associate with various perivascular cells, such as pericytes, astrocytes and microglia[9].They constitute the endothelium and differ from peripheral endothelial cells in four elements[4]: tight junctions at their adjacent margins, lack of fenestration into the cytoplasm, less pinocytic vesicles and more mitochondria. Endothelial cells are connected one to each other through tight junctions at the blood side and adherent junctions at the brain parenchyma side[3] (figure 2). Extremely tight junctions, known as *zonulae occludentes,* are the structural key feature of cerebral endothelial cells, reducing the paracellular diffusion of polar solutes from blood to the brain interstitial fluid [10]. Factors from bloodstream can rapidly modulate tight junctions properties of the BBB[6]. Extra- and intracellular calcium concentrations influence tight junctions assembly, changing the electrical resistance across the endothelial cells [11]. Also, microglia and astrocytes as well as neurons, that are closely associated with the neurovascular unit, can release vasoactive agents and cytokines which can regulate tight junction assembly and BBB permeability [4]. On the other hand, adherent junctions give the structural support to hold the endothelial cells together and are essential for tight junction assembly. In fact, adherent junctions impairment leads to BBB disruption[12]. Also glial cells, pericytes and neurons are important for tight junctions and adherent junctions formation and maintenance of the BBB [13,14]. Furthermore, endothelial cells exert a regulatory function on peripheral immune cells infiltration, through the expression of several adhesion molecules, such as ICAMs, on the luminal side of their membranes [15].

Pericytes are another essential cell type of neurovascular unit ontogenesis, life-span maintenance and functionality[16,17]. These cells are discontinuously distributed along the vascular endothelium, partially surrounding it. They are anchored to endothelial cells through adherent junctions, gap junctions and integrins (figure 2). They produce the basal lamina, known as perivascular extracellular matrix, which completely envelops them and cerebral endothelial cells. Recently, two-photon microscopy *in vivo* experiments have shown that pericytes can regulate blood flow in the brain under pathological conditions, but pre-capillary arterioles are the main responsible of blood flow regulation during normal neural activity[18]. Furthermore, they act as multi-potential stem cells reservoir of neural cells in response to vascular insults or stress signals. Pericytes can also differentiate into macrophage or dendritic perivascular cells[19].

The smooth muscle cells are spread along the arterioles and venules in order to control their caliber through vasodilation and constriction. Together with endothelial cells and pericytes, smooth muscle cells are separated from astrocyte endfeet by a second extracellular matrix, called Virchow-Robin space in humans, with a different molecular composition than the perivascular extracellular matrix[3].

The astrocytes network is important to induce and maintain the barrier functional properties. They envelop brain vessels with their endfeet, constituting an outer physical barrier called *glia limitans*. In the capillaries, *glia limitans* intimately interact with endothelial cells, allowing a direct interaction. On the other hand, astrocytes in pre- and post-capillary vessels are separated from endothelium by the smooth muscle cells and the Virchow-Robin space[8]. Together with axonal projections from neurons, astrocytes can control vessels tone through vasoactive neurotransmitters and peptides, regulating the local cerebral blood flow[14].

In the neurovascular unit of post-capillary venules, lymphocytes and blood-borne perivascular macrophages can cross the endothelium through a highly-regulated mechanism and pass into the Virchow-Robin space[20]. On the outer side of the neurovascular unit, we can also find microglial cells surveying the perivascular environment, which can also release vasoactive agents and cytokines that can affect BBB functions[13].

1.2.2 Functions [3,5,11]

- Ion regulation: the BBB keeps the optimal ionic composition for axonal transmission and synaptic signaling through specific ion channels and transporters.
- Neurotransmitters homeostasis: blood plasma contains several molecules that can act as neurotransmitters. The BBB actively regulates their concentration into the brain and pump-out the potential excitotoxic molecules.
- Macromolecules control: the BBB prevents many macromolecules, including antigens, from entering the brain, because many plasma proteins can cause glial activation.
- Neurotoxins defense: endogenous metabolites or proteins, or xenobiotics from food or environment can damage the CNS. The BBB shields their passage and actively remove them from the neuronal environment.
- CNS metabolism: the BBB has a key role in the regulation of nutrients transport from blood to CNS.
- Leukocytes infiltration: the BBB is the main site where leukocytes can infiltrate into the CNS. Adhesion molecules overexpression at the apical membrane of cerebral endothelial cells can recruit leukocytes from the bloodstream. In this manner infiltration begins and leukocytes can cross the endothelial cells via transcellular diapedesis or they can migrate through a paracellular route by modulating tight junctions.

1.2.3 The routes across the BBB

The endothelial cell presents five pathways to regulate the passage or the transport of substances and cells across the BBB[3] (figure 3):

- Passive diffusion: Passive diffusion of solutes through the cell membrane.
- Active efflux: Active efflux carriers are ATP-binding cassette transporters that pump passively penetrating solutes out of the endothelial cell.
- Solute carrier-mediated influx: Carrier-mediated influx via solute carriers. It can be passive or secondarily active and can transport many essential polar molecules such as glucose, aminoacids and nucleosides into the CNS. The solute carriers may be bi-directional, exchanging one substrate for another and/or depending on electrochemical gradients.
- Receptor-mediated transcytosis: Receptor-mediated transcytosis transports a variety of macromolecules across the cerebral endothelium. Adsorptive-mediated transcytosis appears to be induced in a non-specific manner by positively charged macromolecules. Both transcytosis appear to be vesicular-based systems.

- Diapedesis: Leukocytes can cross the BBB either by a process of diapedesis through the endothelial cells, near the tight junction region, or via modified tight junctions.

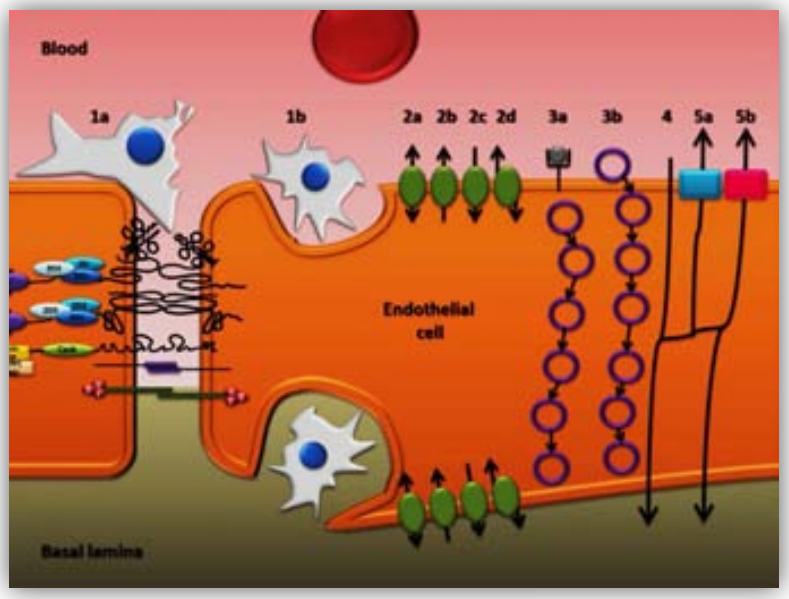

Figure 3: Schematic view of the routes across the BBB[3]. Five different pathways exist to pass from blood stream to brain parenchyma: 1. Mononuclear cells may cross BBB by (1a) modifying tight junctions a diapedesis and opening a paracellular route; or they can cross through endothelial cells by a diapedesis process (1b). 2. Carrier-mediated influx via solute carriers; solute carriers can work bi-directionally (2a) depending on concentration gradient, uni-directionally out (2b) or in (2c), or exchanging one substrate for another or depending on electrochemical anion gradient (2d). 3. Vesicular based transcytosis pathways, receptor-mediated transcytosis (3a) needs the binding of ligand to transport macromolecules, whereas adsorptive-mediated transcytosis occurs only with cationic charged macromolecules (3b). 4. Several molecules[301], such as lipophilic solutes, may passively diffuse through endothelial cells. 5. Active efflux carriers, known as ABC-transporters, pump out from endothelial cells the passively diffused cytoplasmic (5a) or lipophilic solutes (5b).

1.3 The lymphatic drainage in the brain

It has been hypothesized for many years that the absence of a proper lymphatic system inside the CNS was contributing to its immune privilege. Now it is well-known that cerebrospinal fluid and interstitial fluid can be totally or partially drained to nearby lymph nodes, permitting antigen and solute drainage outside the CNS[21].

1.3.1 Lymphatic drainage of cerebrospinal fluid

The cerebrospinal fluid is an aqueous solution of strictly controlled molecules and ions, which is regulated by blood-cerebrospinal fluid-barrier of choroid plexuses. It flows from the ventricles into the subarachnoid space, in order to protect the CNS from any trauma. As we said before, the cerebrospinal fluid in not an immune privileged milieu of the CNS and an innate immune response can occur[21].

In experimental animal models, around 50% of cerebrospinal fluid is eliminated by lymphatic drainage[22,23]. A direct connection exists between the subarachnoid space and lymphatic vessels, which cross the cribriform plate of the ethmoid bone together with olfactory nerves to nasal submucosa. This path can convey cerebrospinal fluid and lymphocytes to cervical lymph nodes where antigen presentation could be triggered[24,25]. The remaining part of the cerebrospinal fluid drains out into the blood through subarachnoid villi or may be expulsed through CNS capillaries (figure 4). In adult humans, most of the cerebrospinal fluid seems to drain mainly through arachnoid villi and granulations in the walls of major venous sinuses, but for human neonates, who do not have developed arachnoid villi, the nasal route can probably be important[26].

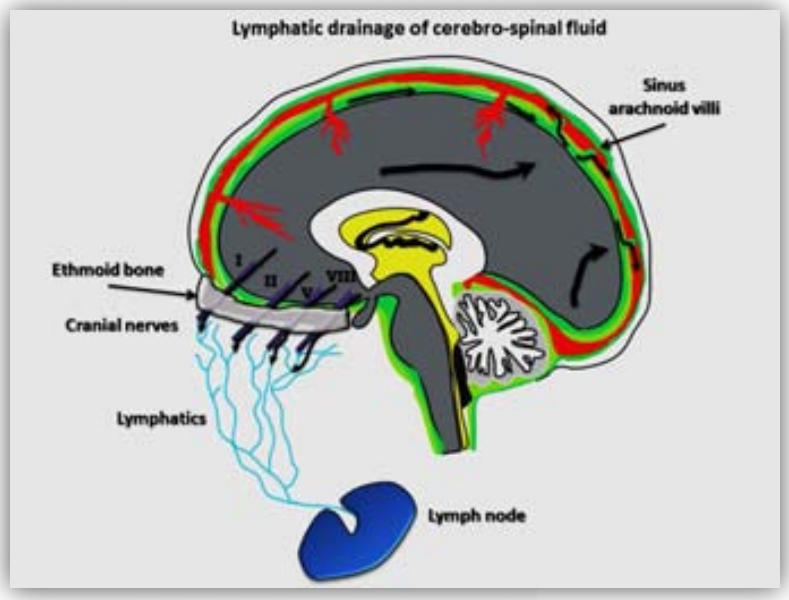

Figure 4: Schematic view of lymphatic drainage paths of subarachnoid cerebrospinal fluid. A number of studies in animal models have proposed perineural pathways for cerebrospinal fluid (black arrows) along cranial nerves (I, II, V and VIII) that cross the cribiform plate of the ethmoid bone. These pathways could allow the recollection of cerebrospinal fluid by peripheral lymphatic system. Another possible path is through arachnoid villi which can drain the fluid into cranial venous blood sinus, allowing a possible contact with cervical lymph nodes.

1.3.2 Interstitial fluid drainage

Interstitial fluid is located in the brain parenchyma which is the immune privileged region of the CNS. Metabolic activity of nervous tissue and blood exudate produce interstitial fluid. Interstitial fluid composition is strictly regulated by the BBB and by neural cells activity. Many studies in experimental animals have shown how several tracers injected into the brain parenchyma, differing in weight and bio-properties, were found to be associated with brain vessel wall and they could be drained out into the ipsi-lateral lymph node. Ultra-structural imaging has identified the tracer into the perivascular extracellular matrix of the vessel at the base of the skull (figure 5). Interestingly, analysis of amyloid-β (Aβ) in human cerebral vessels suggests a similar interstitial fluid drainage system through the walls of middle cerebral and basilar arteries[26]. In conclusion, this path could represent a way-out for endogenous abnormal antigens, such as mutant proteins produced by neural cells, which can activate the peripheral immune response.

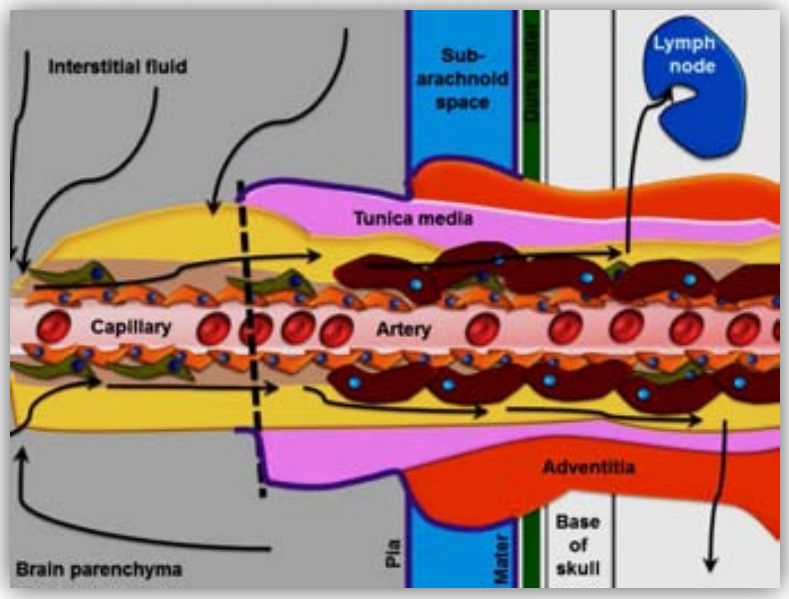

Figure 5: Representation of the hypothetical pathway for interstitial fluid drainage. Interstitial fluid and solutes could drain from the brain parenchyma into the perivascular extracellular matrix of capillaries and then along the basement membranes between smooth muscle cells in the tunica media of arteries. Interstitial fluid and solutes then could enter the adventitia around leptomeningeal arteries and continue through the base of the skull along the carotid artery to cervical lymph nodes. A layer of pia-arachnoid membranes separates the adventitia of the leptomeningeal arteries from the cerebrospinal fluid in the subarachnoid space.

1.4 The immune-inhibitory environment established by neurons

The brain parenchyma is a highly regulated environment towards immune cells, through so-called "off signals". Healthy neurons inform microglia and perivascular macrophages about their physiological condition and functionality, maintaining immune cells activation under control. Microglia can receive these inputs thanks to various constitutively expressed receptors and when the neuronal signals disappear, they interpret this event as danger and start the innate immune response.

Neuronal control of the immune response can be summarized in the following main strategies:

- Neurons can maintain microglia in surveying state[27,28].
- Neurons can decrease the antigen-presenting cell (APC) properties of microglial cells[29].
- Neurons could directly suppress T-cell activation or polarize them to a Th2- instead of Th1-phenotype[30].
- Neurons might promote apoptosis of immune cells[31].

These strategies can be achieved through two co-existing ways of communication; neurons can directly contact with immune-cells and/or they can communicate with these cells through soluble factors.

1.4.1 Contact dependent "off" signals

Neurons present on their membranes several ligands that have their receptors counterparts on microglial membranes, allowing cell-cell communication:

- It has been shown *in vitro* that neuronal NCAM can partially regulate microglia activation following LPS exposure[32].
- Neurons express CD47, an integrin associated protein that is the cellular ligand of the microglial receptor CD172a (also known as SIRPα)[33]. CD172a presents four immunoreceptor tyrosine-based inhibition motifs (ITIMs), suggesting an inhibitory function after its activation. Recently, it has been shown that CD47 is also a component of myelin and its interaction with microglial CD172a decreases phagocytosis activity, acting as a "self" signal[34].
- CD200 is a glycoprotein belonging to the immunoglobulin superfamily constitutively expressed by healthy neurons that binds its receptor CD200R1 on microglia[35]. CD200-null mice show constitutive microglial activation[27]. On the other hand, neuronal overexpression of CD200 diminishes microglial activity and attenuates experimental autoimmune encephalomyelitis (EAE)[36].
- CX3CL1 exists as a membrane-anchored protein in neurons or as a soluble form, but only the membrane CX2CL1 acts as "off" signal interacting with its receptor, CX3CR1, on microglia. CX3CR1-null microglia show increased neurotoxic potential after LPS stimulation[37], whereas CX3CL1 inhibits microglial pro-inflammatory markers production through ERK1/2 and PI3K/Akt pathways[38,39]. Recently, microglial CX3CR1 has been identified as a key pathway in protecting Alzheimer disease (AD)-related cognitive impairments associated with aberrant microglial activation[40].

- CD95 system (also known as Fas/FasL) is also used by neurons to control glial activation through microglial apoptosis[30].

1.4.2 Soluble "off" signals

Microglia express receptors for most neurotransmitters. The role they play is unclear in many cases but some of these receptors send inhibitory signals when they are exposed to physiological concentration of the neurotransmitters. Therefore neurotransmitters act mostly as "off" signals for microglia. A substance is defined as neurotrasmitter when it meets four main criteria[41]:

- It is synthesized in neurons
- It is present in the pre-synaptic terminal and released in a sufficient amount to exert a post-synaptical effect.
- The exogenous administration can mimic the endogenous effect.
- A specific removal mechanism from its action site exists.

We can distinguish four molecular families among "off" neurotransmitters[41]:

1- Aminoacids: GABA is the main inhibitory neurotransmitter in the CNS. The activation of $GABA_B$ receptor in a subpopulation of microglial cells can reduce IL6 and IL12p40 release after LPS stimulation[42].
2- Biogenic amines: Chronic dopamine release can increase microglial motility and reduce LPS-induced NO production by microglia[42]. Also norepinephrine modulates microglial activation suppressing pro-inflammatory response induced by Aβ and promoting phagocytosis[43,44].
3- Neuropeptides[45]: It has been observed that astrocytes can secrete somatostatin (SST) which acts in a paracrine manner on microglia and astrocytes, reducing proliferation and IL6 production respectively. Similarly vasoactive intestinal peptide (VIP) and pituitary adenylyl cyclase-activating polypeptide (PACAP) exert anti-inflammatory effect in brain activating $VPAC_1$ receptor on microglia, reducing several pro-inflammatory markers. Furthermore, the α-melanocyte stimulating hormone can reduce *in vitro* production of NO and TNFα in LPS-treated astrocytes and in Aβ-treated microglia.
4- Neurohormones: Anandamide is an endocannabinoid released massively during brain insults and it triggers the activation of CB_1 and CB_2 receptors on microglia, reducing their neurotoxic effects[46,47].

It has been hypothesized that neurons can also inhibit microglial activation through the cholinergic pathway. In fact, *in vitro* experiments have demonstrated that microglia express the α7nACh receptor subunit[48]. Acetylcholine and nicotine pre-treatment can reduce production of TNFα[48] and reactive oxygen species[49] of activated microglia.

However, at least two non-neurotransmitter systems can also act as soluble "off" signals in CNS, other than neurotransmitters. Neurons express and secrete CD22 which exerts an inhibition of pro-inflammatory microglial activation by binding CD45, a transmembrane

tyrosine phosphatase[50]. In addition, stressed neurons can secrete Sema-3a which interacts with microglial Plexin-1 and Neuropilin-A1, inducing microglial apoptosis[31].

Several studies have shown other molecules that can act as "off" signals but their effects depend on the experimental model, on the concentration and on the activated receptor[30,42,45,51]. Furthermore, neuronal "off" signals could also exert their inhibitory effect on astrocytes, preventing reactive astrogliosis, but this field is poorly investigated.

Table 1: Neuronal "off" signals keep glia, especially microglia, under control. In normal conditions, neurons constantly express molecules and release compounds that can interact with glial receptors counterparts, keeping glia informed of their state and activity.

Ligand	Cell type	Receptor	Cell type	Signalling	Reference
CD200	Neurons	CD200R1	Microglia	cell-cell	27,35,36
CD47	Neurons	CD172a	Microglia	cell-cell	34
CX3CL1	Neurons	CX3CR1	Microglia	cell-cell	39,40,52,53
CD56 (NCAM-1)	Neurons	NCAM	Microglia	cell-cell	32
CD22	Neurons	CD45	Microglia	soluble	50
ICAM-5 (Telencephalin)	Neurons	LFA-1	Microglia	soluble	54
ACh	Neurons	α7nAChR	Microglia	soluble	48,49
GABA	Neurons	GABAb	Microglia	soluble	42
Norepinephrine	Neurons	α1A, α2A; β1, β2	Microglia	soluble	43,44
Anandamide	Neurons	CB1/CB2	Microglia	soluble	46,47
Dopamine (chronic)	Neurons	D1, D2	Microglia	soluble	42

1.5 The resident immune effector cells

1.5.1 Astrocytes

These star-shaped cells represent the most abundant glial population in the CNS with heterogeneous sub-populations and a high variety of morphologies and functions. They form a functional and physical network through the CNS parenchyma, communicating to each other through gap junctions (connexin-30 and -43)[55]. Their massive presence and networking highlight how these cells are essential in the CNS.

Astrocytes have neuro-ectodermic origin and their maturation is a complex process, depending on the CNS location and on the astroglial subpopulation. Radial glial cells are the first group to differentiate and they help neuroblasts migration with their long branching pseudopodes. After this step in CNS ontogenesis, radial glia differentiate into mature astrocytes.

Ramón y Cajal established the first *in vivo* classification dividing the astrocytes into two subgroups: the protoplasmic astrocytes which are localized in the grey matter, characterized by complex dendritic processes, and the fibrous astrocytes, sited into the white matter, having globular morphology with moderate branching. A more complex classification based mainly on astrocytic morphology has been proposed and divides astrocytes in 9 sub-populations[56,57], as shown in table 2. However, it is noteworthy how cell morphology in the CNS can be determined by micro-environment conditions; therefore two astrocytes can be morphologically similar but functionally different.

Table 2: Astroglia classification

Astroglial subpopulation	CNS localization	Functions
Tanycytes	Peri-ventricular organs Hypophyseal infundibulum Velum medullare Raphe region	Blood-cerebrospinal fluid barrier Signaling molecules Reissner's fibers
Radial glia	Hippocampal granular layer of dentate gyrus Hippocampal striatum oriens of the CA1 Mammalian optic nerve (known as Müller cells) Lower vertebrates CNS	Migration guidance for neuroblasts
Bergmann glia	Cerebellum, cell bodies in the Purkinje layer and the processes cross the molecular layer	Synapses on the Purkinje cells dendrites interaction and sheathing
Protoplasmic astrocytes	Gray matter	Perivascular endfeet (BBB)
Fibrous astrocytes	White matter tracts Optic nerve Nerve fiber layer of mammalian vascularized retina	Sheating of retina blood vessels (BBB)
Velate glia	Cerebellum granular layer	Velate sheathing of small neuronal granular cells
Marginal glia	Pia Mater	Glial limiting zone
Perivascular glia	Brain	Endfeet sheathing of blood vessels (BBB)
Ependymal glia	Ventricular border	cerebrospinal fluid barrier

A problem of this high variety of astrocytes is the difficulty to find a unique common marker to identify them all. Historically, GFAP is the main astroglial marker used to stain the intermediate filaments. Although it is a reliable marker for virtually all reactive astrocytes, it is not a marker of all non-reactive astrocytes and some non-astroglial cells can express GFAP.

Astroglial functions in physiological state

Astrocytes support several important neuronal functions throughout all life. During development they assist neuroblasts migration and guidance of developing axons; they also participate in synaptogenesis acting as reservoir of cholesterol and thrombospondins, and modulating microglial synaptic pruning. In addition, astrocytes produce TNFα and activity-dependent neurotrophic factor (ADNF) that promotes maturation of post-synaptic terminals[58].

Astrocytes mediate the uptake of glutamate, glycine and GABA from neuronal synapses since they express the specific transporters for these neurotransmitters. Glutamate uptake from the synaptic space is one of the main functions of astrocytes, avoiding a high concentration that could result toxic. They capture glutamate through sodium-dependent transporters and then convert glutamate into glutamine, which is secreted and then taken up by neurons[59].

Astrocytes also regulate ionic homeostasis of CNS parenchyma through their Na/K channels and ATP-pumps. This key function maintains the ionic equilibrium of extracellular matrix allowing the correct neuronal membrane polarization. In the same context they can control the pH with bicarbonate transporters[59]. Astrocytes also control reactive oxygen species levels with the high quantity of glutathione and ascorbate in their cytoplasm[60].

Besides, astrocytes end-feet form with capillary endothelial cells a glio-vascular complex, which is a key system in the BBB ontogenesis and maintenance. However, this glio-vascular complex is also fundamental for the CNS energy metabolism. In fact, astrocytes are the main glycogen storage in the CNS and they can release lactate which is taken up by neurons and used as energy source[61,62]. Therefore, astroglial glucose uptake from blood is coupled to neuronal activity. In order to exert this function, they can communicate with pericytes and smooth muscle cells through nitric oxide (NO), amino acids or prostanoids secretion, to regulate the volume of blood flow in order to satisfy the nutrient demand[63].

However, astrocytes are not only passive sustainers of neurons; in the last two decades many studies strongly support a key role of astrocytes in modulation of neuronal synaptic transmission[64]. Astrocytes show electrical properties and express a plethora of receptors that allow the detection of neuronal activity. In addition its physical association with synapses is the structural fundament of gliotransmission. Chemical gliotransmitters (glutamate, D-serine, and ATP)[65] can be released from astrocytes through a variety of mechanisms which depend on astrocyte calcium elevations, but also on cytokines like TNFα[66] that suggest an indirect role for microglia.

The relevance of gliotransmission on neuronal activity *in vivo* is still debated, although some studies have shown that astrocyte-derived glutamate plays important roles in behavioral responses to cocaine[61]. Furthermore it has been studied how D-serine from astrocytes

modulates NMDA receptor function, whereas astroglial ATP modulates synaptic transmission and promotes sleep homeostasis[66,67].

It is generally accepted that astrocytes also participate in adult neurogenesis in the subventricular zone of the CNS[69].

The astroglial role in innate immune response during neuroinflammation is very important. They can respond to pathogen-associated molecular patterns or damage-associated molecular products producing inflammatory factors (see §1.6.1). They also communicate with microglia to regulate the CNS immune response.

Reactive astrogliosis

Reactive astrogliosis is defined as astroglial activation in response to various stimuli. This process if finely graded and highly heterogeneous depending on the type, severity, time and duration of the neurological insult. A simplified summary is presented in the following table:

Table 3: Reactive astrogliosis scale[57]

Astrogliosis grade	GFAP level	Hyper-trophy	Spatial distribution	Proliferation	Causes	Physiological outcome
Mild to Moderate	↑	↑	Maintenance of individual domain	—	- Mild non-penetrating, non-contusive trauma; - Viral and bacterial infection; - Distal area of focal CNS lesion	Unknown
Severe diffuse	↑↑	↑↑	Extension of processes into neighboring astrocytes domains	↑	- Penumbra areas of severe focal lesions; - Infections; - Chronic neurodegenerative areas	Long-lasting reorganization of tissue architecture
Severe	↑↑	↑↑	Loss of individual domain	↑↑	- Penetrating trauma; - Severe contusive trauma, - Invasive infections; - Neoplam; - Chronic neurodegeneration; - Auto-immune disease	Compact glial scar; Long-lasting and persisting reorganization of tissue architecture

Reactive astrogliosis shows a complex interplay between detrimental and protective effects. On one side, activated astrocytes can loss physiological functions when participating in neuroinflammation, leaving neurons unsupported. On the other side, they can directly participate in neuronal damage by the release of pro-inflammatory neurotoxic factors, or by the release of high glutamate levels[70] and indirectly by activating microglia[71]. Furthermore, astrocytic-microglial communication uses TNFα, interferon-γ (IFNγ) and many other cytokines

to regulate timing and spatial microglial immune response. Astrocytes also trigger the phagocytic phenotype of microglia through CD95-CD95L T-cell death pathway[72].

Activated astrocytes also trigger neuroprotection through a STAT3 pathway and through the glial scar formation which is essential for spatio-temporal restriction of inflammation and BBB repair[59]. They also secrete TGFβ and NGF to limit microglial activation and to support neurons, respectively[73]. In addition, alerted microglia produce IL1β, CXCL10 and CCL5 promoting astroglial proliferation[2], probably to limit the inflammatory area and to reestablish the physiological environment in order to prevent neuronal loss.

1.5.2 Microglia

Microglia are the resident macrophages of the CNS and represent the primary immune cellular effectors in the brain. They constitute a distinct myeloid cell population with a low turnover[74]. In rodents, microglial ontogenesis starts early on embryonic day 7 (E7) when microglial progenitors migrate from yolk sac to enter the embryo on E8. On E9,5, microglial precursors surround the neuroepithelium and on E10,5 the earliest microglia can be found already in the parenchyma[75–77]. They are immunologically distinguishable from peripheral macrophages due to a low-intermediate expression of CD45 and high expression of CD11b ($CD45^{low}$,$CD11b^{high}$)[78].

Microglia represent 10-20% of all CNS glial population and they are ubiquitously distributed in the CNS but with variable cell density in different brain areas[79]. In addition, microglia display a brain region specific phenotypical diversity[80]. Microglia are not interconnected through gap-junctions as astrocytes, so intercellular communication relies on autocrine and paracrine mechanisms based on a very low threshold of activation. However, activated microglia triggering reactive astrogliosis can promote astrocytic calcium waves, which can transmit microglial activation distally[73].

Microglial immune functions play a major role during CNS development, showing an important role in neurogenesis and in synaptic pruning[33,81]. Microglia can control developmental apoptosis at least through four pathways: CD95/CD95L, TNF-TNFR, CD262 and reactive oxygen species release[33]. Each apoptotic pathway ends with microglial phagocytosis of apoptotic cells and cellular debris, which is an important process in tissue homeostasis[82]. This is a non-inflammatory process that is very finely regulated through anti-inflammatory cytokines[83]. Apoptotic cells express apoptotic cell-associated molecular patterns that promote their rapid clearance by microglia. Phosphatidylserine is the main apoptotic cell-associated molecule, located in the cell membrane, recognized by phosphatidylserine receptor and by bridging molecules, such thrombospondin, milk fat globule, β2-glycoprotein 1 and GAS-6[84]. Alerted microglia show all receptors necessary to directly or indirectly recognize phosphatidylserine and express proteins, such as acute phase reactant C and serum amyloid protein, which are able to opsonize apoptotic cells. Reactive microglia can bind with their own receptors (PSR, CD11b, CD11c, CD14, CD36, CD91 and TREM-2) the complement components deposited on opsonized apoptotic cell and phagocyte them[82,84,85].

Mirroring the macrophage nomenclature[86], microglia show an M1-like pro-inflammatory phenotype when stimulated with IFNγ or LPS, while IL10 and IL4 polarize microglia towards an M2-alternatively activated anti-inflammatory phenotype[87]. However microglia functional phenotypes are really dynamic in terms of CNS region, timing and types of stimulus. For many years most authors distinguished between resting and activated microglia; recently, it is becoming accepted a new classification into surveying, alerted and reactive microglia[88]. Each state shows relatively well-characterized phenotypical features of microglia. In any case all existing classifications are an oversimplification of a four-dimensional reality. In fact, they do not consider the dynamic existence of more functional and phenotypical microglial subpopulations, which can coexist in the same CNS region and change over time.

Surveying microglia

As we have mentioned before, neurons and astrocytes are constantly producing inhibitory signals to avoid microglial activation in normal conditions. Intercellular interactions between microglial cells and neurons, such as CD200-CD200R, CD172a-CD47 and CD22-CD45, represent inhibitory signals for microglia. Moreover, soluble peptides and neurotransmitters inform on neuronal state, while inhibitory signals like anti-inflammatory cytokines IL10, TGFβ or IL1ra are constitutively secreted by glial cells[89].

For many years, this phenotypical appearance of microglia was defined as "resting/quiescent" state, but at present it is generally accepted that it represents a functional phenotype of an active cell. Recently, two-photon microscopy experiments have demonstrated that in fact resting microglia are constantly overseeing the brain parenchyma to keep the CNS under control in order to maintain the homeostasis[90]. In this view, the change between resting and activated states is a change of functional phenotype and some authors suggest that "resting" microglia should be renamed "surveying" microglia[88], definition that we have adopted.

During the surveying state, microglia show a ramified morphology with a large number of long thin and highly dynamic ramifications[90]. Low expression of CD45 and MHC-II and low endocytic and phagocytic activity characterize microglia in normal conditions. In agreement with its surveying role, resting microglial cells present a low expression of all those receptors that can trigger its activation.

Alerted Microglia

Endogenous factors over a critical concentration (glutamate, cytokines, etc.) or with an abnormal conformation (α-synuclein or Aβ) and exogenous molecules (pathogens or toxics) are activating signals for microglia. Furthermore, the disappearance of neuronal or astroglial inhibitory signals also contribute to this process in two different effects. Absence of "off" signals, such as neuronal CD200, can directly trigger pro-inflammatory microglial activation[27], whereas the disappearance of other "off" signals, such as CX3CL1, can serve as a "priming" stimulus for microglia, and upon secondary stimulation, these primed microglia will show a hyper-activated phenotype[37].

The change from surveying to activated states is a highly dynamic transition. The first effect of pro-inflammatory signals is an increase of microglial proliferation. Colony stimulating factor 1 (Csf1/M-CSF) and 2 (Csf2/GM-CSF) from astrocytes, together with microglial interleukin 1β (IL1β) and IL6, are proliferative signals for microglia[91].

Alerted microglia have short hypertrophic ramifications and synthesize a large number of pro-inflammatory molecules (see table 5). But it is important to remember that microglia function is to remove the homeostatic disturbances and to restore the physiological activity in the CNS; for this reason activated microglia produce neurotrophic factors, such as nerve growth factor and neurotrophin 3, to support endangered neurons[92].

Alerted microglia also overexpress enzymes, mainly the inducible NO synthase isoform (NOS2 or iNOS) and the phagocytic NADPH oxidase-2 (PHOX2) that can be important effectors of microglial induced neurotoxicity through the synthesis of reactive oxygen and nitrogen species. These inflammatory effectors can be induced by different signals, like pathogen-associated molecular patterns and damage-associated molecular products[93].

Reactive microglia

Amoeboid morphology characterizes microglia during this activation state, in which a key microglial function is to phagocyte. Phagocytosis is one of the main activities of reactive microglia that allows the removal of pathogens, proteins and cell debris from extracellular matrix.

The pattern of microglial activation depends on the surrounding environment and on what kind of signal is present. As mentioned before, apoptotic cells promote phagocytosis and anti-inflammatory cytokine release. Instead, viral and bacterial pathogens trigger phagocytosis and pro-inflammatory factor production[84].

Microglia express pattern recognition receptors, such as Toll-like receptors (TLRs 1-9), scavenger receptors (SR-A1, SR-B1, CD36 and MARCO) or integrin-αM (Itgam, also known as CD11b, CR3 or CD11b/CD18) that identify and bind pathogen-associated molecular patterns. Some of these receptors are expressed at low levels, but the binding with specific ligands triggers their up-regulation and antibody-independent phagocytic activity[94].

Microglia can also show antibody-mediated phagocytic activity. This type of phagocytosis is mediated by different classes of opsonic receptors, such as Fc receptors (FcγRI, II and III), complement receptors (CR1, CR3 and CR4) and CD93 (C1qRp). All these receptors are expressed at low levels in resting microglia[95].

1.6 Neuroinflammation

Neuroinflammation can be evolutionary defined as an immune response initiated by CNS innate immunity arm and sometimes mediated by the adaptive peripheral arm, which contributes to tissue defense against pathogens by production of cytotoxic substances, chemokines for the recruitment of specific immune cell populations, and cytokines for coordinating the immune responses. Neuroinflammation may also participate in tissue homeostasis by removing dead tissue, promoting the rapid death of irretrievably damaged cells, and promoting wound healing.

The main feature of neuroinflammation is glial activation. Astrocytes and microglia become activated in reaction to CNS homeostasis changes, accompanied by several phenotypical and functional modifications that we have already introduced, known as reactive astrogliosis and microglial activation. In particular, microglia (figure 6) act as active sensors and show a wide range of receptors to mediate a proper immune response.

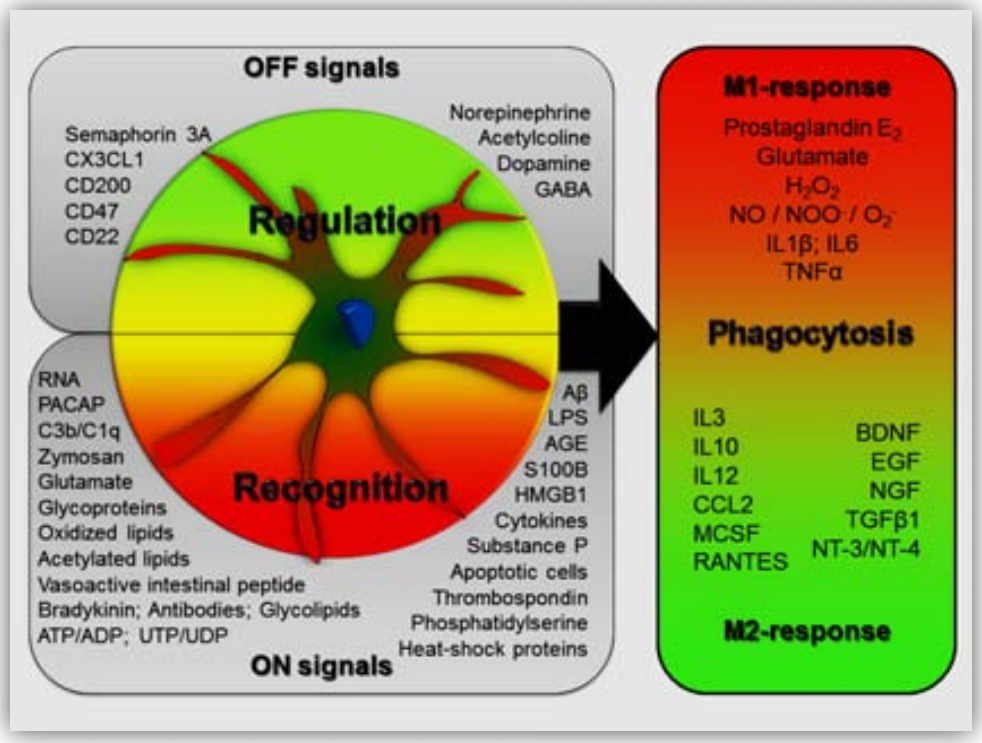

Figure 6: Microglia act as active sensors in CNS parenchyma. Physical interaction between neurons and microglia or soluble neuronal signals can act as inhibitory signals ("OFF") for microglial activation. Disappearance of these inhibitory signals and/or appearance of abnormal endogenous, as well as exogenous, signals ("ON") can activate microglia. Microglial response to activation can be pro-inflammatory (M1) or anti-inflammatory (M2). Both responses can involve microglial phagocytosis activity. This figure was inspired from a review by Lucin and Wyss-Coray[96]

We can distinguish between acute and chronic neuroinflammation. Acute neuroinflammation can be further distinguished by the BBB state. When BBB is compromised, like in cerebrovascular disease, acute neuroinflammation is similar to any peripheral inflammatory response, with antigen presentation, neutrophil and T-cell infiltration and activation. This process leads to neurotoxic and long-lasting effects. On the other hand a "pure" acute

neuroinflammation, in which BBB is preserved, can be exemplified by peripheral axotomy, when only CNS immune cell effectors are involved. In this case, astrocytes and microglia act to re-establish the CNS homeostasis, supporting a neuroprotective environment. Chronic neuroinflammation, independently of stimulus, is an alteration of normal microglial physiology which determines a constant inflammatory environment that leads to neurodegeneration[97].

So far chronic neuroinflammation has been postulated to play a role in neurological disorders, and even if it is not the cause it may represent an amplifier of pathology[94,98]. In this view, it is important to analyze what triggers the immune response and which are the immune-mediators in the CNS.

1.6.1 Neuroinflammation in neurological disorders

The *U.S. National Institute of Neurological Disorders and Stroke* classifies around five-hundred neurological disorders (www.ninds.nih.gov), but neuroinflammation is not involved in all these

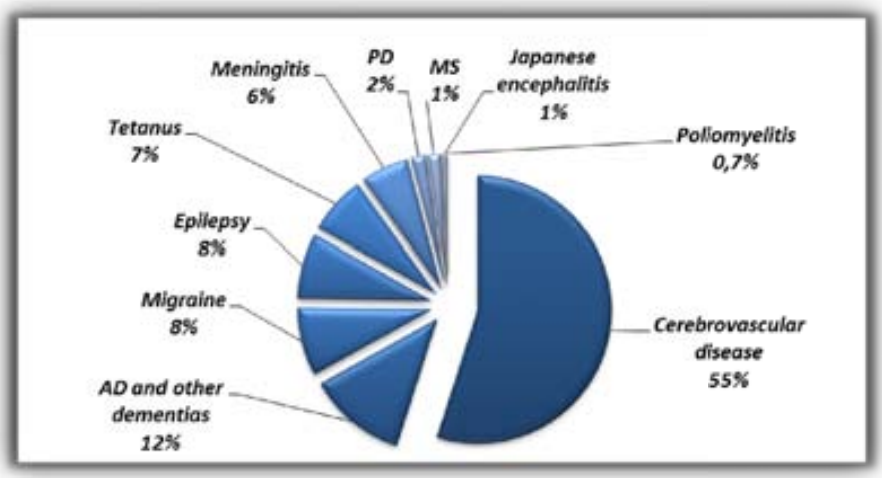

Figure 7: DALYs for individual neurological disorders. Disability-adjusted life years (DALYs) is a time-based measure of the sum of years of life lost because of the premature mortality and years of healthy life lost as a result of disability, weighted by the severity of the disability. Values are expressed as percentage of total neurological disorders in world-wide population[101].

pathologies. However, the main disabling neurological disorders reported by World Health Organization (figure 7) may present a direct or indirect neuroinflammation component. Interestingly, many psychiatric disorders as schizophrenia, post-traumatic stress, autism and depression have also recently shown to have a neuroinflammatory component[99].

Immune response to injuries and infections is considered neuroprotective, because it induces an acute neuroinflammation to limit and resolve damage in the CNS. On the other hand, many dementias show chronic neuroinflammation which can contribute to neurodegeneration. For this reason, many forms of dementias are studied by neuro-immunologists to find an optimal spatio-temporal inhibition of the aberrant immune response. In this view, a working classification for non-cell autonomous participation of glia in neurological diseases was proposed[100]:

- ❖ Neurons → Glia: Changes in neuronal physiology could stimulate glial immune response, but glial cells are not directly damaged by this toxicity or by their own synthesis of the mutant protein (i.e. Cerebral ischemia, HD, PD).
- ❖ Neurons ↔ Glia: Changes in glial physiology could modify a normal glial response, amplifying initial damage to the vulnerable neurons (i.e. AD and ALS).
- ❖ Neurons ← Glia: Changes in glia could disturb normal glial function, thus becoming a primary source of neurotoxicity (i.e. MS, HAD and Meningitis).

Neuroinflammation in cerebral ischemia

Among all neurological disorders, cerebrovascular diseases are the major cause of disability and they are associated mainly with age, diabetes, hypertension and obesity[101]. Cerebral ischemia is the condition when blood flow is below the metabolic threshold to sustain brain tissue function, leading to cerebral infarction, known as stroke. Neurons are the first cells to die during brain ischemia and their necrosis trigger glial activation, which induces secondary neuronal damage. Focusing on pathogenesis, two main regions can be defined after ischemia, the core and the penumbra. Their extension in brain parenchyma depends on the occluded artery. At pathophysiological level, cerebral ischemia can be simplified into three steps. First, the acute phase starts when blood flow drops down. The altered ionic homeostasis causes an extended calcium wave and glutamate release which triggers excitotoxicity in the area. Plasma leaks in this area due to osmotic gradients, resulting in brain edema. Second, a sub-acute phase begins a few hours later characterized by an apoptotic and neuroinflammatory response. A long lasting chronic phase follows in which repair and regeneration take place[102].

Cerebral ischemia involves CNS immune-system in two opposite roles. During the acute phase, microglial cells can resist up to 90 minutes under ischemic conditions; however a constant microglial migration begins from the surrounding areas into the core of the lesion where they show an M1-like phenotype. In the meantime, a severe reactive astrogliosis begins; astrocytes proliferate and migrate, starting glial scar formation. This pro-inflammatory response potentiates pathogenesis especially in the area of penumbra, where microglia shows a sub-chronic activation with neurotoxic consequences[102]. In fact, it has been shown that pharmacological or genetic inhibition of pro-inflammatory microglial activation leads to a smaller penumbra zone[102–105]; however the ablation of proliferating microglia has been shown to exacerbate ischemic injury[106]. In the sub-acute phase, lysis of necrotic neurons activates microglia, which can also be activated in a paracrine manner and possibly through astrocytic calcium waves. On the other hand, microglia also show an M2-like phenotype in the ischemic chronic phase, controlling leukocyte infiltration, promoting apoptotic cell clearance and supporting neuronal viability through neurotrophins and anti-inflammatory cytokine secretion[107].

Neuroinflammation in Alzheimer disease

Alzheimer's disease (AD) is the second cause of disability among neurological disorders[101] and the main risk factor is ageing, affecting 7% of the people older than 65 and approximately 40% of people over the age of 80.

As we can see in figure 8 many risk factors are associated with AD, but nowadays the amyloid cascade hypothesis is the most supported pathogenic mechanism. It is based on the concept of Aβ as causative agent. Aβ deposits activate microglia with subsequent production of neurotoxic pro-inflammatory cytokines, reactive oxygen species and prostanoids[108,109]. Furthermore, this molecular repertoire can increase neuronal Aβ secretion in a positive feed-back, triggering NF-kB translocation in neuronal nuclei activating transcription of APP gene, which contains NF-kB consensus sites[110]. Furthermore, intracellular and extracellular Aβ could trigger Tau protein hyperphosphorylation which is another possible cause of neurodegeneration in AD due to axonal transport impairment[108,111]. The microglial role in AD is controversial; it has been shown that microglial removal does not affect Aβ plaque formation[112], but other groups have demonstrated that inhibition of microglial migration enhances Aβ deposition[113]. Furthermore, norepinephrine decreases microglial pro-inflammatory response in presence of Aβ and stimulates microglial phagocytosis of plaques, but the major source of norepinephrine is the *locus coeruleus* which is often degenerated in AD[44]. On the other hand, disequilibrium between pro- and anti-inflammatory responses associated with age, known as "inflammaging", has been implicated in AD[114,115]. This chronic activation and pro-inflammatory brain environment can also cause senescence and death of microglial cells, reducing Aβ clearance[96,116]. More studies are necessary to clarify the pathogenic mechanisms and the involvement of the various cell types, but AD is probably an example of non-cell autonomous disease.

