Universidade do Minho
Escola de Ciências da Saúde

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The Choroid Plexus as a sensor of peripheral inflammation

Os Plexus Coroideus como sensores da inflamação periférica

Março de 2008

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ABSTRAT

The Choroid Plexus as a sensor of peripheral inflammation

Particular interest has been raised in the last few years, regarding the contribution of the immune system in neurological and psychiatric diseases. It is recognized that an inflammatory component underlies disorders such as Alzheimer's disease and multiple sclerosis. However, whether these are a consequence, a cause, or just a trigger of disease progression remains to be clarified. In this context, understanding how peripheral inflammatory stimulus reach the brain may contribute to better understand the mechanisms involved in such conditions. Most studies on the communication between the periphery and the central nervous system (CNS) focus on the blood-brain barrier, which is composed by the endothelial cells of the brain capillaries. However, the blood-cerebrospinal fluid barrier (BCSFB) may as well convey signals from the periphery into the CSF and backwards. The BCSFB is formed by the choroid plexus (CP) epithelial cells. Given its role as the major producer of the CSF, and the presence of several receptors and transporters both in the apical and in the basolateral membranes, the CP is ideally positioned to transmit signals between the immune and the CNS.

In the present study we address the CP response to inflammatory stimuli induced in the periphery. We show that the CP displays a specific, rapid and transient response to an acute inflammatory stimulus induced by the intraperitoneal administration of the Gram negative bacteria cell wall lipopolysaccharide (LPS). As soon as 1h after the injection, the CP responds by altering the expression of several genes. This response reaches a maximum at 3h, in which the level of 324 (out of 24 000 studied) transcripts are altered, and returns to the basal profile for most of the transcripts at 72h. Most of the up-regulated genes belong to immune-mediated cascades while those down-regulated encode for proteins involved in the maintenance of the CP barrier function.

When LPS is administered repetitively every 2 weeks for 3 months, the CP response persists for longer periods but is attenuated. The biological pathway with higher up-regulation includes genes encoding for proteins that facilitate cells entry into the CSF, which may be particularly relevant in diseases such as multiple sclerosis.

Of notice, in both conditions of acute and sustained inflammatory response, we observed altered expression of genes that participate in the innate immune response to infection by microorganisms, and that are related to iron homeostasis. In both conditions, the CP responds

by decreasing iron availability for bacterial growth. Specifically, lipocalin 2, a sequester for bacteria iron-loaded siderophores is rapidly secreted into the CSF, persisting in the sustained inflammatory conditions. In addition, the CP is here suggested to play an identical role of that played by the liver, in regulating iron uptake and release, through the synthesis and secretion of hepcidin. Of interest, iron deregulation has been implicated in neurodegenerative diseases including Alzheimer's disease and multiple sclerosis.

Taken together, the data presented here highlights the role of the CP in mediating immune signals into the brain. How, this ultimately corresponds to neuroprotective or deleterious consequences for the brain and how this may relate to diseases of the CNS needs to be further investigated.

RESUMO

Os Plexus Coroideus como sensores da inflamação periférica

Nos últimos anos tem aumentado o interesse no estudo da participação do sistema imunológico em doenças neurológicas e psiquiátricas. A inflamação é hoje aceite com estando presente em doenças como a de Alzheimer e a esclerose múltipla. No entanto, ainda está por esclarecer se esta é uma causa, consequência ou estímulo de progressão e agravamento da doença. Neste contexto, a compreensão do modo como estímulos inflamatórios periféricos influenciam o cérebro pode contribuir para a compreensão dos mecanismos envolvidos nestas doenças. A maioria dos estudos sobre a comunicação entre a periferia e o sistema nervoso central têm incidido na barreira hemato-encefálica, que é constituída pelas células endoteliais dos capilares sanguíneos. No entanto, a barreira sangue-líquido céfalo-raquidiano (BCSFB) pode, igualmente, participar na transmissão de sinais entre a periferia e o CSF. A BCSFB é formada pelas células epiteliais dos plexus coroideus (CP). Tendo como função principal a produção de CSF, e uma vez que possuem vários receptores e transportadores, tanto na membrana apical como na basolateral, os CP estão idealmente posicionados para facilitar a interacção entre os sistemas imunológico e nervoso central.

O trabalho desenvolvido nesta tese avalia a resposta dos CP a estímulos inflamatórios periféricos induzido pela administração intraperitoneal do lipopolissacarídeo (LPS) da parede de bactérias de Gram negativo. Quando este estímulo é agudo, observa-se uma resposta específica, rápida e transitória nos níveis de expressão de vários genes. Esta resposta é evidente 1h após a injecção, atinge um máximo às 3h, com a expressão diferencial de 324 (num total de 24 000 estudados) transcritos, e volta ao estado basal para a maioria dos transcritos ao fim de 72h. De entre os genes cuja expressão se encontra alterada os que têm expressão mais aumentada codificam moléculas envolvidas em cascatas imunológicas, enquanto que aqueles com expressão mais diminuída incluem genes que codificam proteínas que participam na manutenção da função de barreira do CP.

Quando o LPS é administrado a cada 2 semanas durante 3 meses, a resposta dos CP persiste mais tempo e é de pequena amplitude. A via metabólica com maior aumento na expressão inclui genes que codificam proteínas que facilitam a migração de células para o CSF, o que pode ser relevante em doenças como a esclerose múltipla.

De realçar, tanto na resposta inflamatória aguda como na continuada, observa-se expressão

alterada de genes que participam na resposta imunológica inata à infecção por microorganismos, e que estão relacionados com o metabolismo do ferro. Em ambos os casos, o CP responde diminuindo a disponibilidade de ferro necessário para o crescimento bacteriano. Especificamente, a lipocalina 2, uma proteína que sequestra sideroforos bacterianos que ligam ferro, é rapidamente segregada para o CSF; uma resposta que persiste na inflamação continuada. Para além disso os resultados obtidos sugerem que o CP desempenha uma função idêntica à do fígado na regulação da captação e libertação de ferro, através da regulação da síntese e segregação de hepcidina. É de notar que alterações no metabolismo do ferro têm sido implicadas em doenças neurodegenerativas como a doença de Alzheimer e a esclerose múltipla.

No seu conjunto, os resultados desta tese apontam para uma função clara do CP na transmissão da resposta inflamatória periférica para o cérebro. Se a consequência final desta resposta é benéfica ou deletéria para o cérebro, ou se contribui para doenças do sistema nervoso central fica ainda por esclarecer.

CONTENTS

ABBREVIATIONS

AD - Alzheimer disease

BBB - Blood-brain barrier

BCSFB - Blood-cerebrospinal fluid barrier

CNS - Central nervous system

CP - Choroid plexus

CSF - Cerebrospinal fluid

FPN Ferroportin

HAMP - Hepcidin

IL - Interleukin

i.p. - Intraperitoneal

LCN2 - Lipocalin 2

LPS - Lipopolysaccharide

L-PTGDS - Lipocalin-type prostaglandin D2 synthase

MS - Multiple esclerosis

PD - Parkinson disease

TF - Transferrin

TFR - Transferrin receptor

TLR - Toll-like receptor

TNF - Tumor necrosis factor

TTR - Transthyretin

THESIS ORGANIZATION

The present dissertation is organized in 7 different chapters. Chapter 1 introduces the subject, chapters 2-6 represent the experimental work (in the form of articles) and chapter 7 the general discussion and future perspectives of the work. The manuscripts presented in chapters 2 and 3 have been already published in the journals *Neuroscience* and *Journal of Cerebral Blood Flow & Metabolism*, respectively. The manuscripts presented in chapters 4, 5 and 6 will soon be submitted for publication.

In chapter 1, a general introduction raises the major questions addressed in this thesis. A brief explanation of the choroid plexus (CP) as part of the barriers system of the brain is given. Its location, morphology, as well as its major functions and those of some of the secreted proteins are described. A review on the relevance of these barriers as mediating the immune response of the central nervous system (CNS) is discussed. A brief comparison between the blood-brain (BBB) and the blood-cerebrospinal fluid (CSF) barriers (BCSFB) is made. Finally, a description of the innate immune response in the periphery, its proposed implications in the CP and its recognized impact in the brain, with particular attention to the alterations of iron metabolism as an immune mechanism of defence, are given.