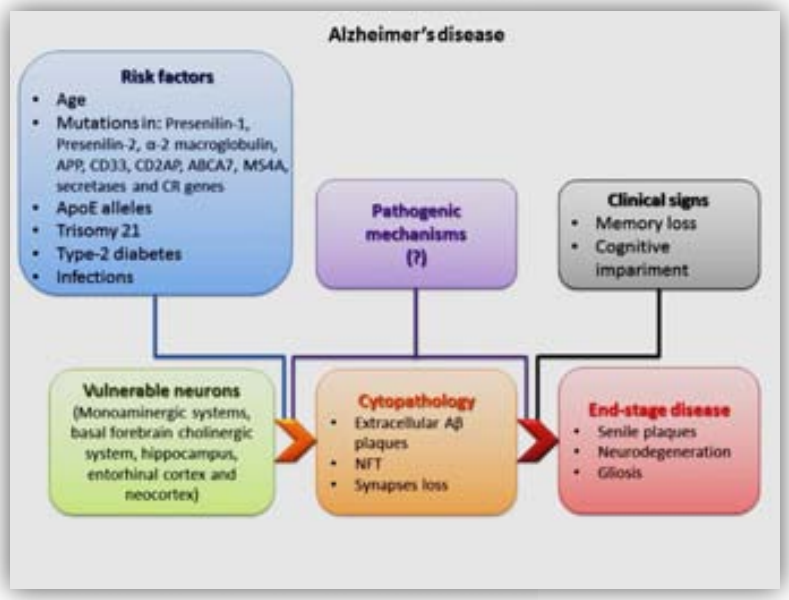

Figure 8: Schematic diagram of AD main features, modified from[302].

Neuroinflammation in amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is the primary disease affecting motor neurons (MNs) in humans. It is characterized by a neurodegenerative process of MNs leading to progressive paralysis until death due to respiratory insufficiency. Ninety percent of cases are sporadic and causes are unknown; the remaining 10% of affected patients show mutations in superoxide dismutase-1 (SOD1) and other genes (figure 9). SOD1 mutation shows a 20% frequency in familial ALS and clinical symptoms are identical to sporadic ALS, for this reason mutant SOD1 (mSOD1) transgenic mice are the favorite animal model of ALS[117].

Human post-mortem tissue analysis shows a clear gliosis by microglial and astrocytic markers upregulation and morphological appearance, suggesting a possible role of neuroinflammation in the pathogenesis. Furthermore, T-cell infiltrates are common and microglial APC-markers up-regulation (CD11c, CD86, MHC-II and ICAM1) is associated; however lymphocytes seem to down-regulate M1-like microglial phenotype[117]. In ALS patients, positron emission tomography imaging has shown microglial activation in motor cortices, in dorso-lateral prefrontal cortices and in thalamus; microglial activation and damage to upper motor neurons, but not to lower motor neurons, was significantly associated[118]. Furthermore, up-regulation of pro-inflammatory markers has been shown *in vitro* and *in vivo* in mSOD1 models, which is exacerbated by peripheral inflammation when compared to wild-type mice[119]. In SOD1 mutant mice, reactive non-proliferative astrogliosis has been shown concomitantly to neurodegeneration; mSOD1 triggers a neurotoxic phenotype of astrocytes and decreased expression of glutamate transporters in the synaptic cleft, which can possibly determine excitotoxicity[117]. Interestingly, MNs can secrete mSOD1 in the extracellular matrix triggering glial activation[120].

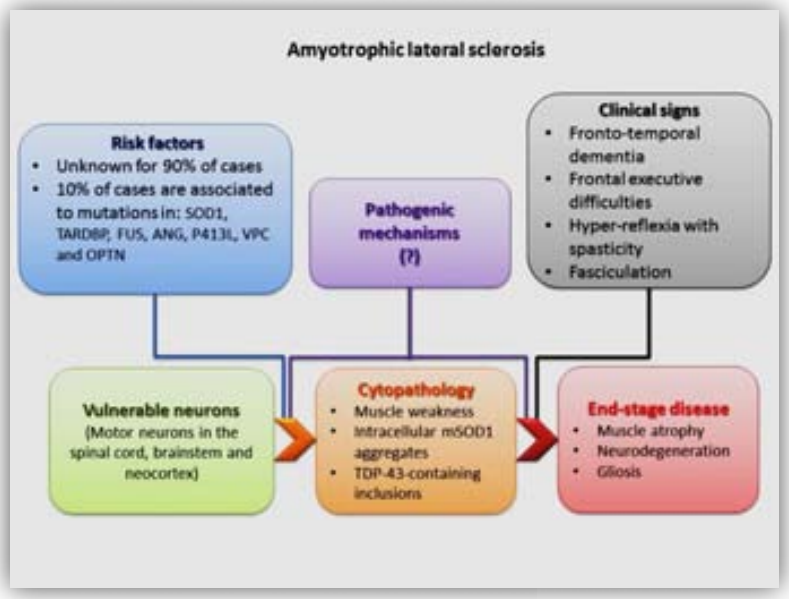

Figure 9: Schematic diagram of ALS main features.

ALS is a prototypical example of non-cell autonomous disease. Several experiments with selective expression or depletion of mSOD1 have shown that there is not a unique cell type necessary to trigger pathogenesis. Several studies (see table 4) suggest that mutant proteins in MN are pathogenic and necessary to early stages, but mSOD1 in glial cells mainly determines disease course.

Table 4: Summary of experiments that have been performed in order to dissect the pathogenic role of the different cell populations in ALS.

Mouse genotype		Cell type	Pathogenesis outcome	Refs.
Wt mice	+	mSOD1 MNs	No MNs degeneration	121–124
		mSOD1 microglia	No MNs degeneration	125
		mSOD1 astrocytes	No MNs degeneration	126
mSOD1 mice	+	Wt MN	Delayed disease onset	127–129
		Wt neurons	Delayed disease onset and early stage	127
		↓ mSOD1 myeloid cells	Deceleration of disease progression	129
		Wt myeloid cells	Deceleration of disease progression	130
		↓ mSOD1 astrocytes	Deceleration of disease progression	127
		Wt astrocytic precursors	MNs rescue	131
	-	T-cells	Disease course worsened	132

Neuroinflammation in multiple sclerosis

Multiple sclerosis (MS) is a complex autoimmune disease that affects young adults with higher incidence in females. Its symptomatological manifestations are cognitive impairment and deficits in motor, sensory, autonomic and visual systems (figure 10). In the early stage of the disease, 85% of patients show a relapsing-remitting form of MS, where patients can have a short time MS manifestation, days to weeks, and then a long relapsing period, months to years. At the cellular level, MS is characterized by abundant antibody-producing plasma cell and T-cell infiltration in the CNS perivascular spaces. An immune response against myelin due to a molecular mimicry mechanism is the most supported hypothesis of MS pathogenesis[32].

EAE is the most extensively used animal model to understand MS mechanisms. In EAE, microglia is a key contributor to determine the onset and progression of pathogenesis. However, infiltrating dendritic cells seem to play a key role in T-cell activation in the CNS. In general, APCs present myelin basic protein antigen through MHC-II to naïve T-cells, which is necessary for peripheral immune system to be in charge of EAE progression[133]. Glial cells can provide cytokine stimulation in order to polarize and differentiate naïve T-cells into effector lymphocytes[134]. It has been shown that previous infections can activate or prime microglia, contributing to EAE[32,135]. Besides, lymphocytic IFNγ production polarizes microglia to M1 phenotype leading to peroxynitrite production which can be toxic to oligodendrocytes[136]. Nevertheless, microglia can also have a neuroprotective role due to its myelin debris clearance capacity, especially during the remission phase[137].

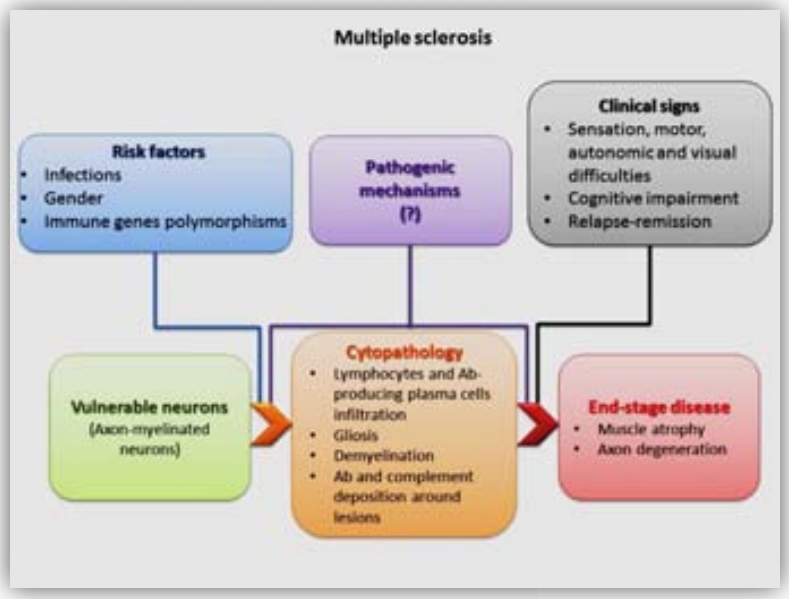

Figure 10: Schematic diagram of MS main features.

1.6.2 Neuroinflammatory effectors

Cytokines

Cytokines are a heterogeneous class of small molecules with pleiotropic immune effects. These proteins interact with their counterpart receptors modulating several cellular responses. Cytokines include at least six super-families, interleukins (ILs), interferons (IFNs), trophic or growth factors, tumor necrosis factors (TNFs), colony stimulating factors (CSFs) and chemokines (see table 5). Many stimuli can induce cytokine production in the CNS and effects can be protective or toxic depending on the nature of the stimulus, its intensity or its timing. Some of the main cytokines involved in neuroinflammation are presented below.

IL1β is highly expressed and secreted by microglia during CNS insults. It triggers glial activation, astroglial proliferation and astroglial release of neurotrophic factors such as NGF *in vivo* and *in vitro*[138,139]. IL1β also induces production of several pro-inflammatory factors such as IL6 and reactive oxygen species in glial cells and its inhibition leads to a reduced neurodegeneration[140].

IL4 is a prototypical anti-inflammatory cytokine that promotes M2-like phenotypical polarization of microglia. IL4 treatment induces up-regulation of arginase-1, CCL2, Fizz1 and Ym1/2 M2-marker genes in rat microglia *in vitro*[86,141]. There are no evidences that CNS-produced IL4 can drive anti-inflammatory activation of microglia. Interestingly glioma cells can secrete IL4, together with IL6 and IL10, promoting a favorable environment for tumor growth[142].

IL6 is classically considered a pro-inflammatory interleukin produced by activated microglia, with paracrine and autocrine effects, inducing astrogliosis. It also participates in the regulation of the BBB permeability. It has been reported that IL6 can induce the expression of neuroprotective molecules in CNS[143].

IFNɣ is the only member of the interferon subtype-II subfamily. It has a priming action sensitizing cells for increased response to subsequent stimuli. It has a clear pro-inflammatory action with a high capacity to induce a M1-like phenotype[88] through a complex gene regulatory network[144]. A recent study shows that microglial IFNɣ counteracts neural progenitor cells proliferation up-regulating p21 and promoting neural progenitor cells death through a caspase-3 dependent pathway[145]. Furthermore, IFNɣ may mediate microglial neurotoxicity towards dopaminergic neurons, suggesting a possible role in PD[146].

TGFβ belongs to the trophic growth factor family. It inhibits microglial proliferation and production of pro-inflammatory cytokines *in vitro*. Interestingly, it shows protective effects in the EAE model[95,142].

TNFα is one of the classical pro-inflammatory cytokines together with IL1β. TNFα forms a trimer with its receptors (TNFR1 and TNFR2) promoting many microglial functions, such as phagocytosis, antigen-presentation, matrix metallo-proteases expression and peripheral immune cells recruitment through chemokine secretion[95].

Chemokines, which means chemo-attractant cytokines, are an enormous superfamily with key functions in cell migration in healthy and endangered CNS. They are 6-12kDa proteins that can

be grouped in four families (C, CC, CXC, CX_3C) depending on the N-terminal cysteine conserved sequence. Astrocytes, neurons and microglia release chemokines to the extracellular matrix creating a chemotactic gradient for cell migration. Furthermore, chemokines can interact with extracellular matrix and cell proteoglycans activating not only immune cells recruitment, but also multiple physiological responses. The cellular effects of chemokines are mediated by 7-transmembrane domain receptors; interestingly, chemokines and their receptors show cross reactivity, one ligand can bind various receptors and vice versa, suggesting a complex network[42].

Table 5: Cytokines known to trigger biological effects in neuroinflammation.

Superfamily	Family	Source	Physiological outcome	Refs.
Interleukins (ILs)				
IL1	**IL1α**	Astrocyte; Microglia	Adhesion molecules and chemokines ↑	147–149
	IL1β	Astrocyte; Microglia		147–149
	IL1ra	Astrocyte; Microglia		147–149
IL3		Neurons; glial cells		91
IL4		Microglia; Th2 cells	CD200↑; TNFα↑; IGF1↑; Microglia apoptosis; anti-inflammatory cytokine↑	87
IL6		Astrocyte; Microglia	B-cell growth and differentiation	95,150
IL10		Astrocyte; Microglia	Pro-inflammatory mediators↑	151
IL12		Microglia	Stimulation of NK and Th1 cells; TNFα↑	152–154
IL13			Microglia apoptosis	155
IL15			Activate NK and CD8+ T cells	156
IL18		Microglia	Stimulation of NK and Th1 cells	91,157
Interferons (IFNs)				
Subtype II	**IFNγ**	Astrocyte; Microglia	Pro-inflammatory mediators↑	91
Tumor Necrosis Factors (TNFs)				
	TNFa	Astrocyte; Microglia	phagocytosis↑; CCL2↑; MMPs↑	140
Trophic or growth factors				
	TGFβ	Astrocyte; Microglia	Pro-inflammatory mediators↓	158
	BDNF	Astrocyte; Microglia	Axonal sprouting↑	159
	GDNF	Microglia	MHC-II ↓	160
	NGF	Astrocyte; Microglia	Neuronal survival	29
	CNTF	Astrocytes	PGE_2↑ Phagocytosis↑	161–163
	NT-3	Microglia	Neuronal survival	92
	IGF-1	Microglia	Proliferation↑	164,165
Colony Stimulating Factors (CSFs)				
	Csf1	Astrocytes	Glial proliferation	
	Csf2	Astrocytes	Glial proliferation	166,167
Chemokines				
CX3C	**CX3CL1**	Neurons	proliferation; migration; cytokine↑	168
CXC	**CXCL1**	Astrocyte	migration; cytokines↑	169
	CXCL2	Microglia	Polymorphonuclear neutrophils recruitment	170
	CXCL8	Microglia	Migration	171
	CXCL10	Microglia	Proliferation; migration	171,172
	CXCL12	Neurons; glial cells	Apoptosis; mitosis; migration; proliferation; cytokines↑	173
CC	**CCL1**	Astrocyte; Microglia	Migration	173
	CCL2	Astrocyte; Microglia	Proliferation; recruitment of neutrophils and monocites	169,174–176
	CCL3	Astrocyte; Microglia	Migration; proliferation	173
	CCL4	Microglia	Cytokines↑	177
	CCL5	Astrocyte; Microglia	Proliferation; pro-inflammatory mediators↑	178
	CCL11	?	Cytokines↑	173
	CCL19	Astrocyte; Microglia	?	179
	CCL20	Astrocytes	Leukocytes subset recruitment	180
	CCL21	?	Pro-inflammatory mediators↑	179,181
	CCL22	Microglia	Th2 chemotaxis	182

microRNA

The recently described microRNAs play important roles in every aspect of eukaryotic cell physiology. They are 21- or 22-base non-coding RNA molecules that regulate decay and translation of specific mRNAs. MicroRNAs have been thought to act only in the cell where they are produced. However, recent studies suggest that microRNAs can be secreted and act in a paracrine or even endocrine manner[183]. Therefore microRNAs can assume a very important role during neuroinflammation. The following table summarizes the relevance of microRNAs in neurological disorders:

Table 6: Human microRNAs (miRs) with possible relevance in neurological disorders are shown. These miRs are also called NeurimmiRs for their possible involvement in neuronal-immune communication. Table is modified from a review by Soreq and Wolf[184].

miR	Neurological disorder	Expression change	Validate target	Physiological outcome
miR-9	Hungtinton's disease	Down-regulation	REST	Disrupted regulation of neuronal gene expression
	Spinal muscular atrophy	Down-regulation	Heavy neurofilament subunit	Disrupted axonal cytoskeleton
	Alzheimer's disease	Down-regulation	?	?
miR-124	EAE	Down-regulation	C/EBPα	Over-activation of microglia and infiltrating macrophages
miR-125b	Alzheimer's disease	Up-regulation	?	?
miR-132	Hungtinton's disease	Up-regulation	?	?
	Alzheimer's disease	Down-regulation	?	?
	Ischemia	Down-regulation	MeCP2	Neuroprotection against subsequent ischemic insults
	Schizophrenia/bipolar disorder	Up-regulation	?	?
miR-146a	Alzheimer's disease	Up-regulation	CFH	Enhanced inflammatory response in neocortex and hippocampus of AD patients
	Epilepsy	Up-regulation	?	?
	HIV-associated dementia	Up-regulation	CCL-8	Regulation of excessive inflammatory response during reaction to viral infection
	Rett syndrome	Down-regulation	IRAK-1	?
miR-155	Down's syndrome	Multiple copies	MeCP2	Broad disruption of gene expression as a result of aberrant expression of transcription factors downstream of MeCP2
	Multiple sclerosis/EAE	Up-regulation	CD47, Inpp5d	Aberrant 'don't eat me signal' to macrophages/ microglia; aberrant regulation of Th17 subset via dentritic cell signaling
miR-212	Schizophrenia/bipolar disorder	Up-regulation	?	?
	Alzheimer's disease	Down-regulation	?	?
miR-326	Multiple sclerosis/EAE	Up-regulation	CD47, Ap1s2	Aberrant 'don't eat me signal' to macrophages/ microglia; deregulated shift of Th0 cells to Th17 cells

Reactive nitrogen species

The main reactive nitrogen species is peroxynitrite ($ONOO^-$), resulting from reaction of NO (•NO) with superoxide ($O_2^{-\bullet}$) (figure 11). NO is normally used by cells as a biological short-life signaling molecule and its synthesis is catalyzed by neuronal or endothelial NOS, NOS1 and NOS3 respectively. During inflammation, NOS2 is up-regulated in microglial mitochondrion and it produces NO at high concentration for an extended period of time. NO at high concentration kills pathogens, but it also exerts many paracellular toxic effects through its nitrosylated products and peroxynitrite, which is the primary cytotoxic mediator for neurons and oligodendrocytes [93,136,185,186].

Reactive oxygen species

NADPH oxidases (NOXs) and mitochondria respiratory chain are the main producers of reactive oxygen species, H_2O_2 and $O_2^{-\bullet}$ (Figure 11). Microglia express a specific NOX isoform typical of phagocytic cells, named NOX2 or PHOX and containing 6 subunits (gp91, p22, p47, p67, p40, and Rac). In basal conditions gp91, the catalytic core, and p22 subunits are in the cell membrane, while the other subunits (cytosolic regulators) are dissociated in the cytoplasm. During microglial activation, pro-inflammatory stimuli induce NOX2 cytosolic subunits translocation. Phosphorylation of p47 triggers its interaction with p22. Once on the membrane, p47 organizes the translocation of the other cytosolic subunits to cell membrane. At the end, when p67 interacts with gp91, NOX2 starts to produce a high concentration of $O_2^{-\bullet}$. At high concentration, this reactive species can directly react with lipids and proteins or indirectly as $ONOO^-$, causing irreversible damage to cells[185,186].

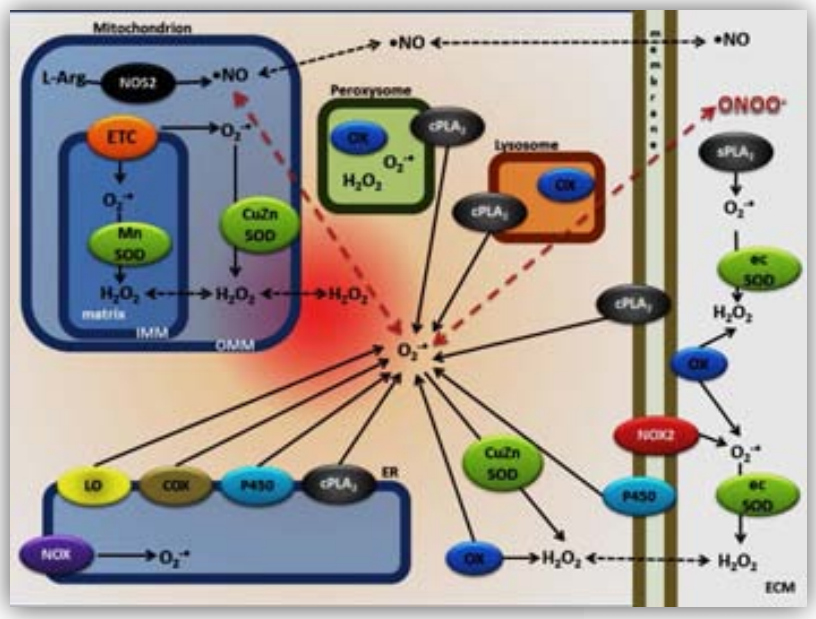

Figure 11: Reactive oxygen and nitrogen species synthesis pathways leading to oxidative stress.

Prostaglandin E2

In the CNS, pro-inflammatory stimuli induce cyclooxygenase 2 (COX-2) expression which catalyzes the first step in prostanoids (prostaglandins, prostacyclins, thromboxanes) synthesis, transforming arachidonic acid into prostaglandin H_2 (PGH_2). PGH_2 is a substrate for several prostaglandin synthases. Under neuroinflammatory conditions, prostaglandin E synthase (PTGES, known as mPGES-1) is up-regulated and produces prostaglandin E_2 (PGE_2) at high concentration. High concentration of PGE_2 exerts neurotoxic as well as neuroprotective effects. Recently, it has been shown in an intrathecal LPS model that acute neuroinflammation triggers a pro-inflammatory downstream effect of PGE_2, whereas chronic neuroinflammation produces a negative feedback through PGE_2 receptors, EP2 and EP4[187,188].

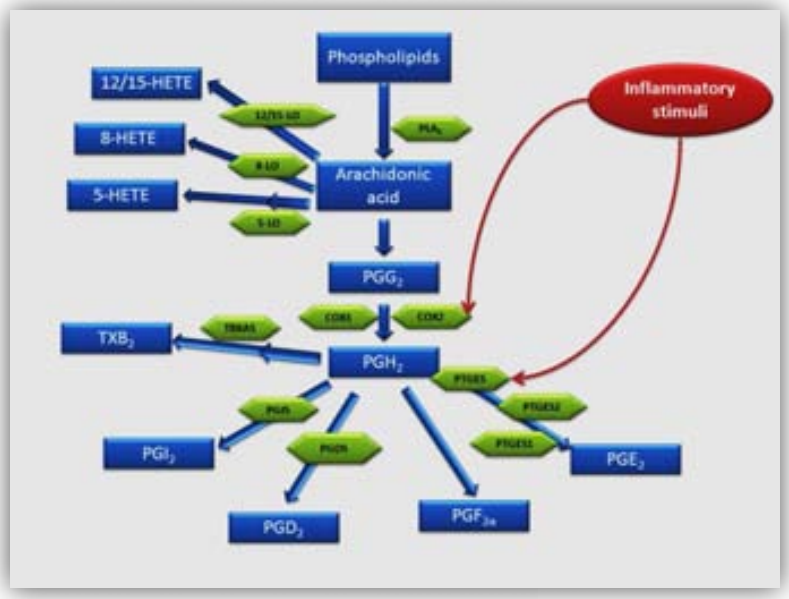

Figure 12: Arachidonic acid synthesis pathway is schematically represented. Recently, the endocannabinoid 2-arachidonoylglycerol has been proposed to be a distinct source of arachidonic acid instead of phospholipids during neuroinflammation. Monoacylglycerol lipase would be the main enzyme to catalyze this hydrolytic reaction[303].

1.7 Transcription factors in glial activation

During glial activation, several transcription factors are fundamental for the fine cell-specific regulation of the significant number of changes occurring in gene expression. The transcription factors most frequently associated with neuroinflammation are briefly introduced:

Nuclear factor kappa B (NF-kB): NF-kB is an early-immediate transcription factor with a pro-inflammatory profile in peripheral immune cells. Activation of glial cells can trigger inhibitory subunit (IkBα) degradation and the subsequent NF-kB translocation from cytoplasm into the nucleus[189,190]. NF-kB role in glial gene promoters is poorly characterized. Astroglial overexpression of the inhibitory subunit IkB triggers IL6 upregulation and the downregulation of CXCL10 and CCL2[191]. It also regulates the expression of ICAM-1[192] and IL6[193] in astroglial cultures.

Activator protein 1 (AP-1): AP-1 is a heterodimer of Fos and Jun oncoproteins that is activated in CNS cells by TNFα and IL1β through the MAPK pathway. The type of cell (microglia or astrocytes) determines the role of this transcription factor during neurodegenerative processes, as it also occurs with NF-kB[194].

Hypoxia-inducible factor-1 (HIF-1): Hypoxic conditions or lipopolysaccharide (LPS) induce HIF-1 heterodimer formation (HIF-1α and HIF-1β) that can activate iNOS gene in the BV2 microglial cell line[195,196]. Furthermore, HIF-1 enhances CXCR4 expression in microglia, promoting migration[197].

Early growth response 1 (Egr1): Different growth factors and environmental stress signals, including hypoxia or vascular injury, can activate Egr-1[198]. This transcription factor regulates several genes encoding cytokines, intercellular adhesion molecules, coagulation proteins, extracellular matrix components and metalloproteases. Egr-1 can act synergistically with HIF-1α to promote the expression of genes such as VEGF and plasminogen activator inhibitor-1[199,200].

Signal transducers and activators of transcription (STATs): IL6 and TNFα exert part of their effects through JAK/STAT signalling[201]. Neuronal increase of STAT1 phosphorylation is known to participate in ischemic neuronal damage and STAT1 knockout (KO) mice show smaller infarts[202], whereas STAT3 silencing prevents inflammation as well as neuronal loss[203].

Peroxisome proliferator activated-receptors (PPARs): PPAR isoforms are expressed in neurons and glial cells[204,205]. PPARs agonists seem to have anti-inflammatory effects[206–209]. In primary mouse microglial and astrocytic cultures, PPARγ agonists can inhibit NO production and cytokine expression[210,211].

Interferon regulatory factor 1 (IRF1): This transcription factor is essential for IFNγ-mediated induction of iNOS in macrophages but not in microglia[212]. However, it was observed that IRF1-null mice have reduced infarct volume and neurological deficit after cerebral ischemia[213].

CCAAT enhancer binding proteins (C/EBPs): C/EBPs are a subfamily of the basic region leucine zipper (bZIP) family of transcription factors. It comprises 6 genes, from C/EBPα to C/EBPζ, with similar structure and evolutionary conservation. C/EBPs have been described in glial cells[214–216], with the exception of C/EBPγ and C/EBPε. C/EBPζ, also known as CHOP, has been implicated in endoplasmic reticulum stress-mediated apoptosis in ALS[214,217]. TLRs activation up-regulates

C/EBPβ and C/EBPδ in astrocytes and microglia[218,219], whereas C/EBPα decreases its expression. The expression of various cytokines and chemokines is controlled by these transcription factors and, on the other hand, some cytokines up-regulate their expression[220,221]. The promoter regions of several pro-inflammatory mediators, such as iNOS and COX-2, have recognition sequences for C/EBPs[222–224]. These evidences highlight C/EBPs as an interesting object of study as well as a possible target to control glial activation.

Table 7: Transcription factors in neuroinflammation.

TFs	Model	Cell type	Stimulus	Effect	Reference
NF-kB	Several	Microglia; Astroglia	LPS; Cytokines	Pro-	191
C/EBPα	EAE	Microglia	?	Pro-	225
C/EBPβ	Ischemia; Inflammation, EAE	Microglia; Astroglia;	LPS; Cytokines	Pro-	105,119,215,226,227
C/EBPδ	EAE; *in vitro* AD; Inflammation	Microglia; Astroglia;	Aβ; LPS; Cytokines	?	215,219,228–230
Egr	Hypoxia; Vascular injury; Stress	Microglia; Astroglia;	Growth factors	Anti-	231
AP-1	Inflammation; Infection	Microglia; Astroglia;	Cytokines	Pro-	194
IRF-1	Ischemia	Microglia; Astroglia;	INFγ	Pro- /Anti-	213
NFATs	Trauma (c3); *in vitro* AD	Astroglia	Aβ	Pro-	232
HIF-1	Hypoxia; Ischemia	Microglia	LPS	Pro-	196,197,233
STAT1	Ischemia	Astroglia; Microglia;	?	Pro-	234,235
STAT3	Spinal cord lesion; Ischemia	Microglia	?	Pro-	203
PU.1	EAE	Microglia	?	Pro-	125,225
ATF2	Ischemia	Microglia	?	Pro-	236
Sp1	Inflammation	BV2	LPS	Pro-	196
PPARs	EAE; Ischemia	Astroglia; Microglia	?	Anti-	237
KLF4	Inflammation	BV2	LPS	Pro-	238
p53	*In vitro* AD	Microglia	Aβ	Pro-	239
NRF-2	MTPT	Astroglia; Microglia	?	Anti-	240
CREB	Ischemia; *in vitro* activation	Astroglia; Microglia	β-adrenergic agonists	Pro- /Anti-	200,236

1.7.1 CCAAT enhancer binding protein β and δ

Gene

C/EBPβ and C/EBPδ are intronless genes with highly conserved sequences between them. The main features are shown in the following table:

Table 8: Essential features of C/EBPβ and C/EBPδ in Mus musculus and Homo sapiens.

	C/EBPβ	C/EBPδ	
Chromosomes	20	8	H. sapiens
	2	16	M. musculus
mRNAs	2113 bp	1269 bp	H. sapiens
	1507 bp	2260 bp	M. musculus
Proteins	345 aa	296 aa	H. sapiens
	269 aa	268 aa	M. musculus

Both genes show a particularly high constitutive expression in intestine, lung and adipose tissue. C/EBPβ also shows high expression in spleen, kidney and myelomonocytic cells, whereas C/EBPδ has constitutive expression in osteoblasts[241].

- *C/EBPβ transcriptional regulation*

cAMP response element-binding protein (CREB) binds to two elements in the C/EBPβ gene promoter in rat liver nuclear extracts and in HepG2 cell line. These CRE binding sites allow stimulation of C/EBPβ by protein kinase A (PKA)[242] and by IL6 through a STAT3 dependent pathway[243].

C/EBPβ shows autoregulation properties in HepG2 cells and in Neuro217 cells through a region near the TATA box. This β-site acts synergistically with CRE-1 site to modulate C/EBPβ gene transcription through CREB[244]. Another example of autoregulation is the binding of C/EBPβ to two ATG-proximal sites during the acute phase response in liver[245].

An analysis of the C/EBPβ promoter has shown that a 104bp region is sufficient to confer strong basal and phorbol 12-myristate 13-acetate-responsive promoter activity in U937 cells. Functionally important binding sites for CREB/ATF and Sp1 families have been described on C/EBPβ promoter[246]. Also Sp3 can regulate human expression of C/EBPβ[247]. In addition, activating transcription factor 2 (ATF2) and c-Jun activate C/EBPβ gene expression in LPS-treated mouse liver nuclear extracts[248].

- *C/EBPδ transcriptional regulation*

C/EBPδ promoter contains a STAT3 consensus site that has been shown to be functional in rat fibroblasts and HepG2 cell line treated with IL6[249], whereas in mouse macrophages Sp1, c-Rel and c-Jun are responsible for the induction of C/EBPδ expression after LPS or peptidoglycan stimuli[250]. However, it has been hypothesized that C/EBPδ can induce its expression after the initial stimulus[251]. In this view, C/EBPs deficient for transactivation domain (TAD) may down-regulate C/EBPδ expression.

Protein

Several C/EBPβ N-terminally truncated isoforms can be generated from a single mRNA as result of alternative translation initiation (ATI) at the four downstream in-frame AUGs codons. C/EBPβ mRNA has been reported to produce at least four isoforms: full-length 38-kDa (Full), 35-kDa LAP (liver-enriched transcriptional activator protein), 21-kDa LIP (liver-enriched transcriptional inhibitory protein) and a less experimentally observed 14-kDa isoform[252,253]. On the other hand, C/EBPδ shows only one isoform of 28kDa, with a proteic structure similar to Full isoform of C/EBPβ.

- Structure

The N-terminal structure of C/EBPβ diverges between isoforms. Full and LAP have TADs that interact with components of the transcriptional complex and stimulate transcription[241,251], but they differ in the presence of a 21 aa sequence (CR1) at the N-terminal (Figure 13). CR1 function is to accomplish the interaction of TADs with SWI/SNF complex to remodel chromatin[254]. TADs lack in LIP and in the 14-kDa isoform; for this reason they act as dominant negative inhibitors[241,251]. C/EBPδ crystallographic structure has not been determined and the N-terminal structure is postulated to be similar to C/EBPβ-Full.

At C-terminal of all C/EBPβ isoforms and C/EPBδ, the bZIP domain consists of a basic-aa-rich DNA-binding region (basic domain) followed by a dimerization domain, the leucine zipper (Figure 13). The leucine zipper is a heptad repeat of four or five leucine residues that assumes an α-helical conformation. Dimerization is fundamental for the interaction of the basic domain with DNA and the dimer assumes an inverted Y-shaped structure. Each arm of the Y represents a basic domain which binds to one half of a palindromic recognition sequence in the DNA major groove. An optimal C/EBPβ binding site is a palindrome repeat of RTTGCGYAAY (R = A or G; Y = C or T)[241,251]. Due to the high conservation in the bZIP domain, C/EBPs are able to form not only homodimers, but they can also heterodimerize with different members of the C/EBP family, the CREB/ATF and the Fos/Jun families of transcription factors [241,251].

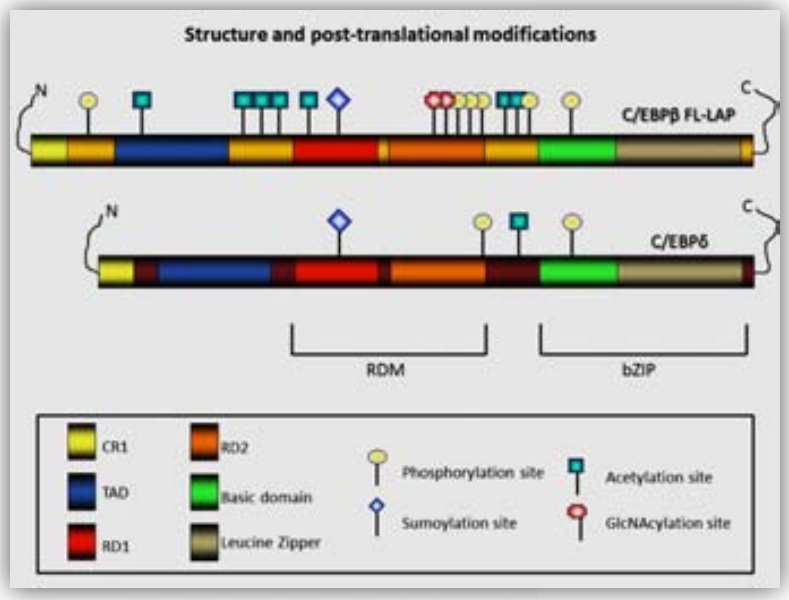

Figure 13: C/EBPβ (Full) and C/EBPδ proteins organization and post-translational modifications. Modified from a review by Nerlov[255]

The activity of TADs and bZIP is controlled by the regulatory domain motif (RDM) that regulates the conformational changes in the protein structure through post-translational modifications. RDM is a bipartite domain located within the 116-191 region in mouse (figure 13). Regulatory domain 1 (RD1) is located adjacent to TADs and regulatory domain 2 (RD2) lies just upstream of the bZIP domain[255,256].

- Translational regulation of C/EBPβ

C/EBPβ shows a complex translational regulation. The RNA CUG repeat binding protein (CUGBP1) and calreticulin (CRT) are two RNA binding proteins that recognize the same sequence. Both proteins can interact with the 5'-region of C/EBPβ mRNA which contains two of these binding sites, located side by side between the first and the second AUG codons. Although CUGBP1 and CRT interact with the same sequence, the consequences of these interactions are different. CRT binds and stabilizes GC-rich stem-loop (SL) structure (figure 14A), leading to the inhibition of translation of C/EBPβ[257]. On the other hand, CUGBP1 interaction increases the translation of small isoforms[258] (figure 14B). Phosphorylation of CUGBP1 allows the interaction with eukaryotic initiation translation factor 2 (eIF2). CUGBP1-eIF2 complex binds to 5'-region of C/EBPβ mRNA and replaces CRT (figure 14B), leading to recruitment of ribosomes on a short out-of-frame open reading frame (sORF). A portion of

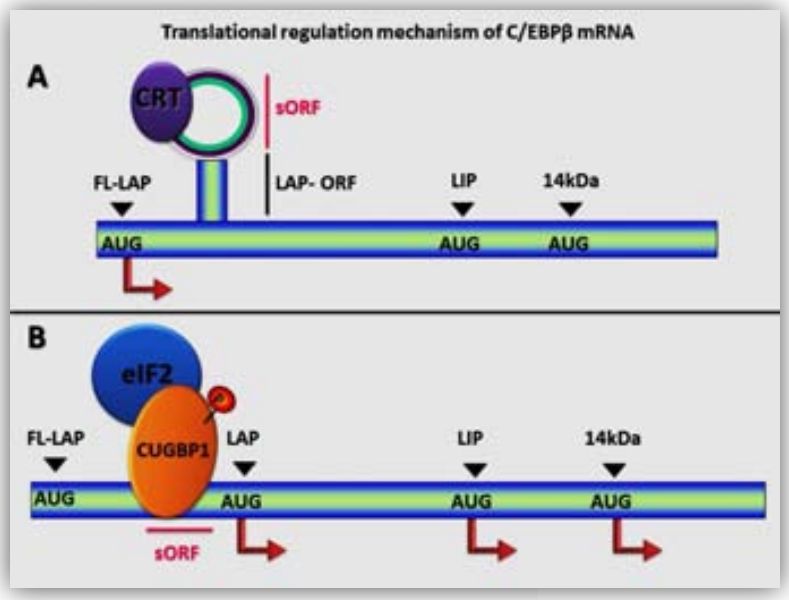

Figure 14: Interactions and conformational changes in translational regulation mechanism of C/EBPβ mRNA (see text for details).

ribosomes initiates translation at the AUG codon of the sORF, synthesizes a 9-aa peptide, reaches the stop codon of the sORF and dissociates. Since there is a very close proximity of the stop codon of sORF and the initiating AUG codon of LAP, the ribosomes are not able to start translation of LAP ("leaky scanning" mechanism) and continue scanning until they meet the third (LIP specific) AUG codon[253,258–260]. It is noteworthy that "leaky scanning" translational mechanism is poorly understood, especially in eukaryotic cells, and C/EBPβ mRNA is being used to clarify this biochemical process.

- *Post-translational regulation*

C/EBPs are mainly regulated by *de novo* protein synthesis; however a fine regulation through post-translational modifications is also present. C/EBPδ is less studied than C/EBPβ and it has been hypothesized, due to the high homology between family members, that post-translational modifications observed in common domains could also occur in the other members[241,255,261]. For this reason, C/EBPβ post-translational modifications will be presented as example.

Phosphorylation: Different groups have demonstrated *in vitro* that the main regulation of C/EBPβ activity is by phosphorylation. Many serine/threonine residues of C/EBPβ protein are phosphorylated by several protein kinases affecting responses such as protein shuttling, transactivation and dimerization. The positive or negative effects on C/EBPβ functions are determined by P-residue location in the protein structure[241,251].

Sumoylation: This post-translational modification consists of an isopeptide bond formed between the C-terminal Gly residue of a small ubiquitin-related modifier protein (SUMO) and a Lys residue in the substrate[262]. Sumoylation of lysine-133 has been shown to decrease the inhibitory function of C/EBPβ RDM[263]. Functional studies have shown that sumoylation regulates NOS-2 expression in astrocytes[264] and COX-2 promoter activity[265]. Other studies have shown the effect of C/EBPβ sumoylation on T-cell proliferation and gene expression[266]. Mutation of sumoylation site prevents C/EBPβ-mediated repression of the Ccnd1 promoter[267], or enhance synergy between multiple C/EBP molecules[268]. Sumoylation also induces the localization of C/EBPβ to pericentromeric heterochromatin, suggesting that the subnuclear localization of C/EBPβ determines its function[266].

Acetylation: C/EBPβ has been shown to undergo acetylation on specific lysine residues which increases or decreases DNA binding activity and protein interactions[255]. Acetylation of C/EBPβ K39 by CREB binding protein potentiates Fos and C/EBPα promoter activity *in vitro*[269]. Other studies have demonstrated how glucocorticoid stimulation determines C/EBPβ acetylation at K98, K101, and K102 by the acetyl transferases GCN5 and PCAF. This modification decreases the binding affinity between C/EBPβ and the histone deacetylase HDAC1 or the co-repressor mSin3a[270]. PU.1 overexpression determines the acetylation of C/EBPβ and a subsequent increase of COX-2 gene expression[271]. On the other hand, a STAT5 dependent-pathway determines HDAC1 activation and the consequent deacetylation of C/EBPβ K215 and K216 residues, increasing its affinity for the Id1 promoter[272].

Glycosylation: Protein glycosylation with O-linked N-acetylglucosamine (OGlcNAc) is a reversible post-translational modification of serines/threonines on proteins via an O-linked glycosidic bond. GlcNAcylation regulates signal transduction, protein expression, degradation and trafficking[273]. Both *in vitro* and *ex vivo* experiments have shown that GlcNAcylation in the RDM of C/EBPβ prevents its phosphorylation and affects the DNA binding activity[274].

Methylation: Radioactive labelling of HEK-293 cells has shown that C/EBPβ can be methylated on lysine-39. The histone-lysine N-methyltransferase, H3 lysine 9-specific 3 (G9a), interacts with C/EBPβ TAD-region in order to promote methylation and the subsequent inhibition of C/EBPβ transactivation potential[275].

Physiological functions

- Cellular differentiation:

A key role of C/EBPβ in cellular differentiation has been well characterized in adipocytes, hepatocytes and myeloid cells. During adipogenesis and hepatogenesis, C/EBPβ and C/EBPδ proteins are overexpressed after hormonal stimulation and participate in the induction of cell differentiation. In early myeloid progenitors C/EBPβ expression is low whereas it is up-regulated during macrophage differentiation[241].

- Cellular proliferation

In many tumors C/EBPβ expression is increased and its absence in knock-out mice abolishes skin tumour[276] or liver regeneration[277]. On the contrary, C/EBPδ down-regulation is implicated in tumorigenesis and its overexpression blocks cell proliferation[278]. Phosphorylation plays a critical role in C/EBPβ proliferative action, as shown by Hunter's group in the case of hepatocyte proliferation[279].

- Control of metabolism

Approximately half of the C/EBPβ-null mice die after birth due to hypoglycemia because insulin and glucocorticoids regulate gluconeogenesis through C/EBPβ. In the surviving animals, stimulation with glucagon and adrenaline induces fasting hypoglycemia, reduced blood lipids, impaired hepatic glucose production and adipose-tissue lipolysis. This has mainly been attributed to altered levels of hepatic cAMP production and to the activity of protein kinase A[241,251].

- Inflammation

C/EBPβ was first identified as a transcription factor able to regulate gene transcription in response to IL6 or IL1[280] . Several pro-inflammatory cytokines (IL1β, IL6, TNFα and IFNγ) and LPS induce C/EBPβ and C/EBPδ binding activity and gene overexpression in different cells, such as hepatocytes, macrophages and glial cells[218,220,221,227]. Furthermore, C/EBPβ regulates transcription of many cytokine genes during macrophage activation, such as IL1β, IL6, IL8, IL12, CCL2 and TNFα. C/EBPβ deficiency causes lymphoproliferative disorder and abnormal T-helper macrophage response in mice. Knock-out mice also show impaired expression of serum amyloid A and P, α1-acid glycoprotein, complement component 3, TNFα, IL12 and NO products[281,282]. TLRs stimulation in double knock-out macrophages shows redundant and complementary roles for C/EBPβ and C/EBPδ in TNFα and IL6 expression[283].

C/EBPβ and C/EBPδ in CNS

During brain development, C/EBPβ phosphorylation has a key role in biasing cortical precursors to become neurons or astroglia through a MEK-ERK dependent pathway[284]. At behavioural level, the process of memory consolidation in hippocampus involves C/EBPβ and C/EBPδ overexpression mediated by CREB[285–287].

Pahan's group has shown a role of C/EBPβ in gene expression of different inflammatory mediators in BV2 microglial cell line, highlighting its possible role in microglial activation[288–291]. Our group has shown C/EBPδ expression in glial cultures[219] and the preferential C/EBPβ overexpression in microglia after LPS stimulus *in vitro* and *in vivo*[227]. Recently, we have demonstrated that C/EBPδ inhibition decreases pro-inflammatory response and microglial neurotoxic potential[292].

In astrocytes, C/EBPβ activation promotes glycogen metabolism gene expression[215] and in reactive astrogliosis, the overexpression of C/EBPβ is related with the gene regulation of C3 and Serpine3K[221], Cytochrome P450 2E1[293], CCL-2[294] and NOS-2[295]. Furthermore, C/EBPβ-null mice develop smaller infarcts and decreased apoptosis after middle cerebral artery occlusion (MCAO)[105] as well as a pronounced reduction in kainic acid-induced glial activation and neuronal damage[226]. C/EBPβ and C/EBPδ have been implicated in inflammaging[296,297] and in AD[228,229,297], whereas C/EBPβ up-regulation has also been observed in ALS patients[119].

2. AIM OF THE THESIS

2. AIM OF THE THESIS

Our aim is the characterization of C/EBPβ and C/EBPδ role in pro-inflammatory *in vitro* and *in vivo* models of glial activation.

Focusing on this, we use two different approaches to study C/EBPβ and C/EBPδ:

1. Mixed glial cultures prepared from mouse cerebral cortex have been stimulated with LPS alone or in combination with IFNγ.
2. Adult mice have been treated systemically with LPS.

The following specific objectives were addressed:

- Analysis of inflammatory gene promoters for C/EBPs consensus sites.
- Characterization of the expression and DNA binding activity of C/EBPβ and C/EBPδ in activated mixed glial cultures under pro-inflammatory stimuli.
- Characterization of C/EBPβ- and C/EBPδ-null mixed glial cultures cell populations under pro-inflammatory stimuli.
- Analysis of inflammatory mediators in activated C/EBPβ- and C/EBPδ-null glial cultures.
- Analysis of microglial neurotoxic potential when C/EBPβ or C/EPBδ functions are abrogated.
- Characterization of pro-inflammatory gene expression in cerebral cortices of C/EBPβ- and C/EBPδ-null mice in the systemic LPS *in vivo* model.

3. EXPERIMENTAL DESIGN

3.1 C/EBPβ MIXED GLIAL CULTURES

Animals	Mating		2	Days	~ 51 days
	Pregnancy		19		
Cultures	Primary culture and genotyping		16		
	Secondary culture		12		
Treatments	DNA Binding activity	(EMSA, qChIP)	2	Hours	
	mRNA analysis	(qRT-PCR)	6		
	Protein analysis	(WB, ICC, ELISA, PGE2 assay)	16		
	Bio-assays	(Griess assay)	48		

Diagram 1: Experimental design followed in C/EBPβ study *in vitro* is schematically presented.

3.2 C/EBPδ MIXED GLIAL CULTURES

Animals	Mating		2	Days	~ 45 days
	Pregnancy		21		
	Post-natal		2		
Cultures	Primary culture		19		
Treatments	DNA Binding activity	(EMSA, qChIP)	2	Hours	
	mRNA analysis	(qRT-PCR)	6		
	Protein analysis	(WB, ICC)	16		
	Bio-assays	(Griess assay)	48		

Diagram 2: Experimental design followed in C/EBPδ study *in vitro* is schematically presented.

3.3 *IN VIVO* EXPERIMENTS

C/EBPβ

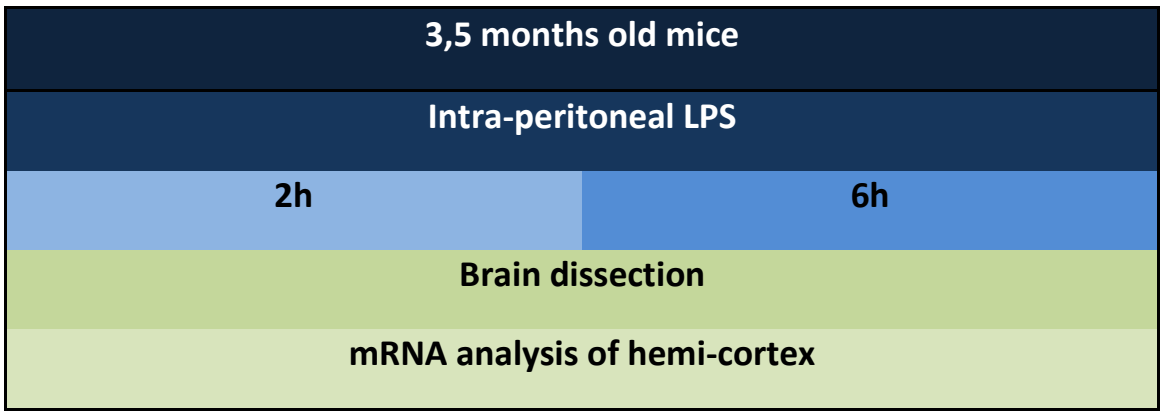

Diagram 3: Experimental design followed in C/EBPβ *in vivo* study is schematically presented.

C/EBPδ

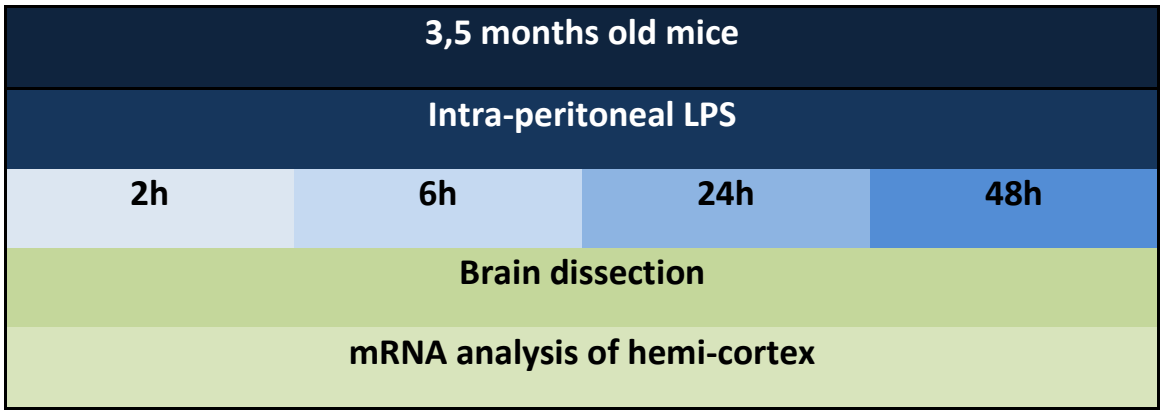

Diagram 4: Experimental design followed in C/EBPδ *in vivo* study is schematically presented.

4. EXPERIMENTAL INSIGHTS

4. EXPERIMENTAL INSIGHTS

The table below is a summary of the number of animals and some technical features related to the mixed glial cultures presented in this thesis.

Table 9: Experimental data about animals and technical information on glial cultures usage. (DIV= days in vitro; E= embrionic; P=Postnatal; n.a.= not available)

	Transgenic colony	C/EBPβ	C/EBPδ
Animals	Total mice	149	199
	Wild-types	27,5%	61,8%
	Heterozygotes	45,0%	n.a.
	Knockouts	27,5%	38,2%
	Age of mice	E18-P1	P0-P3
Cultures	Type of culture	Secondary	Primary
	Nº of cultures	19	15
	Cells per mouse cortex	n.a.	5.614.151 ±1.728.216
	Glial cells before subculturing per mouse cortex	2.055.868 ±433.370	-
	DIV before subculturing	16 ± 2	-
	DIV before treatment	12 ± 3	19 ± 3

5. MATERIALS

5. MATERIALS

In this chapter summarizing tables with primers, antibodies and software used in this thesis are presented. All the other materials as well as methods that have been used along this thesis are described in the Material and Methods section of each article (chapter 6).

5.1 PRIMERS

Quantitative real time PCR primers

Target Gene	Primer	Sequence	bp	Tm	GC %	Product lenght (bp)
C/EBPα	Fwd	5'-Tgg ACA AgA ACA gCA ACg AgT AC-3'	23	55,2	47,8	144
NM_007678.3	Rev	5'-TgC gCA ggC ggT CAT T-3'	16	54,0	62,5	
C/EBPβ	Fwd	5'-AAg CTg AgC gAC gAg TAC AAg A-3'	22	55,9	50,0	116
NM_009883.3	Rev	5'-gTC AgC TCC AgC ACC TTg Tg-3'	20	55,5	60,0	
C/EBPδ	Fwd	5'-CTC CAC gAC TCC TgC CAT gT-3'	20	56,0	60,0	121
NM_007679.4	Rev	5'-gAA gAg gTC ggC gAA gAg TTC-3'	21	55,0	57,1	
NOS2	Fwd	5'-ggC AgC CTg TgA gAC CTT Tg-3'	20	55,5	60,0	72
NM_010927.3	Rev	5'-gCA TTg gAA gTg AAg CgT TTC-3'	21	53,0	47,6	
IL1β	Fwd	5'-Tgg TgT gTg Acg TTC CCA TTA-3'	21	53,7	47,6	71
NM_008361.3	Rev	5'-CAg CAC gAg gCT TTT TTg TTg-3'	21	53,6	47,6	
IL4	Fwd	5'-CgA ggT CAC Agg AgA Agg gA-3'	20	54,7	60,0	101
NM_021283	Rev	5'-AAg CCC TAC AgA CgA gCT CAC T-3'	22	57,1	54,6	
IL6	Fwd	5'-CCA gAg ATA CAA AgA AAT gAT gg-3'	23	49,3	39,1	88
NM_031168.1	Rev	5'-ACT CCA gAA gAC CAg Agg AAA T-3'	22	52,5	45,5	
TNFα	Fwd	5'-TgA TCC gCg ACg Tgg AA-3'	17	53,0	58,8	72
NM_013693.2	Rev	5'-ACC gCC Tgg AgT TCT ggA A-3'	19	54,9	57,9	
TGFβ1	Fwd	5'-TgC gCT TgC AgA gAT TAA AA-3'	20	50,6	40,0	186
NM_011577	Rev	5'-AgC CCT gTA TTC CgT CTC CT-3'	20	54,1	55,0	
PTGS1	Fwd	5'-gTg CTg ggg Cag TgC Tgg Ag-3'	20	60,0	70,0	281
NM_008969.3	Rev	5'-Tgg ggC CTg AgT AgC CCg Tg-3'	20	60,3	70,0	
COX2	Fwd	5'-TgC AGA ATT gAA AgC CCT CT-3'	20	55,3	51,5	95

NM_011198.3	Rev	5'-CCC CAA AgA TAg CAT CTg gA-3'	20	57,3	50,8	
PTGES	Fwd	5'-Agg CCA gAT gAg gCT gCg gA-3'	20	60,0	65,0	278
NM_022415.3	Rev	5'-AgCgAAggCgTgggTTCAgC-3'	20	60,0	65,0	
POR	Fwd	5'-ACA CTT CTT CAg CCA CTg CC-3'	20	54,7	55,0	108
NM_008898,1	Rev	5'-ggT gTg TgA TCT ggT Cgg TA-3'	20	53,3	55,0	
ALOX5	Fwd	5'-CAC Cag TTC CTg gCT gCC CC-3'	20	59,6	70,0	260
NM_009662.2	Rev	5'-gCA ggC AgC Tgg Cgg TAC AT-3'	20	59,8	65,0	
PTGIS	Fwd	5'-gTg gAg gCC TCA CCA CgC AC-3'	20	60,0	70,0	280
NM_008968.3	Rev	5'-CCC ggg CCT gCA TCT CCT CT-3'	20	60,0	70,0	
PTGDS	Fwd	5'-Tgg TCC TCC Tgg gTC TCT Tgg gAT-3'	24	60,1	58,3	417
NM_008963.2	Rev	5'-TgT AgA ggg Tgg CCA TgC ggA-3'	21	59,6	61,9	
TBXAS1	Fwd	5'-CAC ACg ggA ggC AgC ACA gg-3'	20	60,0	70,0	198
NM_011539.3	Rev	5'-ggg CCA gCT CCA Aag ggC Ag-3'	20	60,0	70,0	

Quantitative chromatin immunoprecipitation primers

Target Gene	C/EBPβ binding site sequence (5'→3') Consensus: ATTGCGCAAT	Primer forward (5'→3')	Primer reverse (5'→3')
NOS2	ggagTGaaGCAATga	TTA TgA gAT gTg CCC TCT gC	CCA CCT Aag ggg AAC AgT gA
IL1β	tgtgTgaaGaAAgaa	TCA ggA ACA gTT gCC ATA gC	AgA CCT ATA CAA Cgg CTC CT
IL6	gTttCCAATcagccc	gTT gTg ATT CTT TCg Atg CT	ggA ATT gAC TAT CgT TCT Tg
IL10	aggATTGaGaAATaa	TgA CTT CCg AgT CAg CAA gA	AgA ggC CCT CAT CTg Tgg AT
TNFα	agggTTtgGaAAgtt	TCT CAT TCA ACC CTC ggA AA	CAC ACA CAC CCT CCT gAT Tg
PTGES	gcTTTCAaaagaa	AAC AAT ggT CCT gAg CCA Ag	AAg TCC TgA gTA ggC ggT CA
	acaTTGAGaaagtc	TAA CAg ggC CAg gCT gTA CT	ggT TTT gTT CTg CCA TgT gA
	gccTTACAgaaaac	gCA Tgg CTg TCC CTC TAC AT	TTT CTg Agg CTC Tgg TCC AT
COX2	ttcttgcGCAACtc	Cgg CTT CCT TCg TCT CTC AT	ACA ACT ggC TgC TAA Tgg gg
	caaTTACAaaatact	TCT TgA TTT ggT TTg ggA CAg	AgA CCT ggA ggA CAA gAg CA
	tgtTTGAAttaagg	gAA CTg ACT gCT ATC AAA TgC AC	ACA TgC Tgg gCT TgA AgA CT

Primers for genotyping PCRs

Target Gene	Primer	Sequence	bp	Tm	GC %	Product length (bp)
C/EBPβ-1s	Fwd	5'-AAg ACg gTg gAC AAg CTg Ag-3'	20	54,2	55,0	
C/EBPβ-NeoAs	Rev	5'-CAT CAg AgC AgC CgA TTg TC-3'	20	53,4	55,0	396
C/EBPβ-4As	Rev	5'-ggC AgC TgC TTg AAC AAg TTC-3'	21	54,9	52,4	212
C/EBPδ-wt	Fwd	5'-CTC CAg gCT Tgg ACg gCT AAg TAg g-3'	25	60,3	60,0	
	Rev	5'-AAg TTg gCT gTC ACC TCg CC-3'	20	57,2	60,0	205
C/EBPδ-ko	Fwd	5'-gCT CCA gAC TgC CTg ggA AAA gC -3’	23	60,0	60,9	
	Rev	5'-CAg TCC AgT gCC CAA gCT gC -3’	20	58,2	65,0	305

5.2 ANTIBODIES

Antibody	Provider	Reference code	Type	Animal Source	WB	ICC/IF	IHC	qChIP
mC/EBPα	SantaCruz	sc-61	Poly	rb	1/300	-	-	-
mC/EBPβ	SantaCruz	sc-150	Poly	rb	1/300-1/500	1/500	-	-
mC/EBPβ	SantaCruz	sc-150X	Poly	rb	-	-	-	2µg
mC/EBPδ	SantaCruz	sc-151	Poly	rb	1/300-1/500	-	-	2µg
(h-m)C/EBPδ	Rockland	600-401-A61	Poly	rb	-	-	1/500(h) 1/1000(m)	-
mNOS2	BD transd. Lab.	610431	Mono	m	1/300-1/500	1/200 1/300	-	-
mNF-kB (p65)	SantaCruz	Sc-372G	Poly	g	1/1000	-	-	-
mCOX2	SantaCruz	sc-1747	Poly	rb	1/500-1/2000	1/500-1/1000	-	-
mPTGES	Agrisera	AS03031	Poly	rb	1/1000	1/1000	-	-
mGFAP	DakoCytomation	Z0334	Poly	rb	1/10^4	1/1000 1/2000	1/1000	-
mCD11b	Serotec	MCA711G	Mono	r	-	1/300	1/300	-
hHLA-DP (CR3)	DakoCytomation	M0775	Mono	m	-	-	1/500	-
mMAP2	Sigma-Aldrich	M1406	Mono	m	-	1/2000	-	-
mβ-Actin	Sigma-Aldrich	A1978	Mono	m	1/4x10^4-1/3x10^5	-	-	-
mLaminB	SantaCruz	sc-6217	Poly	G	1/5000	-	-	-
rbHRP	GE	NA934	Poly	d	1/5000	-	-	-
mHRP	SantaCruz	sc-2055	Poly	g	1/5000	-	-	-
mAlexa 546	Molecular Probes	A-11018	Poly	g	-	1/1000	-	-
rbAlexa 546	Molecular Probes	A-11010	Poly	g	-	1/1000	1/500	-
mAlexa 488	Molecular Probes	A-11070	Poly	g	-	1/1000	-	-
rAlexa 488	Molecular Probes	A-11006	Poly	g	-	1/500	1/500	-
mAlexa 488	Molecular Probes	A-11001	Poly	g	-	1/500	1/500	-

HRP-biotinylated	Vector	BA-2000(m) BA-1000(rb) BA-9004 (r)	Poly	g	-	1/200-1/500	1/200-1/500	-
ExtrAvidin-HRP	Sigma-Aldrich	E-2886			-	1/500	1/500	-

(m = mouse, r = rat, rb = rabbit, h = human, g = goat, d = donkey, Poly = polyclonal, Mono = monoclonal)

5.3 SOFTWARES

Software	Provider	Version	Application
GraphPad	GraphPad software Inc.	4.02	Statistical analysis
MATCH	BIOBASE GmbH		Gene promoter analysis
MatInspector	Genomatix		Gene promoter analysis
Quantity One	Bio-Rad	5.4.1	Gel and membrane analysis
iQ5	Bio-Rad	2.0	Real time PCR data analysis
CFX Manager	Bio-Rad		Real time PCR data analysis
Office profesisonal plus 2010	Microsoft	14	Offimatica
Reference Manager 10	Thomson Reuter		Bibliography management
EndNoteX4	Thomson Reuter		Bibliography management
Mendeley	Mendeley	1.3.1	Bibliography management
PDF-Xchange Viewer	Tracker software Ltd.	2.5	PDF management
ImageJ	NIH		Image analysis
Cell F	Olympus Soft Imaging Solution	2.6	Image acquisition
Illustrator	Abobe	CS4	Illustration

6. RESULTS

6.1 PAPER 1

Pro-inflammatory gene expression and neurotoxic effects of activated microglia are attenuated by absence of CCAAT/enhancer binding protein beta.

Straccia M., Gresa-Arribas N., Dentesano G., Ejarque-Ortiz A., Tusell J.M., Serratosa J., Sola C., Saura J.

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Straccia et al. Journal of Neuroinflammation 2011, 8:156
http://www.jneuroinflammation.com/content/8/1/156

JOURNAL OF NEUROINFLAMMATION

RESEARCH Open Access

Pro-inflammatory gene expression and neurotoxic effects of activated microglia are attenuated by absence of CCAAT/enhancer binding protein β

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Abstract

Background: Microglia and astrocytes respond to homeostatic disturbances with profound changes of gene expression. This response, known as glial activation or neuroinflammation, can be detrimental to the surrounding tissue. The transcription factor CCAAT/enhancer binding protein β (C/EBPβ) is an important regulator of gene expression in inflammation but little is known about its involvement in glial activation. To explore the functional role of C/EBPβ in glial activation we have analyzed pro-inflammatory gene expression and neurotoxicity in murine wild type and C/EBPβ-null glial cultures.

Methods: Due to fertility and mortality problems associated with the C/EBPβ-null genotype we developed a protocol to prepare mixed glial cultures from cerebral cortex of a single mouse embryo with high yield. Wild-type and C/EBPβ-null glial cultures were compared in terms of total cell density by Hoechst-33258 staining; microglial content by CD11b immunocytochemistry; astroglial content by GFAP western blot; gene expression by quantitative real-time PCR, western blot, immunocytochemistry and Griess reaction; and microglial neurotoxicity by estimating MAP2 content in neuronal/microglial cocultures. C/EBPβ DNA binding activity was evaluated by electrophoretic mobility shift assay and quantitative chromatin immunoprecipitation.

Results: C/EBPβ mRNA and protein levels, as well as DNA binding, were increased in glial cultures by treatment with lipopolysaccharide (LPS) or LPS + interferon γ (IFNγ). Quantitative chromatin immunoprecipitation showed binding of C/EBPβ to pro-inflammatory gene promoters in glial activation in a stimulus- and gene-dependent manner. In agreement with these results, LPS and LPS+IFNγ induced different transcriptional patterns between pro-inflammatory cytokines and NO synthase-2 genes. Furthermore, the expressions of IL-1β and NO synthase-2, and consequent NO production, were reduced in the absence of C/EBPβ. In addition, neurotoxicity elicited by LPS +IFNγ-treated microglia co-cultured with neurons was completely abolished by the absence of C/EBPβ in microglia.

Conclusions: These findings show involvement of C/EBPβ in the regulation of pro-inflammatory gene expression in glial activation, and demonstrate for the first time a key role for C/EBPβ in the induction of neurotoxic effects by activated microglia.

Background

Glial activation is an inflammatory process that occurs in astrocytes and microglia to re-establish homeostasis of the CNS after a disequilibrium of normal physiology. Microglia are tissue-associated macrophages that keep the CNS under dynamic surveillance. Most insults to the CNS switch microglia into an M1-like phenotype, characterized by production of pro-inflammatory cytokines, reactive oxygen/nitrogen species and prostanoids. Scavenger receptors and chemokines are also upregulated and phagocytic activity increases. An M2-like phenotype usually follows, characterized by production of interleukin-4 (IL-4), IL-10, transforming growth factor-β and neurotrophic factor [1]. Glial activation requires massive and fine-tuned re-

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arrangements in gene transcription. The transcription factors behind this process include nuclear factor-kB, which seems to mediate early-immediate cytokine and chemokine gene responses in glial activation [2,3], and other transcription factors with a pro-inflammatory profile such as AP-1 [4], STATs [5], HIF-1 [5-7], Egr-1 [8], IRF1 [9]. On the other hand, transcription factors such as PPARs [10] or Nrf2 [11,12] play an anti-inflammatory role in glial activation.

CCAAT/enhancer binding protein β (C/EBPβ) is a candidate to regulate pro-inflammatory gene expression in glial activation. C/EBPβ is one of seven members of the C/EBP subfamily of bZIP transcription factors. At least three N-terminally truncated isoforms are known: 38-kDa Full, 35-kDa LAP and 21-kDa LIP [13,14]. C/EBPβ transcriptional functions in cell energy metabolism, cell proliferation and differentiation are well-characterized [15,16]. C/EBPβ also plays a role in inflammation [17]. Promoters of many pro-inflammatory genes contain putative C/EBPβ consensus sequences [18-20] and C/EBPβ levels are upregulated in response to pro-inflammatory stimuli in macrophages [21] and glial cells [22-25]. Interestingly, C/EBPβ deficiency provides neuroprotection following ischemic [26] or excitotoxic injuries [27].

Several lines of evidence suggest that glial activation is involved in the pathogenesis of many neurological disorders. The present study stems from this hypothesis and from the hypothesis that there is a regulatory role for C/EBPβ in pro-inflammatory gene expression in neuroinflammation. To define the transcriptional role of C/EBPβ in glial activation we have here studied pro-inflammatory gene profiles and neurotoxicity in glial cultures from C/EBPβ-null mice. Our results show for the first time that absence of C/EBPβ attenuates pro-inflammatory gene expression and abrogates neuronal loss induced by activated microglia.

Methods

Animals

A colony of C/EBPβ$^{+/-}$ [28] mice on a C57BL/6-129S6/SvEv background was maintained. Animals from this colony showed no serological evidence of pathological infection. The animals were group-housed (5-6) in solid floor cages and received a commercial pelleted diet and water ad libitum. Experiments were carried out in accordance with the Guidelines of the European Union Council (86/609/EU) and following the Spanish regulations (BOE 67/8509-12, 1988) for the use of laboratory animals, and were approved by the Ethics and Scientific Committees from the Hospital Clínic de Barcelona.

DNA extraction and genotyping

Genomic DNA was isolated from 2 mg liver samples using Extract-N-AmpTissue PCR Kit (Sigma-Aldrich, XNAT2) following kit instructions. PCR amplification was performed in 20 μl total volume, using 1 μl of tissue extract, 0.8 μM C/EBPβ-1s forward primer (AAgACggTggACAAgCTgAg), 0.4 μM C/EBPβ-NeoAs (CATCAgAgCAgCCgATTgTC) and 0.4 μM C/EBPβ-4As (ggCAgCTgCTTgAACAAg TTC) reverse primers. Samples were run for 35 cycles (94°C for 30 s, 59°C for 30 s, 72°C for 90 s).

Cortical mixed glial culture from a single embryo

C/EBPβ+/- mice were crossed and pregnant females were sacrificed on the 19th day of gestation by cervical dislocation. Embryos (E19) were surgically extracted from the peritoneal cavity. Their livers were dissected and used to genotype the animal, whereas their brains were dissected and processed as previously described [29] with minor modifications. Cultures reached confluence after 16 ± 3 days in vitro (DIV) and were then subcultured.

Mouse mixed glial subculture

Each flask was washed in serum-free medium and was digested with 0.25% trypsin-EDTA solution for 5 min at 37°C. Trypsinization was stopped by adding an equal volume of culture medium with FBS 10%. Cells were pelleted (7 min, 180 g), resuspended in 1 mL culture medium, and brought to a single cell suspension by repeated pipetting. Cells were seeded at 166000 cells/mL. These were therefore secondary cultures and they were used at 12 ± 3 DIV. Astrocytes were the most abundant cell type and microglial cells were approximately 20%.

Microglial culture

Microglial cultures were prepared by mild trypsinization from mouse mixed glial culture as previously described [30].

Primary cortical neuronal culture

Cortical neuronal cultures were prepared from C57BL/6 mice at embryonic day 16 as described [31]. Neuronal cultures were used at 5 DIV.

Primary neuronal-microglial co-cultures

Microglial cultures were obtained as described [31]. After astrocyte removal, microglial cells were incubated with 0.25% trypsin for 10 min at 37°C. Trypsinization was stopped by adding the same volume of culture medium with 10% FBS. Cells were gently scraped and centrifuged for 5 min at 200 g. Pellets were resuspended in neuronal culture medium and aliquots of the cell suspension (10 μL/well) were seeded on top of 5 DIV primary neuronal cultures at a final density of 4×10^5 cells/mL (1.3×10^5 cells/cm^2).

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In vitro treatments

Mixed glial cultures: The culture medium was replaced 24 h prior to treatment. Mixed glial cultures were treated with 100 ng/mL lipopolysaccharide (LPS, Sigma-Aldrich, L-2654, E. coli serotype 026:B6) and 0.1 ng/mL recombinant mouse interferon-γ (IFNγ, Sigma-Aldrich, I4777) prepared from x10 solutions.

Neuronal-primary microglia co-cultures: 100 ng/mL LPS and 30 ng/mL IFNγ were added to the culture medium one day after seeding primary microglial cells on top of neuronal cultures.

Nitrite assay

NO production was assessed by the Griess reaction. Briefly, 50 μL aliquots of culture supernatants were collected 48 h after LPS+IFNγ treatment, and incubated with equal volumes of Griess reagent (1% sulphanilamide, 0.1% N-(1-naphthyl)ethylendiamine dihydrochloride, and 5% phosphoric acid) for 10 min at room temperature (RT). Optical density at 540 nm was determined using a microplate reader (Multiskan spectrum, Thermo Electron Corporation). Nitrite concentration was determined from a sodium nitrite standard curve.

Electrophoretic mobility shift assay

Nuclear extracts were prepared as described [32] with a few modifications. Nuclear protein was extracted from mixed glial cultures after 2 h LPS or LPS+IFNγ treatment. Cells from two wells of 6-well plate were scrapped into cold 0.01 M phosphate-buffered saline (PBS, pH 7.4) and centrifuged for 4 min, 4500 g at +4°C. The resulting pellet was resuspended in 400 μL of buffer A: 10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.5 mM phenylmethylsulphonyl fluoride (PMSF) and 1 mM dithiothreitol (DTT) and cells were swollen on ice for 15 min. After addition of 25 μL of 10% Igepal CA-630 (Sigma-Aldrich, I8896), cells were vigorously vortexed for 10 s and incubated for 10 min on ice, then a 10-min centrifugation at 13200 g was performed and the pellets were resuspended in 50 μL of buffer C consisting of 20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF and 1 mM DTT. Solutions A, B, C and PBS were supplemented with protease inhibitor cocktail Complete® (Roche, 1836145). After 2 h of shaking at 4°C, nuclei were pelleted by a 5 min spin at 2000 g. The supernatant containing nuclear proteins was collected and protein amount was determined by the Lowry assay (Total Protein kit micro-Lowry, Sigma-Aldrich, TP0300). Oligonucleotides containing C/EBP consensus sequences (Santa Cruz Biotechnology, sc-2525) were labelled at their 3'-end using [α-^{33}P]dATP (3000 Ci/mmol; Dupont-NEN, NEG-612H) and terminal deoxynucleotidyltransferase (TdT; Oncogene Research Products, PF060), and purified using illustra MicroSpin G-50 Columns (GE, 27-5330-01). Five micrograms of nuclear proteins were incubated for 30 min at RT with the labelled oligonucleotides (25000 cpm/reaction assay) in binding buffer (20% glycerol, 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM DTT, 50 mM Tris-HCl, 250 mM NaCl and 0.2 mg/mL Poly(dI:dC)). After the addition of Hi-Density TBE buffer to samples (15% Ficoll type 400, 1x TBE, 0.1% Bromophenol Blue, 0.1% Xylene Cyanol), proteins were separated by electrophoresis on a 6% DNA retardation gel (Invitrogen, EC6365BOX) at 4°C, 90 min at 100 V in 0.5x TBE buffer. In supershift assay, 0.5 μg of rabbit anti-mouse C/EBPβ (Santa Cruz Biotechnology, sc-150) or IgG (Santa Cruz Biotechnology, No.sc-2027) were added 10 min before oligonucleotide incubation.

Total protein extraction

Protein levels were determined in primary mixed glial cells 16 h after treatments. For isolation of total proteins, two wells from 6-well plates were used per condition. After a cold PBS wash, cells were scrapped and recovered in 100 μL per well of RIPA buffer (1% Igepal CA-630, 5 mg/mL sodium deoxycholate, 1 mg/mL sodium dodecyl phosphate (SDS) and protease inhibitor cocktail Complete® in PBS). The content of the wells was pooled, sonicated, centrifuged for 5 min at 10400 g and stored at -20°C. Protein amount was determined by the Lowry assay.

Western blot

Fifty micrograms of denatured (2.5 mM DTT, 100°C for 5 min) total protein extracts were subjected to 10% SDS-PAGE and transferred to a PVDF membrane (Millipore, IPVH00010) for 90 min at 1 mA/cm^2. After washing in Tris-buffered saline (TBS: 20 mM Tris, 0.15 M NaCl, pH 7.5) for 5 min, dipping in methanol for 10 s and air drying, the membranes were incubated with primary antibodies overnight at 4°C: polyclonal rabbit anti-C/EBPβ (1:500, Santa Cruz Biotechnology, sc-150), monoclonal mouse anti-NO synthase-2 (NOS2; 1:200, BD Transduction Laboratories, 610431), monoclonal mouse anti-βactin (1:100000, Sigma-Aldrich, A1978) and polyclonal rabbit anti-GFAP (1:10000, DakoCytomation, Z0334) diluted in immunoblot buffer (TBS containing 0.05% Tween-20 and 5% no-fat dry milk). Then, the membranes were washed twice in 0.05% Tween-20 in TBS for 15 s and incubated in horseradish peroxidase (HRP)-labelled secondary antibodies for 1 h at RT: donkey anti-rabbit (1:5000, GE, NA934) or goat anti-mouse (1:5000, Santa Cruz Biotechnology, sc-2055). After extensive washes in 0.05% Tween-20 in TBS, they were incubated in ECL-Plus (GE, RPN2132) for 5 min. Membranes were then exposed to the camera of a VersaDoc System (Bio-Rad), and pixel intensities of the

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immunoreactive bands were quantified using the percentage adjusted volume feature of Quantity One 5.4.1 software (Bio-Rad). Data are expressed as the ratio between the intensity of the protein of interest band and the loading control protein band (β-actin).

Quantitative real time PCR (qPCR)

mRNA expression was determined in mouse mixed glial cells 6 h after treatments. For isolation of total RNA, 2 wells of 24-well plates were used per experimental condition. Total RNA was isolated using an Absolutely RNA Miniprep kit (Agilent Technologies-Stratagene 400.800) and 100 ng of RNA for each condition was reverse-transcribed with random primers using Sensiscript RT kit (Qiagen, 205213). cDNA was diluted 1/25 and 3 μL were used to perform qPCR. The primers (Roche) were used at a final concentration of 300 nM (Table 1). β-Actin and Rn18s mRNAs levels are not altered by treatments (data not shown). qPCR was carried out with IQ SYBR Green SuperMix (Bio-Rad, 170-8882) in 15 μL of final volume using iCycler MyIQ equipment (Bio-Rad). Primer efficiency was estimated from standard curves generated by dilution of a cDNA pool. Samples were run for 40 cycles (95°C for 30 s, 60° C for 1 min, 72°C for 30 s). Amplification specificity was confirmed by analysis of melting curves. Relative gene expression values were calculated with the comparative Ct or ΔΔCt method [33] using iQ5 2.0 software (Bio-Rad). Ct values were corrected by the amplification efficiency of the respective primer pair which was estimated from standard curves generated by dilution of a cDNA pool.

Quantitative chromatin immunoprecipitation (qChIP)

qChIP was performed as previously described [34] with modifications. Briefly, primary mixed glial cultures were cross-linked in 1% formaldehyde for 10 min at RT, quenched with 125 mM glycine for 5 min a RT. Cells were washed in PBS with 1 mM PMSF and protease inhibitor mix, then the cells were resuspended with 150 mM NaCl, 50 mM Tris-HCL pH7.5, 5 mM EDTA, 0.5% vol/vol NP-40, 1% vol/vol Triton X-100, 1% wt/vol SDS, 1 mM PMSF, protease inhibitor mix (IP Buffer). Chromatin shearing was obtained from 2×10^5 cells using Labsonic M sonicator (7 × 30 s on and 30 s off; cycle 0.8; 100% amplitude). In parallel, an aliquot of chromatin sheared from each sample was separated as a loading control for the experiment (input). The protocol for chromatin immunoprecipitation (ChIP) was as follows: first, 10 μL of Dynabeads® protein A (Invitrogen, 100.01D) were washed twice with 22 μL of cold IP Buffer (without SDS). Then the beads were resuspended in 11 μL of IP Buffer. Next, 90 μL of IP Buffer was added to a PCR tube with 10 μL of pre-washed protein A-beads. Two micrograms of polyclonal rabbit C/EBPβ antibody (Santa Cruz Biotechnology, sc-150X) or with 2 μg of rabbit IgG (Santa Cruz Biotechnology, sc-2027) as negative control were added and the mixture was incubated at 40 rpm on a rotating wheel for at least 2 h at 4°C. Then, the tube was placed on a magnetic rack for 1 min. The supernatant was discarded and 100 μL of sheared chromatin was added. Samples were incubated overnight at 40 rpm rotation at 4°C. Finally, the tube was placed on the magnetic rack for 1 min. The supernatant was discarded and the immunoprecipitation complex was washed three times with 100 μL of IP Buffer for 4 min on a rotating wheel and placed in the magnetic rack again for 1 min to discard the supernatant. The fourth wash was done with 10 mM Tris-HCl pH 8.0 and 10 mM EDTA buffer. Protein was degraded by a 2-h incubation at 68°C in 200 μL of IP Buffer complemented with 50 μg/mL of proteinase K. DNA was isolated with phenol-chloroform-isoamylalcohol 25:24:1 (Sigma-Aldrich, 25666 and P4556) extraction. Input and ChIP samples were analyzed with qPCR using SYBR green (Bio-Rad). Three microliters of input DNA (diluted 1/50) and ChIP were amplified in triplicate in 96-well optical plates using a MyIQ Bio-Rad Real Time Detection System. The C/EBPβ binding site in the IL-10 promoter was used as a positive control [35]. MatInspector was used to identify the proximal C/EBPβ consensus sequence in each analyzed promoter. The sequences for each amplified locus are indicated in the table 2. Samples were run for 45 cycles (95°C for 30 s,

Table 1 Primers used in quantitative real time PCR.

Target Gene	Accession	Primer forward (5→3')	Primer reverse (5→3')
NOS2	NM_010927.3	ggCAgCCTgTgAgACCTTTg	gCATTggAAgTgAAgCgTTTC
IL1β	NM_008361.3	TggTgTgTgACgTTCCCATTA	CAgCACgAggCTTTTTTgTTg
IL6	NM_031168.1	CCAgTTTggTAgCATCCATC	CCgCAgAggAgACTTCACAg
TNFα	NM_013693.2	TgATCCgCgACgTggAA	ACCgCCTggAgTTCTggAA
TGFβ1	NM_011577.1	TgCgCTTgCAgAgATTAAAA	AgCCCTgTATTCCgTCTCCT
IL4	NM_021283, 2	CgAggTCACAggAgAAgggA	AAgCCCTACAgACgAgCTCACT
Actin	NM_007393.3	CAACgAgCggTTCCgATg	gCCACAggATTCCATACCCA
Rn18s	NR_003286.2	gTAACCCgTTgAACCCCATT	CCATCCAATCggTAgTAgCg

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Table 2 C/EBPβ binding sites and primers used in quantitative ChIP assay.

Target Gene	C/EBPβ binding site sequence (5→3') Consensus: ATTGCGCAAT	Genomic localization respect to ATG	Primer forward (5→3')	Primer reverse (5→3')
NOS2	ggagTGaaGCAATga	-892/-907	TTATgAgATgTgCCCTCTgC	CCACCTAAggggAACAgTgA
IL1β	tgtgTgaaGaAAgaa	-16/-31	TCAggAACAgTTgCCATAgC	AgACCTATACAACggCTCCT
IL6	gTttCCAATcagccc	-173/-188	gTTgTgATTCTTTCgATgCT	ggAATTgACTATCgTTCTTg
TNFα	agggTTtgGaAAgtt	-336/-351	TCTCATTCAACCCTCggAAA	CACACACACCCTCCTgATTg
IL10	aggATTGaGaAATaa	-463/-448	TgACTTCCgAgTCAgCAAgA	AgAggCCCTCATCTgTggAT

62°C for 1 min, 72°C for 30 s), for further details see qPCR methods.

Immunocytochemistry

Cultured cells were fixed with 4% paraformaldehyde in PBS for 20 min at RT. For immunocytochemistry using fluorescence labelling, cells were permeated with chilled methanol for 7 min, then washed with PBS. Cells were incubated overnight at 4°C with 7% normal goat serum (Vector, S-1000) in PBS containing 1% Thimerosal (Sigma-Aldrich, T5125) and primary antibodies: polyclonal rabbit anti-C/EBPβ (1:500, Santa Cruz Biotechnology, sc-150), monoclonal mouse anti-NOS2 (1:200, BD Transduction Laboratories, 610431), polyclonal rabbit anti-GFAP (1:1000, DakoCytomation, Z0334) and monoclonal rat anti-CD11b (1:300, Serotec, MCA711G, clone 5C6). After rinsing in PBS, cells were incubated for 1 h at RT with secondary antibodies: goat anti-mouse Alexa 546 (1:1000, Molecular Probes, A-11018), goat anti-rabbit Alexa 546 (1:1000, Molecular Probes A-11010), Alexa 488 (1:1000, Molecular Probes, A-11070) or goat anti-rat Alexa 488 (1:500, Molecular Probes, A-11006). After secondary antibody incubation, cells were stained with Hoechst 33258 for 7 min. For immunocytochemistry using peroxidase labelling, cells were permeated and endogenous peroxidase activity was blocked by incubation with 0.3% H_2O_2 in methanol for 10 min. Non-specific staining was blocked by incubating the cells with 10% normal goat serum in PBS containing 1% BSA for 20 min at RT. The cells were then incubated with monoclonal mouse anti-MAP2 primary antibody (1:2000, Sigma-Aldrich, M1406) overnight at 4°C. In MAP2 staining, biotinylated horse anti-mouse secondary antibody (1:200, Vector, BA-2000) for 1 h at RT. Following incubation with ExtrAvidin®-Peroxidase (1:500, Sigma-Aldrich, E2886) for 1 h at RT, colour was developed with diaminobenzidine (Sigma-Aldrich, D5637). The antibodies were diluted in PBS containing 1% BSA and 10% normal horse serum (Vector, S-2000). Microscopy images were obtained with an Olympus IX70 microscope and a digital camera (CC-12, Soft Imaging System GmbH).

Assessment of neuronal viability (MAP2/ABTS/ELISA)

Neuronal viability was evaluated by MAP2 immunostaining using ABTS (2, 3'-azinobisethylbenzothiazoline-6-sulphonic acid) and absorbance analysis [31]. Neuronal viability was expressed as a percentage of control levels.

Cell counting

Hoechst-33258- and CD11b-positive cells were semi-automatically counted from 20x photomicrographs using ImageJ 1.42I NIH software. For each experiment (n = 4), three wells per condition were used and four fields per well were counted in a blind manner. NOS2-positive cells were counted manually from 20x photomicrographs. For each experiment (n = 11), two wells per condition were used and two fields per well were counted.

Statistical analysis

Data were analyzed using GraphPad 4.02. Two-way analysis of variance (ANOVA) followed by Bonferroni post-test was used when the effect of genotype on treatment was studied and vice versa. One-way ANOVA was used followed by Dunnet's post-test when comparing versus control or Bonferroni's post-test when comparing versus different experimental conditions. Values of $p < 0.05$ were considered statistically significant. Error bars are presented in all graphs as standard error of the mean (SEM).

Results

Characterization of C/EBPβ$^{+/+}$ and C/EBPβ$^{-/-}$ single embryo secondary mixed glial cultures

To study the role of C/EBPβ in glial activation we used C/EBPβ-null mice. Because of the infertility of C/EBPβ-null females and a perinatal death rate of approximately 50% for C/EBPβ-null neonates, we have modified the standard procedures to prepare mixed glial cultures from CNS tissue pools of several mouse neonates and designed a protocol to prepare secondary mixed glial cultures from the cerebral cortex of one single E19-E20 mouse embryo (see Methods for details). Forty-one C/EBPβ-null mice and forty-one wild-type littermates were

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used during this study. To ensure that wild-type and C/EBPβ-null glial cultures were comparable, we first analyzed total cell density and abundance of their two main cell types, astrocytes and microglia, in both cultures. No differences were observed between wild-type and C/EBPβ-null cultures in total cell density as assessed by automatic counting of Hoechst 33258-stained nuclei (Figure 1A), but a moderate increase in total cell number was induced by LPS and LPS+IFNγ. C/EBPβ absence did not affect microglial density as assessed by CD11b-positive cell counting (Figure 1B). Estimation of astrocytes number in these cultures is not trivial. Astrocytes are densely packed, almost all nuclei are surrounded by GFAP-positive filaments, and it is often difficult to discern whether a given nucleus belongs to a GFAP-positive cell or, in fact, the GFAP signal belongs to a neighbor astrocyte. We therefore analyzed total GFAP content by western blot as an indirect estimation of astroglial number and no differences were observed between wild-type and C/EBPβ-null glial cultures (Figure 1D, E). Neither CD11b nor GFAP immunocytochemistry revealed differences between wild-type or C/EBPβ-null cultures in morphology of microglial cells or astrocytes, respectively (Figure 1C, F). These results indicate that wild-type and C/EBPβ-null mixed glial cultures do not differ in total cell density or in proportions or morphology of their two major cell types, astrocytes and microglia.

LPS and LPS+IFNγ upregulate C/EBPβ in secondary mixed glial cultures

In this study, we have used LPS and LPS+IFNγ to study the role of C/EBPβ in glial activation in secondary cultures. The effects of both stimuli on C/EBPβ expression in glial cultures have not been compared before. As seen in Figure 2A-D, both LPS and LPS+IFNγ induced strong increases in C/EBPβ mRNA levels 6 h after treatment, and in nuclear levels of both activating (Full/LAP) and inhibitory (LIP) C/EBPβ isoforms 24 h after treatment. The increases in C/EBPβ mRNA and protein induced by LPS and LPS+IFNγ were of similar magnitude.

Differential C/EBPβ activation is triggered by LPS and LPS +IFNγ

Since the mRNA or protein levels of a transcription factor are of relative importance to study its functionality, we studied the DNA binding activity of C/EBPβ in LPS- or LPS+IFNγ-treated glial cells. Electrophoretic mobility shift assays showed that binding of nuclear proteins to a DNA oligonucleotide containing the C/EBPs consensus sequence was increased by LPS and LPS+IFNγ treatments (Figure 3A, lanes 1-3). Supershift experiments showed the presence of C/EBPβ in shifted complexes I to III (Figure 3A lanes 4-6). The specificity of the supershift is demonstrated by the lack of supershift elicited by the same concentration of IgG (Figure 3A lanes 7-9). This indicates that C/EBPβ is a key component of C/EBPs DNA binding complexes during LPS- and LPS +IFNγ-induced glial activation.

Next, we estimated the binding of C/EBPβ to the promoters of four major pro-inflammatory genes: nitric oxide synthase 2 (NOS2), IL-1β, IL-6 and TNFα, in mixed glial cultures using a qChIP assay (Figure 3B). In untreated glial cultures, no specific binding of C/EBPβ was measurable in any of the four promoters analyzed. However, 2 h after LPS treatment, C/EBPβ binding was observed in the NOS2 promoter. Interestingly, in LPS +IFNγ-treated glial cultures C/EBPβ binding was observed in all four promoters analyzed and, in the case of the NOS2 promoter, C/EBPβ binding was significantly higher than in LPS-treated glial cultures (Figure 3B).

C/EBPβ regulates pro-inflammatory gene expression in glial activation

To study the involvement of C/EBPβ in the regulation of pro-inflammatory gene expression, mRNA levels of NOS2, IL-1β, IL-6 and TNFα were analyzed by qPCR in wild-type and C/EBPβ-null cultures treated with LPS or LPS+IFNγ for 6 h. In wild-type cultures all four mRNAs were strongly upregulated by LPS. This effect was exacerbated by co-treatment with IFNγ in the case of NOS2 (+92.3%), but not in the case of IL-1β, IL-6 or TNFα (Figure 4). In C/EBPβ-null cultures LPS induced upregulation of IL-1β, IL-6 and TNFα mRNAs, which was similar to that observed in wild-type cultures. However, as expected from qChIP results, the LPS-induced increase in NOS2 mRNA levels was significantly lower in C/EBPβ-null than in wild-type glial cultures (-67.4%, $p < 0.05$). The pattern of gene expression induced by LPS+IFNγ was more affected by lack of C/EBPβ. Thus, LPS+IFNγ-induced mRNA levels of NOS2 and IL-1β were significantly lower in C/EBPβ-null than in wild-type cultures. TNFα and IL-6 mRNA levels did not differ statistically between the two genotypes (Figure 4). In contrast to the pro-inflammatory gene pattern, mRNA levels of the anti-inflammatory cytokines IL-4 and transforming growth factor β (TGFβ1) were not altered by LPS or LPS+IFNγ treatments and no significant changes in IL-4 or TGFβ1 mRNA levels were observed between wild-type and C/EBPβ-null glial cultures under any experimental condition (Figure 4).