In chapter 2, the work "The choroid plexus response to peripheral inflammatory stimulus", shows that the expression of major CP proteins, in particular of lipocalin-type prostaglandin D2 synthase (L-PTGDS), is increased upon peripheral administration of lipopolysaccharide (LPS). This is the first evidence of a CP constitutively expressed protein whose secretion is up-regulated by peripheral inflammation. Given the role of L-PTGDS in prostaglandin metabolism it is suggested that this might constitute a mediator of immune signals between the periphery and the brain.

In chapter 3, the work "Lipocalin 2 is a choroid plexus acute-phase protein", shows that lipocalin2 (LCN2) is produced by the CP as a component of the innate immune response. It is proposed that LCN2 may protect the CNS from infection. The relevance of LCN2, at the BCSFB, as a bacteriostatic component in situations of bacterial infections of the brain is discussed.

In chapter 4, the work “Altered iron metabolism is part of the choroid plexus response to peripheral inflammation” shows that the CP alters iron metabolism during inflammation, probably as a mechanism to decrease iron availability for the brain. The relevance of such response for bacteria access to and distribution within the brain is discussed.

In chapter 5, the work “The choroid plexus displays an acute-phase response to peripheral inflammation”, shows a rapid and transient response of the CP transcriptome to peripheral inflammation, as revealed by microarray analysis. How this response is established and what are its consequences for the brain are discussed.

In chapter 6, the work “The choroid plexus response to a sustained peripheral inflammatory stimulus” shows that prolonged exposition to LPS leads to an adaptative response when compared to an acute stimulation, even though the expression of key mediators of the inflammatory response are maintained.

The general discussion of the present dissertation, as well as the final conclusions and future perspectives, is presented in chapter 7.

1

Introduction

1.1. The brain as an immune-privileged organ

1.1.1. The central nervous system barriers

The brain is an unusual tissue since it is protected from free exchange with the blood by blood-brain barriers. Due to these barriers, that inhibit the free diffusion of hydrophilic molecules across the brain capillaries, the central nervous system (CNS) was traditionally described as an immune-privileged site. This view was additionally based on observations such as that: allografts survive longer in the CNS than in other organs (Fuchs and Bullard, 1988); the CNS lacks lymphatic vessels; the CNS is devoided of classical antigen-presenting cells, such as dendritic cells (Matyszak and Perry, 1996) and; the CNS lacks constitutive expression of major histocompatibility complex class I and II molecules on parenchymal cells (Perry, 1998).

The CNS homeostasis is essential for the proper function of neuronal cells and is the presence of blood-brain barriers that protects the brain from the constant oscillation observed in the concentrations of blood constituents. There are two main barriers: the blood-brain barrier (BBB) and the blood-cerebrospinal fluid (CSF) barrier (BCSFB) (Abbott, 2005). In the context of this dissertation BBB and BCSFB will be extensively discussed. Particularly, the focus will rely on how the BCSFB provides signals into the brain in conditions of peripheral inflammation.

1.1.2. The blood-brain barrier

The BBB is formed by the endothelium lining the brain capillaries. This endothelium differs from that of most other capillaries in the rest of the body by the presence of extensive intercellular tight junctions, by the absence of fenestrations and by sparse pinocytic transport (Ballabh et al., 2004). The BBB impedes the influx of most components from the blood into the brain. Small lipophilic molecules such as O_2 and CO_2 can diffuse freely across endothelial cells along their concentration gradient. Nutrients like glucose and amino acids enter into the brain trough specific transporters. In addition, endothelial cells have receptors that bind certain molecules and transport them by receptor-mediated endocytosis, as is the case for molecules like insulin, leptin and transferrin (TF) (Pardridge et al., 1985; Zhang and Pardridge, 2001).

The circumventricular organs, that include the area postrema, median eminence, pineal gland, subfornical organ, and lamina terminalis, are specialized brain regions devoided of BBB. The circumventricular organs

are characterized by their small size, high permeability and fenestrated capillaries. The exchanges with the periphery in those regions is higher, the capillary endothelium is permeable, but those regions do not form an open door into the brain parenchyma by virtue of tight junctions between specialized ependymal cells, tanycytes, that surround them (Brightman and Kadota, 1992).