C/EBPβ-null glial cultures show a marked reduction in NO production

The important reduction in NOS2 mRNA levels in activated C/EBPβ-null glial cultures prompted us to analyze

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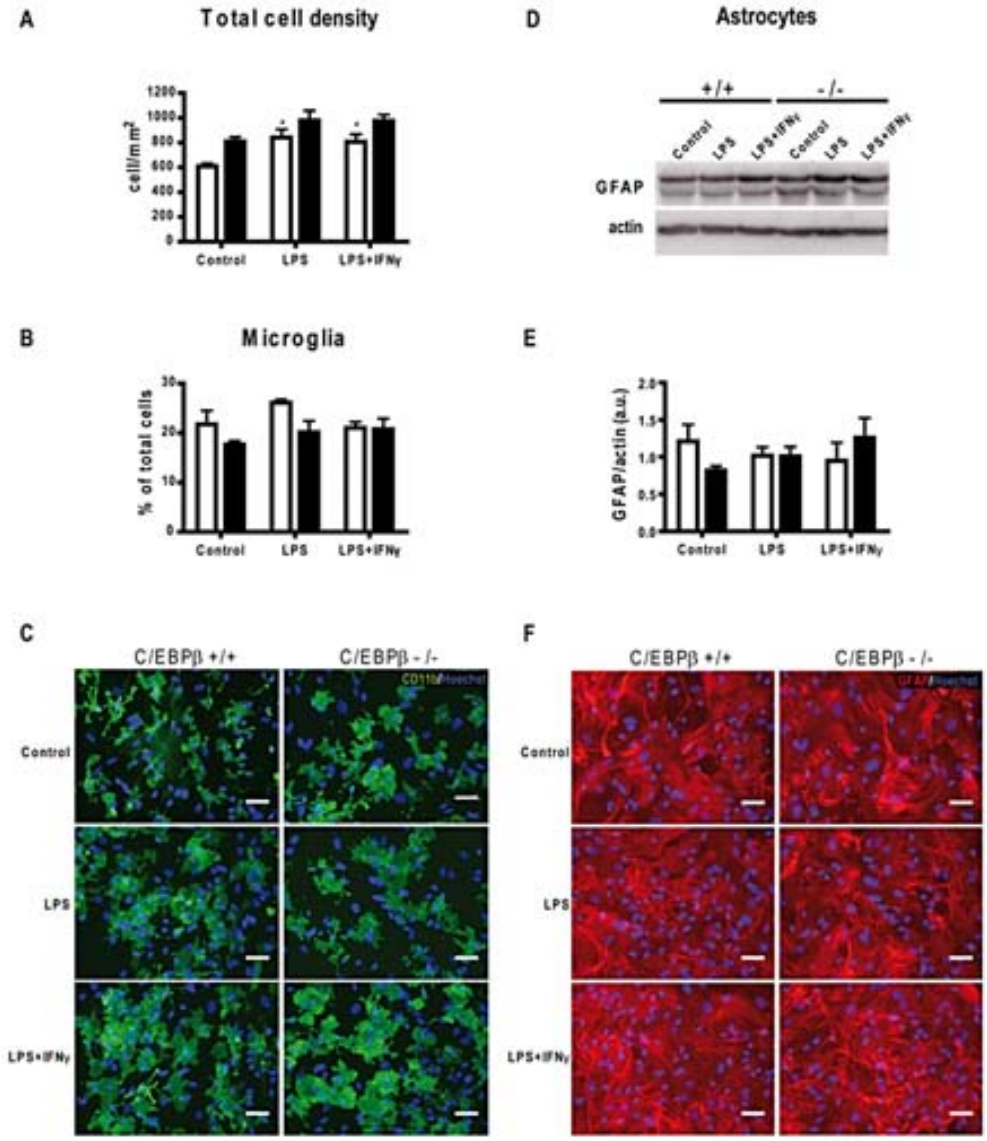

Figure 1 Basic characterization of C/EBPβ$^{-/-}$ mixed glial cultures. Secondary mixed glial cultures from C/EBPβ$^{+/+}$ (white bars) and C/EBPβ$^{-/-}$ (black bars) show similar total cell numbers and microglial density in control conditions and after 16 h of LPS or LPS+IFNγ. **A**. C/EBPβ$^{+/+}$ and C/EBPβ$^{-/-}$ total cell density was estimated by Hoechst-33258-positive nucleus counting. No significant differences were observed between genotypes. Wild-type cultures show a statistically significant increase of cell density after 16 h of LPS and LPS+IFNγ treatment compared to control; C/EPBβ-null cultures show no difference after treatments. Two-way ANOVA, followed by Bonferroni's test was applied, *$p < 0.05$; compared to C/EBPβ$^{+/+}$ control. (n = 4). **B**. Microglia as a percentage of total cells was estimated by CD11b-positive cell counting in C/EBPβ$^{+/+}$ and C/EBPβ$^{-/-}$ cultures after 16 h treatments with LPS, LPS+IFNγ or vehicle. Significant differences among treatments groups or genotypes are not observed. Two-way ANOVA, followed by Bonferroni's test was applied. (n = 4). **C**. Secondary mixed glial cultures were immunostained for CD11b (green). Nuclei are stained with Hoechst-33258 (blue). Microglial cell numbers were similar for C/EBPβ$^{+/+}$ and C/EBPβ$^{-/-}$ cultures. LPS and LPS+IFNγ induced morphological changes in microglial cells in both genotypes. Bar = 50 μm. **D**. A representative western blot shows levels of GFAP in C/EBPβ$^{+/+}$ and C/EBPβ$^{-/-}$ mixed glial protein extracts 16 h after vehicle (control), LPS and LPS+IFNγ treatments. β-Actin was used for normalization. **E**. Densitometric analysis was used to quantify GFAP protein levels versus β-actin in 4 independent western blots in arbitrary units (a.u.). Changes in GFAP protein levels are not observed. Two-way ANOVA, followed by Bonferroni's test was applied. (n = 4). **F**. Secondary mixed glial cultures were immunostained for GFAP (red) showing a confluent astrocytic layer. Overlapping of astroglial cell bodies makes counting very difficult and imprecise. No differences in astroglial morphology or density among genotypes are observed. Nuclei are stained with Hoechst-33258 (blue). Bar = 50 μm. **E**. Lack of NOS2 expression in activated astrocytes. C/EBPβ$^{+/+}$ and C/EBPβ$^{-/-}$ secondary mixed glial cultures were immunostained for GFAP (green) and NOS2 (red), and stained for Hoechst 33258 (blue), after 16 h of LPS+IFNγ treatment. A marked reduction in number of NOS2-positive cells is seen in C/EBPβ-null cultures. The representative merge images show clearly that NOS2-positive cells do not colocalize with GFAP-positive cells. Bar = 50 μm.

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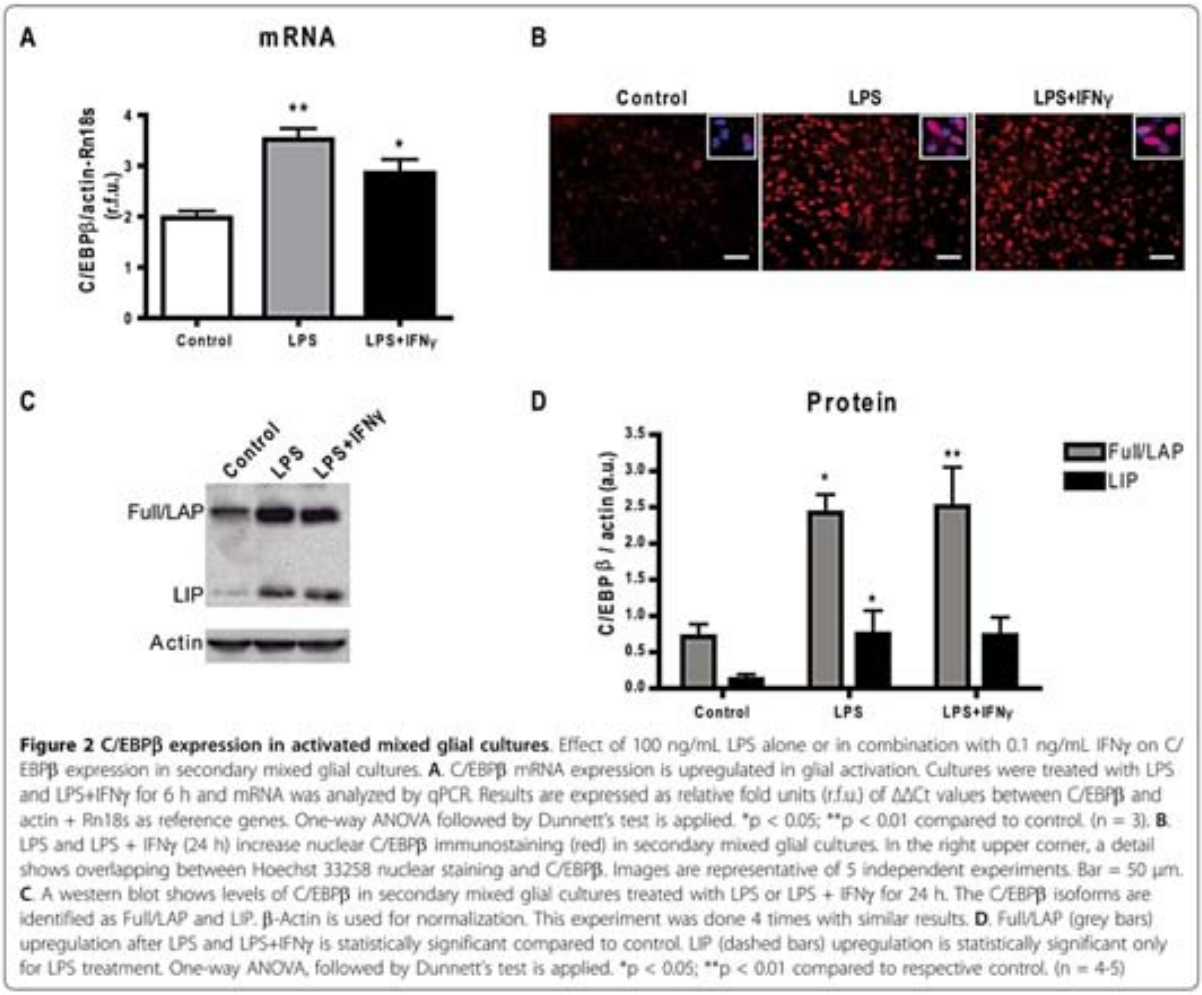

Figure 2 C/EBPβ expression in activated mixed glial cultures. Effect of 100 ng/mL LPS alone or in combination with 0.1 ng/mL IFNγ on C/EBPβ expression in secondary mixed glial cultures. **A**. C/EBPβ mRNA expression is upregulated in glial activation. Cultures were treated with LPS and LPS+IFNγ for 6 h and mRNA was analyzed by qPCR. Results are expressed as relative fold units (r.f.u.) of ΔΔCt values between C/EBPβ and actin + Rn18s as reference genes. One-way ANOVA followed by Dunnett's test is applied. *$p < 0.05$; **$p < 0.01$ compared to control. (n = 3). **B**. LPS and LPS + IFNγ (24 h) increase nuclear C/EBPβ immunostaining (red) in secondary mixed glial cultures. In the right upper corner, a detail shows overlapping between Hoechst 33258 nuclear staining and C/EBPβ. Images are representative of 5 independent experiments. Bar = 50 μm. **C**. A western blot shows levels of C/EBPβ in secondary mixed glial cultures treated with LPS or LPS + IFNγ for 24 h. The C/EBPβ isoforms are identified as Full/LAP and LIP. β-Actin is used for normalization. This experiment was done 4 times with similar results. **D**. Full/LAP (grey bars) upregulation after LPS and LPS+IFNγ is statistically significant compared to control. LIP (dashed bars) upregulation is statistically significant only for LPS treatment. One-way ANOVA, followed by Dunnett's test is applied. *$p < 0.05$; **$p < 0.01$ compared to respective control. (n = 4-5)

NOS2 protein levels by western blot and immunocytochemistry, and generation of NO by colorimetric detection of nitrites (Griess assay). In wild-type cultures NOS2 protein expression was induced by LPS and more markedly by LPS+IFNγ. In C/EBPβ-null cultures LPS-induced NOS2 levels were not significantly different from wild-type whereas LPS+IFNγ-induced NOS2 protein levels were markedly reduced (-77.4%, $p < 0.0001$) (Figure 5A, B). NO levels correlated well with the NOS2 protein data and a strongly significant attenuation in NO production induced by LPS+IFNγ was seen in C/EBPβ-null cultures (Figure 5C).

The reduction in LPS+IFNγ-induced NOS2 expression in C/EBPβ-null glial cultures seen by western blot was confirmed by immunocytochemistry. We did not observe by immunocytochemistry any NOS2-positive cells in untreated cultures (not shown), whereas in LPS- (not shown) and LPS+IFNγ-treated wild-type cultures, NOS2 immunoreactivity was observed in 14.0 ± 3.6% of total cells (Figure 5D, E). The vast majority of NOS2-positive cells in LPS+IFNγ-treated wild type mixed glial cultures also expressed CD11b (99.3 ± 1.4%; n = 11) and very rarely NOS2-positive cells expressed GFAP (0.6 ± 1.2%; n = 11) indicating that in these conditions NOS2 expression in mouse cortical mixed glial cultures is predominantly microglial. In C/EBPβ-null cultures the number of NOS2 cells was dramatically reduced after either LPS (not shown) or LPS+IFNγ treatments (Figure 5D, E). As seen in Figure 5D, the reduction of NOS2-positive cells could not be attributed to a reduction in microglial density.

C/EBPβ deficiency in activated microglia abrogates neurotoxicity

Activated microglia have strong neurotoxic potential [36]. The observations of reduced expression of pro-inflammatory mediators in LPS+IFNγ-activated C/EBPβ-null glial cells, particularly microglia, prompted us to

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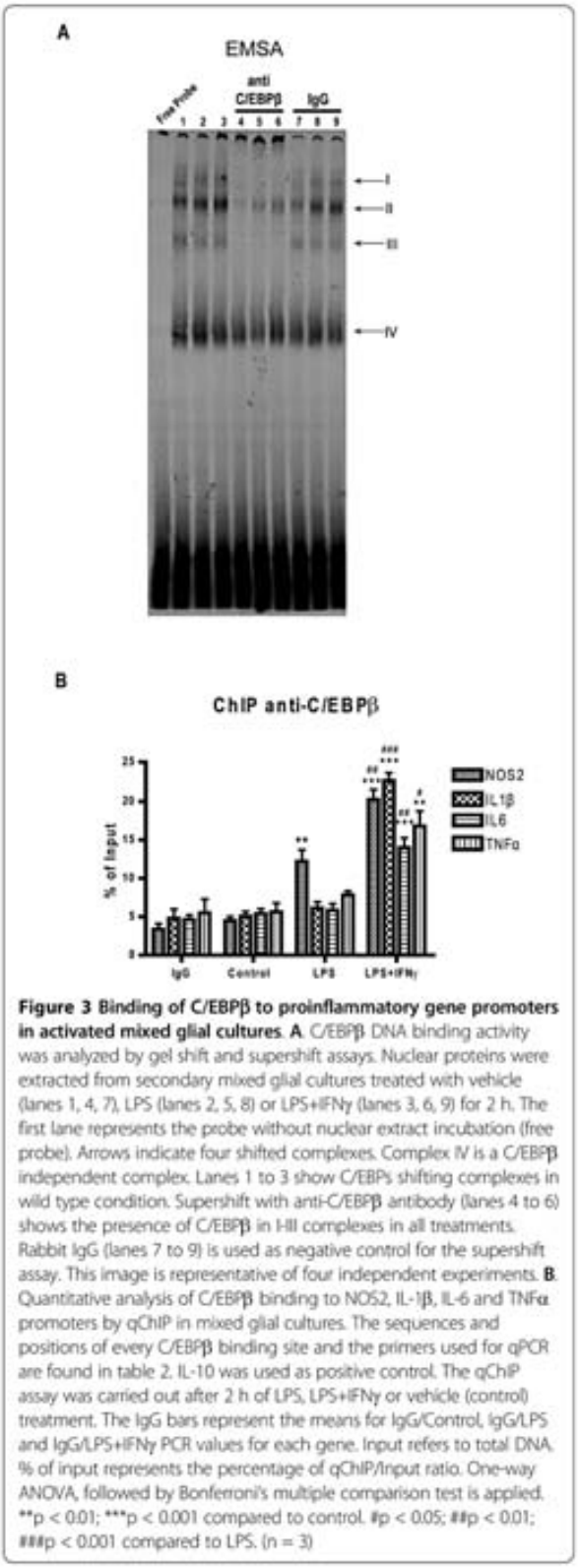

Figure 3 Binding of C/EBPβ to proinflammatory gene promoters in activated mixed glial cultures. A. C/EBPβ DNA binding activity was analyzed by gel shift and supershift assays. Nuclear proteins were extracted from secondary mixed glial cultures treated with vehicle (lanes 1, 4, 7), LPS (lanes 2, 5, 8) or LPS+IFNγ (lanes 3, 6, 9) for 2 h. The first lane represents the probe without nuclear extract incubation (free probe). Arrows indicate four shifted complexes. Complex IV is a C/EBPβ independent complex. Lanes 1 to 3 show C/EBPs shifting complexes in wild type condition. Supershift with anti-C/EBPβ antibody (lanes 4 to 6) shows the presence of C/EBPβ in I-III complexes in all treatments. Rabbit IgG (lanes 7 to 9) is used as negative control for the supershift assay. This image is representative of four independent experiments. **B**. Quantitative analysis of C/EBPβ binding to NOS2, IL-1β, IL-6 and TNFα promoters by qChIP in mixed glial cultures. The sequences and positions of every C/EBPβ binding site and the primers used for qPCR are found in table 2. IL-10 was used as positive control. The qChIP assay was carried out after 2 h of LPS, LPS+IFNγ or vehicle (control) treatment. The IgG bars represent the means for IgG/Control, IgG/LPS and IgG/LPS+IFNγ PCR values for each gene. Input refers to total DNA. % of input represents the percentage of qChIP/Input ratio. One-way ANOVA, followed by Bonferroni's multiple comparison test is applied. **p < 0.01; ***p < 0.001 compared to control. #p < 0.05; ##p < 0.01; ###p < 0.001 compared to LPS. (n = 3)

analyze whether the neurotoxic effects of LPS+IFNγ-activated microglia could be attenuated by C/EBPβ absence. To this aim, wild-type and C/EBPβ-null microglial cells were isolated and co-cultured with wild-type neurons. No neuronal death was observed when neurons not co-cultured with microglia were treated with LPS+IFNγ or when neuron/wild-type microglia co-cultures were treated with LPS alone (data not shown). In contrast, LPS+IFNγ treatment of neuron/wild-type microglia co-cultures resulted in death of 51.2% of neurons, as estimated by MAP2/ABTS/ELISA (Figure 6). Interestingly, in neuron/C/EBPβ-null microglia co-cultures treated with LPS+IFNγ, MAP2 immunoreactivity levels were equal to control levels (Figure 6) indicating that the neurotoxicity induced by LPS+IFNγ-treated microglia was completely abolished in the absence of C/EBPβ. In this model, NO production plays a major role in the neurotoxicity elicited by activated microglia since the NOS2 inhibitor 1400W (10 μM) completely abolished neuronal death in LPS+IFNγ-treated neuron/microglia co-cultures (Gresa-Arribas et al, unpublished observations).

Discussion

The transcription factor C/EBPβ is expressed in glia but no direct evidence exists for its involvement in glial activation. In the present study we show that both LPS and LPS+IFNγ upregulate C/EBPβ expression in mixed glial cultures to a similar extent. Both stimuli also induce C/EBPβ binding to proinflammatory gene promoters but this binding is stronger when induced by LPS+IFNγ. Lack of C/EBPβ results in attenuated expression of proinflammatory genes and, again, this effect is more pronounced when glial cells are activated with LPS +IFNγ than when LPS alone is the activating stimulus. Finally, we describe for the first time that neurotoxicity elicited by LPS+IFNγ-treated microglial cells is completely abrogated by lack of C/EBPβ.

In this study we have used mixed glial cultures composed mainly of astrocytes and microglia. This culture system is our model of choice to study glial activation because it allows cross-talk between the two cell types, which is extremely important in glial activation [37]. Working with astrocytes or microglia in isolation may yield misleading results and there are numerous examples of astroglial or microglial responses that are markedly affected by the absence of the other cell type [37-39]. Regarding C/EBPβ, we have previously shown in experiments with mixed glial and astroglial- or microglial-enriched cultures that, upon activation, C/EBPβ is primarily expressed by microglia with a lesser upregulation in astrocytes [24]. This suggests that the data here reported on C/EBPβ in glial activation mainly reflects C/EBPβ changes in microglia although part of the

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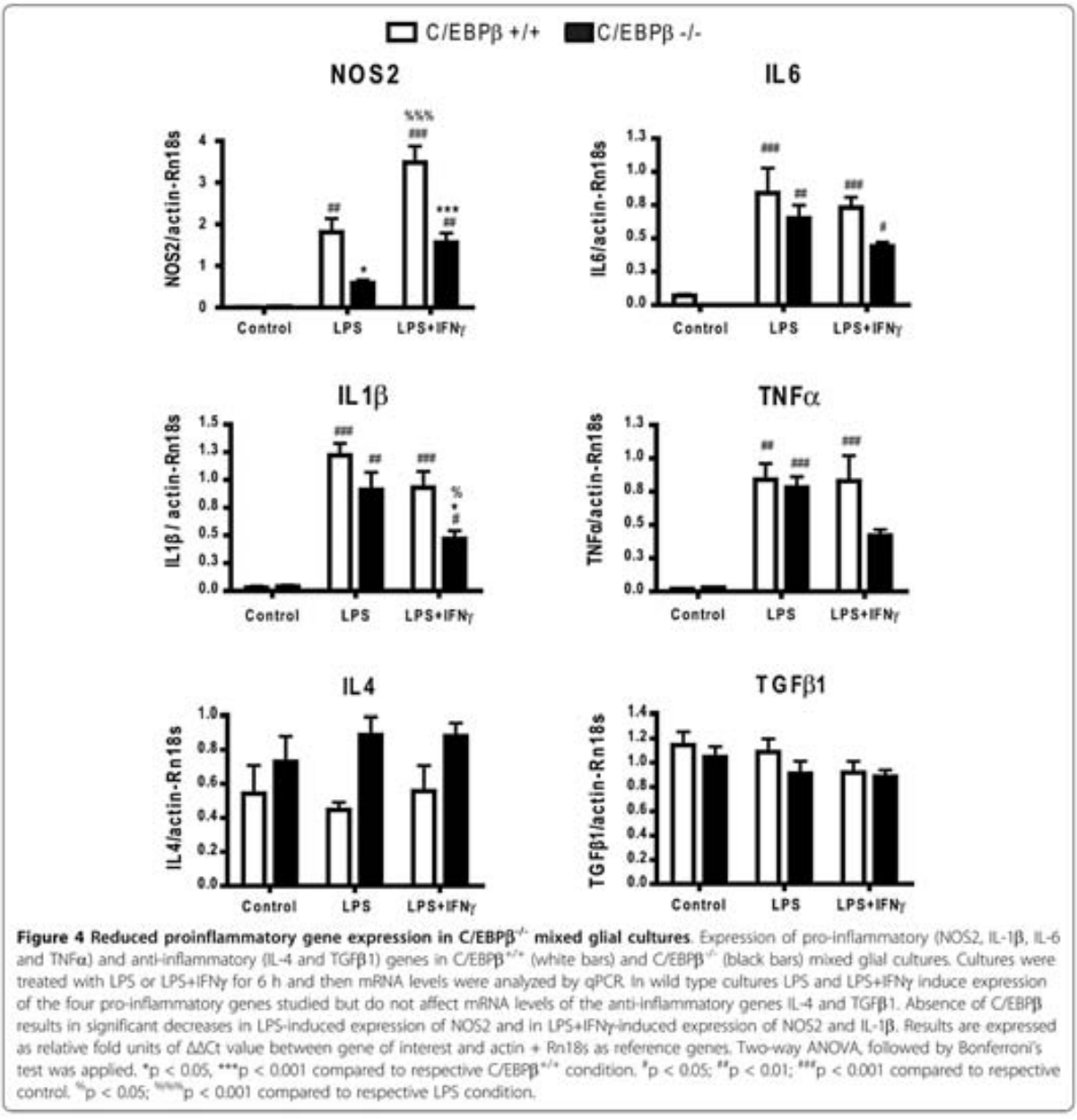

Figure 4 Reduced proinflammatory gene expression in C/EBPβ$^{-/-}$ mixed glial cultures. Expression of pro-inflammatory (NOS2, IL-1β, IL-6 and TNFα) and anti-inflammatory (IL-4 and TGFβ1) genes in C/EBPβ$^{+/+}$ (white bars) and C/EBPβ$^{-/-}$ (black bars) mixed glial cultures. Cultures were treated with LPS or LPS+IFNγ for 6 h and then mRNA levels were analyzed by qPCR. In wild type cultures LPS and LPS+IFNγ induce expression of the four pro-inflammatory genes studied but do not affect mRNA levels of the anti-inflammatory genes IL-4 and TGFβ1. Absence of C/EBPβ results in significant decreases in LPS-induced expression of NOS2 and in LPS+IFNγ-induced expression of NOS2 and IL-1β. Results are expressed as relative fold units of ΔΔCt value between gene of interest and actin + Rn18s as reference genes. Two-way ANOVA, followed by Bonferroni's test was applied. *p < 0.05, ***p < 0.001 compared to respective C/EBPβ$^{+/+}$ condition. $^{\#}$p < 0.05; $^{\#\#}$p < 0.01; $^{\#\#\#}$p < 0.001 compared to respective control. $^{\%}$p < 0.05; $^{\%\%\%}$p < 0.001 compared to respective LPS condition.

observed effects could be of astroglial origin. However, in the case of the effects of C/EBPβ absence on NOS2 expression and neurotoxicity, the observed effects are clearly microglial, as shown by the microglial localization of NOS2 immunoreactivity and by the use of isolated microglia, respectively.

Most protocols to prepare primary mixed glial cultures from rodents use pools of tissue from several neonates, generally one or two litters. Since C/EBPβ females are sterile [40] litters of C/EBPβ-null neonates cannot be obtained. Furthermore, approximately 50% of C/EBPβ-null pups die perinatally [28] which favors the use of late embryos instead of neonates to ensure a maximum number of available C/EBPβ-null mice. Therefore, we established for this study a new protocol of secondary mixed glial cultures by subculturing primary glial cultures prepared from the cerebral cortex of a single E19-E20 embryo. The use of secondary cultures was particularly suitable for this project because we could prepare mixed glial cultures that were very similar to

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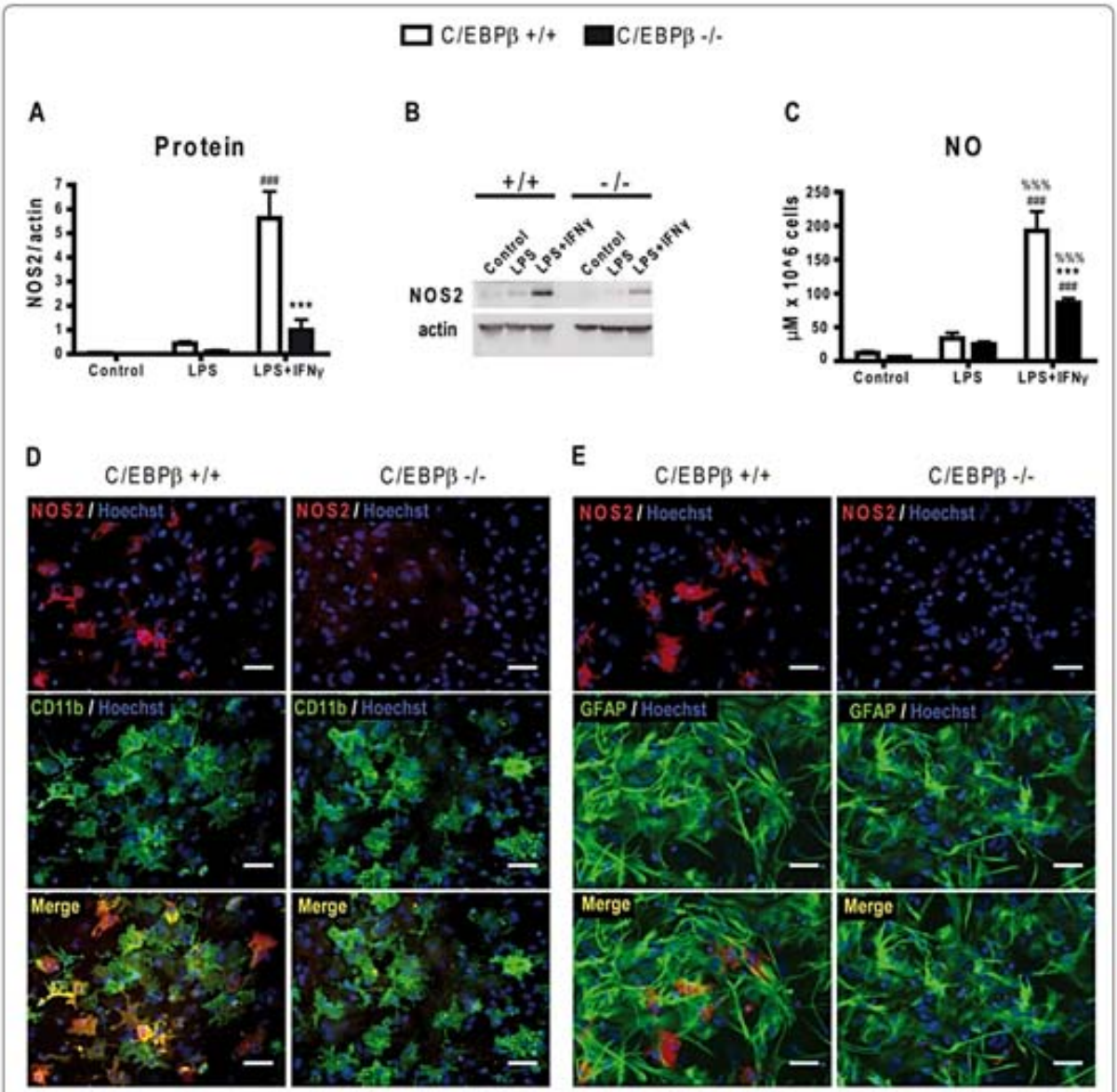

Figure 5 Absence of C/EBPβ dramatically decreases microglial NOS2 protein expression and NO production in activated mixed glial cultures. A. NOS2 protein levels in total protein extracts from mixed glial cultures were analyzed by western blot, followed by densitometry. Data is expressed as NOS2 versus β-actin band intensities. Cultures were treated for 16 h with LPS, LPS+IFNγ or vehicle. In C/EBPβ$^{+/+}$ cultures (white bars) NOS2 protein levels were detected after LPS treatment, but LPS+IFNγ induced a clear upregulation, in agreement with mRNA expression levels. In C/EBPβ$^{-/-}$ mixed glial cultures (black bars), NOS2 protein levels decreased in LPS and LPS+IFNγ compared to C/EBPβ$^{+/+}$ cultures. Two-way ANOVA, followed by Bonferroni's test was applied. ***$p < 0.001$ compared with C/EBPβ$^{+/+}$ condition. ###$p < 0.001$ compared with respective control condition. ($n = 5$). A representative western blot is shown in **B**. **C**. NO production is decreased in activated C/EBPβ$^{-/-}$ glial cultures. NO levels were measured by colorimetric analysis 48 h after treatments and normalized per cell number. Values are reported as micromolar concentration $\times 10^6$ cells. NO levels in C/EBPβ$^{+/+}$ (white bars) cultures were upregulated after LPS and LPS+IFNγ treatments compared to controls. In C/EBPβ$^{-/-}$ glial cultures (black bars), NO production is reduced in LPS+IFNγ treatment compared to wild-type NO levels. Two-way ANOVA, followed by Bonferroni's test was applied. ***$p < 0.001$ compared to C/EBPβ$^{+/+}$ condition. ###$p < 0.001$ compared to respective control condition. %%%$p < 0.001$ compared to respective LPS condition. ($n = 7$-9). **D**. NOS2 is expressed by activated microglia. C/EBPβ$^{+/+}$ and C/EBPβ$^{-/-}$ secondary mixed glial cultures were immunostained for CD11b (green) and NOS2 (red), and stained for Hoechst 33258 (blue) after 16 h of LPS+IFNγ treatment. The C/EBPβ$^{+/+}$ merged image shows colocalization of NOS2-positive cells and CD11b-positive cells. Bar = 50 μm.

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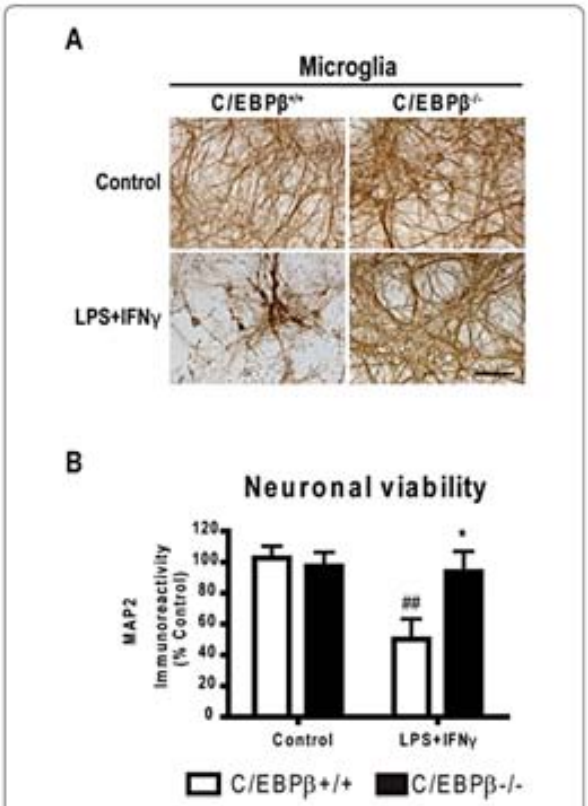

Figure 6 Lack of C/EBPβ in activated microglia completely abolishes the neurotoxic effects of activated microglia in neuronal-microglial co-cultures. A. MAP2 immunostaining of wild-type neurons co-cultured with C/EBPβ$^{+/+}$ or C/EBPβ$^{-/-}$ microglia was performed 48 h after LPS+IFNγ treatment. MAP2 staining shows a clear decrease of network fibres caused by activated C/EBPβ$^{+/+}$ microglia, but not by C/EBPβ$^{-/-}$ microglia or by vehicle-treated microglia. Images are representative of 5 independent experiments. (Bar = 100 μm). **B**. Evaluation of neuronal viability by MAP2/ABTS/ELISA assay 48 h after treatment with LPS+IFNγ or vehicle (control). Results are presented as % of MAP2 immunostaining in control cultures. Treatment with LPS+IFNγ reduces MAP2 immunostaining in neurons cocultured with C/EBPβ$^{+/+}$ microglia, but not with C/EBPβ$^{-/-}$ microglia. Two-way ANOVA, followed by Bonferroni's test was applied. $^{\#\#}p < 0.01$ compared with C/EBPβ$^{+/+}$ control; $^{*}p < 0.05$ compared to C/EBPβ$^{+/+}$ LPS+IFNγ. (n = 5).

primary cultures in terms of cell density and proportions with a more-than-2-fold higher yield. Besides, the use of siblings eliminates any genetic background effect. Altogether, this makes the use of secondary mixed glial cultures from a single embryo or neonate a useful approach when working with mouse strains of compromised fertility.

LPS is a toll-like receptor 4 agonist that induces marked changes in gene expression in astrocytes and microglia [1]. The combination of LPS, a pathogen factor, with IFNγ, a host factor, potentiates some of the LPS-induced effects [41]. Here we report for the first time a proper comparison between LPS and LPS+IFNγ effects on C/EBPβ and on pro-inflammatory markers in glial cells. We have observed that both LPS and LPS +IFNγ induce similar increases in C/EBPβ mRNA and protein levels as well as in DNA binding. Time-course analyses have revealed that upregulation of the C/EBPβ activating isoforms Full/LAP often precedes upregulation of the inhibitory isoform LIP [21,24,42]. When a single time-point is analyzed, as in the present study, the simultaneous increase in activating and inhibitory C/EBPβ isoforms is a common observation. EMSA analysis with supershift experiments showed the presence of C/EBPβ in bands I, II and III. These bands may contain different C/EBPβ isoforms (Full, LAP or LIP) with various post-translational modifications (phosphorylation, SUMOylation or acetylation has been described [43]). It is likely that some of these bands contain more than one complex (e.g. band II since it is only partially supershifted by anti-C/EBPβ) and that some of these complexes contain other transcription factors, p65-NFκB [44] and C/EBPδ [45,46] being two of the most likely candidates to form complexes with C/EBPβ in neuroinflammation. An extensive biochemical analysis would be necessary to characterize the transcriptional C/EBPβ complexes in activated glial cells.

This study shows for the first time in glial cells an analysis of mRNA levels for the pro-inflammatory genes NOS2, IL-1β, IL-6 and TNFα, comparing LPS and LPS +IFNγ as activating stimuli. In this model, IFNγ alone did not trigger any effect (data not shown) whereas LPS and LPS+IFNγ upregulated all four pro-inflammatory genes analyzed. LPS and LPS+IFNγ increased expression of IL-1β, IL-6 and TNFα to the same extent, as reported for macrophages [47], whereas LPS-induced upregulation of NOS2 was markedly potentiated by cotreatment with IFNγ, in agreement with previous observations in microglia [48] and macrophages [19]. Even though transcriptional levels of cytokine genes in LPS-treated glial cultures are not modulated by cotreatment with IFNγ, their promoter regions undergo a remodeling of transcriptional complex as proved by qChIP assay. mRNA analysis showed that absence of C/EBPβ does not affect LPS-induced upregulation of the three cytokines, in agreement with absence of C/EBPβ binding to IL-1β, IL-6 or TNFα promoters in LPS-treated glial cultures, as seen by qChIP. Although we cannot exclude the presence of C/EBPβ in other promoter regions, because we focused our promoter analysis on the C/EBPβ consensus sequence most proximal to the translation start site, these data strongly suggest that C/EBPβ does not participate in the LPS-induced expression of these three genes in the present model. It may seem contradictory that strong C/EBPβ binding to IL-1β, IL-6 and TNFα

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promoters was induced by LPS+IFNγ, but not by LPS alone, whereas the levels of these cytokine mRNAs were similar after treatment with either LPS or LPS+IFNγ. In our opinion, this indicates that different sets of transcription factors act on these promoters after LPS or LPS+IFNγ treatment or, in other words, that there is IFNγ-induced chromatin remodeling on these promoters [49]. This is also suggested by the qPCR data showing that LPS+IFNγ-induced expression of IL-1β is reduced in the absence of C/EBPβ, and that there is also a tendency toward reduced expression of TNFα and IL-6. These data demonstrate for the first time that C/EBPβ plays a role in transactivation of pro-inflammatory cytokine genes in glial cells induced by LPS+IFNγ but not by LPS alone.

In our glial activation model, the NOS2 gene shows a different transcription pattern when compared with the pro-inflammatory cytokines. On the one hand, as mentioned before, LPS-induced NOS2 expression is potentiated by co-treatment with IFNγ. On the other hand, C/EBPβ binding to the NOS2 promoter is already seen after LPS treatment alone and, interestingly, this binding is potentiated by IFNγ treatment. As observed in macrophage cell lines, IFNγ can trigger C/EBPβ phosphorylation, modulating its capacity to form transcriptional complexes with p300 [50] or Med1 [51]. Also, IFNγ can promote C/EBPβ DNA binding activity to IFN-stimulated regulatory elements (ISREs) which we have found tightly associated with C/EBPβ consensus sequences on the mouse NOS2 promoter (unpublished observations). Finally, both LPS- and LPS+IFNγ-induced increases in NOS2 expression are attenuated in the absence of C/EBPβ. These findings suggest that C/EBPβ plays a functional role both in LPS-induced NOS2 expression and in the potentiation of this effect elicited by IFNγ. In accordance with the multiple stage glial activation model [52], we can hypothesize that LPS alone activates the glia, but that only with a host warning signal, such as IFNγ, are glia totally committed to a hyper-reactive phenotype. We propose that C/EBPβ could trigger this shift through the executive phase of glial activation.

The hypothesis of a pathogenic role for exacerbated glial activation, particularly activation of microglia, is based on the known in vitro neurotoxic effects of activated microglia [53,54], on the protective effects of anti-inflammatory treatments or genetic modifications in animal models of neurodegenerative disorders [55,56] and on epidemiological data [57-59]. Since we have shown in this study that C/EBPβ deficiency attenuates expression of potentially neurotoxic pro-inflammatory mediators but not that of anti-inflammatory cytokines, we were interested to test the hypothesis that C/EBPβ plays a key role in the induction of detrimental effects by microglial activation. Reduced neuronal damage after ischemic [26] or excitotoxic insults [27] has been observed in C/EBPβ-null mice. Even though C/EBPβ expression has been reported in activated glial cells [22-24], C/EBPβ is known to be also expressed in the adult mouse by neurons [60] and peripheral cells [16]. Consequently, the neuroprotective effect observed in C/EBPβ-null mice could be mediated by lack of C/EBPβ in any of these cells. We show here that the neurotoxicity elicited by activated wild-type microglial cells co-cultured with wild-type neurons is completely abolished by the absence of C/EBPβ specifically in microglia. This strongly supports a role of C/EBPβ in the regulation of potentially neurotoxic effects of microglia and suggests that the neuroprotective effects of total C/EBPβ absence in vivo [26,27] are due to microglial C/EBPβ deficiency. Specific microglial C/EBPβ deletion would be very informative to clarify the role of microglial C/EBPβ in neurodegeneration in *in vivo* models of neurological disease.

Conclusions

In summary, this study shows that LPS and LPS+IFNγ induce expression of C/EBPβ in mixed glial cultures, and both stimuli also induce differential binding of C/EBPβ to proinflammatory gene promoters. A functional role for C/EBPβ in glial activation is demonstrated by the attenuated gene expression and abrogation of neurotoxicity in microglial cells devoid of C/EBPβ. Altogether, these findings point to C/EBPβ as a key transcription factor in the molecular reprogramming that occurs in microglial activation and suggest that C/EBPβ is a possible therapeutic target to ameliorate neuronal damage of neuroinflammatory origin.

List of abbreviations

ABTS: 2, 3'-azinobisethylbenzothiazoline-6-sulphonic acid; ANOVA: Analysis of variance; C/EBPβ: CCAAT/enhancer binding protein β; DIV: Days in vitro; GFAP: Glial fibrillary acidic protein; HRP: Horseradish peroxidase; IFNγ: Interferon γ; IL: Interleukin; LPS: Lipopolysaccharide; NOS2: NO synthase-2; qChIP: Quantitative chromatin immunoprecipitation; qPCR: Quantitative real time PCR; RT: Room temperature; TGFβ1: Transforming growth factor β1; TNFα: Tumour necrosis factor-α

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Authors' contributions

MS carried out most experiments and drafted the manuscript. NGA carried the experiments involving neuron/microglia cocultures. GD carried out the

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qChIP experiments. AEO set the C/EBPβ-null colony and carried out the preliminary experiments. JMT participated in the preparation of primary cultures. JSe participated in immunocytochemistry experiments. CS designed and participated in the neuron/microglia cocultures experiments and participated in the statistical analysis. JSa conceived and coordinated the study and drafted the manuscript. All authors critically revised and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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6.2 PAPER 2

CCAAT/Enhancer Binding Protein β regulates PTGES expression and PGE2 production in activated microglial cells

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Submitted

Title:

CCAAT/Enhancer Binding Protein β regulates PTGES expression and PGE_2 production in activated microglial cells.

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ABSTRACT

The eicosanoid prostaglandin E_2 (PGE_2) plays important roles in neuroinflammation and it is produced by the sequential action of the enzymes cyclooxygenase-2 (COX-2) and prostaglandin E synthase (PTGES). The expression of both enzymes and the production of PGE2 are increased in neuroinflammation. The objective of this study was to elucidate whether the transcription factor CCAAT/enhancer binding protein β (C/EBPβ) regulates the expression of prostaglandin synthesis enzymes in neuroinflammation. To this aim, the expression of these enzymes in wild-type and C/EBPβ-null mice was analyzed in vitro and in vivo. Lipopolysaccharide (LPS) ± interferon γ (IFNγ) induced C/EBPβ binding to COX-2 and PTGES promoters in mixed glial cultures. In the absence of C/EBPβ, LPS±IFNγ-induced increases in PTGES expression, which was basically of microglial origin, and in PGE_2 production were abrogated. Also, increased brain PTGES expression induced by systemic LPS administration was markedly reduced in C/EBPβ-null mice. In contrast to PTGES, the induction of COX-2 expression *in vitro* or *in vivo* was not clearly affected by C/EBPβ absence. These results demonstrate that C/EBPβ regulates PTGES expression and PGE_2 production by activated microglial cells and strengthen the proposed role of C/EBPβ as a key player in the orchestration of gene response in neuroinflammation.

Keywords: microglia, C/EBP, prostaglandin E 2, PTGES, COX-2, glial activation, neuroinflammation.

Running title: C/EBPbeta regulates microglial PTGES

INTRODUCTION

Prostaglandin E2 (PGE2) is an important bioactive lipid with pleiotropic effects in the CNS through a paracrine action on neurons (Li et al. 2008), glial cells (Chaudhry et al. 2008, Levi et al. 1998) and smooth muscle cells of brain blood vessels (Baenziger et al. 1979, Whalley et al. 1989). The homeostatic PGE2 level is usually low and it is maintained mainly by the ubiquitous enzymes prostaglandin-endoperoxide synthase 1 (PTGS1, also known as COX-1) and prostaglandin E synthase 3 (PTGES3, also known as cPGES) activities (Tanioka et al. 2000). In neuroinflammation, microglial cells become the main PGE2 producers in the CNS through the up-regulation of COX-1/COX-2 and PTGES (also known as mPGES-1) enzymes (Choi et al. 2009, Ikeda-Matsuo et al. 2010, Tomimoto et al. 2000). Once released, PGE2 can interact with four G-coupled receptors, named E Prostanoids receptors (EP1-4), each of them having a distinct structure (Narumiya et al. 1999), function, expression and distribution in the brain (Andreasson 2010, Cimino et al. 2008).

PGE_2 paracrine action is dual and the response can be anti- or pro-inflammatory (Milatovic *et al.* 2011). There is a strong interest in finding strategies to promote the neuroprotective effects and to attenuate the neurotoxicity of PGE_2 in neuroinflammation (Andreasson 2010). Epidemiological studies strongly suggest a possible neuroprotective effect of COXs inhibition (Vlad *et al.* 2008) but recent data seem to refute this hypothesis (Driver *et al.* 2011). However, it is important to take into account that COX-2 activity results in the production of PGH_2 which is the precursor of not only PGE_2, but also PGD_2, PGF, PGI_2 and Tromboxanes, whereas the sole product of PTGES activity is PGE_2. In this view, it is conceivable that the negative outcomes of COX-2 inhibition (Cheng *et al.* 2006) could be caused by altered levels of the other prostanoids. Hence, PTGES is a more selective target to inhibit PGE_2 during inflammation. We have been therefore interested to study the expression of the two inducible enzymes in the PGE_2 pathway, COX-2 and PTGES, in glial activation and in particular in the possible involvement of the transcription factor CCAAT/Enhancer Binding Protein β (C/EBPβ) in the regulation of their transcription.