1.1.3. The blood-cerebrospinal fluid barrier

Although most studies performed in the barrier systems of the brain address the BBB, other important barrier is the BCSFB constituted by the choroid plexus (CP) epithelial cells. Of notice, the blood vasculature that irrigates the CP is also fenestrated, thus not restricting the cellular movement of molecules. Their diffusion into the CSF is prevented by the tight junctions that characterize the CP epithelial monolayer. The CP epithelial cells, responsible for producing most of the CSF, separate, therefore, the blood from the CSF. Similarly to the BBB, the BCSFB restricts passage of blood-born molecules, but displays transporters (for instance for glucose and amino acids) and receptors, that condition the composition of the CSF (Chodobski and Szmydynger-Chodobska, 2001). In addition, the CP in itself contributes to the composition of the CSF by secreting several carrier proteins and growth factors.

1.1.4. Two barriers: two major cell types (endothelial versus epithelial cells)

The characteristic hallmark of the BBB and BCSFB is the presence of intercellular junctions between endothelial cells (Pachter et al., 2003; Persidsky et al., 2006) of the brain capillaries and between epithelial cells of the CP (Szmydynger-Chodobska et al., 2007) that physically restrict the passage of substances between cells. Nevertheless the morphological distribution of the proteins that constitute the referred intercellular junctions differs between these two barrier systems.

The intercellular junctions of the BBB endothelial cells and of the CP epithelial cells are composed by tight junctions and the adherens junctions (Kniesel and Wolburg, 2000; Vorbrodt and Dobrogowska, 2003). The tight junctions are constituted by three transmembranar proteins: claudins, occludins, and junctional adhesion molecule. The adherens junctions are composed by the cadherins. The associated cytoplamatic accessory proteins namely zonula occludens proteins, in the case of the tight junctions, and catenins, for the adherens junctions, connect the transmembranar proteins with the cell cytoskeleton (Vorbrodt and Dobrogowska, 2003; Kniesel and Wolburg, 2000) (Figure 1.). This binding is responsible for the maintenance of the structural integrity of the epithelium and the endothelium.