C/EBPβ is a b-zip transcription factor that is expressed in activated astrocytes and microglia (Ejarque-Ortiz *et al.* 2007). We and others have demonstrated that C/EBPβ plays a key role in the regulation of pro-inflammatory genes in glial activation (Cortes-Canteli *et al.* 2008, Straccia *et al.* 2011). Interestingly, C/EBPβ deficiency provides neuroprotection following ischemic (Kapadia *et al.* 2006) or excitotoxic injuries (Cortes-Canteli et al. 2008), two models where COX-2 inhibition has shown to be beneficial (Ikeda-Matsuo et al. 2010, Nakayama *et al.* 1998). We have undertaken this study to analyze whether COX-2 and PTGES expressions are regulated by C/EBPβ in glial activation. We show for the first time that C/EBPβ plays a key role in PGE_2 production in microglia. We clearly demonstrate a PGE_2 reduction in C/EBPβ-null glial cultures that is mainly due to decreased levels of PTGES and how this enzyme is under the transcriptional control of C/EBPβ in *in vitro* and *in vivo* models

MATERIALS AND METHODS

Animals

A colony of C/EBPβ+/- (Screpanti et al. 1995) mice on a C57BL/6-129S6/SvEv background was maintained. Animals from this colony showed no serological evidence of pathological infection. The animals were group-housed (5-6) in solid floor cages and received a commercial pelleted diet and water ad libitum. Experiments were carried out in accordance with the Guidelines of the European Union Council (86/609/EU) and following the Spanish regulations (BOE 67/8509-12, 1988) for the use of laboratory animals, and were approved by the Ethics and Scientific Committees from the University of Barcelona and Hospital Clínic.

DNA extraction and genotyping

Genomic DNA was isolated from 2 mg liver samples using Extract-N-AmpTissue PCR Kit (Sigma-Aldrich, XNAT2) following kit instructions. PCR amplification was performed as previously described (Straccia et al. 2011).

Cortical mixed glial culture from a single embryo

C/EBPβ+/- mice were crossed and pregnant females were sacrificed at 19th day of gestation by cervical dislocation. Embryos (E19) were surgically extracted from the peritoneal cavity. Their livers were dissected and used for genotyping, whereas their brains were dissected and processed to prepare primary mixed glial cultures as previously described (Straccia et al. 2011).

Mouse mixed glial subculture

Each flask was washed in serum-free medium and was digested with 0.25% trypsin-EDTA solution for 5 min at 37°C. Trypsinization was stopped by adding an equal volume of culture medium with FBS 10%. Cells were pelleted (7 min, 180 g), resuspended in 1 mL culture medium, and brought to a single cell suspension by repeated pipetting. Cells were seeded at 166000 cells/mL. Cultures were used at 12 ± 3 DIV. Astrocytes were the most abundant cell type and microglial cells were approximately 20-25%.

Microglial culture

Microglial cultures were prepared by mild trypsinization from mouse mixed glial culture as previously described (Saura et al. 2003). Briefly, 19-21 DIV mixed glial subcultures were treated for 30 min with 0.06% trypsin in the presence of 0.25 mM EDTA and 0.5 mM Ca2+. This resulted in the detachment of an intact layer of cells containing virtually all the astrocytes, leaving a population of firmly attached cells identified as >98% microglia.

In vivo treatments

Male C/EBPβ-null mice (Screpanti et al. 1995) and their wild type (14-16 weeks, 20–25 g) littermates were used. Mice (5-6/group) were injected i.p. with 100 µg LPS per mouse dissolved in 100 µL of NaCl 0.9%. Controls received the same volume of vehicle. Animals were anesthetized and sacrificed by decapitation 2 h or 6 h after treatment. From every animal cerebral cortex was dissected, quickly removed and frozen with dry ice. The isolation of RNA was performed as indicated below.

In vitro treatments

Culture medium was replaced 24 h prior to treatment. Mixed glial cultures were treated with 100 ng/mL lipopolysaccharide (LPS, Sigma-Aldrich, L-2654, E. coli serotype 026:B6) and 0.1 ng/mL recombinant mouse IFNγ (Sigma-Aldrich, I4777) prepared from x10 solutions.

Total protein extraction

Protein levels were determined in primary mixed glial cells 16 h after treatments. For isolation of total proteins, two wells from 6-well plates were used per condition. After a cold PBS wash, cells were scrapped and recovered in 100 µL per well of RIPA buffer (1% Igepal CA-630, 5 mg/mL sodium deoxycholate, 1 mg/mL sodium dodecyl phosphate (SDS) and protease inhibitor cocktail Complete® in PBS). The content of the wells was pooled, sonicated, centrifuged for 5 min at 10400 g and stored at -20°C. Protein concentration was determined by the Lowry assay.

Western blot

Thirty micrograms of denatured (2.5 mM DTT, 100ºC for 5 min) total protein extracts were subjected to 10% SDS-PAGE and transferred to a PVDF membrane (Millipore, IPVH00010) for 90 min at 1 mA/cm2, as previously described (Straccia et al. 2011). Membranes were incubated with the following primary antibodies: polyclonal rabbit anti-COX-2 (1:500, Santa Cruz Biotechnology, sc-1747), polyclonal rabbit anti-PTGES (1:1000, Agrisera, AS03031), monoclonal mouse anti-β-actin (1:3x105, Sigma-Aldrich, A1978), diluted in immunoblot buffer (TBS containing 0.05% Tween-20 and 5% no-fat dry milk). Data are expressed as the ratio between the intensity of the protein of interest band and the loading control protein band (β-actin).

RNA isolation and quantitative real time PCR (qPCR)

mRNA expression was determined 6 h after treatments in mouse mixed glial cell cultures and in microglial enriched cultures; mRNA expression from in vivo experiments was determined 2 and 6 h after treatment. For isolation of total RNA, 2 wells of 24-well plates were used per

experimental condition in mixed glial cultures, 1 well of 6-well plate was used per experimental condition for microglial enriched cultures and the whole left cortex for in vivo experiments. Total RNA was isolated with High Pure RNA isolation kit (Roche, 11828665001) for mixed glial cultures, with PureLink RNA Micro Kit (Invitrogen, 12183-016) for microglial cultures. Tri-Reagent (Sigma-Aldrich, T9424) was used, following product instruction, to isolate total RNA from mouse hemi-cortex. 100 ng of RNA for each cell condition and 1,5 µg per cortex was reverse transcribed with random primers using Transcritor Reverse Transcriptase (Roche, 03531287001). cDNA was diluted 1/25 and 2 µL were used to perform qPCR. Primers (Roche) were used at final concentration of 400 nM (Table 1). β-Actin, Rn18s and HPRT1 mRNA levels were not altered by treatments (data not shown) and were used as reference genes. qPCR was carried out with Express Sybr®GreenER™ qPCR Supermix Universal (Invitrogen, 1178401K) in 6 µL of final volume using CFX384-C1000 Thermal Cycler equipment (Bio-Rad). Primer efficiency was estimated from standard curves generated by dilution of a cDNA pool. Samples were run for 45 cycles (95ºC for 30 s, 60ºC for 1 min, 72ºC for 30 s). Amplification specificity was confirmed by the analysis of melting curves. Relative gene expression values were calculated with the comparative Ct or ΔΔCt method (Livak & Schmittgen 2001) using Bio-Rad CFX manager software (Bio-Rad). Ct values were corrected by the amplification efficiency of the respective primer pair which was estimated from standard curves generated by dilution of a cDNA pool.

Quantitative chromatin immunoprecipitation (qChIP)

MatInspector (matrix library version 8.4) was used to identify putative C/EBPβ consensus sequences in the 5000 bp region upstream of the transcription start site of the COX-2 and PTGES mouse genes. MatInspector was set as follows: matrix group = vertebrates; core similarity = 0,75; matrix similarity = optimized. qChIP was performed as previously described (Straccia et al. 2011). Primer sequences to amplify each consensus site regions are indicated in table 1.

Immunocytochemistry

Cultured cells were fixed with 4% paraformaldehyde in PBS for 20 min at RT. For fluorescence labelling, cells were incubated overnight at 4ºC with 7% normal goat serum (Vector, S-1000) in PBS containing 1% Thimerosal (Sigma-Aldrich, T5125), 0.03% Triton-X100 (Sigma-Aldrich, X100) and primary antibodies: polyclonal rabbit anti-COX-2 (1:500, Santa Cruz Biotechnology, sc-1747), polyclonal rabbit anti-PTGES (1:1000, Agrisera, AS03031), monoclonal mouse anti-GFAP (1:1000, Sigma-Aldrich, G3893) and monoclonal rat anti-CD11b (1:300, Serotec, MCA711G, clone 5C6). After rinsing in PBS, cells were incubated for 1 h at RT with secondary antibodies: goat anti-mouse Alexa 546 (1:1000, Molecular Probes, A-11018), goat anti-rabbit Alexa 546 (1:1000, Molecular Probes A-11010), Alexa 488 (1:1000, Molecular Probes, A-11070) or goat anti-rat Alexa 488 (1:500, Molecular Probes, A-11006). After secondary antibody incubation, cells were stained with Hoechst 33258 for 5 min. Microscopy images were obtained with an Olympus IX70 microscope and a digital camera (CC-12, Soft Imaging System GmbH).

PGE_2 quantification

PGE_2 was quantified by using the High Sensitivity PGE_2 Kit (Assay Design, 930-001) based on the competitive binding technique. A 1:100 dilution of culture medium from LPS-, LPS+IFNγ- and vehicle-treated mixed glial cultures was used following the protocol provided by the manufacturer. Each condition was done in triplicate.

Statistical analysis

Data were analyzed using GraphPad 4.02. Two-way ANOVA followed by Bonferroni post-test was used when the effect of genotype on treatment was studied and vice versa. Values of $p<0.05$ were considered statistically significant. Error bars are presented as SEM.

RESULTS

C/EBPβ binds COX-2 and PTGES gene promoters in activated glial cells

Our preliminary goal was to demonstrate the existence of C/EBPβ binding to COX-2 and PTGES promoters in activated glial cells. Bioinformatics analysis of the 5000bp region upstream of the transcription start site revealed the presence of three putative C/EBPβ binding sites in the COX-2 and PTGES promoters (Fig 1A, B). By ChIP analysis with an anti-C/EBPβ antibody we did not detect C/EBPβ binding to any of these putative C/EBPβ sites in untreated mixed glial cultures. However, 2 h after LPS or LPS+IFNγ treatments C/EBPβ binding was observed in the COX-2 promoter region containing the most proximal C/EBPβ site (C 1) and in two regions of the PTGES promoter containing the most proximal (P 1) and the most distal (P 3) C/EBPβ sites (Fig 1C, D). LPS-induced C/EBPβ binding to the C1 site in the COX-2 promoter was significantly potentiated by IFNγ (Fig 1 A) whereas C/EBPβ binding to P1 and P3 sites in the PTGES promoter was similar after LPS or LPS+IFNγ treatments (Fig 1 B). These results indicate that C/EBPβ binds to COX-2 and PTGES promoters in glial activation.

Absence of C/EBPβ markedly reduces PTGES expression and PGE_2 production during glial activation

qChIP results prompted us to analyze the functional role of C/EBPβ on COX-2 and PTGES expression by comparing their expressions in wild-type and C/EBPβ-null mixed glial cultures. We have recently reported that mixed glial cultures prepared from the cerebral cortices of C/EBPβ-null late embryos develop normally in vitro and do not differ from their sibling wild-type cultures in terms of total cell density, time to confluency, microglial or astroglial content (Straccia et al. 2011). Wild-type and C/EBPβ-null mixed glial cultures were treated with LPS, LPS+IFNγ or vehicle. In wild-type cultures LPS and LPS+IFNγ induced a strong upregulation of COX-2 (3.6 and 4.6 fold increase respectively) and PTGES (4.3 and 4.0 fold increase, respectively) mRNA levels 6 h after treatment. No IFNγ potentiation of the LPS effect was observed (Fig 2). In C/EBPβ-null glial cultures LPS and LPS+IFNγ induced a similar COX-2 mRNA upregulation as that seen in wild-type cultures. In contrast, the LPS and LPS+IFNγ-induced upregulation of PTGES mRNA levels was completely abrogated in the absence of C/EBPβ (Fig 2). We also analyzed mRNA expression of other six main enzymes in arachidonic acid downstream pathway (Supplementary Table 1). In wild-type cultures, TBAXS1 and ALOX5 mRNAs showed significant decrease after LPS and LPS+IFNγ treatments. In C/EBPβ-null mixed glial cultures TBAXS1 mRNA showed the same significant decrease as in wild-type. Among these six enzymes, the only significant difference between genotypes was a lower level of PTGS1 mRNA in wild-type versus C/EBPβ-null glial cells after LPS+IFNγ treatment.

Next we analyzed by Western blot COX-2 and PTGES protein levels 16 h after treatment with LPS or LPS+IFNγ. LPS increased significantly PTGES protein levels and this effect did not occur in C/EBPβ-null cultures. In contrast, COX-2 protein levels were not significantly affected by LPS neither in wild-type nor C/EBPβ-null cultures. On the other hand, LPS+IFNγ increased significantly both PTGES and COX-2 protein levels and these increases were not observed in C/EBPβ-null cultures (Fig 3). To determine whether the changes observed in COX-2 and PTGES

proteins translate into changes in the concentrations of the main product of their combined action, PGE_2 levels were analyzed by competitive binding assay in the conditioned medium of mixed glial cultures 16 h after treatments. LPS and LPS+IFNγ significantly increased PGE_2 levels in wild-type cultures (vehicle 12.5 ± 8.2 ng/mL; LPS 82.5 ± 33.0 ng/mL, $p<0.001$; LPS+IFNγ 88.0 ± 30.4 ng/mL, $p<0.001$). In C/EBPβ-null glial cultures LPS- and LPS+IFNγ-induced upregulation of PGE_2 levels were attenuated by 45.7% ($p<0.05$) and 48.0% ($p<0.05$), respectively. Altogether these data demonstrate that the absence of C/EBPβ clearly attenuates PTGES expression and PGE_2 production in activated mixed glial cultures.

COX-2 and PTGES are expressed in microglia in mixed glial cultures

The experiments reported so far were carried out with mixed glial cultures consisting basically of astrocytes (approximately 60-75%) and microglia (approximately 20-25%). This is our biological preparation of choice to study glial activation *in vitro* because it allows the cross-talk between astrocytes and microglia. To study whether observed effects were produced by astrocytes, microglial cells or both, mixed glial cultures treated with LPS+IFNγ for 16 h were immunostained with COX-2 and PTGES and the nature of positive cells was ascertained by double immunofluorescence with the astroglial and microglial markers GFAP and CD11b, respectively. In untreated cultures COX-2 immunoreactivity was not detected (not shown) whereas LPS and LPS+IFNγ clearly induced COX-2 immunoreactivity in a subpopulation of cells. COX-2 positive cells were clearly delineated with COX-2 immunoreactivity filling the whole cytoplasm. COX-2 positive cells were never positive for GFAP whereas all COX-2 positive cells were also CD11b positive (Fig 5A), although there were CD11b-positive cells devoid of COX-2 immunoreactivity. The same pattern was observed for PTGES namely, absence of PTGES in untreated cells and strong upregulation by LPS±IFNγ in a subpopulation of cells defined as microglia by CD11b immunostaining. PTGES immunoreactivity showed perinuclear localization (Fig 5B). Once established that COX-2 and PTGES expression in mixed glial cultures is basically of microglial origin, we analyzed the effect of the absence of C/EBPβ on COX-2 and PTGES in microglial-enriched cultures. As reported in mixed glial cultures, COX-2 and PTGES mRNA levels were low in untreated microglial cultures (0.09 ± 0.03 and 0.09 ± 0.07) and were strongly upregulated by LPS (0.69 ± 0.15, $p<0.001$ and 0.92 ± 0.23, $p<0.001$) and LPS+IFNγ (1.47 ± 0.27, $p<0.001$ and 0.61 ± 0.15, $p<0.001$) (Fig 5C). LPS-induced COX-2 expression was significantly potentiated by IFNγ but this was not observed for PTGES expression. Interestingly, in C/EBPβ-null microglial cultures we observed a marked reduction in the LPS+IFNγ-induced COX-2 mRNA upregulation (-43.26%, $p<0.001$) and in the LPS- and LPS+IFNγ-induced upregulation of PTGES mRNA (-90.64%, $p<0.001$, and –89.79%, $p<0.001$, respectively).

C/EBPβ absence reduces early cortical mRNA expression of PTGES after systemic LPS

To determine whether the prior in vitro observations also occur in vivo, wild-type and C/EBPβ-null mice were treated systemically with LPS (100 µg/mice, i.p.) and COX-2 and PTGES mRNA cortical levels were analyzed by qPCR 2 h and 6 h after treatment. In wild-type mice COX-2 mRNA levels were upregulated by LPS (vehicle 0.39 ± 0.11; LPS 2 h 1.19 ± 0.17, $p<0.001$; LPS 6

h 0.72 ± 0.41, p<0.05) and a similar response was observed in C/EBPβ-null mice (Fig 6). PTGES mRNA levels were also strongly upregulated in the cerebral cortex by LPS i.p. in wild-type mice (vehicle 0.24 ± 0.05; LPS 2 h 0.71 ± 0.13, p<0.001; LPS 6 h 0.72 ± 0.28, p<0.001) but in this case this response was significantly lower in C/EBPβ-null mice (vehicle 0.30 ± 0.10; LPS 2 h 0.49 ± 0.07; LPS 6 h 0.45 ± 0.20, p<0.05). By analyzing mRNA expression of the other abovementioned arachidonic acid downstream pathway enzymes (Supplementary Table 2) in cerebral cortices, we did not found any significant difference, neither between genotypes nor among LPS-treatment time-points.

DISCUSSION

We have recently demonstrated that the transcription factor C/EBPβ regulates pro-inflammatory gene expression and neurotoxicity elicited by activated microglia (Straccia et al. 2011). Given the important roles played by prostaglandins in neuroinflammation (Milatovic et al. 2011) in the present study we have investigated the involvement of C/EBPβ in the regulation of prostaglandin-synthesis enzymes expression, with a particular focus on the two inducible enzymes COX-2 and PTGES. Our results show the induction of COX-2 and PTGES expression and PGE_2 production in neuroinflammation and the abrogation of PTGES upregulation and PGE_2 over-production in the absence of C/EBPβ.

COX-2 expression can be regulated by C/EBPβ in many cell types and stimulation paradigms (Healy *et al.* 2008, Kutchera *et al.* 1996, Liu *et al.* 2008, Wadleigh *et al.* 2000, Zhao *et al.* 2009). In fact, C/EBPβ has been proposed as a transcription-based strategy target to inhibit COX-2 (Wu 2006). The regulatory effects of C/EBPβ on COX-2 transcription seem to be mediated by two proximal C/EBP elements, –117/-109 and -162/-153, in the mouse COX-2 promoter (Wadleigh et al. 2000, Gorgoni *et al.* 2001) although distal sites have been shown to be important in some experimental settings (Lee *et al.* 2005). C/EBPβ binding to COX-2 promoter in astrocytes or microglia has not been shown to date. Using a restrictive search strategy we identified three putative C/EBP sites in the COX-2 promoter. ChIP assays showed no C/EBPβ binding to regions containing the C2 and C3 distal sites but in LPS- and LPS+IFNγ-treated mixed glial cultures C/EBPβ binding was observed to a -213/-19 region containing the proximal C1 site -165/-152, which overlaps the abovementioned -162/-153 site. The importance of the C1 site is supported by data showing that in mouse macrophages LPS-induced COX-2 expression is reduced by mutation in this site (Wadleigh et al. 2000) and by the binding of C/EBPβ to an oligonucleotide containing the C1 site (Gorgoni et al. 2001). The C1 containing-region amplified by ChIP contains another putative C/EBP site (-117/-109) not identified by our analysis but shown to be functionally important in some experimental conditions (Wadleigh et al. 2000). We therefore cannot exclude that the ChIP data showing C/EBPβ binding to the COX-2 promoter shows binding not only to the C1 site -165 /-152 sites but also to the -117/-109 site.

Despite the demonstration of C/EBPβ binding to the COX-2 promoter in activated glial cells, LPS-induced COX-2 expression was not affected by the absence of C/EBPβ in vitro or in vivo (see Table 2 for summary). On the other hand, LPS+IFNγ-induced COX-2 protein levels were decreased in C/EBPβ-null mixed glial cultures despite the lack of changes at the mRNA level. Furthermore, LPS+IFNγ-induced COX-2 mRNA levels were decreased in C/EBPβ-null microglial cultures. The fact that in both cases the activating stimulus is LPS+IFNγ suggests a possible specific involvement of C/EBPβ in the regulation of COX-2 expression induced by IFNγ synergy with LPS but not by LPS alone, as previously suggested for IL-1β, IL-6 and TNFα expression (Straccia et al. 2011). The lack of marked effects on LPS-induced COX-2 expression caused by C/EBPβ absence is surprising given the large number of cell types/experimental paradigms in which this regulation has been observed (see references above). However, the regulation of COX-2 expression by C/EBPβ in activated glial cells has not been demonstrated. Our ChIP data showing C/EBPβ binding to COX-2 promoter in activated glial cells probably indicates that C/EBPβ participates in the regulation of COX-2 expression in neuroinflammation through

proximal C/EBP sites. The unaffected COX-2 expression when C/EBPβ is absent suggests that C/EBPβ role is taken over by other transcription factors. NF-kB and C/EBPδ are strong candidates to play redundant roles with C/EBPβ. C/EBPδ can bind the same DNA consensus sequence as C/EBPβ (Tsukada et al. 2011) and it has been shown to bind the COX-2 promoter (Wang et al. 2006, Ejarque-Ortiz et al. 2010), triggering gene transactivation (Wadleigh et al. 2000, Caivano et al. 2001). On the other hand NF-kB subunit, p65, has been shown to partially regulate COX-2 gene expression in activated primary astroglia (Font-Nieves et al. 2012).

Much less known than COX-2, PTGES is the terminal enzyme responsible for the synthesis of PGE_2. Since its discovery in the late 90's (Jakobsson et al. 1999) it is the subject of a very active research because it constitutes a more selective target than COX-2 to regulate PGE_2 levels (Koeberle & Werz 2009). Putative binding sites for various transcription factors have been identified in the mouse PTGES promoter (Naraba et al. 2002). To our knowledge only Early growth response protein 1 and NFκB have been shown to bind the PTGES promoter and to regulate PTGES expression induced by pro-inflammatory stimuli (Naraba et al. 2002, Ackerman et al. 2008, Diaz-Munoz et al. 2010). PTGES expression is attenuated in C/EBPβ-deficient macrophages suggesting the involvement of this factor in PTGES expression in this model (Uematsu *et al.* 2002) but the direct binding of C/EBPβ to the PTGES promoter or the identification of functional C/EBP sites in this promoter have not been demonstrated. In this study we show for the first time in any cell type or experimental condition the binding of C/EBPβ to the PTGES promoter. By bioinformatics analysis we identified three potential C/EBP sites in the mouse PTGES promoter. ChIP analysis demonstrated strong C/EBPβ binding to a region containing a proximal -201/-188 site and a weaker but significant binding to the region containing a distal -2565/-2552 C/EBP site. C/EBPβ binding to PTGES promoter was only observed in activated glial cells suggesting a direct regulation of PTGES expression by C/EBPβ in neuroinflammation. In order to explore the functional role of C/EBPβ in PTGES expression in activated glial cells, PTGES expression was analyzed *in vitro* and *in vivo* in C/EBPβ-null mice. The absence of C/EBPβ resulted in reduced LPS- and LPS+IFNγ-induced PTGES expression in mixed glial and microglial cultures as well as in the CNS of LPS-treated mice (Table 2). These data overwhelmingly supports a functional role for C/EBPβ in the regulation of PTGES expression in neuroinflammation which in our opinion constitutes the main finding of this study. It is of note that the expression of the two main inducible PGE-synthesis enzymes, COX-2 and PTGES, can be regulated independently. Thus, resveratrol (Candelario-Jalil *et al.* 2003) minocycline (Silva Bastos *et al.* 2011) or C/EBPβ-deficiency (Uematsu et al. 2002) are able to attenuate LPS-induced macrophage or microglial PTGES expression without affecting COX-2 expression. In fitting with this, we have also observed that IFNγ does not synergize with LPS to further increase PGE_2 production in activated glial cultures, as reported in peripheral human monocytes (Hart *et al.* 1989). This mirrors PTGES but not COX-2 expression pattern, again highlighting the uncoupled regulation of these two enzymes.

PGE_2 is the most common eicosanoid derived from arachidonic acid metabolism. PGE_2 levels increase in the brain and CSF in various neurological disorders and animal models thereof (Candelario-Jalil et al. 2003, Aktan et al. 1991, Almer et al. 2002, Combrinck et al. 2006, Minghetti & Pocchiari 2007, Mattsson et al. 2009). Although the role of PGE_2 in these disorders is controversial, the evidence for a detrimental role for PGE_2 in some experimental models is strong (Li et al. 2008, Ikeda-Matsuo et al. 2010, Takemiya et al. 2010). E prostanoid receptors

and COXs are actively pursued as potential targets to regulate PGE_2 production and function. Our results show that C/EBPβ deficiency results in a decrease in PGE_2 production by activated glial cells which parallels PTGES but not COX-2 expression. These findings support the concept that the inhibition of PTGES can reduce PGE_2 levels in the absence of COX-2 inhibition.

Interestingly, C/EBPβ deficiency did not affect basal PTGES expression and PGE_2 levels in activated glial cells, as we have recently shown for NO synthase 2 expression and NO release (Straccia et al. 2011). The unaltered physiological levels of pro-inflammatory effectors and the abrogation of their overproduction in C/EBPβ absence, point to this factor as a candidate to mediate the transcriptional switch from alerted to reactive/neurotoxic microglia.

In this study we have identified microglial cells and not astrocytes as COX-2 and PTGES expressing cells in mouse mixed glial cultures upon LPS or LPS+IFNγ activation. The expression of COX-2 (Ajmone-Cat *et al.* 2003, Minghetti & Pocchiari 2007) and PTGES (Candelario-Jalil et al. 2003) in activated microglial cells in culture has already been reported. There are also reports showing COX-2 mRNA or protein in murine astroglial cultures but the presence of COX-2 in astrocytes in these cultures has not been unequivocally demonstrated. As we have previously discussed, the presence of microglial cells is often underestimated in primary astroglial cultures and there are examples of microglia being the cell type responsible for effects observed in so-called astroglial cultures (Saura 2007). The absence of detectable immunoreactivity for COX-2 or PTGES in astrocytes in our model suggests lack of astroglial expression and in any case, it clearly demonstrates that activated microglial cells are the main COX-2 and PTGES expressing cells in this model. *In vivo* we have observed a marked upregulation of brain COX-2 and PTGES mRNA levels after systemic LPS injection and an attenuated expression of PTGES but not COX-2 in C/EBPβ-null mice, which is in complete correspondence with the data obtained in mixed glial cultures. Certainly, the CNS is much more complex than the mixed glial culture and the direct stimulation of the cultures differs in many aspects from the stimulation of the CNS cells by systemic LPS. With this in mind and although activated microglial cells can express COX-2 and PTGES in vivo (Ikeda-Matsuo et al. 2010, Ikeda-Matsuo et al. 2005, Campuzano et al. 2008, Kihara et al. 2009, Font-Nieves et al. 2012), we cannot discard that perivascular cells (Cao *et al.* 1995, Grill *et al.* 2008), endothelial cells (Yamagata *et al.* 2001) or neurons (Cao et al. 1995) can contribute to COX-2 or PTGES in vivo expression. Of particular interest, the upregulation of brain COX-2 and PTGES mRNA levels is a rapid event, occurring only 2 hours after systemic LPS administration in agreement with previous data (Cao et al. 1995, Yamagata et al. 2001). Independently of the cellular type, this data implies PGE_2 in the early phase of neuroinflammation.

In summary, we here demonstrate the regulation of PTGES expression by the transcription factor C/EBPβ in activated microglial cells and confirm the independent regulation of COX-2 and PTGES in neuroinflammation. Since both C/EBPβ deficiency (Kapadia et al. 2006, Cortes-Canteli et al. 2008, Straccia et al. 2011) and PTGES deficiency are neuroprotective, these data suggest that attenuation of PTGES expression in activated microglia could participate in the beneficial effects of C/EBPβ deficiency. Altogether, these findings strengthen the proposed role of C/EBPβ as a key player in the regulation of gene expression in neuroinflammation.

AUTHORS CONTRIBUTIONS

MS carried out most experiments and drafted the manuscript. GD carried out the qChIP experiments. TV participated in *in vivo* experiments. CS participated in the statistical analysis. JSa coordinated the study and drafted the manuscript. All authors critically revised and approved the final manuscript.

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CONFLICT OF INTERESTS

The authors declare that they have no competing interests.

Table 1

The qPCR primers used for mRNA amplification and for qChIP are shown.

Assay	Target Gene	Accession	Forward primer (5'→3')	Reverse primer (5'→3')
qPCR	PTGES	NM_022415.3	AggCCAgATgAggCTgCggA	AgCgAAggCgTgggTTCAgC
	COX-2	NM_011198.3	TgCAGAATTgAAAgCCCTCT	CCCCAAAgATAgCATCTggA
	HPRT1	NM_013556.2	ATCATTATgCCgAggATTTgg	gCAAAgAACTTATAgCCCCC
	β-Actin	NM_007393.3	CAACgAgCggTTCCgATg	gCCACAggATTCCATACCCA
	Rn18s	NR_003286.2	gTAACCCgTTgAACCCCATT	CCATCCAATCggTAgTAgCg
Assay	**Target Gene**	**BOX**	**Forward primer (5'→3')**	**Reverse primer (5'→3')**
qChIP	PTGES	P 1	AACAATggTCCTgAgCCAAg	AAgTCCTgAgTAggCggTCA
		P 2	TAACAgggCCAggCTgTACT	ggTTTTgTTCTgCCATgTgA
		P 3	gCATggCTgTCCCTCTACAT	TTTCTgAggCTCTggTCCAT
	COX-2	C 1	CggCTTCCTTCgTCTCTCAT	ACAACTggCTgCTAATgggg
		C 2	TCTTgATTTggTTTgggACAg	AgACCTggAggACAAgAgCA
		C 3	gAACTgACTgCTATCAAATgCAC	ACATgCTgggCTTgAAgACT

Table 2

Summary of changes induced by C/EBPβ deficiency reported in this study. = denotes no changes and ↓ denotes decrease in COX-2, PTGES or PGE_2 expression in C/EBPβ-null primary cultures/mice when compared to wild-type; nd: not determined.

		COX-2		PTGES	
		LPS	LPS+IFNγ	LPS	LPS+IFNγ
Mixed glia	mRNA	=	=	↓	↓
	protein	=	↓	=	↓
	PGE_2			↓	↓
Microglia	mRNA	=	↓	↓	↓
CNS *in vivo* (Cortex)	mRNA	=	nd	↓	nd

Figure 1

A, B. Schematic representation of COX-2 and PTGES promoters showing the sequences and positions with respect to the transcription start site (TSS) of the C/EBPβ binding sites identified and analysed by ChIP. **C, D.** Quantitative analysis of C/EBPβ binding to COX-2 and PTGES promoters by qChIP in mixed glial cultures. qChIP assay is carried out after 2 h of LPS, LPS+IFNγ and vehicle (control) treatment. IL-10 was used as positive control. The Mock bars represent the mean of IgG/Control, IgG/LPS and IgG/LPS+IFNγ PCR values for each gene. Input refers to total DNA. % of input represents the percentage of qChIP/Input ratio. One-way ANOVA, followed by Bonferroni's multiple comparison test is applied. *p<0.05; **p<0.01; ***p<0.001 compared to control. ##p<0.01 compared to LPS. (n = 3-4).

Figure 2

Expression of COX-2 and PTGES genes in wild-type (white bars) and C/EBPβ-null (black bars) mixed glial cultures. Cultures were treated with LPS and LPS+IFNγ for 6 h and mRNA levels were analyzed by qPCR. In wild type cultures LPS and LPS+IFNγ induce the expression of both genes. The absence of C/EBPβ results in significant decreases in the LPS- and LPS+IFNγ-induced expression of PTGES whereas no differences in COX-2 mRNA levels were observed between the two genotypes. Results are expressed as relative fold units of ΔΔCt value between gene of interest and actin + Rn18s as reference genes. Two-way ANOVA, followed by Bonferroni's test was applied. ***p<0.001 compared to respective wild-type condition. $^{\#\#}$p<0.01; $^{\#\#\#}$p<0.001 compared to respective control. (n= 4-6)

Figure 3

The absence of C/EBPβ decreases COX-2 and PTGES proteins expression in activated mixed glial cultures. **A.** COX-2 and PTGES protein levels in total protein extracts from mixed glial cultures were analyzed by western blot, followed by densitometry. Data is expressed as target protein versus β-actin band intensities. Cultures were treated for 16 h with LPS, LPS+IFNγ or vehicle. In wild-type cultures (white bars) COX-2 protein levels were detected after LPS treatment, but LPS+IFNγ induced a clear up-regulation. On the contrary, the effect of LPS or LPS+IFNγ on PTGES protein up-regulation was similar. In C/EBPβ-null mixed glial cultures (black bars), COX-2 and PTGES protein levels after LPS+IFNγ treatment decreased when compared to wild-type cultures. Two-way ANOVA, followed by Bonferroni's test was applied. *p<0.05, ***p<0.001 compared with the respective C/EBPβ$^{+/+}$ condition. $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 compared with the respective control condition. (n = 4-7). Representative western blots are shown in **B**.

Figure 4

PGE_2 production is decreased in activated C/EBPβ-null glial cultures. PGE_2 levels were measured by colorimetric analysis 16 h after treatments. Values are reported as ng PGE_2/ml. PGE_2 levels in wild-type (white bars) cultures were upregulated by LPS and LPS+IFNγ. In C/EBPβ-null glial cultures (black bars), PGE_2 production is reduced in LPS and LPS+IFNγ treatments compared to wild-type levels. Two-way ANOVA, followed by Bonferroni's test was applied. *p<0.05 compared to wild-type condition. $^{###}$p<0.001 compared to respective control condition. (n = 5)

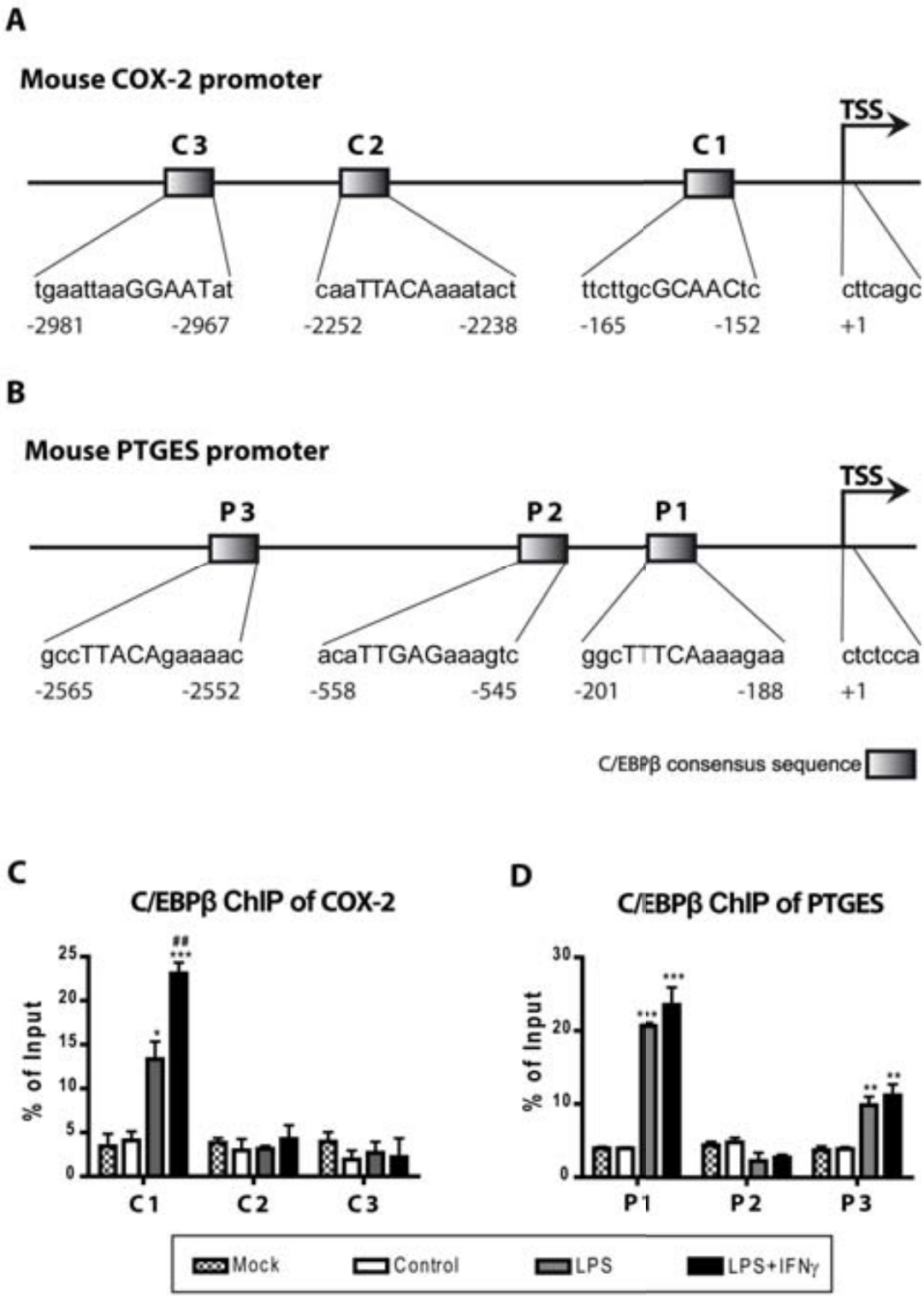
Figure 5

COX-2 and PTGES are expressed by activated microglia. **A, B.** Wild-type and C/EBPβ-null mixed glial cultures were immunostained for CD11b or GFAP (green), COX-2 (red in **A**) or PTGES (red in **B**) and stained for Hoechst 33258 (blue) after 16 h of LPS+IFNγ treatment. Wild-type merged images show colocalization of COX-2 and PTGES-positive cells and CD11b-positive cells. These representative merged images clearly show that COX-2 and PTGES positive cells are not GFAP positive. Bar = 50 μm. **C.** Expression of COX-2 and PTGES genes in wild-type (white bars) and C/EBPβ-null (black bars) microglial cultures. Cultures were treated with LPS and LPS+IFNγ for 6 h and then the mRNA levels were analyzed by qPCR. In wild-type cultures (white bars) COX-2 mRNA levels were detected after LPS treatment, but LPS+IFNγ induced a clear upregulation, in agreement with qChIP data. PTGES gene was up-regulated at the same extent in wild type cultures by LPS and LPS+IFNγ. The absence of C/EBPβ results in significant decreases in LPS+IFNγ-induced expression of COX-2 and in LPS- and LPS+IFNγ-induced expression of PTGES, compared to wild-type. Results are expressed as relative fold units of ΔΔCt value between gene of interest and β-actin + Rn18s as reference genes. Two-way ANOVA, followed by Bonferroni's test was applied. ***p<0.001 compared to respective wild-type condition. $^{#}$p<0.05; $^{###}$p<0.001 compared to respective control. $^{\%}$p<0.05; $^{\%\%}$p<0.01; $^{\%\%\%}$p<0.001 compared to respective LPS condition. (n= 3-5)

Figure 6

Expression of COX-2 and PTGES genes in wild-type (white bars) and C/EBPβ-null (black bars) LPS-treated male mice cortex. Mice were treated intraperitoneally with LPS or vehicle for 2 h or 6 h and mRNA levels in cerebral cortex were analyzed by qPCR. In wild type mice LPS induces already at 2 h the expression of both genes. The absence of C/EBPβ results in significant decreases in expression of PTGES 6 h after LPS, whereas no differences in COX-2 mRNA levels were observed between genotypes. Results are expressed as relative fold units of ΔΔCt value between gene of interest and HPRT1 + Rn18s as reference genes. Two-way ANOVA, followed by Bonferroni's test was applied. *p<0.05 compared to respective wild-type condition. $^{#}$p<0.05; $^{###}$p<0.001 compared to respective control. (n= 5-6)

Figure 1


A
Mouse COX-2 promoter
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C3
C2
C1
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caaTTACAaaatact
ttcttgcGCAACtc
cttcagc
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-2967
-2252
-2238
-165
-152
+1
B
Mouse PTGES promoter
TSS
P3
P2
P1
gccTTACAgaaaac
acaTTGAGaaagtc
ggcTTTCAaaagaa
ctctcca
-2565
-2552
-558
-545
-201
-188
+1
C/EBPβ consensus sequence
C
C/EBPβ ChIP of COX-2
% of Input
C1
C2
C3
D
C/EBPβ ChIP of PTGES
% of Input
P1
P2
P3
Mock
Control
LPS
LPS+IFNγ

Figure 2

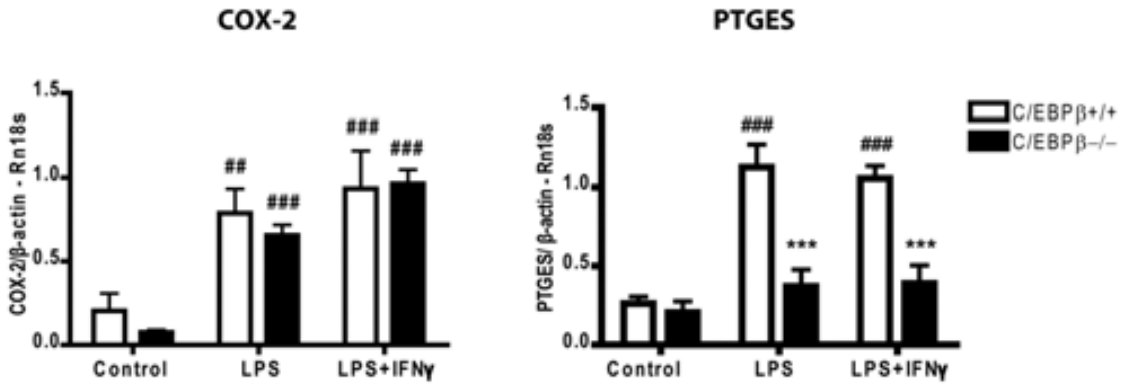

COX-2
PTGES
COX-2/β-actin - Rn18s
PTGES/ β-actin - Rn18s
1.5
1.0
0.5
0.0
##
###
###
###
###
***
***
Control
LPS
LPS+IFNγ
C/EBPβ+/+
C/EBPβ−/−

Figure 3

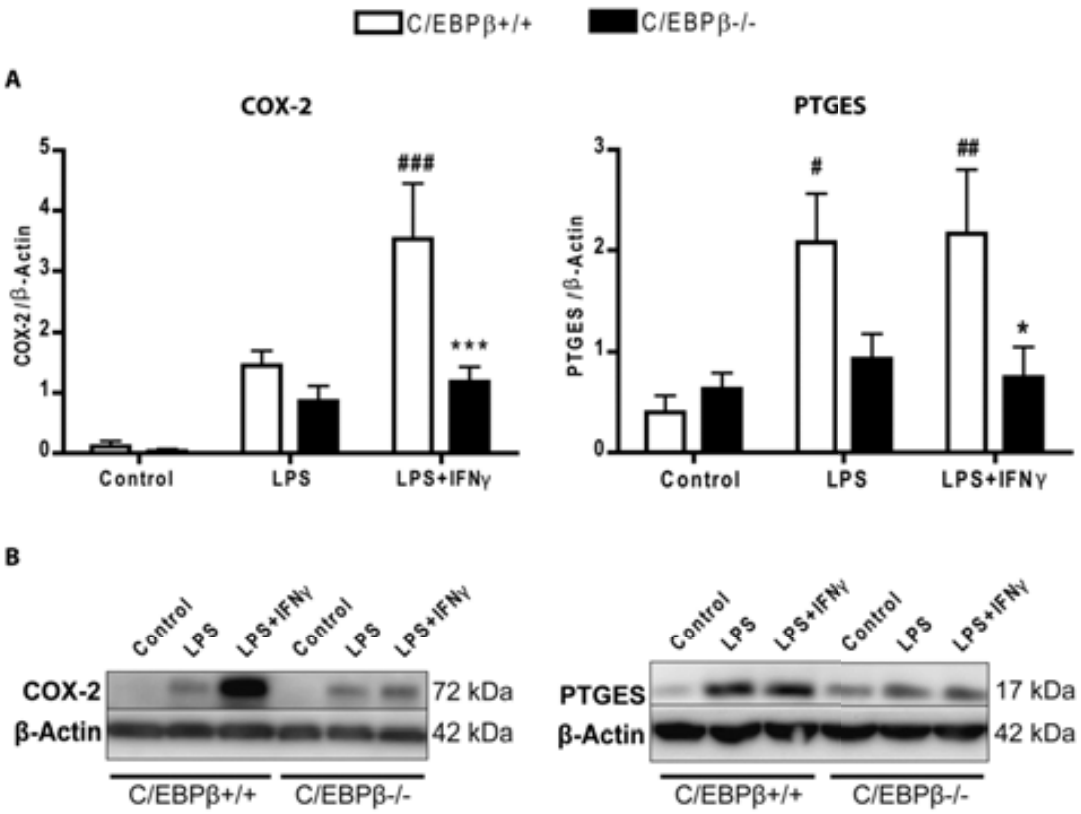

C/EBPβ+/+
C/EBPβ-/-
A
COX-2
PTGES
COX-2 /β-Actin
PTGES /β-Actin
###
***
#
##
*
Control
LPS
LPS+IFNγ
B
COX-2
β-Actin
PTGES
72 kDa
42 kDa
17 kDa
C/EBPβ+/+
C/EBPβ-/-

Figure 4

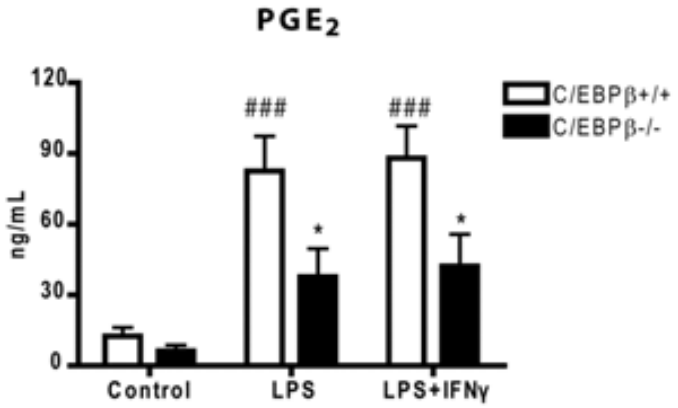
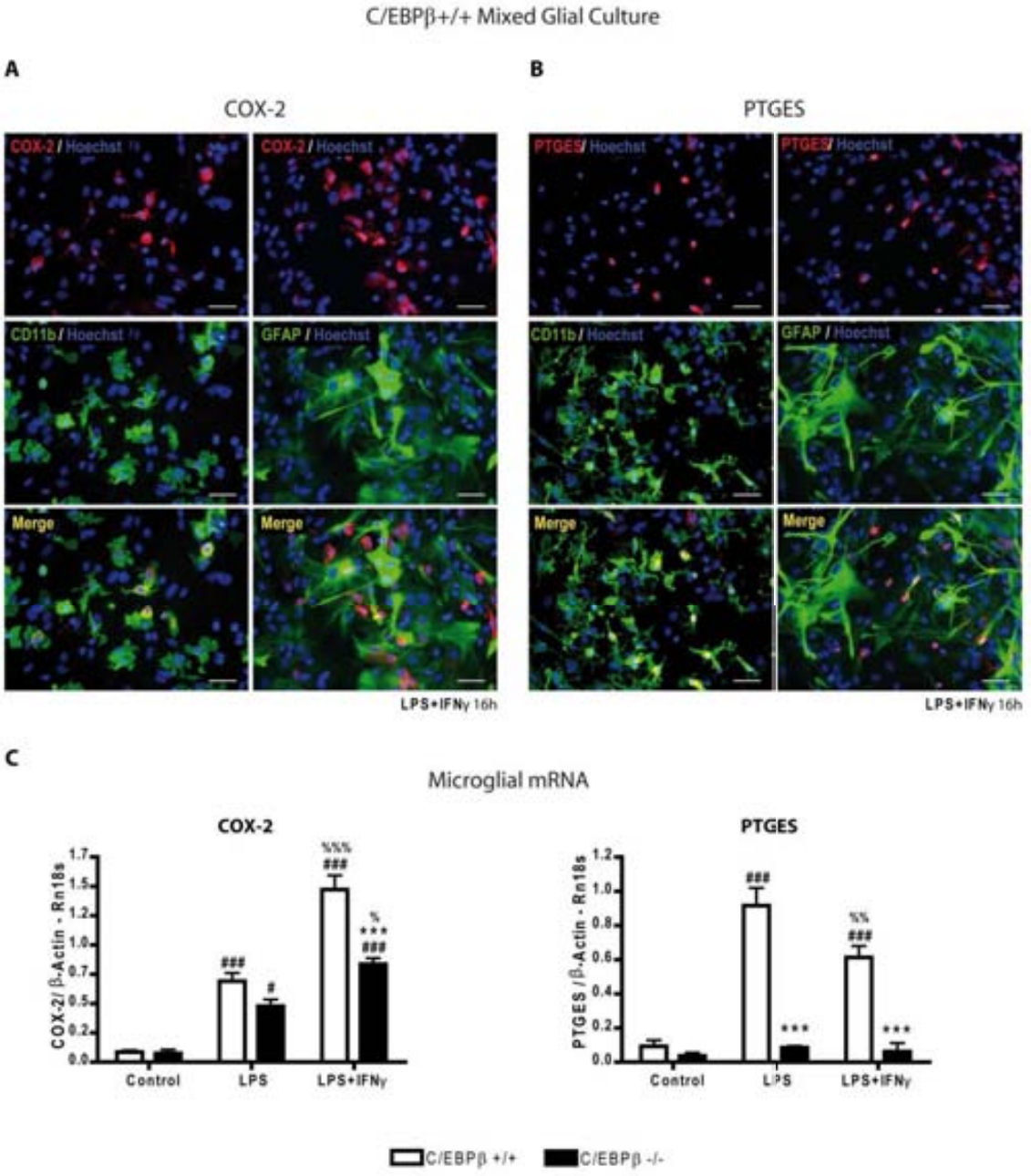


Figure 5


C/EBPβ+/+ Mixed Glial Culture
A
COX-2
B
PTGES
COX-2 / Hoechst
PTGES / Hoechst
CD11b / Hoechst
GFAP / Hoechst
Merge
LPS+IFNγ 16h
C
Microglial mRNA
COX-2
PTGES
COX-2/ β-Actin - Rn18s
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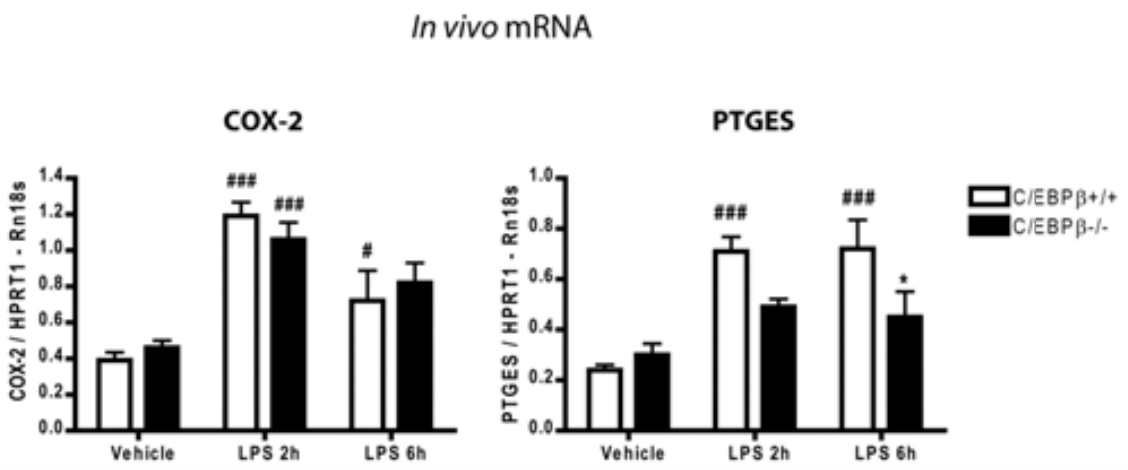
LPS+IFNγ
C/EBPβ +/+
C/EBPβ -/-

Figure 6


In vivo mRNA
COX-2
PTGES
COX-2 / HPRT1 - Rn18s
PTGES / HPRT1 - Rn18s
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
###
###
#
###
###
*
Vehicle
LPS 2h
LPS 6h
C/EBPβ+/+
C/EBPβ-/-

Supplementary Table 1

Mus musculus			*In vitro* mRNA expression					
			C/EBPβ+/+			C/EBPβ-/-		
GENE NAME	OFFICIAL SYMBOL	mRNA	Control (n=4)	LPS (n=4)	LPS+IFNγ (n=4)	Control (n=6)	LPS (n=6)	LPS+IFNγ (n=6)
Prostaglandin-endoperoxide synthase 1	PTGS1	NM_008969.3	1,00±0,25	0,83±0,29	0,83±0,29	1,12±0,24	1,02±0,30	1,10±0,19*
Thromboxane A synthase 1	TBXAS1	NM_011539.3	1,38±0,38	0,56±0,16####	0,66±0,21####	1,16±0,27	0,60±0,12####	0,65±0,07####
Prostaglandin D2 synthase	PTGDS	NM_008963.2	0,35±0,37	0,21±0,19	0,24±0,07	0,75±0,76	0,35±0,23	0,46±0,23
Prostaglandin I2 (prostacyclin) synthase	PTGIS	NM_008968.3	1,18±0,56	0,75±0,32	0,81±0,39	0,83±0,29	0,65±0,27	0,47±0,07
P450 (cytochrome) oxidoreductase	POR	NM_008898.1	0,96±0,38	0,88±0,44	0,69±0,30	1,01±0,25	0,62±0,27	0,64±0,24
Arachidonate 5-lipoxygenase	ALOX5	NM_009662.2	1,10±0,46	0,50±0,11#	0,50±0,33#	0,95±0,37	0,71±0,17	1,70±0,14

Expression of the other six main enzymes in arachidonic acid pathway was analyzed in wild-type and C/EBPβ-null mixed glial cultures. Cultures were treated with LPS and LPS+IFNγ for 6 h and then the mRNA levels were analyzed by qPCR. LPS and LPS+IFNγ induce a down-regulation of the TBXAS1 in both genotypes. In wild type cultures LPS and LPS+IFNγ induce a down-regulation of the ALOX5 gene expression. The absence of C/EBPβ results in a significant difference between the LPS+IFNγ condition of PTGS1 compared to wild-type. Results are expressed as relative fold units of ΔΔCt value between gene of interest and β-Actin + Rn18s as reference genes. Two-way ANOVA, followed by Bonferroni's test was applied. #p<0.05; ###p<0.001 compared to respective control condition. *p<0.05 compared to respective wild-type condition.

Supplementary Table 2

Mus musculus			*In vivo* mRNA expression					
			C/EBPβ+/+			C/EBPβ-/-		
GENE NAME	OFFICIAL SYMBOL	mRNA	Vehicle (n=6)	LPS 2 h (n=5)	LPS 6 h (n=6)	Vehicle (n=5)	LPS 2 h (n=5)	LPS 6 h (n=4)
prostaglandin-endoperoxide synthase 1	PTGS1	NM_008969.3	0.89 ±0,16	0,74 ±0,40	0,96 ±0,26	0,82 ±0,28	0,64 ±0,52	0,48 ±0,48
thromboxane A synthase 1	TBXAS1	NM_011539.3	0,59 ±0,10	1,12 ±0,57	0,97 ±0,28	2,06 ±1,50	0,95 ±0,39	1,78 ±1,76
prostaglandin D2 synthase	PTGDS	NM_008963.2	0,94 ±0,26	0,69 ±0,17	0,70 ±0,27	0,93 ±0,21	0,67 ±0,29	0,83 ±0,32
prostaglandin I2 (prostacyclin) synthase	PTGIS	NM_008968.3	0,97 ±0,44	0,87 ±0,27	0,86 ±0,54	2,31 ±1,94	1,37 ±1,01	1,20 ±1,16
P450 (cytochrome) oxidoreductase	POR	NM_008898.1	1,02 ±0,13	1,03 ±0,27	0,88 ±0,17	0,83 ±0,15	0,83 ±0,22	0,91 ±0,23
arachidonate 5-lipoxygenase	ALOX5	NM_009662.2	0,72 ±0,17	0,8 ±0,24	1,06 ±0,28	1,58 ±1,06	0,91 ±0,45	1,02 ±0,81

Expression of the other six main enzymes in arachidonic acid pathway was analyzed in wild-type and C/EBPβ-null mice hemi-cortex. Mice were treated with vehicle or LPS for 2 h or 6 h and then the mRNA levels were analyzed by qPCR. Two-way ANOVA, followed by Bonferroni's test was applied. No significant difference were observed between genotypes, neither between time points.

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6.3 PAPER 3

CCAAT/enhancer binding protein δ regulates pro-inflammatory gene expression in glial activation: potential role in amyotrophic lateral sclerosis

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Submitted

Title:

CCAAT/enhancer binding protein δ regulates pro-inflammatory gene expression in glial activation: potential role in amyotrophic lateral sclerosis.

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Conflict of Interest

The authors declare that they have no competing interests.

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ABSTRACT

Changes in the CNS environment can induce a response in astrocytes and microglia, known as glial activation, aimed at minimizing damage and restoring homeostasis. However, exacerbated and/or chronic glial activation can be detrimental to the surrounding tissue. The transcription factor CCAAT/enhancer binding protein δ (C/EBPδ) is expressed in activated astrocytes and microglia and can regulate the expression of potentially detrimental pro-inflammatory genes. The objective of this study was to analyze the role of C/EBPδ in glial activation in vitro and in vivo. To this end, primary glial cultures were prepared from wild type and C/EBPδ -/- and adult male mice of both genotypes received systemic injection of lipolysaccharide (LPS). In vitro studies showed that the expression of pro-inflammatory genes NO synthase-2, cyclooxygenase-2 and interleukin (IL)-6 in mixed glial cultures and the neurotoxicity elicited by microglial cells in neuron-microglia co-cultures were reduced by the absence of C/EBPδ. Systemic LPS injection induced C/EBPδ expression in microglia and astrocytes. In C/EBPδ -/- mice, LPS-induced expression of pro-inflammatory genes NO synthase-2, tumor necrosis factor-α, IL-1β and IL-6 was attenuated. Finally, increased C/EBPδ nuclear expression was observed in microglial cells from amyotrophic lateral sclerosis patients and G93A-SOD1 mice spinal cord. These results clearly demonstrate that C/EBPδ plays a key role in the regulation of pro-inflammatory gene expression in glial activation and suggest that C/EBPδ inhibition could be of interest in the treatment of neurodegenerative disorders and in particular, in amyotrophic lateral sclerosis.

INTRODUCTION

Neuroinflammation is a complex response to infections, foreign substances and cell damage (Schmidt et al., 2005) in which activated astrocytes and microglial cells always participate and occasionally also infiltrating cells such as lymphocytes or macrophages. Glial activation plays a central role in CNS inflammatory processes and it is characterized by morphological changes, increased proliferation, migration and phagocytic activity, and enhanced production of cytokines (Ransohoff et al., 2007; Kettenmann et al., 2011), growth factors (Liu et al., 2011) and potentially toxic molecules (Block et al., 2007). Increased production of pro-inflammatory mediators and altered microglial phagocytic activity could contribute to neuronal damage in chronic neuroinflammation (Block et al., 2007).

Glial activation requires a precise regulation of gene expression and transcription factors play a central role in this process. Transcription factors such as NF-κB (Kaltschmidt et al., 2005), AP-1 (Kwon et al., 2004) or STATs (Kim et al., 2002) mediate a pro-inflammatory response in glial activation, whereas others such as PPARs (Drew et al., 2006) or Nrf2 (Vargas and Johnson, 2009) mediate an anti-inflammatory response. Recent studies suggest that transcription factors of the CCAAT/enhancer binding protein (C/EBP) family play also a role in the regulation of gene expression in neuroinflammation (Poli, 1998; Ramji and Foka, 2002; Saha and Pahan, 2006). The present study is focused on the functional role of C/EBPδ in glial activation. C/EBPδ regulates gene expression by binding to C/EBP binding sites as homo- or heterodimers. Basal C/EBPδ expression is low in most cell types and tissues but it is rapidly induced in response to multiple stimuli (Cardinaux and Magistretti, 1996; Cardinaux et al., 2000; Sekine et al., 2002). C/EBPδ participates in the regulation of important physiological processes including energy metabolism (Cardinaux and Magistretti, 1996), growth and differentiation of specific cell types (Yu et al., 2010) and inflammation (Ramji and Foka, 2002; Litvak et al., 2009; Lu et al., 2009). In the CNS, neuronal C/EBPδ participates in the regulation of learning and memory (Sterneck et al., 1998), (Yukawa et al., 1998) whereas glial C/EBPδ is primarily involved in the neuroinflammatory response. C/EBPδ expression is induced in cultured astrocytes or microglia by pro-inflammatory cytokines (Cardinaux et al., 2000; Samuelsson et al., 2008) or LPS (Ejarque-Ortiz et al., 2010) and C/EBPδ is upregulated in human neurological disorders, such as Alzheimer's disease (Colangelo et al. 2002; Li et al. 2004) and spinocerebellar ataxia type 3 (Evert et al., 2006) where it is expressed by astrocytes and neurons, respectively. There are to our knowledge no reports of microglial C/EBPδ expression in human CNS.

Although it has been clearly shown that C/EBPδ is upregulated in activated astrocytes and microglia, there is little evidence on its functional role in glial activation. Under the hypothesis that C/EBPδ regulates pro-inflammatory gene expression in neuroinflammation we have here characterized glial activation in the absence of C/EBPδ in vitro and in vivo. We show that C/EBPδ absence attenuates expression of pro-inflammatory genes and reduces neuronal damage induced by activated microglia. Furthermore, we show microglial C/EBPδ expression in amyotrophic lateral sclerosis (ALS) spinal cord, which constitutes the first description to our knowledge of microglial C/EBPδ expression in human disease.

MATERIALS AND METHODS

Animals

All animal experiments were done in accordance with the Guidelines of the European Union Council (86/609/EU) and Spanish Government (BOE 67/8509-12), and approved by the Ethics and Scientific Committees from the University of Barcelona and registered at the "Departament d'Agricultura, Ramaderia i Pesca de la Generalitat de Catalunya".

C/EBPδ mice and genotyping

A colony of C/EBPδ -/- and C/EBPδ +/- mice (Sterneck et al., 1998) on a C57BL/6 background was maintained at the specific pathogen-free animal facilities of the School of Medicine, University of Barcelona, under regulated light and temperature conditions.

For genotyping of C/EBPδ -/- mice, genomic DNA was isolated from ears punches and polymerase chain reactions (PCR) were performed according to Extract-N-Amp Tissue Kit protocol (Sigma-Aldrich, St. Louis, MO, USA). DNA products were amplified using the allele-specific primers designed by Dr. Esta Sterneck: C/EBPδwt forward (5'-CTCCAGGCTTGGACGGCTAAGTAGG-3') and reverse (5'- AAGTTGGCTGTCACCTCGCC-3') primers amplify a 205 bp fragment of the C/EBPδ coding region. C/EBPδko forward (5'-GCTCCAGACTGCCTGGGAAAAGC-3') and reverse (5'- CAGTCCAGTGCCCAAGCTGC-3') primers amplify a 305 bp fragment of the pGKneo promoter and 3'UTR of C/EBPδ to detect knockout fragment. Samples were run for 35 cycles: 95 ºC for 30 sec., 63.4 ºC for 30 sec. and 72 ºC for 30 sec.

G93A-SOD1 mice

G93A-SOD1 mice (Gurney et al., 1994) were generously provided by Dr. Manuel Portero (School of Medicine, University of Lleida, Spain) as described previously (Valente et al., 2011). For analysis of C/EBPδ mRNA levels during development, wild-type and G93A-SOD1 mice of embryonic day 17 (n=4) and postnatal days 7 (n=3), 30 (n=3) and 130 (n=4) were deeply anesthetized, sacrificed and spinal cords and brains were carefully removed and frozen in carbonic ice.

Mixed Glial Cultures

Mixed glial cultures were prepared from P0-P3 C/EBPδ-/- or wild-type mice both on C57BL/6 genetic background. Briefly, mice cerebral cortices were dissected, their meninges were totally removed and cortices were digested with 0.25% trypsin for 25 min at 37°C. Trypsinization was stopped by adding an equal volume of culture medium (Dulbecco's modified Eagle medium-F-12 nutrient mixture, fetal bovine serum 10%, penicillin 100 U/mL, streptomycin 100 µg/mL and amphotericin B 0.5 µg/mL (all from (Invitrogen, Carlsbad, CA, USA) with 0.02% deoxyribonuclease I (Invitrogen). The solution was pelleted (5 min, 200g), resuspended in culture medium and brought to a single cell suspension by repeated pipetting followed by passage through a 100 µm pore mesh. Glial cells were seeded at a density of 3.0 x 10^5 cells/mL and cultured at 37°C in humidified 5% CO_2–95% air. For characterization of C/EBPδ-/- mixed glial cultures and for phagocytosis, cells were seeded in 96-well plates. For immunocytochemistry and nitrites assay, cells were seeded in 48-well plates. For protein or

RNA isolation cells were seeded in 6-well plates or T25 flasks. Medium was replaced every 5–7 days.

Microglial cultures

Microglial cultures were prepared by mild trypsinization from mouse mixed glial culture (Saura et al., 2003). Microglial cultures were treated 24 h after isolation by this procedure.

Primary cortical neuronal cultures

Primary cortical neuronal cultures were prepared from C57BL/6 mice at embryonic day 16 as described (Gresa-Arribas et al., 2010). Briefly, cells were seeded at a density of 8×10^5 cells/mL in 48-well culture plates coated with poly-D-lysine (Sigma-Aldrich) and cultured at 37°C in a 5% CO_2 humidified atmosphere. Neuronal cultures were used at 5 days in vitro (DIV).

Primary neuronal-microglial co-cultures

Microglial cultures were obtained as described above. After isolation, microglia-enriched cultures were incubated with 0.25% trypsin for 10 min at 37°C. Trypsinization was stopped by adding the same volume of culture medium with 10% fetal bovine serum. Cells were gently scraped and centrifuged for 5 min at 200 g. Pellet was resuspended in neuronal culture medium and aliquots of the cell suspension (50 µL/well) were seeded on top of DIV5 primary neuronal cultures at a final density of 4×10^5 cells/mL and were used on the following day.

In vitro treatments

After 19-21 DIV, mixed glial cultures were treated with of lipopolysaccharide (LPS, 100 ng/ml; 026:B6; Sigma-Aldrich) ± interferon γ (IFNγ, 0.1 ng/ml; Sigma-Aldrich) for 2 or 6 h for mRNA extraction, 6 or 16 h for protein extraction, and 16 or 48 h for nitrites assay and immunocytochemistry. Control cells were treated with an equivalent volume of culture medium. Primary neuronal-microglial co-cultures were treated with LPS (100 ng/mL) + IFNγ (30 ng/mL) for 24 h.

Immunocytochemistry

Cultured cells were fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 20 min at room temperature. Cells were permeated with 0.3% H_2O_2 in methanol for 10 min, washed in PBS, and incubated overnight at 4°C with one of the following primary antibodies: monoclonal rat anti-CD11b (1:300, Serotec, Oxford, UK), polyclonal rabbit anti-GFAP (1:1000, DAKO, Glostrub, Denmark) or monoclonal mouse anti-MAP2 (1:2000, Sigma-Aldrich). Then, cells were sequentially incubated with biotinylated goat anti-rabbit, anti-rat or anti-mouse secondary antibody (1:200, Vector, Peterborough, UK), and with ExtrAvidin-HRP (1:500, Sigma-Aldrich). Immunostaining was developed with 0.05% diaminobenzidine (Sigma-Aldrich) and 0.1% H_2O_2. For immunocytofluorescence cells were permeated with methanol for 10 min, washed in PBS and incubated with a mixture of two primary antibodies: monoclonal rat anti-CD11b (1:300, Serotec) or mouse anti-GFAP (1:2000, DAKO) mixed with polyclonal rabbit anti-NOS2 (1:300, BD Biosciences, San Diego, CA, USA) or anti-COX-2 (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA). After that cells were incubated with goat anti-rabbit Alexa 546 (1:1000) and goat anti-rat or anti-mouse Alexa 488 (1:500) secondary antibodies (Molecular Probes, Eugene, OR, USA). Microscopy images were obtained with a Zeiss Axio

observer Z1 microscope (Carl Zeiss, Jena, Germany) and a digital camera (QImaging Retiga Exi, QImaging, Surrey, BC, Canada).

Estimation of neuronal viability in neuronal-microglial co-cultures

Neuronal viability was evaluated by MAP2 immunocytochemistry with the antibodies above described using ABTS (2, 3'-azinobisethylbenzothiazoline-6-sulphonic acid) as substrate and followed by absorbance analysis as described (Gresa-Arribas et al., 2010).

Nitrite assay

NO production was assessed by Griess reaction. Briefly, culture supernatants were collected 16 and 48 hours after LPS and LPS/IFN-γ treatment, and incubated with equal volumes of Griess reagent for 10 min at room temperature. Optical density at 540 nm was determined using a microplate reader (Multiskan spectrum, Thermo Electron Corporation, Waltham, CA, USA). Nitrite concentration was determined from a sodium nitrite standard curve.

Phagocytosis assay

Phagocytosis was quantified by measuring the uptake of Red fluorescent FluoSpheres® beads (Invitrogen, F8826). Briefly, DIV19-21 mixed glial cultures were treated with LPS/IFNγ or vehicle for 16 h and then incubated with 0.02% of Red fluorescent FluoSpheres® beads for 1 hour. Intracellular fluorescence was read with a fluorescence plate reader (Spectra MAX Gemini XS, Molecular Devices, Sunnyvale, CA, USA) at 580 nm excitation and 605 nm emission.

***In vivo* LPS treatment of C/EBPδ-/- mice**

Eight-ten weeks old male C/EBPδ-/- (n=30) and wild-type (n=60) mice were used for in vivo LPS treatment. Mice were injected intraperitoneally with 100 µl LPS (055:B5, Sigma-Aldrich; 100 µg per animal) or vehicle. For qRT-PCR, mice were deeply anesthetized with isofluorane, sacrificed and the cerebral cortex, hippocampus and striatum were carefully removed and frozen in carbonic ice. For in situ hybridization or immunohistochemistry, wild-type mice were deeply anesthetized with isofluorane at 6, 16 or 24 h after treatment, perfused in 4% of PFA and the brains was carefully removed, post-fixed in PFA for 48 h and cryoprotected in 30% sucrose- 4% PFA solution. Then, the samples were frozen in carbonic ice and twenty-five µm-thick cryostat coronal sections were cut and stored at -20°C in a cryoprotected solution (30% ethyleneglycol and 30% glycerol in PBS solution).

***In vivo* LPS treatment of G93A-SOD1 mice**

G93A-SOD1 mice were used when symptoms of severe hindlimb motor deficits were observed (130 days) as described (Valente et al., 2011). Age-matched wild-type C57Bl/6 mice were used. Twenty-eight mice (4 groups, 7 mice per group) were used. Mice were injected intraperitoneally with 200 µg per animal of LPS (055:B5, Sigma-Aldrich) or vehicle. For qRT-PCR, mice were deeply anesthetized with isofluorane, sacrificed and the thoracic and lumbar regions of the spinal cord were carefully removed and frozen in carbonic ice. For immunohistochemistry, mice were deeply anesthetized, sacrificed and the spinal cords were treated as described above for brains in LPS-treated mice.

Isolation of nuclear and total proteins

Nuclear protein and total protein extraction from cell cultures and tissues were performed as described (Valente et al., 2011). For mixed glial cultures, nuclear and total proteins were extracted from two wells of 6-well plates. For total protein extraction for in vivo western blots, 100-150 mg of brain tissue was sonicated at 4º C in RIPA buffer containing protease inhibitor cocktail (1 ml of ice cold RIPA buffer per g of tissue). After 30 min in ice, samples were centrifuged at 1500 g for 5 min at 4° C and supernatants were collected. Protein concentration was determined in vitro and in vivo samples by Lowry assay (Total Protein kit micro-Lowry, Sigma-Aldrich).

Western blot

Western blots for total and nuclear extracts were performed as described (Valente et al., 2011). Briefly, around 50 µg of denatured protein (95°C for 5 min) was subjected to electrophoresis on a 10-12% polyacrylamide gel, and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). After washing in Tris-buffered saline (TBS: 20 mM Tris, 0.15 M NaCl, pH 7.5) for 5 min, dipping in methanol for 10 s and air dry, membranes were incubated with primary antibodies overnight at 4°C: polyclonal rabbit anti-C/EBPα (1:300, Santa Cruz Biotechnology), polyclonal anti-C/EBPβ (1:300, Santa Cruz Biotechnology), polyclonal anti-C/EBPδ (1:300, Santa Cruz Biotechnology), polyclonal rabbit anti-p65-NFκB (1:1000, Santa Cruz Biotechnology), polyclonal rabbit anti-COX-2 (1:2000, Santa Cruz Biotechnology), polyclonal rabbit anti-NOS2 (1:300, Chemicon, Temecula, CA, USA), monoclonal mouse anti-β actin (1:40000, Sigma-Aldrich) or polyclonal goat anti-lamin B (1:5000, Santa Cruz Biotechnology) diluted in immunoblot buffer (TBS containing 0.05% Tween-20 and 5% non-fat dry milk). Membranes were then washed twice in 0.05% Tween-20 in TBS for 15 s and incubated in horseradish peroxidase (HRP)-labelled secondary antibodies for 1 h at room temperature: donkey anti-rabbit (1:5000, Amersham/GE Healthcare Waukesha, WI, USA), goat anti-mouse (1:5000, Santa Cruz Biotechnology) or mouse anti-goat/sheep (1:2000, Sigma-Aldrich). After extensive washes in 0.05% Tween-20 in TBS, they were incubated in ECL-Plus (Amersham/GE Healthcare) for 5 min. Membranes were then exposed to the camera of a VersaDoc System (Bio-Rad Laboratories, Hercules, CA, USA), and pixel intensities of the immunoreactive bands were quantified using the % adjusted volume feature of Quantity One 5.4.1 software (Bio-Rad Laboratories). Data are expressed as the ratio between the intensity of the protein of interest band and the loading control protein band (lamin B for nuclear extracts and β-actin for total extracts).

In situ hybridization

Antisense and sense riboprobes were labeled with digoxigenin-d-UTP (Boehringer-Mannheim GmbH, Mannheim, Germany). In situ hybridization was performed on free-floating tissue sections as previously described (Valente et al., 2005). Briefly, sections were pre-treated with H_2O_2 and HCl, hybridized overnight at 65 ºC with antisense or sense digoxigenin-d-UTP-labeled riboprobes (mouse C/EBPδ cDNA, gift by Dr Knut Steffensen, Karolinska Institute, Sweden), stringently washed in 50% formamide solutions at 65 ºC and incubated with 100 mg/ml RNase A (at 37 ºC). Sections were then blocked with 10% normal goat serum and incubated overnight at 4 ºC with an alkaline phosphatase-labeled antidigoxygenin antibody (1:2000; Boehringer-

Mannheim). Alkaline phosphatase activity was visualized with nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indolyl-phosphate toluidinium salt.

Immunohistochemistry

Briefly, free-floating sections were treated with 0.5 % H_2O_2, blocked with 10 % of fetal bovine serum and incubated overnight at 4 ºC with a polyclonal rabbit anti-C/EBPδ antibody (1:1000, Rockland Immunochemicals, Gilbertsville, PA, USA). After that, sections were sequentially incubated with biotinylated goat anti-rabbit antibody (1:200, Vector), and then with ExtrAvidin-HRP (1:500, Sigma-Aldrich) and the immunostaining was developed with 0.05% diaminobenzidine (Sigma-Aldrich) and 0.01% H_2O_2.

Double immunofluorescence

For double immunohistofluorescence, free-floating sections were washed in PBS, blocked with 10% of normal goat serum and incubated overnight at 4°C with polyclonal rabbit anti-C/EBPδ (1:1000, Rockland Immunochemicals) mixed with monoclonal rat anti-CD11b (1:300, Serotec) or monoclonal mouse anti-GFAP (1:1000, DAKO) primary antibodies. After rinsing in PBS, sections were incubated for 1 hour at room temperature with goat anti-rabbit Alexa 546 (1:500) mixed with goat anti-rat or anti-mouse Alexa 488 (1:500) secondary antibodies (Molecular Probes).

Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from mixed glial cultures with RNA Miniprep kit (Roche Diagnostics, Basel, Switzerland) and from tissues with Trizol method (TriRReagent, Sigma-Aldrich). One µg of RNA for each condition was reverse transcribed with random primers using Transcriptor Reverse Transcriptase (Roche Diagnostics). In vivo and in vitro cDNA was diluted 1/25 and 1/250, respectively, to perform real-time PCR as described (Valente et al., 2011). Primers used to amplify mouse mRNA are described in table 1. For normalization of cycle threshold (Ct), 2 or 3 housekeeping genes were used for in vivo and in vitro samples, respectively (see Table 1). Real-time PCR was carried out with IQ SYBR Green SuperMix (Bio-Rad Laboratories) and iCycler MyIQ equipment (Bio-Rad Laboratories). Samples were run for 50 cycles (95°C for 15 s, 60°C for 30 s, 72°C for 15 s). Relative gene expression values were calculated with the comparative Ct or ΔΔCt method (Livak and Schmittgen, 2001) using iQ5 2.0 software (Bio-Rad Laboratories).

Quantitative chromatin immunoprecipitation (qChIP)

MatInspector (matrix library version 8.4) was used to identify the proximal C/EBP consensus sequence in each analyzed promoter. MatInspector was set as follows: matrix group = vertebrates; core similarity = 0,75; matrix similarity = optimized. The sequences for each amplified locus are indicated in Table 2. qChIP was performed as previously described (Straccia et al., 2011). Briefly, chromatin sheared was incubated at 40 rpm on a rotating wheel for at least 2 h at 4°C with two micrograms of polyclonal rabbit C/EBPδ antibody (Santa Cruz Biotechnology, sc-636) or rabbit IgG (Santa Cruz Biotechnology, sc-2027) as negative control. DNA was isolated with phenol-chloroform-isoamylalcohol 25:24:1 (Sigma-Aldrich) extraction. ChIP samples were analyzed with qPCR using SYBR green (Bio-Rad Laboratories). Samples were run for 45 cycles (95°C for 30 s, 62°C for 1 min, 72°C for 30 s).

Human spinal cord samples

Post-mortem human spinal cord paraffin sections used in this study were supplied by the human neurological tissue bank at the Hospital Clínic (Barcelona, Spain) in accordance with the Helsinki Declaration, Convention of the Council of Europe on Human Rights and Biomedicine and Ethical Committee of Barcelona University. Post-mortem histological spinal cord samples were obtained from control (n=5; 3 ♀, 2 ♂; age range 66-81; post-mortem delay range 3.5-23.5 h) and sporadic ALS (n=6; 1 ♀, 5 ♂; age range 58-79; post-mortem delay 7-19 h) patients.

Human immunohistochemistry

Immunohistochemistry was performed as described (Valente et al., 2011). Briefly, after deparaffinization and antigen retrieval in citrate buffer, sections were treated with 2 N HCl at 37 ºC for 30 min and with 2% H_2O_2 in methanol for 10 min. Sections were blocked with 10% fetal bovine serum, incubated overnight with the monoclonal mouse anti-CR3 antibody (1:500, DAKO) and then with alkaline phosphatase goat anti-mouse antibody (1:500, Sigma-Aldrich). Sections were developed with 5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine Salt and Nitro-Blue Tetrazolium Chloride, until a specific blue color was observed. After that, sections were rinsed for 1 hour in PBS 0.1% Tween-20 and incubated with the polyclonal anti-C/EBPδ antibody (1:500, Rockland Immunochemicals). After washes, sections were sequentially incubated with biotinylated goat anti-rabbit antibody (1:500, Sigma-Aldrich) and with ExtrAvidin-HRP (1:500, Sigma-Aldrich), and developed with 0.05% diaminobenzidine-0.01% H_2O_2 (brown color stain). For each human sample (control, n=5; sporadic ALS n=6), 3 sequential spinal cord sections were used for cell counts. CR3+ C/EBPδ+ cells and CR3- C/EBPδ+ in the whole white matter that was determined by hematoxylin-eosin staining in an adjacent section.

Data presentation and statistical analysis

All results are presented as mean ± SD. Statistical analyses were performed using one-way ANOVA followed by Newman–Keuls post-hoc test or two-way ANOVA followed by Bonferroni's multiple comparisons test. Values of $p < 0.05$ were considered statistically significant. GraphPad Prism 4.02 (GraphPad Software, Inc., La Jolla, USA) was used.

RESULTS

Cell density and cellular composition of wild type and C/EBPδ -/- mixed glial cultures

First, we analyzed whether wild type and C/EBPδ -/- primary mixed glial cultures were similar in cellular density, composition and growth. Counting of Hoechst-33258-stained nuclei showed that total cell number increases with days in culture and reaches a plateau when astrocytes reach confluency which approximately occurs at DIV12-14. No differences in total cell density between both genotypes were observed at any time point (Fig 1A). The number of microglial cells, estimated by counting CD11b-positive cells, increases also over time and represents between 16.7% and 19.5% of total cells at DIV14-21. No differences in microglial density were observed between the two genotypes (Fig 1A). Both cultures are composed of a majority of astrocytes and approximately 20% of microglial cells at confluency with no qualitative differences in terms of cellular composition or morphology (Fig 1B). Astroglial density is difficult to estimate precisely in these cultures in which astrocytes are densely packed and it is often difficult to discern whether a given nucleus is GFAP-positive or in fact the GFAP signal belongs to a neighbor astrocyte. As an indirect measure of astroglial density the GFAP content was analyzed by western blot. No differences in GFAP content between wild type and C/EBPδ -/- cultures were observed neither in control nor in activated (LPS or LPS+IFNγ) conditions (Fig 1C,D) which, in agreement with the qualitative observations of GFAP immunocytochemistry, suggests that the number and morphology of astrocytes are not altered by the absence of C/EBPδ.

We were next interested to analyze whether the absence of C/EBPδ results in compensatory changes in the protein levels of the transcription factors C/EBPα, C/EBPβ and NF-κB which either have a similar functional profile to C/EBPδ (C/EBPβ or NF-κB) or can bind the same DNA consensus sequences as C/EBPδ (C/EBPα or C/EBPβ). In the absence of activating stimuli, no differences between wild type or C/EBPδ -/- cultures were observed in the protein levels of any of the transcription factors analyzed (Fig 2). Treatment of wild type cultures with LPS or LPS+IFNγ increased the levels of the three C/EBPβ isoforms, Full, LAP and LIP, and of the NF-κB protein p65 (Fig 2A-B). No significant differences between wild type and C/EBPδ -/- cultures were observed with the exception of LIP levels that were lower in C/EBPδ -/- cultures after LPS or LPS+IFNγ treatments (Fig 2A). C/EBPα protein levels decreased after LPS or LPS+IFNγ treatment and no changes were observed between wild type and C/EBPδ -/- cultures (Fig 2C)

Attenuated pro-inflammatory gene expression in C/EBPδ -/- cultures

To test the hypothesis that C/EBPδ regulates pro-inflammatory gene expression in glial activation we analyzed the mRNA levels of 5 key pro-inflammatory genes in wild type and C/EBPδ -/- cultures treated with vehicle, LPS or LPS+IFNγ. As expected, both LPS and LPS+IFNγ induced a robust upregulation of the mRNA levels of all 5 genes analyzed, namely NOS2, COX-2, TNFα, IL-1β and IL-6 (Fig 3). In wild type cultures, IFNγ potentiated the LPS-induced upregulation of NOS2 and COX-2, but not that of TNFα, IL-1β and IL-6. LPS-induced expression of these 5 genes was not significantly different between wild type and C/EBPδ -/- cultures. In contrast, LPS+IFNγ-induced expression of NOS2 (-34.8%, $p<0.05$), COX-2 (-57.3% , $p<0.01$) and IL-6 (-43.1%, $p<0.01$) was significantly lower in C/EBPδ -/- than in wild type cultures (Fig 3A,B,E).

We next studied whether the attenuation of NOS2 and COX-2 expression in LPS+IFNγ-treated C/EBPδ -/- glial cultures could also be observed at the protein level. In accordance with the mRNA data, western blot analysis revealed the induction of NOS2 protein by LPS in wild type cultures which was markedly potentiated by IFNγ (8.2-fold increase; $p<0.001$) and a significant attenuation in LPS+IFNγ-induced NOS2 protein levels was observed in C/EBPδ -/- cultures when compared with wild type cultures (-55.2%, $p<0.001$) (Fig 4A). In agreement with this, NO levels in conditioned media were lower in C/EBPδ -/- than in wild type glial cultures treated with LPS+IFNγ (wild type 6.61 $\pm$ 1.44 µM; C/EBPδ -/- 3.48 $\pm$ 1.05 µM, $p<0.001$) whereas NO levels did not differ between LPS-treated wild type and C/EBPδ -/- glial cultures (Fig 4B). A similar pattern of changes was seen for COX-2 protein levels analyzed by western blot (Fig 4C). COX-2 protein levels were induced by LPS in wild type cultures and this effect was potentiated by IFNγ. As seen for NOS2, COX-2 protein levels in LPS-treated C/EBPδ -/- glial cultures were similar to wild type levels whereas in LPS+IFNγ-treated C/EBPδ -/- cultures COX-2 levels were lower (-52.7%, $p<0.05$) than in wild type cultures (Fig 4C).

The primary cortical murine mixed glial cultures used in this study are mainly composed of astrocytes and microglia. We used immunocytochemistry to identify the cell types expressing NOS2 and COX-2. In untreated cultures NOS2 and COX-2 immunoreactivities were not detected. After LPS or LPS+IFNγ treatments, NOS2 and COX-2 immunoreactivities were observed in cells identified as microglia by their CD11b immunostaining (Fig 4D). No colocalization of NOS2 or COX-2 with GFAP was observed (data not shown) indicating the absence of astroglial NOS2 or COX-2 expression in these conditions. In agreement with the western blot data, immunocytochemistry showed reduced expression of NOS2 and COX-2 in LPS+IFNγ-treated C/EBPδ -/- cultures when compared with LPS+IFNγ-treated wild type cultures (Fig 4D).

Binding of C/EBPδ to pro-inflammatory gene promoters

The reduced expression of NOS2, COX-2 and IL-6 in activated glial cultures could indicate a direct effect of C/EBPδ as an activator of transcription in these genes. To investigate whether C/EBPδ binds to these promoters in glial cultures quantitative chromatin immunoprecipitation (qChIP) was performed. Chromatin from untreated, LPS- and LPS+IFNγ-treated mixed glial cultures was immunoprecipitated with an anti-C/EBPδ antibody and quantitative PCR was performed with primers targeting the putative C/EBP site closest to the translation initiation site in NOS2, COX-2 and IL-6 genes. No C/EBPδ binding to any of the 3 promoters analyzed was observed in untreated mixed glial cultures (Fig 5). Compared to untreated cultures, LPS induced a significant increase of C/EBPδ binding to the NOS2 promoter and LPS+IFNγ induced a significant increase of C/EBPδ binding to the NOS2 and COX-2 promoters (Fig 5).

Neurotoxicity induced by activated microglia is abrogated by the absence of C/EBPδ

Activated microglial cells have a strong neurotoxic potential that is mediated, at least partly, by production of inflammatory mediators. Given the attenuated pro-inflammatory gene expression profile of activated C/EBPδ -/- glial cultures we were interested to study whether the neurotoxic effects of activated microglia were decreased in the absence of C/EBPδ. To this end, wild type neurons were cocultured with wild type or C/EBPδ -/- microglial cells and treated with LPS+IFNγ as described in methods. In the absence of microglial cells treatment of

neuronal cultures with LPS+IFNγ did not result in any neurotoxic effect (data not shown). In contrast, marked reduction of neuronal viability, estimated by quantification of solubilized MAP2 immunostaining, was induced by LPS+IFNγ when neurons were cocultured with wild type microglial cells (100.0 ± 23.5 (Control) vs 56.9 ± 14.1 (LPS+IFNγ); $p<0.01$). Interestingly, this neurotoxicity was abrogated when the same treatment was applied to neurons cocultured with C/EBPδ -/- microglial cells (104.3 ± 34.0 (Control) vs 89.3 ± 33.6 (LPS+IFNγ); $p>0.05$). These results clearly demonstrate the involvement of C/EBPδ in the neurotoxic effects induced by activated microglia in this model.

Phagocytosis in the CNS is a distinctive microglial feature. In opposition to the expression of pro-inflammatory mediators, phagocytosis is regarded as a beneficial effect of microglial activation. We were therefore interested to analyze whether the microglial ability to phagocytize was affected by the absence of C/EBPδ. To this end, wild type and C/EBPδ -/- mixed glial cultures were treated with vehicle or LPS+IFNγ and then incubated with fluorescent beads as described in methods. Phagocytosis was estimated by measuring the intracellular fluorescence with a fluorescence plate reader. Microscopic examination of PFA-fixed cultures revealed that intracellular beads were accumulated in a population of cells identified as microglia by CD11b immunostaining (data not shown). In contrast to the neurotoxicity findings, no differences in phagocytosis were observed between wild type and C/EBPδ -/- glial cultures neither in the untreated condition (137,98±43,46 rfu(wild type) vs 125,87±37,38 rfu (C/EBPδ -/-), $p>0.05$) nor in the LPS+IFNγ condition (195,23±73,17 rfu (wild type) vs 168,54±75,06 rfu (C/EBPδ -/-), $p>0.05$).

C/EBPδ expression is induced in the CNS by systemic LPS

After demonstrating the involvement of C/EBPδ in the regulation of microglial pro-inflammatory gene expression and neurotoxicity in an in vitro model we were interested to analyze the effects of C/EBPδ deficiency in glial activation in vivo. To induce glial activation in vivo we used systemic injection of LPS. We first analyzed the pattern of brain C/EBPδ expression in this model. A time-course analysis revealed the increase of C/EBPδ mRNA levels in the cerebral cortex 2 h and 6 h after LPS i.p. injection (Control 0.166±0.118; 2 h LPS 1.139±0.284, $p<0.001$ vs control); 6 h LPS 1.700±0.740, $p<0.001$ vs control) but not at 24 or 48 h post-injection (Fig 7A). The increase in C/EBPδ mRNA levels in the cerebral cortex 6 h after LPS i.p. was also observed in striatum and hippocampus (Fig 7B). In situ hybridization histochemistry experiments also demonstrated induction of brain C/EBPδ mRNA by systemic LPS injection. Thus, in vehicle-treated mouse brain C/EBPδ mRNA was only detected in hippocampal granule cells, whereas in mice treated with LPS i.p. for 6 h numerous C/EBPδ mRNA-positive cells were observed in all areas analyzed including cerebral cortex (Fig 7C), hippocampus, striatum and hypothalamus. To investigate whether these changes in C/EBPδ mRNA levels translate into changes at the protein level we performed an LPS i.p. time-course study of C/EBPδ protein levels by western blot. C/EBPδ protein was barely detectable by western blot in the cerebral cortex of untreated mice or 6 h after LPS i.p. whereas 24-48 h after LPS i.p. a marked increase in the 35 kDa band corresponding to C/EBPδ was observed (Fig 7D) (Control 3.47 ± 1.97; 24 h LPS 21.03 ± 4.97 ($p<0.001$ vs control); 48 h LPS 16.59 ± 8.34 ($p<0.001$ vs control). Immunohistochemistry experiments confirmed the induction of C/EBPδ protein by systemic LPS injection. In brain sections of untreated or vehicle-treated mice C/EBPδ immunoreactivity was not observed in brain parenchyma (Fig 7E) with the exception of

the dentate gyrus where C/EBPδ immunoreactive neuronal nuclei were observed (not shown). C/EBPδ positive nuclei were observed in hippocampus, striatum and cerebral cortex 16 h, 20 h and 24 h after LPS i.p. injection, respectively. Colocalization of C/EBPδ with glial and neuronal markers was studied by immunofluorescence 16 h after LPS i.p.. C/EBPδ positive nuclei colocalized with GFAP and CD11b in cerebral cortex (Fig 7F), hippocampus and striatum, whereas C/EBPδ colocalization with NeuN was observed in some cells in the hippocampus and striatum, but not in most brain regions including the cerebral cortex (Fig 7F). These results clearly demonstrate that systemic LPS induces the expression of C/EBPδ in the mouse brain which occurs in activated astrocytes and microglia.

Pro-inflammatory gene expression induced by peripheral LPS injection is attenuated in the cerebral cortex of C/EBPδ -/- mice

We were next interested to analyze whether the absence of C/EBPδ results in compensatory changes in the in vivo brain expression of other C/EBPs. As seen in Fig 8 no differences in the mRNA levels of C/EBPα or C/EBPβ were observed between vehicle-treated wild type and C/EBPδ -/- mice. A time-course analysis of systemic LPS-induced changes revealed that 6 h after LPS C/EBPα mRNA brain levels were significantly higher in C/EBPδ -/- mice (wt 0.767 ± 0.217, n=4; C/EBPδ -/- 1.122 ± 0.138, n=5; $p < 0.001$) and the same effect was observed for C/EBPβ mRNA brain levels 16 h after LPS (wt 0.636 ± 0.136, n=3; C/EBPδ -/- 1.512 ± 0.575, n=3; $p<0.001$). No significant differences were observed in C/EBPα or C/EBPβ brain mRNA levels between the two genotypes at any of the other time-points analyzed (Fig 8).

To test our hypothesis that C/EBPδ regulates pro-inflammatory gene expression in the brain, we compared the mRNA levels for NOS2, COX-2, TNFα, IL-1β and IL-6 after systemic LPS injection in wild type and C/EBPδ -/- mice (Fig 9). A time-course analysis was performed with 2 h, 6 h, 16 h and 24 h as time-points post-LPS. NOS2 mRNA levels were upregulated by LPS at 6 h, 16 h and 24 h. A significant reduction in NOS2 mRNA levels was observed in C/EBPδ -/- mice 16 h after LPS (wt 0.598 ± 0.141, n=3; C/EBPδ -/- 0.372 ± 0.104, n=3, $p>0.05$) (Fig 9A). The expression of COX-2 was upregulated by LPS at 2 h in wild type mice but not at later time-points. No differences were observed in COX-2 mRNA levels between wild type and C/EBPδ -/- mice at any time-point (Fig 9B). Systemic LPS increased TNFα mRNA levels in wild type mice at all time-points analyzed. A significant reduction in TNFα mRNA levels was observed in C/EBPδ -/- mice 6 h after LPS (wt 0.613 ± 0.206, n=4; C/EBPδ -/- 0.215 ± 0.070, n=4; $p<0.01$) (Fig 9C). IL-1β mRNA levels were also induced by LPS in wild type mice at 2h, 6h and 16h and, as seen for TNFα, a significant reduction in IL-1β mRNA levels was observed in C/EBPδ -/- mice 6 h after LPS (wt 0.881 ± 0.245, n=4; C/EBPδ -/- 0.156 ± 0.077, n=5; $p<0.001$) (Fig 9D). Finally, IL-6 mRNA levels were upregulated by LPS in wild type mice at all time-points analyzed. In C/EBPδ -/- mice, a significant reduction in IL-6 mRNA levels was observed 2 h (wt 0.801 ± 0.231, n=4; C/EBPδ -/- 0.390 ± 0.134, n=6; $p<0.01$), 6 h (wt 0.590 ± 0.291, n=4; C/EBPδ -/- 0.161 ± 0.082, n=5; $p<0.01$) and 16 h (wt 0.684 ± 0.218, n=3; C/EBPδ -/- 0.205 ± 0.120, n=3; $p<0.01$) after LPS (Fig 9E).

C/EBPδ expression in G93A-SOD1 mice and in human ALS samples

After showing the upregulation of C/EBPδ in LPS-induced glial activation in vivo we were interested to analyze C/EBPδ expression in G93A-SOD1 mice, an animal model of ALS. In these animals, there is a neuroinflammatory process which is most likely mediated by neuronal

damage signals. C/EBPδ mRNA levels were analyzed in spinal cord of G93A-SOD1 and wild type mice of various ages. In both genotypes C/EBPδ mRNA levels were maximal at embryonic age and decreased with age (Fig 10A). In embryonic (E17), post-natal (P7) or young asymptomatic (P30) animals no differences in C/EBPδ mRNA levels were observed between genotypes. Interestingly, in G93A-SOD1 mice of 130 days of age, which were symptomatic, spinal cord C/EBPδ mRNA levels were higher than in wild type mice (0.131 ± 0.006 wild type, 0.368 ± 0.031 G93A-SOD1, p<0,001, Student t-test; Fig 10A). Systemic injection of LPS in 130 days old mice induced C/EBPδ mRNA upregulation in wild type mice spinal cord and this effect was exacerbated in G93A-SOD1 mice (Fig 10B). This LPS-induced exacerbation of C/EBPδ expression in G93A-SOD1 mice was also observed at the protein level by immunohistochemistry (Fig 10C). Double immunofluorescence experiments revealed that in LPS-treated G93A-SOD1 mice C/EBPδ immunoreactive nuclei colocalized with CD11b and GFAP indicating C/EBPδ expression in both activated microglia and astrocytes in this model (Fig 10D). We then analyzed C/EBPδ expression in primary microglial cultures from wild type and G93A-SOD1 mice. Western blot experiments revealed that LPS+IFNγ increased C/EBPδ protein levels in wild type microglial cultures and this effect was exacerbated in microglial cultures expressing the G93A-SOD1 transgene (Fig 10E). Similar results were obtained by C/EBPδ immunocytochemistry (Fig 10F) with the exception of an increase in C/EBPδ immunoreactivity in untreated G93A-SOD1 vs wild type microglia that was not observed by Western blot.

Finally, C/EBPδ expression was analyzed by immunohistochemistry in human spinal cord sections from ALS patients and non-neurological controls. C/EBPδ immunoreactivity was observed in small, compact cell nuclei found in grey and white matter. A diffuse, cytoplasmic staining was also observed in some neurons in grey matter. The number of C/EBPδ positive nuclei was 4.7-fold higher in ALS spinal cord than in controls (1.61 ± 0.72 cells/mm^2, control; 7.65 ± 2,27 cells/mm^2, ALS; p<0.001; Fig 11A,B). Approximately 40% of C/EBPδ immunoreactive nuclei colocalized with the microglial/macrophage CR3 and the number of C/EBPδ-positive microglial cells was also increased 3.1 fold in ALS (0.99 ± 0.28 cells/mm^2, control; 3.11 ± 0.98 cells/mm^2, ALS; p<0.05; Fig 11A,C).

DISCUSSION

The attenuation of detrimental effects of glial activation has therapeutic potential in a large number of neurological disorders (Block et al., 2007). A strategy to globally modulate a complex response such as glial activation is to act on the master regulators of gene expression, i.e. the transcription factors (Wu, 2006). The present work is focused on role of the transcription factor C/EBPδ in neuroinflammation with the aim of establishing proof of concept for neuroprotective effects of C/EBPδ inhibition in activated glial cells. C/EBPδ has recently emerged as a key transcription factor in inflammation (Litvak et al., 2009; Medzhitov and Horng, 2009; Yan et al., 2011), but little is known about its involvement in glial activation. C/EBPδ is upregulated by a variety of stimuli in activated astrocytes (Cardinaux and Magistretti, 1996; Cardinaux et al., 2000; Li et al., 2004; Ramberg et al., 2011; Ko et al., 2012) and microglia (Paglinawan et al., 2003; Ejarque-Ortiz et al., 2010), but data is scant on the functional role of this transcription factor in glial activation: on one hand the flavonoid chrysin, which reduces C/EBPδ but not C/EBPβ or NFκB levels, attenuates microglial activation (Gresa-Arribas et al., 2010) and, on the other hand, C/EBPδ deficiency is neuroprotective in EAE and the C/EBPδ effects in this model are mainly attributed to dendritic cells (Tsai et al., 2011). These data suggest that C/EBPδ plays a regulatory role in neuroinflammation. However, no study has directly addressed this question by analyzing pro-inflammatory gene expression, phagocytosis or neurotoxicity in activated glial cells in the absence of C/EBPδ, be it by using C/EBPδ -/- glial cultures or by RNAi.

The results here presented clearly demonstrate that C/EBPδ plays a functional role in glial activation. In mixed glial cultures LPS+IFNγ-induced expression of key pro-inflammatory genes such as NOS2, COX-2 and IL-6 was reduced in the absence of C/EBPδ and in a neuronal/microglial co-culture model the neurotoxicity elicited by microglia was abolished when C/EBPδ was absent. It is interesting to note that all these effects were observed after LPS+IFNγ and not after LPS alone even though in the same model LPS markedly upregulated C/EBPδ expression. We think that despite the lack of effects seen in its absence, C/EBPδ probably plays a role in LPS-induced neuroinflammation but that in the absence of C/EBPδ this role is taken over by other transcription factors, particularly C/EBPβ. C/EBPβ and C/EBPδ share identical sequence in their DNA binding motifs (Tsukada et al., 2011), are both upregulated by LPS in activated mixed glial cultures (Ejarque-Ortiz et al., 2007; Ejarque-Ortiz et al., 2010) and have common functional targets. Thus, B lymphoblasts acquire LPS-inducible expression of MCP-1 and IL-6 by exogenous expression of C/EBPβ or C/EBPδ (Hu et al., 1998) and a similar redundancy has also been observed for NOS-2 (Kolyada and Madias, 2001) or IL-10 (Liu et al., 2003) expression. In line with this, we have observed in the absence of C/EBPδ a downregulation of the C/EBPβ inhibitory isoform LIP that is probably a compensatory mechanism resulting in increased availability of C/EBPβ activating isoforms Full/LAP to counteract the lack of C/EBPδ.

In contrast to LPS alone, LPS+IFNγ-induced pro-inflammatory gene expression and neurotoxicity were reduced in C/EBPδ-deficient microglial cells. This indicates that C/EBPδ plays a role in IFNγ-triggered neuroinflammation that cannot be taken over by C/EBPβ or other transcription factors. Our ChIP analysis indicates that IFNγ does not increase C/EBPδ DNA

binding activity (data not shown) and in fitting with this there are no reports on IFNγ-mediated C/EBPδ post-translational modifications that could mediate such an increase. Alternatively, after LPS+IFNγ treatment C/EBPδ could activate pro-inflammatory gene transcription by interacting with IFNγ-induced coactivators or transcription factors in a way C/EBPβ could not. There are very interesting reports showing the IFNγ-mediated C/EBPβ phosphorylation and binding to GATE sequences (Li et al., 2007). Unfortunately, the field of IFNγ-mediated effects on C/EBPδ function is almost unexplored. We are not aware of interactions described between C/EBPδ and classical IFNγ-induced transcription factors such as STATs or IRFs. Of note, C/EBPδ can interact with p204 (Xiao et al., 2010), an interferon-induced coactivator. p204 expression in the CNS has not been described but since it plays a role in macrophage differentiation (Dauffy et al., 2006), its microglial expression is likely.

The comparison of the effects of the absence of C/EBPβ (Straccia et al., 2011) and C/EBPδ in glial activation in vitro shows some common themes. Absence of C/EBPβ or C/EBPδ has little effect on LPS-induced pro-inflammatory gene expression but a clear effect on LPS+IFNγ-induced expression of different subsets of pro-inflammatory genes and on LPS+IFNγ-induced microglial neurotoxicity. It would be very informative to study the effects of double C/EBPβ/δ deficiency in glial activation. Experiments with embryonic or immortalized double C/EBPβ/δ deficient macrophages show a marked reduction in pro-inflammatory gene expression and therefore suggest C/EBPβ/δ redundancy (Lu et al., 2009; Yan et al., 2011).

Unlike pro-inflammatory gene expression, phagocytosis, viability or proliferation were not affected by C/EBPδ deficiency in mixed glial cultures. These findings suggest that C/EBPδ does not participate in all cellular programs that are launched in neuroinflammation. This is of interest when considering the inhibition of C/EBPδ as a pharmacological target to attenuate detrimental effects of neuroinflammation.

After demonstrating the involvement of C/EBPδ in glial activation in vitro we used the systemic LPS model (Perry et al., 2007) to analyze whether C/EBPδ plays an analogous role in vivo. In this model, LPS penetrance to the brain parenchyma is minimal at the doses here used (Banks and Robinson, 2010) but a robust glial activation occurs that is caused by a combination of factors including upregulation of circulating pro-inflammatory mediators (Murray et al., 2011), afferent nerve stimulation ((Blatteis, 2007) and direct LPS stimulation of endothelial cells (Singh and Jiang, 2004) and circumventricular organs ((Laflamme and Rivest, 2001). Systemic LPS is therefore not a strict correlate of LPS or LPS+IFNγ treatment of glial cultures. The results here presented clearly show that systemic LPS increases C/EBPδ mRNA and protein levels in various CNS regions. Previous reports had described C/EBPδ mRNA increased levels in the CNS by systemic LPS (Alam et al., 1992) but this is the first study to show increased C/EBPδ protein levels and its cellular localization in this model. In fact, this is one of the first studies to show C/EBPδ expression in activated glial cells in vivo. To date C/EBPδ protein has only been observed in the cytoplasm of activated astrocytes of Alzheimer patient brains (Li et al., 2004) and in the nucleus of astrocytes and dendritic cells in experimental autoimmune encephalomyelitis (Tsai et al., 2011). This study is therefore the first demonstration of C/EBPδ expression in microglial cells in vivo.

Systemic LPS induced a marked upregulation of pro-inflammatory gene expression in the cerebral cortex. In the absence of C/EBPδ, LPS-induced upregulation of NOS2, TNFα, IL-1β and, most markedly, IL-6 was attenuated. This is a clear demonstration that C/EBPδ plays a functional role in glial activation in vivo and consequently it should be included in the short list of key transcription factors activating pro-inflammatory gene expression in neuroinflammation together with NFκB, AP-1, STATs, PPARγ, CREB, NFATs and C/EBPβ, among others. It is of note that the subset of genes significantly affected by C/EBPδ absence is different in the in vitro and in vivo models here used. Thus, NOS2 and IL-6 were affected in both cases, COX-2 only in vitro and TNFα and IL-1β only in vivo. This reflects the different nature of the models used but also the potential of C/EBPδ to regulate all these genes in a given condition. Somewhat surprisingly, the effect of C/EBPδ absence in vivo was observed for most genes before C/EBPδ increase at the protein level. This suggests the presence of basal C/EBPδ levels that soon after systemic LPS injection activate pro-inflammatory gene transcription. This effect could be mediated by a synergistic interaction between C/EBPδ binding and early-response transcription factors or by post-translational modifications in C/EBPδ.

C/EBPδ was finally studied in neuroinflammation associated to a neurodegenerative process, namely ALS. We observed C/EBPδ upregulation in the spinal cord of symptomatic G93A-SOD1 mice, an animal model of ALS, and in primary microglial cultures prepared from G93A-SOD1 mice. The increased C/EBPδ levels in G93A-SOD1 microglia could be involved in the increased pro-inflammatory profile and neurotoxic potential of these cells (Weydt et al., 2004; Xiao et al., 2007). We also observed that in G93A-SOD1 mice treated systemically with LPS C/EBPδ expression was exacerbated and the pattern of changes was very similar to that of pro-inflammatory genes and C/EBPβ (Valente et al., 2011). Finally, the finding that C/EBPδ is expressed by microglia in human ALS spinal cord samples is the first demonstration of C/EBPδ in activated microglial cells in human disease. In ALS, the neuroinflammatory process leads to increased expression of pro-inflammatory genes shown here to be regulated by C/EBPδ (Philips and Robberecht, 2011). This strongly supports the involvement of C/EBPδ in microglial activation in human CNS. The recent finding that C/EBPδ can activate the expression of SOD1 (Hour et al., 2010), a gene that is often mutated in familial ALS and that shows increased levels in sporadic ALS (Gagliardi et al., 2010), places C/EBPδ inhibition in a unique position in ALS since it could be beneficial by decreasing SOD1 levels and by attenuating a detrimental neuroinflammatory response.

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FIGURE LEGENDS

Figure 1. Characterization of C/EBPδ -/- primary mixed glial cultures. A) Total cell number, assessed by Hoechst 33258 staining, and microglial cell number, assessed by CD11b immunocytochemistry, in primary mixed glial cultures from wild type and C/EBPδ -/- mice at various DIV. Data show mean + SD of 3 independent experiments and is expressed as cells/mm2. No significant differences in total or microglial cell number were observed between both genotypes at any time point. Two-way ANOVA followed by Bonferroni test. B) The two main cell types in mixed glial cultures, astrocytes and microglia, were labeled with anti-GFAP and anti-CD11b antibodies respectively at DIV21. No qualitative differences were observed between wild type and C/EBPδ -/- cultures in density and morphology of astrocytes or microglia. Images are representative of 7 independent experiments. Magnification bar, 100 µm. C) GFAP levels were analyzed by western blot and were used as an indirect estimate of astroglial content. No differences in GFAP levels were observed between wild type and C/EBPδ -/- cultures in control or in activated conditions (LPS 100 ng/ml ± IFNγ 0.1 ng/ml, 16 h). Data show mean + SD of 5 independent experiments and are expressed as GFAP/actin band density ratio. No significant differences between both genotypes were observed. Two-way ANOVA followed by Bonferroni test. D) A representative GFAP western blot is shown.

Figure 2. **C/EBPα/β and NF-κB expression in C/EBPδ -/- glial cultures**. Primary mixed glial cultures from wild type (white bars) and C/EBPδ -/- mice (grey bars) were treated with vehicle, LPS (100 ng/ml) ± IFNγ (0.1 ng/ml) for 6 h and nuclear proteins were analyzed by western blot. Data show mean + SD of 5 independent experiments and are expressed as the ratio of the band density of the protein of interest to band density of the constitutive nuclear protein Lamin B. * p<0.05; ** p<0.01; *** p<0.001 vs respective control. & p<0.05; && p<0.01 vs respective LPS. ## p<0.01; ### p<0.001 show significant differences between genotypes. Two-way ANOVA followed by Bonferroni test. A representative western blot image is shown below the respective graph. A) No differences in the levels of C/EBPβ isoforms Full and LAP were observed between wild/type and C/EBPδ -/- glial cultures. In contrast, LIP levels were significantly lower in C/EBPδ -/- cultures after LPS and LPS+IFNγ treatments. B) Nuclear levels of the NFκB-p65 protein were increased by LPS or LPS+IFNγ and were not affected by genotype. C) Nuclear levels of C/EBPα were decreased by LPS or LPS+IFNγ and were not affected by genotype.

Figure 3. Pro-inflammatory gene expression in C/EBPδ -/- primary glial cultures. Wild type (white bars) and C/EBPδ -/- (grey bars) primary mixed glial cultures were treated with vehicle, LPS (100 ng/ml) ± IFNγ (0.1 ng/ml) for 6 h. The mRNAs of the pro-inflammatory genes NOS2 (A), COX-2 (B), TNF-α (C), IL-1β (D) and IL-6 (E) were analyzed by qRT-PCR as described in methods. Data show mean + SD of 7 independent experiments. Results are expressed as relative fold units of ΔΔCt value between gene of interest and actin + Rn18s as reference genes. * p<0.05; ** p<0.01; *** p<0.001 vs respective control. & p<0.05; &&& p<0.001 vs respective LPS. ## p<0.01 show significant differences between genotypes. Two-way ANOVA followed by Bonferroni test.

Figure 4. Reduced NOS2 and COX-2 expression in C/EBPδ -/- primary glial cultures. Mixed glial cultures from wild type (white bars) and C/EBPδ -/- mice (grey bars) were treated with vehicle, LPS (100 ng/ml) ± IFNγ (0.1 ng/ml). A) NOS2 protein levels were analyzed by western blot 16 h after treatment. Graph shows the quantification of 6 independent experiments as mean + SD. Data are expressed as the NOS2/actin ratio of band densities. *** p<0.001 vs respective control. ### p<0.001 show significant differences between genotypes. Two-way ANOVA followed by Bonferroni test. A representative western blot experiment is shown below the graph. B) NO production was estimated by measuring nitrites accumulation in the conditioned medium by the Griess method 48 h after treatments. LPS+IFNγ-induced NO production is significantly lower in C/EBPδ -/- than in wild type cultures*** p<0.001 vs respective control. ### p<0.001 show significant differences between genotypes. Two-way ANOVA followed by Bonferroni test. C) COX-2 protein levels were analyzed by western blot 16 h after treatment. Graph shows the quantification of 6 independent experiments as mean + SD. Data is expressed as the COX-2/actin ratio of band densities. ** p<0.01 vs respective control. # p<0.05 show significant differences between genotypes. Two-way ANOVA followed by Bonferroni test. A representative western blot experiment is shown below the graph. D) Representative images of wild type and C/EBPδ -/- primary mixed glial cultures treated for 16 h with LPS+IFNγ and double immunostained for NOS2 or COX-2 (in red) and CD11b (in green). Nuclei are stained in blue with Hoechst 33258. NOS2 and COX-2 immunopositive cells are always positive for CD11b. Note also the lower number of NOS2 and COX-2 positive cells in C/EBPδ -/- vs wild type. Magnification bar 200 µm.

Figure 5. C/EBPδ binds to pro-inflammatory gene promoters in activated glial cells in vitro. Mixed glial cultures were treated with vehicle and LPS (100 ng/ml) + IFNγ (0.1 ng/ml) for 2 h and chromatin was immunoprecipitated with anti-C/EBPδ antibodies. As a negative control chromatin from a pool of the three conditions was immunoprecipitated with rabbit IgG. Regions containing C/EBP sites in the NOS2, COX-2 and IL-6 promoters were amplified by qRT-PCR. Data shows mean + SD of 3 independent experiments and is expressed as the % input. * p<0.05; ** p<0.01; *** p<0.001 vs respective control. Two-way ANOVA followed by Bonferroni test.

Figure 6. Microglial neurotoxicity is abrogated by C/EBPδ deficiency in neuronal-microglial co-cultures. Wild type cortical neurons were co-cultured with wild type or C/EBPδ -/- microglial cells and treated for 24 h with vehicle or LPS (100 ng/ml) + IFNγ (30 ng/ml). A) The content of the neuronal protein MAP2 was semiquantified as described in methods. Activated wild type but not C/EBPδ -/- microglia are neurotoxic. Data show mean + SD of 6 independent experiments. ** p<0.01 vs respective control. Two-way ANOVA followed by Bonferroni test. B) Representative images of MAP2 immunocytochemistry of neurons co-cultured with wild type or C/EBPδ -/- microglia and treated with vehicle (control) or LPS+IFNγ. Note the decrease in MAP2 immunoreactivity only when neurons are cocultured with wild type microglia and treated with LPS+IFNγ.

Figure 7. C/EBPδ expression in mouse brain after systemic LPS. A) Adult mice were treated with 100µg LPS i.p. or vehicle and the C/EBPδ mRNA levels in the cerebral cortex were analyzed by qRT-PCR at various times post-injection. Data show mean + SD of 5-9 mice/group.

Results are expressed as relative fold units of ΔΔCt value between gene of interest and actin + Rn18s as reference genes. *** p<0.001 vs control. One-way ANOVA followed by Neumann-Keuls test. B) C/EBPδ mRNA levels were analysed as described in A in cerebral cortex, hippocampus and striatum of mice treated i.p. with vehicle or LPS (100 µg/mouse) for 6 h. Data show mean + SD with 5 mice/group. ** p <0.01; *** p<0.001 vs respective control. One-way ANOVA followed by Neumann-Keuls test. C) Representative images of C/EBPδ mRNA in situ hybridization histochemistry in brain sections from mice treated i.p. with vehicle or 100 µg LPS for 6 h. Note the induction of C/EBPδ mRNA by LPS in the outer cortical layers. This experiment was repeated in 5 mice/group with similar results. Magnification bar 200 µm. D) Adult male mice were treated with 100 µg LPS i.p. or vehicle and C/EBPδ protein levels in cerebral cortex were analysed by western blot at various times post-injection. Data show mean + SD of 7 mice/group and are expressed as the C/EBPδ/lamin B ratio of band densities. *** p<0.001 vs control. One-way ANOVA followed by Neumann-Keuls test. A representative western blot image is shown below the graph. E) C/EBPδ immunohistochemistry in brain sections from mice treated i.p. with vehicle or 100 µg LPS for 16 h or 24 h. Note the induction of C/EBPδ by LPS in cerebral cortex. These images are representative of 7 mice/group. Magnification bar 200 µm. F) C/EBPδ immunofluorescence (red) in brain sections from mice treated with LPS i.p. for 16 h and double immunostained with the microglial marker CD11b (green), the astroglial marker GFAP (green) or the neuronal marker NeuN (green). Images correspond to cerebral cortex and are representative of 7 mice/group. Note the colocalization of C/EBPδ with CD11b and GFAP, but not with NeuN. Magnification bar 50 µm.

Figure 8. C/EBPα/β expression in C/EBPδ -/- mice after systemic LPS. Adult wild-type (white bars) or C/EBPδ -/- mice (grey bars) were treated i.p. with vehicle or 100 µg LPS for 2 h, 4 h, 6 h, 16 h or 24 h and mRNA levels for C/EBPα (A) and C/EBPβ (B) were analyzed by qRT-PCR. Data show mean + SD of 3-5 mice/group. Results are expressed as relative fold units of ΔΔCt value between gene of interest and actin + Rn18s as reference genes. * p<0.05; ** p<0.01; *** p<0.001 vs respective control. ### p<0.001 show significant differences between genotypes. Two-way ANOVA followed by Bonferroni test.

Figure 9. Pro-inflammatory gene expression in C/EBPδ -/- brain after systemic LPS. Adult wild-type (white bars) or C/EBPδ -/- mice (grey bars) were treated i.p. with vehicle or 100 µg LPS for 2 h, 6 h, 16 h or 24 h and cerebral cortex mRNA levels for NOS2 (A), COX-2 (B), TNFα (C), IL-1β (D) and IL-6 (E) were analyzed by qRT-PCR. Data show mean + SD of 3-5 mice/group. Results are expressed as relative fold units of ΔΔCt value between gene of interest and actin + Rn18s as reference genes. * p<0,05; ** p<0,01; *** p<0.001 vs respective control. # p<0.05; ## p<0.001; ### p<0.001 show significant differences between genotypes. Two-way ANOVA followed by Bonferroni test.

Figure 10. **C/EBPδ expression in G93A-SOD1 mice**. A) C/EBPδ mRNA levels were analyzed by qRT-PCR in spinal cord of wild type and G93A-SOD1 mice at embryonic day 17 (ED17) and post-natal days (PND) 17, 30 and 130. Data show mean + SD of 3-4 mice/group. Results are expressed as relative fold units of ΔΔCt value between gene of interest and actin + Rn18s as reference genes. No statistical differences between genotypes were observed by two-way ANOVA followed by Bonferroni test. &&&, p<0.001 vs respective wild-type. Student t-test. B)

C/EBPδ mRNA levels were analysed in the spinal cord of adult (130 days) wild type or G93A-SOD1 mice treated for 6 h with LPS (100 µg/mice, i.p.) or vehicle (CT). Data show mean + SD of 3-4 mice/group and are expressed as in A. ***, p<0.001 vs respective control; ###, p<0.001 vs respective wild type condition. Two-way ANOVA followed by Bonferroni test. &&&, p<0.001 vs respective wild-type. Student t-test. C) C/EBPδ immunohistochemistry in spinal cord sections of wild type or G93A-SOD1 mice treated with LPS (100 µg/mice, i.p.) or vehicle (CT) for 24 h. Images are representative of 4 mice/group. Magnification bar, 100 µm. D) C/EBPδ immunofluorescence (red) in spinal cord sections of wild type or G93A-SOD1 mice treated for x h with LPS (100 µg/mice, i.p.) and double immunostained in green with the microglial marker isolectin B4 (left column) or the astroglial marker GFAP (right column). Images are representative of 4 mice/group. Magnification bar, 100 µm. E, F) C/EBPδ protein levels were analyzed in primary microglial cultures prepared from wild type and G93A-SOD1 mice. Cultures were treated with vehicle (Ct) or LPS (100 ng/ml) + IFNγ (0.1 ng/ml) for 24 h. Data shows mean + SD of 4 independent experiments and are expressed as C/EBPδ/lamin B ratio of band densities. * p<0.05; *** p<0.001 vs respective vehicle condition. # p<0.05 show significant differences between genotypes. Two-way ANOVA followed by Bonferroni test. A representative Western blot image is shown. F) C/EBPδ immunocytochemistry staining in primary microglial cultures prepared and treated as described in E. Magnification bar, 20 µm

Figure 11. **C/EBPδ in human ALS spinal cord.** A) Total C/EBPδ immunoreactive cells and cells with double C/EBPδ and CR3 immunoreactivity were quantified in spinal cord white matter of non-neurological controls (n=4) and ALS (n=6) cases. Data show mean + SD. *, p<0,05; ***, p<0,001 vs respective control. Two-way ANOVA followed by Bonferroni test. B) C/EBPδ immunohistochemistry in representative non-neurological control and ALS spinal cord sections. Note the increase in C/EBPδ immunoreactive cells in ALS. Magnification bar, 200 µm. C) Representative image of an ALS spinal cord section immunostained for C/EBPδ (brown) and the microglial marker CR3 (purple). Note the presence of C/EBPδ immunoreactive nuclei in various microglial cells (arrows). Magnification bar, 50 µm.

Figure 1

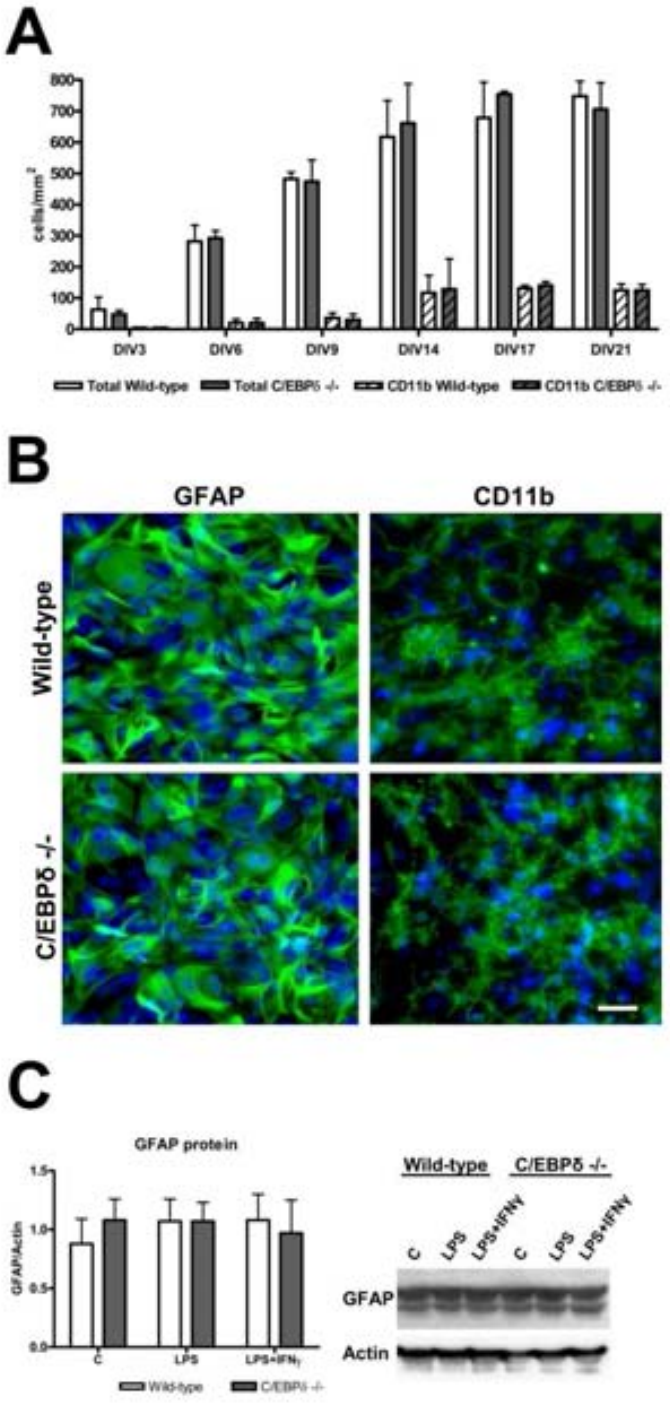
A
cells/mm²
800
700
600
500
400
300
200
100
0
DIV3
DIV6
DIV9
DIV14
DIV17
DIV21
Total Wild-type
Total C/EBPδ -/-
CD11b Wild-type
CD11b C/EBPδ -/-
B
GFAP
CD11b
Wild-type
C/EBPδ -/-
C
GFAP protein
GFAP/Actin
1.5
1.0
0.5
0.0
C
LPS
LPS+IFNγ
Wild-type
C/EBPδ -/-
Wild-type
C/EBPδ -/-
C
LPS
LPS+IFNγ
C
LPS
LPS+IFNγ
GFAP
Actin

Figure 2

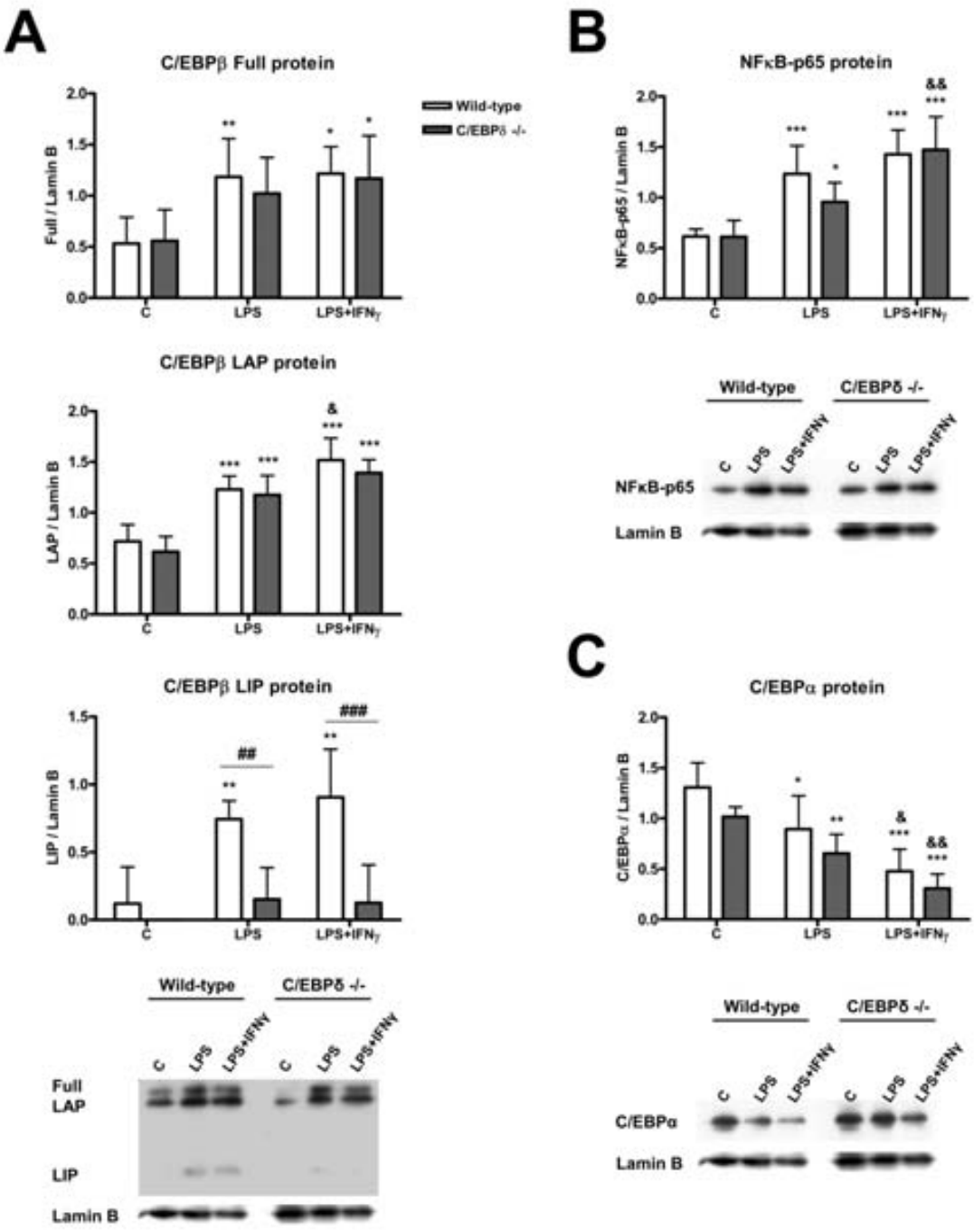
A
C/EBPβ Full protein
Wild-type
C/EBPδ -/-
Full / Lamin B
C
LPS
LPS+IFNγ
C/EBPβ LAP protein
LAP / Lamin B
C/EBPβ LIP protein
LIP / Lamin B
Wild-type
C/EBPδ -/-
Full
LAP
LIP
Lamin B
B
NFκB-p65 protein
NFκB-p65 / Lamin B
NFκB-p65
Lamin B
C
C/EBPα protein
C/EBPα / Lamin B
C/EBPα
Lamin B

Figure 3

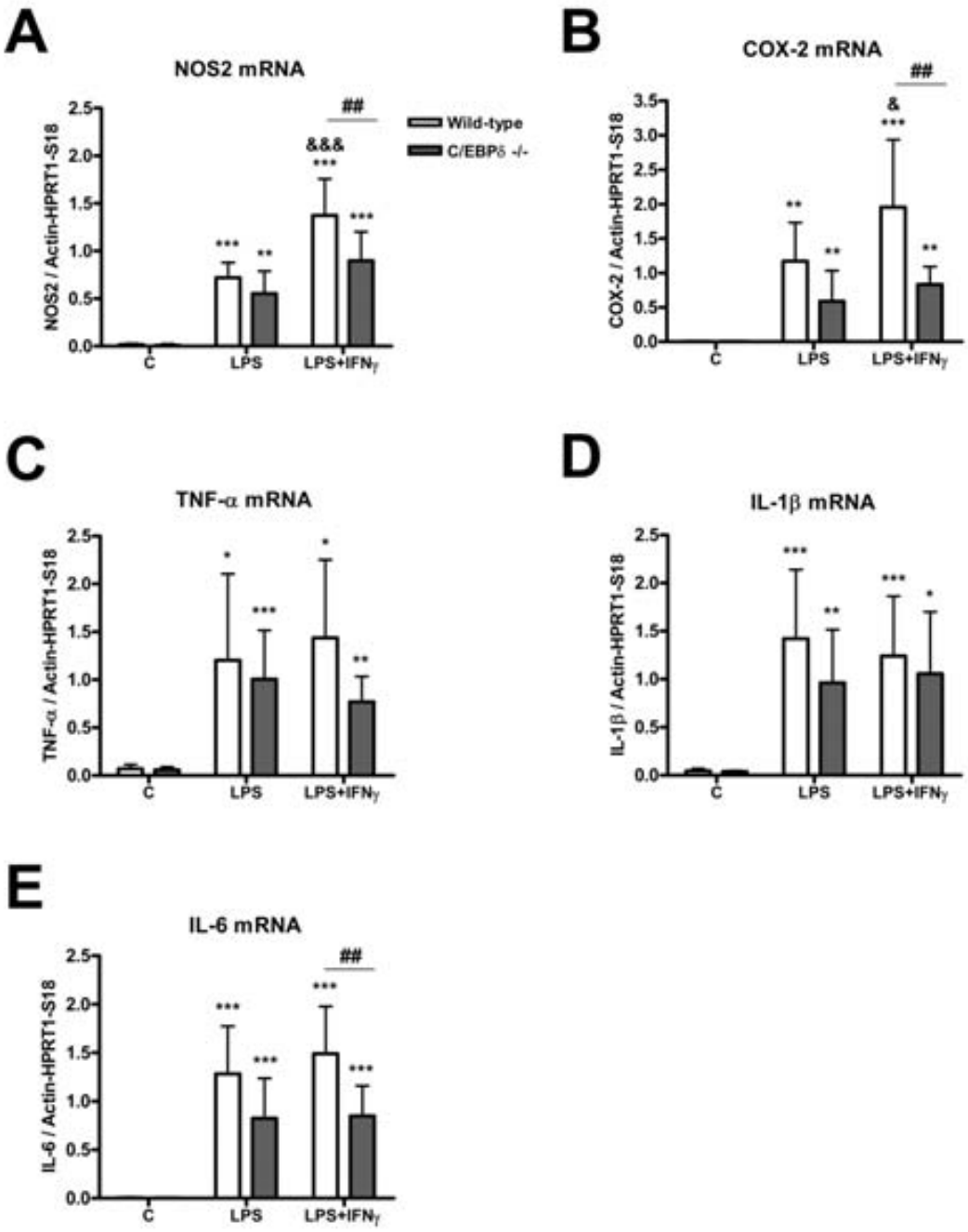
A
NOS2 mRNA
NOS2 / Actin-HPRT1-S18
Wild-type
C/EBPδ -/-
##
&&&
***
C
LPS
LPS+IFNγ
B
COX-2 mRNA
COX-2 / Actin-HPRT1-S18
##
&
C
LPS
LPS+IFNγ
C
TNF-α mRNA
TNF-α / Actin-HPRT1-S18
C
LPS
LPS+IFNγ
D
IL-1β mRNA
IL-1β / Actin-HPRT1-S18
C
LPS
LPS+IFNγ
E
IL-6 mRNA
IL-6 / Actin-HPRT1-S18
##
C
LPS
LPS+IFNγ

Figure 4

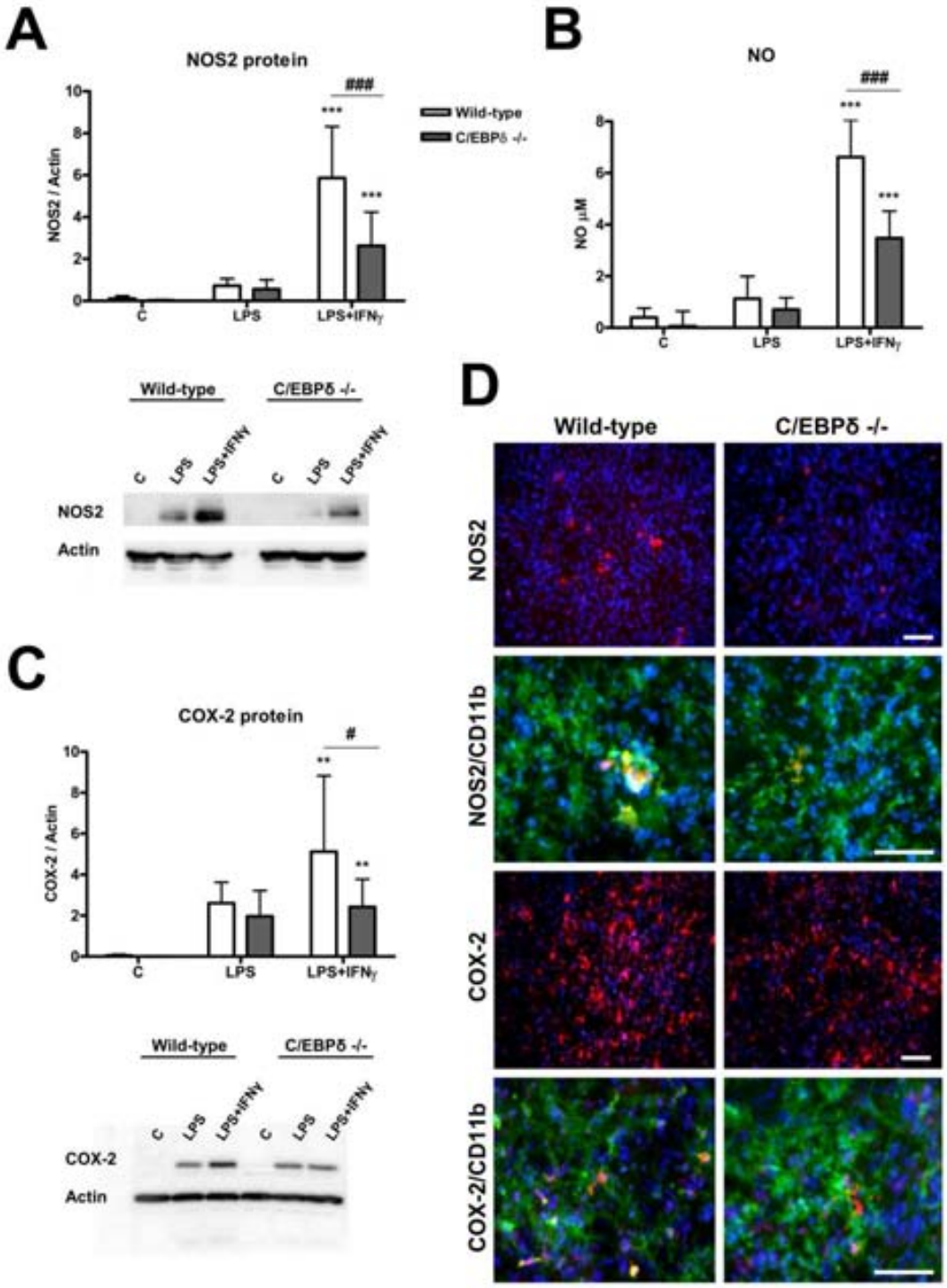
A
NOS2 protein
NOS2 / Actin
Wild-type
C/EBPδ -/-
C
LPS
LPS+IFNγ
###
***
Wild-type
C/EBPδ -/-
NOS2
Actin
B
NO
NO µM
C
D
Wild-type
C/EBPδ -/-
NOS2
NOS2/CD11b
COX-2
COX-2/CD11b
C
COX-2 protein
COX-2 / Actin
#
**
Wild-type
C/EBPδ -/-
COX-2
Actin

Figure 5

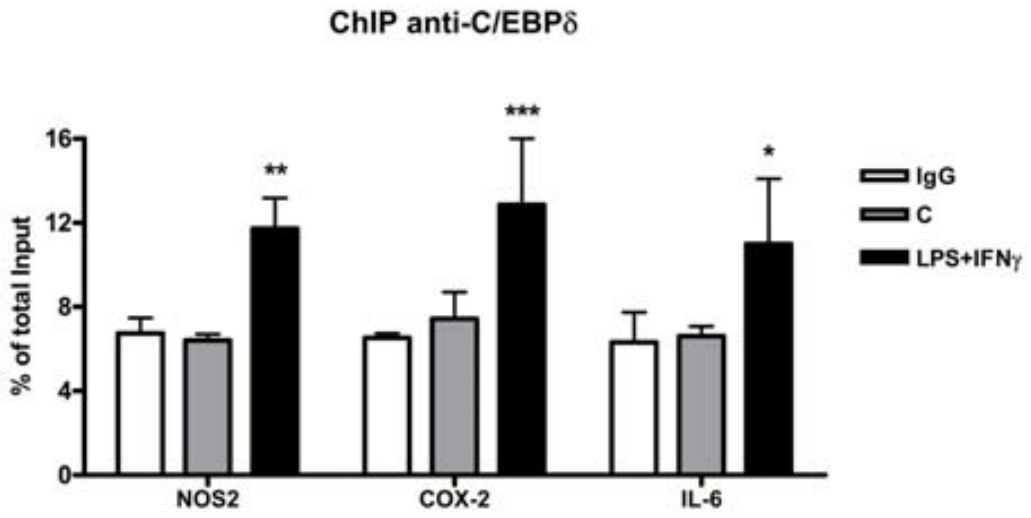
ChIP anti-C/EBPδ
% of total Input
16
12
8
4
0
**
***
*
NOS2
COX-2
IL-6
IgG
C
LPS+IFNγ

Figure 6

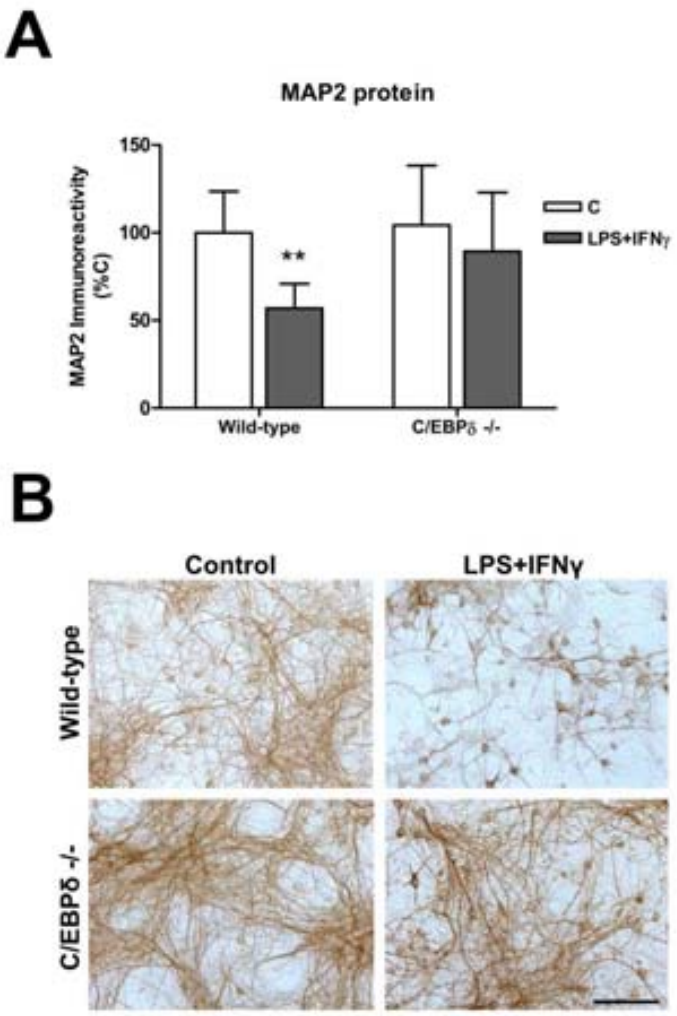
A
MAP2 protein
MAP2 Immunoreactivity (%C)
150
100
50
0
**
C
LPS+IFNγ
Wild-type
C/EBPδ -/-
B
Control
LPS+IFNγ
Wild-type
C/EBPδ -/-

Figure 7

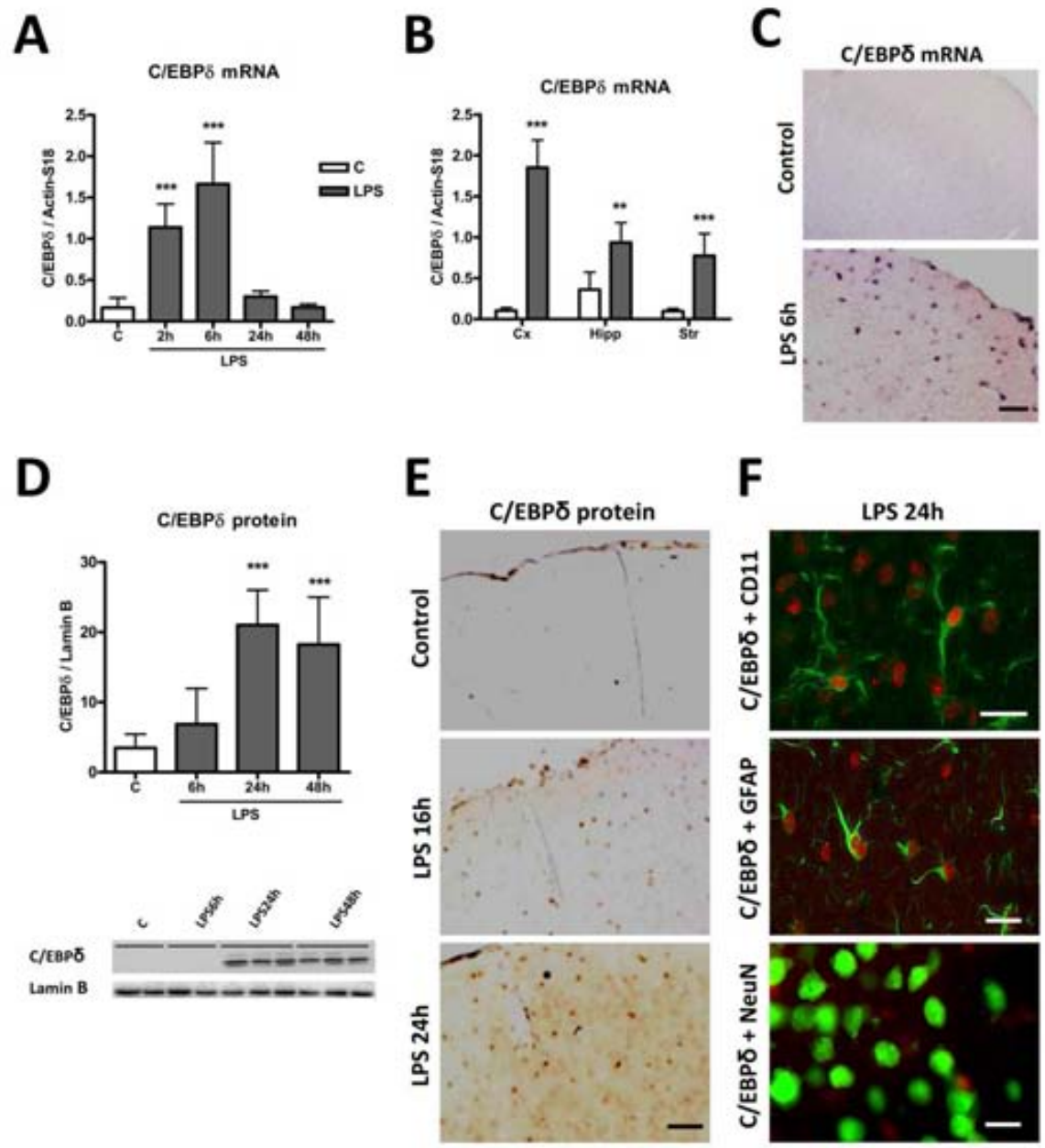

Figure 8

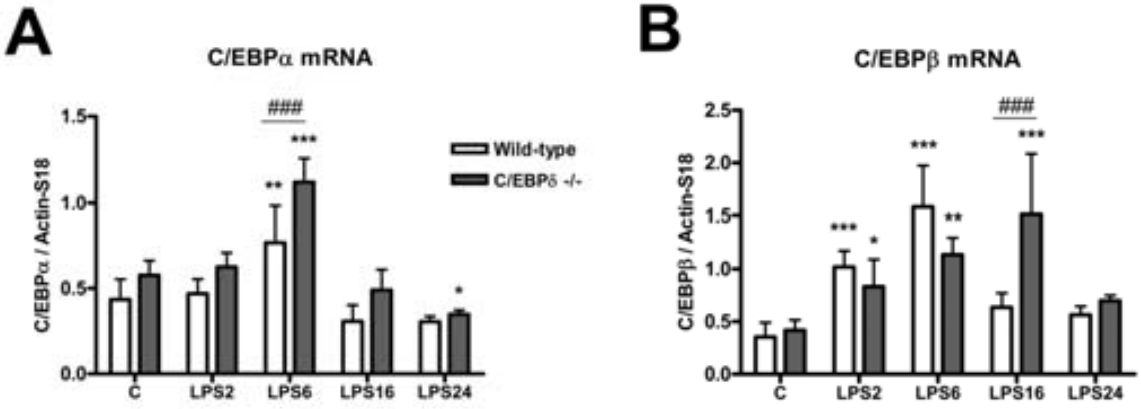

Figure 9

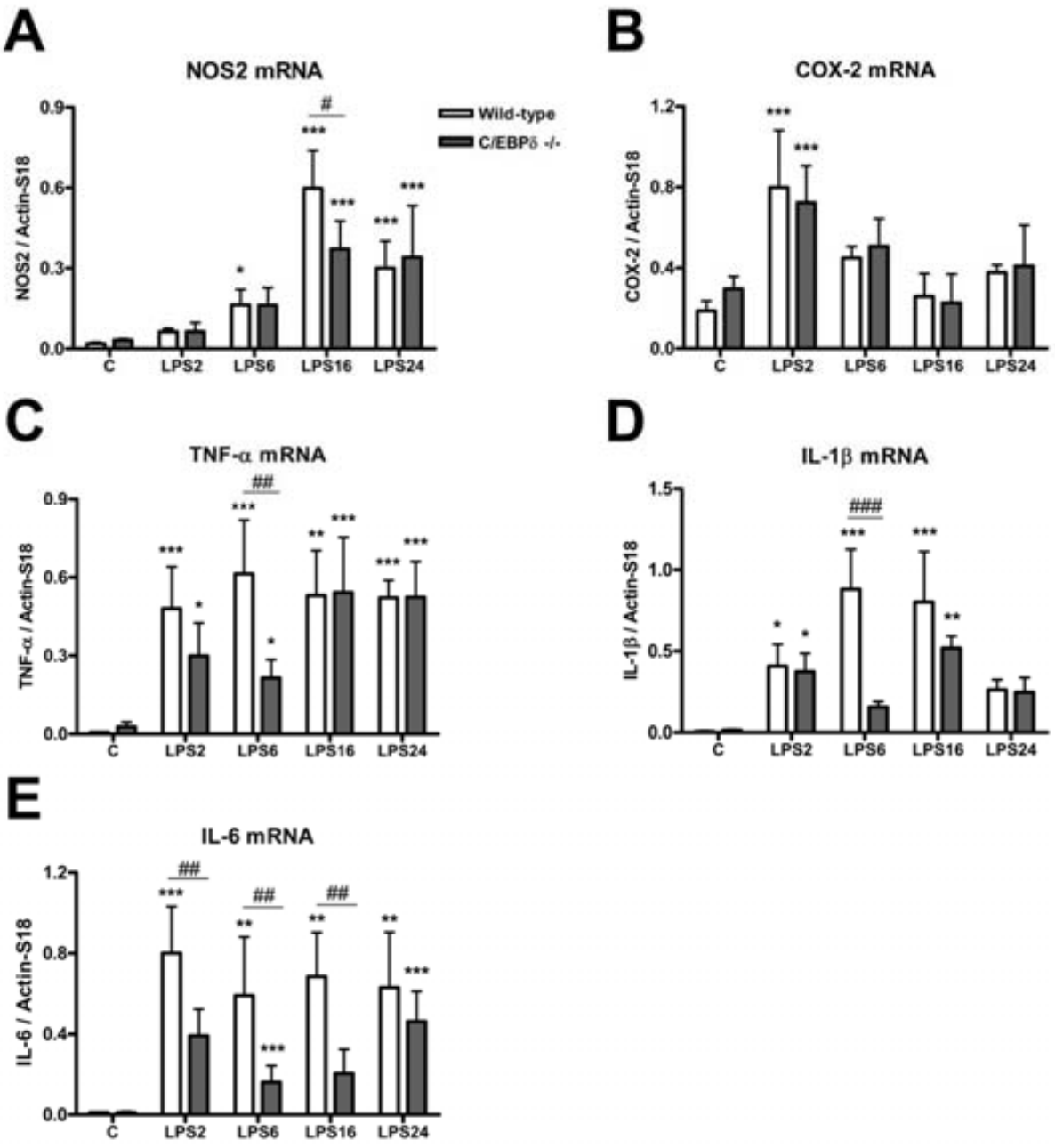
A
NOS2 mRNA
NOS2 / Actin-S18
Wild-type
C/EBPδ -/-
C
LPS2
LPS6
LPS16
LPS24
B
COX-2 mRNA
COX-2 / Actin-S18
C
TNF-α mRNA
TNF-α / Actin-S18
D
IL-1β mRNA
IL-1β / Actin-S18
E
IL-6 mRNA
IL-6 / Actin-S18

Figure 10

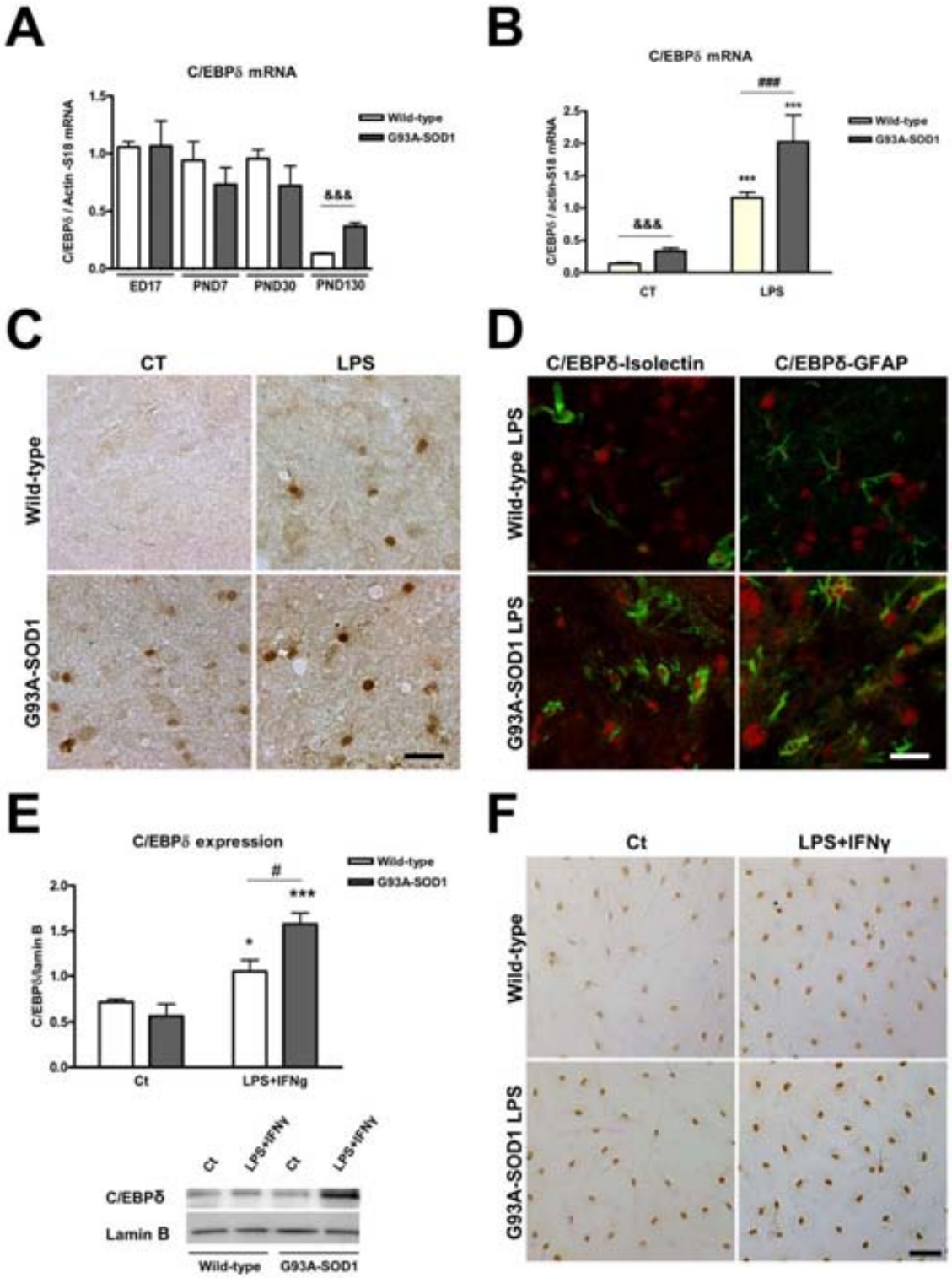
A
C/EBPδ mRNA
C/EBPδ / Actin -S18 mRNA
Wild-type
G93A-SOD1
&&&
ED17
PND7
PND30
PND130
B
C/EBPδ mRNA
###
***
***
&&&
C/EBPδ / actin-S18 mRNA
Wild-type
G93A-SOD1
CT
LPS
C
CT
LPS
Wild-type
G93A-SOD1
D
C/EBPδ-Isolectin
C/EBPδ-GFAP
Wild-type LPS
G93A-SOD1 LPS
E
C/EBPδ expression
Wild-type
G93A-SOD1
#
***
*
C/EBPδ/lamin B
Ct
LPS+IFNg
Ct
LPS+IFNγ
Ct
LPS+IFNγ
C/EBPδ
Lamin B
Wild-type
G93A-SOD1
F
Ct
LPS+IFNγ
Wild-type
G93A-SOD1 LPS

Figure 11

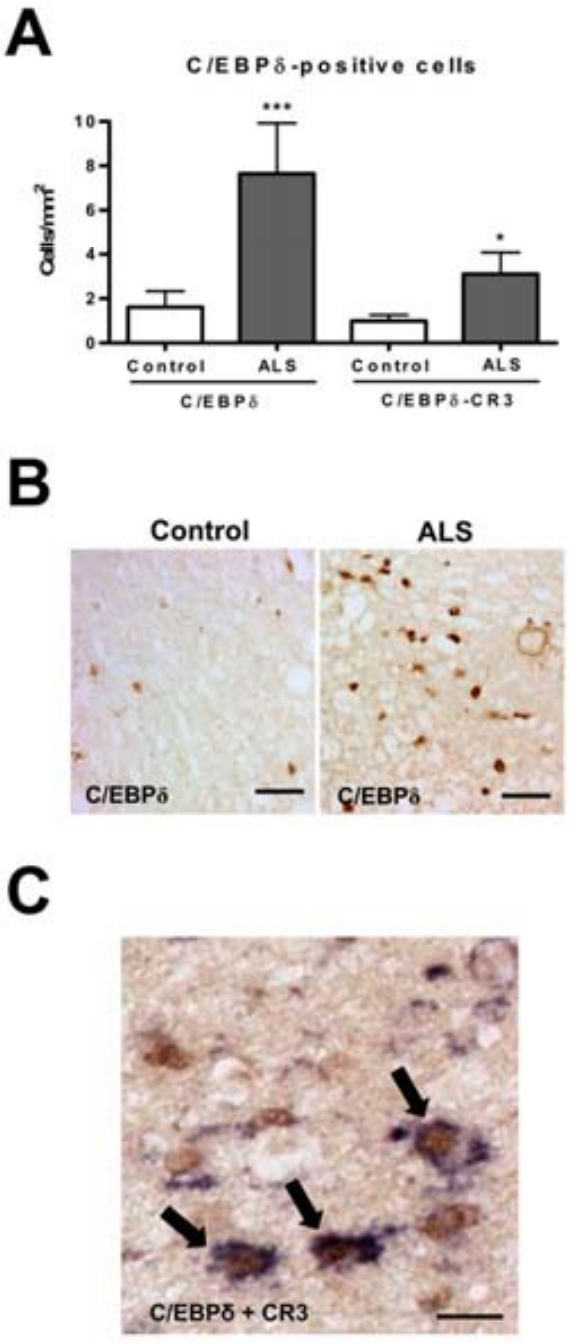
A
C/EBPδ-positive cells
***
*
Cells/mm²
10
8
6
4
2
0
Control
ALS
Control
ALS
C/EBPδ
C/EBPδ-CR3
B
Control
ALS
C/EBPδ
C/EBPδ
C
C/EBPδ + CR3

Table 1

Primers used in qRT-PCR experiments. Housekeeping genes used for normalization are marked with an asterisk.

Target Gene	Accession number	Forward primer (5→3')	Reverse primer (5→3')
NOS2	NM_010927.3	GGCAGCCTGTGAGACCTTTG	GCATTGGAAGTGAAGCGTTTC
COX-2	NM_011198.3	TGCAGAATTGAAAGCCCTCT	CCCCAAAGATAGCATCTGGA
TNFα	NM_013693.2	TGATCCGCGACGTGGAA	ACCGCCTGGAGTTCTGGAA
IL-1β	NM_008361.3	TGGTGTGTGACGTTCCCATTA	CAGCACGAGGCTTTTTTGTTG
IL-6	NM_031168.1	CCAGTTTGGTAGCATCCATC	CCGGAGAGGAGACTTCACAG
C/EBPα	NM_007678.3	TGGACAAGAACAGCAACGAGTAC	TGCGCAGGCGGTCATT
C/EBPβ	NM_009883.3	AAGCTGAGCGACGAGTACAAGA	GTCAGCTCCAGCACCTTGTG
C/EBPδ	NM_007679.4	CTCCACGACTCCTGCCATGT	GAAGAGGTCGGCGAAGAGTTC
Actin*	NM_007393.3	CAACGAGCGGTTCCGATG	GCCACAGGATTCCATACCCA
HPRT1*	NM_013556.2	ATCATTATGCCGAGGATTTGG	GCAAAGAACTTATAGCCCCC
Rn18s*	NR_003286.2	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG

Table 2

C/EBPδ binding sites and primers used in quantitative ChIP assay

Target Gene	C/EBP box	Forward primer (5→3')	Reverse primer (5→3')
NOS2	GGAGTGAAGCAATGA	TTATGAGATGTGCCCTCTGC	CCACCTAAGGGGAACAGTGA
COX-2	TTCTTGCGCAACTC	CGGCTTCCTTCGTCTCTCAT	ACAACTGGCTGCTAATGGGG
IL-6	GTTTCCAATCAGCCC	GTTGTGATTCTTTCGATGCT	GGAATTGACTATCGTTCTTG

7. DISCUSSION

7. DISCUSSION

Glial activation represents the CNS innate immune response during neuroinflammation. In glial activation, astrocytes and microglia show morphological changes, proliferation and production of a wide range of molecules, such as neurotrophic factors, reactive oxygen/nitrogen molecules or pro- and anti-inflammatory cytokines. These enormous changes in glia are the result of an important transcriptional reprogramming of gene expression. We have undertaken this study to elucidate whether C/EBPβ and δ are responsible to orchestrate the gene expression reprogramming in activated glial cells.

The analysis of around 5000 bp in the upstream region of major pro-inflammatory gene promoters (NOS2, COX2, IL1β, IL6, TNFα and PTGES) shows that the frequency of C/EBP consensus sites is significantly higher than that of other transcription factor binding sites (data not shown). This observation highlights the relevance of C/EBP family, suggesting an important role in the transcriptosome re-organization during the pro-inflammatory response. In agreement with this, a role for C/EBPβ and C/EBPδ in inflammatory mediators expression has been demonstrated in microglial cell lines and their upregulation has been shown in glial cells *in vitro* and *in vivo* during glial activation[218,219,227,290,291]. In addition, C/EBPβ and C/EBPδ have been implicated in brain tumors[298], cerebral ischemia[226], ALS[119] and AD[228,229,297], which are neurological disorders where neuroinflammation is highly involved. However, the direct involvement of C/EBPβ and C/EBPδ in glial pro-inflammatory gene expression or the relative contribution of these transcription factors to the neurotoxic phenotype of microglial cells had not been demonstrated until now. In this context, our aim was the analysis of C/EBPβ and C/EBPδ role in neurotoxic glial activation in inflammatory *in vitro* and *in vivo* models.

7.1 LPS versus LPS+IFNγ

In this study, we have analyzed the glial response under two different stimuli, LPS and LPS+IFNγ. To our knowledge, this is the first time that these two activation protocols are used in the same study and their effects are compared in mixed glial cultures. In general, the two stimuli exert similar effects on our glial cultures, but it is interesting to focus on the few differences we have found, because they could explain some aspects of glial physiology. First of all, LPS and LPS+IFNγ produce different morphological changes on glial cells, especially on microglia (Straccia et al., unpublished results). We have observed that LPS induces a transition from a microglia with small ramifications to an amoeboid morphology. On the other hand, after LPS+IFNγ treatment some amoeboid cell bodies can still be found, but hypertrophic ramified microglia predominate. These qualitative morphological observations are concomitant with quantitative results of glial activity, as summarized in table 10.

Table 10: Summary of changes induced by LPS and LPS+IFNγ in wild-type mixed glial cultures. = denotes no changes compared to control condition. ↑ denotes an increase of target molecule levels, whereas ↓ denotes decreased expression of target molecules compared to control. ↑↑ denotes a significant increase compared to LPS condition. In no case, LPS induced higher expression than LPS+IFNγ.

			LPS	LPS+IFNγ
Wild-type Mixed glia cultures	mRNA	IL1β	↑	↑
		IL4	=	=
		IL6	↑	↑
		TNFα	↑	↑
		NOS2	↑	↑↑
		PTGS1	=	=
		COX2	↑	↑
		PTGES	↑	↑
		PTGIS	=	=
		PTGDS	=	=
		ALOX5	↓	↓
		TBAXS1	↓	↓
		TGFβ1	=	=
		POR	=	=
	Protein	IL1β	↑	↑
		NOS2	↑	↑↑
		COX2	↑	↑↑
		PTGES	↑	↑
		gp91PHOX	↑	↑↑
	Bio-products	NO	↑	↑↑
		PGE2	↑	↑

LPS+IFNγ stimulus is able to upregulate NOS2 mRNA and protein levels more than LPS treatment alone. This phenomenon is also true for COX2 and gp91, the catalytic subunit of PHOX2 (Straccia et al., unpublished results). We therefore observe a potentiation of some LPS effects by IFNγ, in particular on those enzymes that are fundamental for reactive oxygen and nitrogen species production. The synergy of IFNγ with LPS was already observed in peripheral macrophages[299], but how this potentiation is triggered and why it is specific only for some effectors has been poorly investigated.

We can roughly classify the pro-inflammatory markers we have studied based on their LPS±IFNγ responsiveness. In this view, cytokines seem not to be affected by IFNγ, whereas LPS induction of NOS2, COX2 and gp91 is clearly potentiated by IFNγ. However, we cannot exclude that expression kinetics is different after LPS of LPS+IFNγ because we have analyzed only a time point. In addition, this does not exclude other changes, occurring at other levels of transcriptional regulation as we will discuss later. We have also studied the phagocytic activity

of microglia in mixed glial culture and we have found that LPS+IFNγ induces a significant increase of phagocytosis compared to LPS treatment alone (Straccia et al., unpublished results). Now, focusing on the neuronal viability assay we have performed, we showed only the LPS+IFNγ treatment. Our choice is due to the lack of microglial neurotoxic effect after stimulation with LPS alone. We can hypothesize that neurotoxicity triggered by LPS+IFNγ-activated microglia can be due to the observed higher production of NO. In addition, we have observed the LPS+IFNγ-induced over-expression of PHOX2 catalytic subunit (Straccia et al., unpublished results), which could be an indirect evidence of a higher production of reactive oxygen species compared to LPS-activated microglia, as previously proposed[93]. It is noteworthy to remember that the production of the analyzed cytokines does not change between LPS and LPS+IFNγ. These results show that glial response induced by these two stimuli is not equal, especially for microglia. Therefore, we can hypothesize that the corresponding gene transcriptional re-programming will also be different. Consequently the composition and the relative contribution of the involved transcription factor complexes could also change. In this view, we could give a cautionary advice to other groups who use these two stimuli without distinction.

7.2 C/EBPβ and C/EBPδ roles in LPS- or LPS+IFNγ-induced glial activation

Glial activation, as we already mentioned, is a very heterogeneous process with an enormous variety of triggering stimuli and several phenotypical responses. In this thesis, we have demonstrated that LPS and LPS+IFNγ are not equivalent and they can induce two different types of glial activation phenotypes. We are mainly interested in C/EBPβ and C/EBPδ role in neurotoxic glial activation that we have shown to be triggered by LPS+IFNγ stimulus in this study. However, depending on the model, LPS alone can be neurotoxic. Hence, it is interesting to analyze the possible contribution of C/EBP transcription factors in different patterns of glial activation, especially because we do not know which one can better mimic an *in vivo* situation.

In the three articles that constitute this thesis, we have already discussed in detail the data obtained in each study. Now we would like to overview and discuss all results as a whole. In order to simplify the overview of all results coming from the analysis of C/EBPβ- and C/EBPδ-null glial cultures, a summarizing table follows:

Table 11: Summary of changes induced by C/EBPβ and C/EBPδ deficiency reported in this thesis. = denotes no changes. ↑ denotes an increase of C/EBPβ- or C/EBPδ-binding to target promoters in wild-type glial cultures. ↓ denotes a decreased expression of target molecules in C/EBPβ- or C/EBPδ-null primary cultures when compared to wild-type; n.d.: not determined.

			C/EBPβ		C/EBPδ	
			LPS	LPS+IFNγ	LPS	LPS+IFNγ
Mixed glia cultures	qChIP	IL1β	=	↑	n.d.	n.d.
		IL6	=	↑	=	=
		TNFα	=	↑	n.d.	n.d.
		NOS2	↑	↑↑	↑	↑
		COX2	↑	↑↑	=	↑
		PTGES	↑	↑	n.d.	n.d.
	mRNA	IL1β	=	↓	=	=
		IL6	=	=	=	↓
		TNFα	=	=/↓	=	=
		NOS2	↓	↓	=	↓
		PTGS1	=	↑	=	=
		COX2	=	=	=	↓
		PTGES	↓	↓	=	=
		PTGIS	=	=	=	=
		PTGDS	=	=	n.d.	n.d.
		ALOX5	=	=	n.d.	n.d.
		TBAXS1	=	=	n.d.	n.d.
		POR	=	=	n.d.	n.d.
	Protein	IL1β	=/↓	=/↓	n.d.	n.d.
		NOS2	=	↓	=	↓
		COX2	=	↓	=	↓
		PTGES	=/↓	↓	n.d.	n.d.
	Bio-products	NO	=	↓	=	↓
		PGE2	↓	↓	n.d.	n.d.

At first sight, table 11 shows that the most relevant effects of these two transcription factors are produced when glial cultures are activated by LPS+IFNγ. This is intriguing, considering the high neurotoxic potential of microglia activated by LPS+IFNγ and that C/EBPβ or C/EBPδ deficiency can prevent neuronal death, as demonstrated by neuronal viability assays. We must highlight that the LPS+IFNγ-induced cytokine expression is also affected by the absence of C/EBPβ and C/EBPδ, not only the reactive oxygen/nitrogen species enzymes. This means that even though cytokine levels are not differentially affected by the two glial activation protocols, the transcription factor composition changes on their promoters. In fact, C/EBPs do not seem to be involved in LPS-induced transcription of cytokine genes, but when IFNγ is also present

their transcription depends on C/EBPβ and C/EBPδ activity, as clearly shown by decreased expression of cytokine genes in C/EBPβ- or C/EBPδ-null glial cultures. Therefore we can hypothesize that C/EBPs are involved in the phenotypical change of glia, especially of microglia, from an activated to a neurotoxic state, which in our model is triggered by the synergy between IFNγ and LPS. The potentiation and changes in transcriptional regulation triggered by IFNγ are noteworthy, because IFNγ is an endogenous cytokine. We can hypothesize that during a bacterial infection, LPS can induce IFNγ release by peripheral immune system and both molecules could cross the BBB and trigger an exacerbated glial response. Our study sheds some light on whether similar stimuli can trigger distinct glial activation and on whether not all glial activation states can be detrimental for neurons.

7.3 C/EBPβ versus C/EBPδ

Table 11 shows that C/EBPβ deficiency has a major effect in pro-inflammatory glial activation compared to C/EBPδ. Focusing on these differences, we can observe how C/EBPδ deficiency has no effect on LPS-induced pro-inflammatory glial expression, whereas C/EBPβ is involved in NOS2 and PTGES expression. Under LPS+IFNγ stimulus C/EBPβ binds to more pro-inflammatory gene promoters than C/EBPδ. Furthermore C/EBPβ deficiency produces a transcriptional decrease in more genes than C/EBPδ. We could explain this difference from a biochemical point of view. As we observed in EMSA experiments in the first article, C/EBPβ supershift removes most of the complexes, suggesting that C/EBPδ homodimers are not as abundant as C/EBPβ-containing complexes. However, it does not mean that C/EBPδ is not present in those shifted complexes as a C/EBPβ partner in heterodimers. C/EBPδ supershift would be a key experiment to understand how abundant C/EBPδ-containing complexes are. An alternative explanation exists; C/EBPδ-null mixed glial cultures show a significant decrease of C/EBPβ-LIP protein levels in activated mixed glial cultures, as shown in the third article. C/EBPβ-LIP is an inhibitory isoform which can homo- or hetero-dimerize with other leucine zipper transcription factors, preventing their interaction with DNA. In this view, decreased C/EBPβ-LIP protein levels could mean that more C/EBPβ activating isoforms are able to interact with DNA and to induce gene transcription. Therefore, C/EBPδ deficiency could be compensated by an increase of available C/EBPβ activating isoforms and the final effect on pro-inflammatory effector levels would be less evident. It is important to note that in C/EBPβ-null mixed glial cultures the genetic deletion eliminates all C/EBPβ isoforms, including C/EBP-LIP. Hence, in this model where an inhibitory isoform is also absent, an increased number of bZIP transcription factors are available to interact with the DNA and could partly compensate the lost function of C/EBPβ-activating isoforms. However *in vitro* results of C/EBPβ study clearly show the effects of C/EBPβ deficiency, consequently even if a compensation mechanism by other bZIP transcription factor exists, it is not enough to fully compensate C/EBPβ absence from a functional point of view.

There are also technical issues that could be important in the comparison analysis of C/EBPβ- and C/EBPδ-null glial culture studies. Mixed glial cultures used in the two studies are processed with different protocols. As mentioned in the first article, C/EBPβ-null female mice are sterile and 50% of mice die perinatally, consequently the number of available mice as well as the

number of glial cells obtained from one embryonic cortex is low. For these reasons, we had to optimize a primary culture protocol for the C/EBPβ study. The optimized protocol consists of processing each embryonic cortex separately; when confluent, each primary culture is subcultured to final culture plates. On the other hand, primary mixed glial cultures were processed for the C/EBPδ study from a pool of cortices coming from post-natal animals of the same genotype and directly seeded in the final culture plates. We are currently comparing the different methods of primary glial culturing used in the laboratory. Early results show that the subculturing process leads to an increase in the number of Iba1-positive microglia. However microglial density and total cell number in C/EBPβ and C/EBPδ studies is very similar. In addition, when the same amount of C/EBP deficient microglial cells are used in the neurotoxicity assay, C/EBPδ-null microglia show a minor protective effect compared to C/EBPβ-null microglia. Therefore we can hypothesize that C/EBPβ has a major role than C/EBPδ in the activation of microglia.

7.4 C/EBPβ and C/EBPδ show overlapping activity

In activated mixed glial cultures, the absence of C/EBPβ or C/EBPδ results in different effects in IL1β, IL6 and PTGES gene expression. In the case of NOS2 and COX2 similar effects are observed, suggesting a complementary function. Both transcription factors bind to NOS2 and COX2 promoters and their absence especially affects LPS+IFNγ-induced protein levels. Hence we can hypothesize that both factors regulate the transcription of NOS2 and COX2 synergistically, probably as heterodimers, as our previous results have shown[219]. In experiments not included in this thesis we have observed that LPS- or LPS+IFNγ-induced NO production is completely abrogated in C/EBPβ-null mixed glial cultures treated with chrysin, a flavonoid that decreases C/EBPδ levels (Straccia et al., unpublished results). These observations indicate that C/EBPβ and C/EBPδ are key contributors to NOS2 expression in this model. It would be interesting to analyze COX2 and cytokine expression in the same double inhibition experiment. These partially overlapping functions suggest that both transcription factors are necessary to trigger the full transcriptosome re-programming in order to induce a neurotoxic microglial phenotype.

7.5 Mixed glial cultures limitations and possible solutions

In our opinion mixed glial culture is a better cell biology model to study microglial activation compared to enriched microglial cultures. The presence of astrocytes and microglia in the same preparation allows cross-talk between both cell types which is of physiological importance[300]. However from a biochemical point of view, this system has limitations to dissect signaling always at a molecular level and to distinguish which is the cell type responsible for the observed effect. The alternatives to mixed glial cultures are microglial- or astroglial-enriched cultures, in which yield is a real limitation to perform certain experiments, or astroglial/microglial cell lines, in which the yield problem is solved but distortions of the cell cycle can lead to artifactual results. Regarding mixed glial cultures, an interesting aspect that we have not addressed in this thesis is the difference in microglial cells located in different

layers of culture and therefore exposed to a different environment. Thus, we have observed that microglial cells under or in the astrocytic layer and in contact with the flask or plate plastic behave differently than microglia on top of the astrocytic layer (Straccia et al., unpublished observations).

7.6 Systemic LPS in C/EBP deficient mice

In this thesis, we have analyzed pro-inflammatory gene expression in the cerebral cortex of systemic LPS-treated mice to study C/EBPs contribution to neuroinflammation *in vivo*. The following table summarizes the results obtained:

Table 12: Summary of changes in mRNA expression induced by C/EBPβ and C/EBPδ deficiency in mouse cortex in the systemic LPS model. = denotes no changes; ↑ and ↓ denote an increased or decreased expression of mRNAs, respectively, in C/EBPβ- or C/EBPδ-null mice when compared to wild-type; n.d.: not determined; @: undetectable.

		C/EBPβ					C/EBPδ				
		Control	LPS				Control	LPS			
			2h	6h	16h	24h		2h	6h	16h	24h
CNS *in vivo* (Cortex)	IL1β	=	=	=	n.d.	n.d.	=	=	↓	=	=
	IL6	=	=	=	n.d.	n.d.	=	↓	↓	↓	=
	TNFα	=	=	=	n.d.	n.d.	=	=	↓	=	=
	NOS2	=	=	=	n.d.	n.d.	=	=	=	↓	=
	PTGS1	=	=	=	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	COX2	=	=	=	n.d.	n.d.	=	=	=	=	=
	PTGES	=	=/↓	↓	n.d.	n.d.	=	=	=	=	=
	PTGIS	=	=	=	n.d.	n.d.	=	=	=	n.d.	n.d.
	PTGDS	=	=	=	n.d.	n.d.	↑	=	=	n.d.	n.d.
	ALOX5	=	=	=	n.d.	n.d.	=	=	=	n.d.	n.d.
	TBAXS1	=	=	=	n.d.	n.d.	↓	↓	=	n.d.	n.d.
	POR	=	=	=	n.d.	n.d.	=	=	=	n.d.	n.d.
	C/EBPα	=	=	=	n.d.	n.d.	=	=	↑	=	=
	C/EBPβ	@	@	@	@	@	=	=	=	↑	=
	C/EBPδ	=	=	=	n.d.	n.d.	@	@	@	@	@

The analysis of gene expression in C/EBPβ-null mice could only be performed at two time-points. This is a consequence of the intrinsic limitation of this mouse colony, from which is extremely difficult to obtain adult mice. In any case, table 12 shows that C/EBPβ deficiency affects only PTGES expression in this *in vivo* model. Nevertheless we cannot exclude that effects of C/EBPβ absence could be observed at longer time points. On the other hand, C/EBPδ deficiency *in vivo* affects gene expression of more pro-inflammatory mediators in mouse brain cortex than C/EBPβ absence. A limitation of this study is that we cannot distinguish which cell types are contributing to the effects caused by C/EBPβ or C/EBPδ deficiency *in vivo*; regarding this we have started to generate a microglial conditional knock-out mouse for C/EBPβ.

7.7 C/EBPβ or C/EBPδ as therapeutical target

A remarkable effect is that C/EBPβ or C/EBPδ absence does not completely abrogate the pro-inflammatory response, supporting their complementary role in the pro-inflammatory neurotoxic glial activation. This complementary aspect of C/EBPβ and C/EBPδ is interesting when considering their pharmacological inhibition as a therapy for neurological diseases. In fact, we can imagine inhibiting selectively one of these two transcription factors in order to ameliorate inflammation-associated neurodegeneration, without the complete abrogation of microglial innate immune response, especially phagocytosis activity. However, the analysis of M2-polarized C/EBPβ- or C/EBPδ-null glial culture is still necessary to verify that the anti-inflammatory response is not compromised by inhibition of these C/EBPs.

7.8 A model for C/EBPβ and C/EBPδ in microglial neurotoxicity.

As conclusion, we would like to present a model for C/EBPβ and C/EBPδ roles in microglial activation integrating previous data with the results of this thesis (figure 15). In this model, LPS induces a pro-inflammatory activation of microglia through several receptors as TLRs[219,227]. In this situation, the transcriptosome re-programming from surveying to reactive microglia is only partially dependent on C/EBPβ and C/EBPδ. On the other hand, the combination of external stimuli, such as LPS, with endogenous ones, such as IFNγ, potentiates the innate immune response of microglia, triggering a stronger pro-inflammatory response with neurotoxic effects. In this case, the IFNγ potentiation of pro-inflammatory enzymes as well as the induction of pro-inflammatory cytokines depends on C/EBPβ and C/EBPδ DNA binding activity, which probably leads to a functional substitution of other transcription factors on target gene promoters.

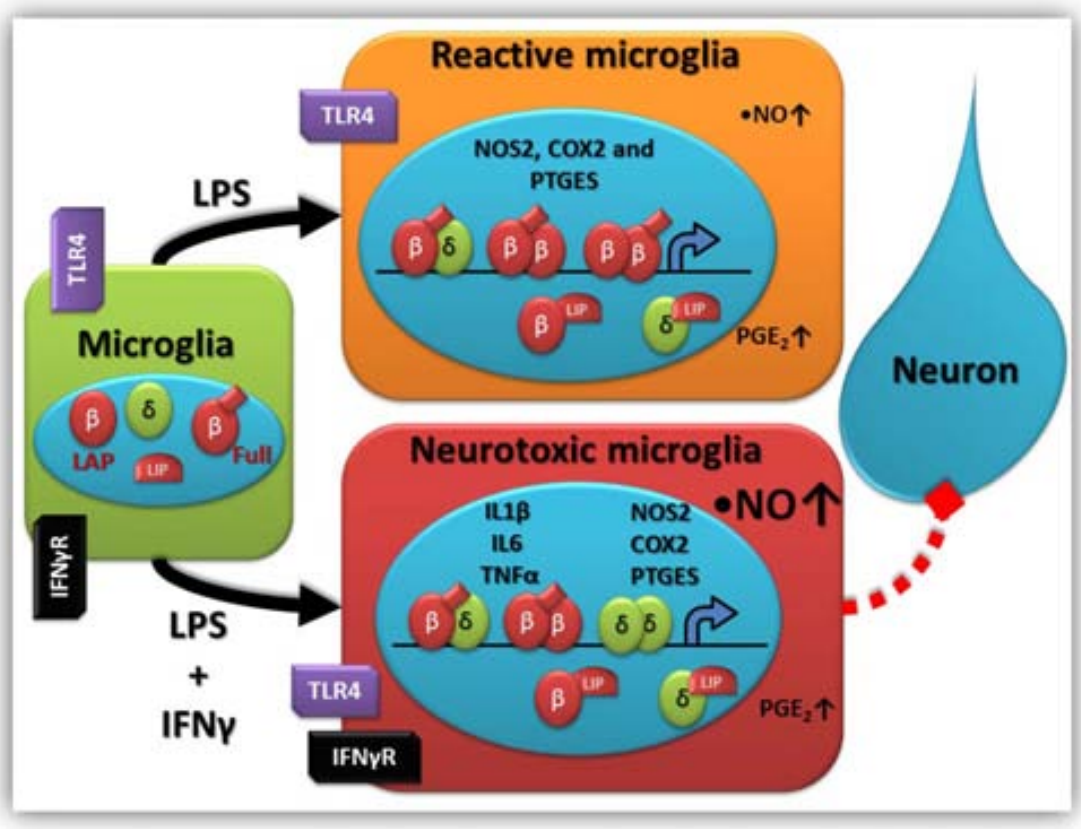

Figure 15: Proposal of a new microglial activation model. LPS induces the change from surveying microglia (green) to reactive pro-inflammatory microglia (orange) through TLR4 activation. TLR4 signaling pathway activates C/EBPβ and C/EBPδ that can bind to PTGES, COX2 and NOS2 gene promoters. At the same time, C/EBPβ and C/EBPδ gene transcription is up-regulated through a MAPKs-dependent mechanism. LPS-induced cytokine gene expression is independent of C/EBPβ and C/EBPδ. On the other hand, LPS and IFNγ can hyper-activate microglia through a C/EBPβ- and C/EBPδ-dependent transcriptional reprogramming. C/EBPβ and C/EBPδ activities replace other transcription factor function, directly controlling NOS2, COX2, PTGES and cytokine gene expression. This pro-inflammatory hyper-activation has toxic effects on neurons, which could participate in neurodegeneration in many neurological diseases.

8. CONCLUSIONS

8. CONCLUSIONS

1. LPS and LPS+IFNγ treatments induce different patterns of glial activation in mixed glial cultures. LPS-induced NOS2 and COX2 expressions are potentiated by IFNγ, whereas cytokine genes are not. However results from C/EBP deficient mice have shown that both treatments cause a different transcriptional regulation of analyzed cytokine genes.

2. Only LPS+IFNγ stimulus is able to induce a neurotoxic phenotype of microglia *in vitro*, whereas LPS alone induces pro-inflammatory glial activation which does not show detrimental effects for neurons.

3. Microglia are the main NOS2, COX2 and PTGES expressing cells in mixed glial cultures.

4. The bioinformatic analysis has shown a high frequency of C/EBPs consensus sites in inflammatory gene promoters. For the first time, qChIP experiments have shown that C/EBPβ and C/EBPδ rapidly bind many of these consensus sites, proximal to the transcription starting site, in activated mixed glial cultures.

5. C/EBPβ and C/EBPδ levels and early DNA binding activity are increased to the same extent by LPS and LPS+IFNγ in mixed glial cultures, but interestingly their ability to interact with inflammatory gene promoters is potentiated by IFNγ.

6. C/EBPβ and C/EBPδ do not play a role in astrocyte or microglia proliferation in mixed glial cultures. The absence of C/EBPβ or C/EBPδ does not affect cell composition or total cell number neither in untreated nor in activated mixed glial cultures.

7. C/EBPβ and C/EBPδ play a key role in the transcriptional regulation of specific pro-inflammatory effectors. C/EBPβ or C/EBPδ deficiency diminishes the up-regulation of IL1β, IL6, NOS2, COX2 and PTGES in activated mixed glial cells, especially after LPS+IFNγ stimulation.

8. Microglial neurotoxic potential depends on C/EBPβ or C/EBPδ activity *in vitro*. C/EBPβ or C/EBPδ deficiency abrogates the neurotoxic effects of microglia in neuronal-microglial co-cultures.

9. C/EBPβ and C/EBPδ play also an important role in pro-inflammatory response *in vivo*. Pro-inflammatory gene expression induced by systemic LPS in cerebral cortex is reduced in C/EBPβ- and C/EBPδ-null mice.

9. BIBLIOGRAPHY

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10. INDEXES

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"Carpe diem quam minimum credula postero"

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