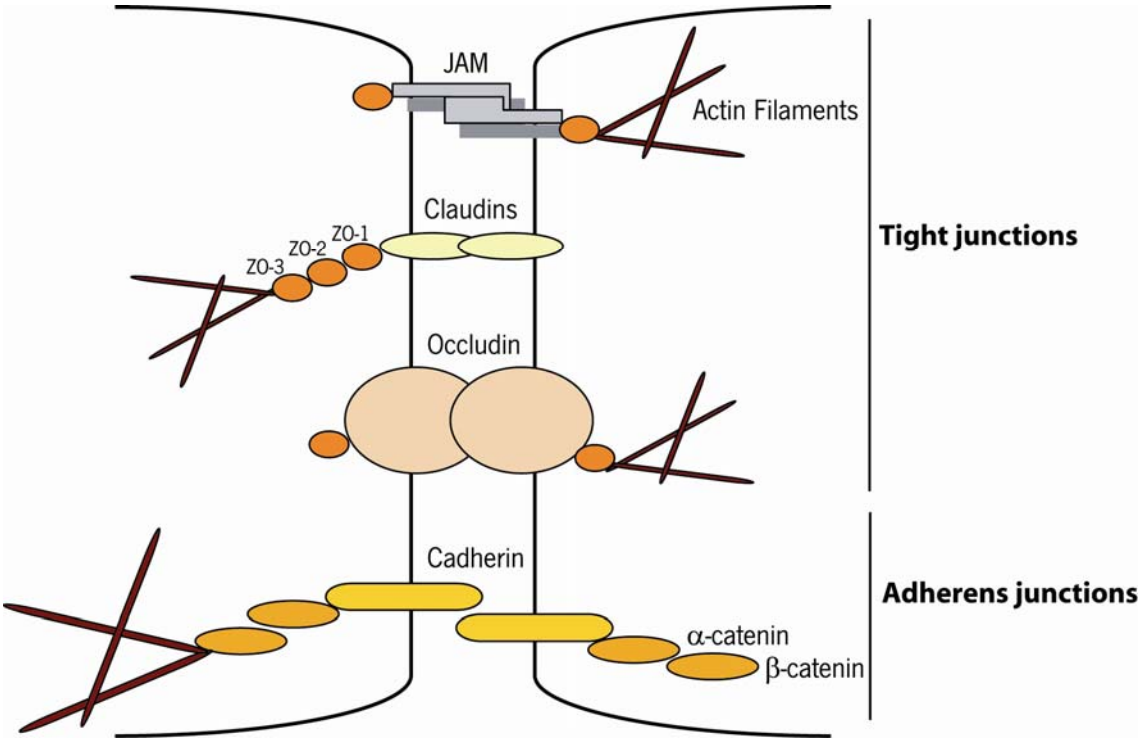

Figure 1. Schematic representation of the distribution of intercellular junctions. The localization of the proteins responsible for the intercellular junctions is different between endothelial cells of the BBB and epithelial cells of the CP. JAM - junctional adhesion molecule; ZO-1, -2, -3 - zonula occludens protein 1, 2, 3.

When considering the morphological distribution of the intercellular junctions, the tight junctions in the CP epithelial cells are more concentrated in a short length of the apical side, close to the CSF, while the tight junctions of the BBB are localized along the entire length of the contacting membrane of the endothelial cells (Vorbrodt and Dobrogowska, 2003). As an example, immunostainning for occludin appears solely in the area of tight junctions formed between the apical segments of adjacent epithelial cells, but their concentration in the BBB is high along the contacting membrane of the cell both in humans and in mice (Vorbrodt and Dobrogowska, 2003). However there is much less information available on the presence and localization, at the ultrastructural level, of various intercellular junctions proteins in the CP epithelium than in the BBB endothelium.

When the transfer of blood-born molecules or cells into the brain is considered, leakage of the barrier function is necessary. Therefore, the proteins that constitute the barriers of the brain are expected to be influenced by conditions such as inflammation, which will be further discussed in chapter 5.

Although the endothelial cells (of the BBB) and the epithelial cells (of the BCSFB) differ on the morphological distribution of the intercellular junctions they share some common features. For instance, a fundamental function of both the epithelium and endothelium is to separate different compartments within the organism and to regulate the exchange of substances between them. In

fact, the endothelium is one type of epithelium; it is a highly specialized active interface between blood and the underlying tissues, maintaining vascular tone, thrombo-resistance and selective permeability to cells and proteins (Methe et al., 2007). Particular to epithelial cells is the presence of polarity and the higher complexity in terms of cytoplasmatic organelles in comparison with endothelial cells.

The epithelial cells have basal lamina and have a higher secretory capacity than endothelial cells, as revealed by the higher number of mitochondria in their cytoplasm. Epithelial cells play an important role in physiological and pathophysiological situations, with organ-, tissue-, type-, and function-specific patterns. The complete set of functions of the CP epithelium is far from clearly understood. However, given its location, it is expected that it can fulfil some of the features attributed to other types of epithelial cells: physical protection (e.g. epiderme, internal recovery superficies of the organs); absorption (small intestine), secretion (caliciformes cells), secretion and transport (renal tubs, vesicular biliar), and immune response (respiratory epithelium) (Chignard et al., 2001; Nemes and Steinert, 1999; Thompson et al., 1995).

1.2. Choroid plexus

1.2.1. Choroid plexus morphology

The CP is a highly phylogenetically and ontogenetically conserved structure. The CP develops early during embryogenesis and already constitutes a functional barrier within the first weeks of gestation (Dziegielewska et al., 2001). During embryogenesis its permeability is higher allowing low molecular weight compounds to enter the brain more easily than in adulthood. During embryonic development, the CP occupies a proportional larger volume of the total brain and it is essential for the proper CNS formation due to the secretion, into the CSF, of morphogens, mitogens and specific growth factors (Bondy et al., 1990; Yamamoto et al., 1996; Figueiredo et al., 1993; Strazielle and Ghersi-Egea, 2000). The CP is positioned within the ventricles of the brain: one in each lateral, one in the third and one in the fourth ventricles. Grossly, the CP is lobulated with a unique and continuous line of epithelial cells originating from the ependymal line of the ventricles and floats into the CSF space inside the ventricles. The CP is essentially constituted by a continuous strand of cuboidal epithelial cells, which rests upon a basal lamina and contains a central core formed by connective and highly vascularised tissue. The apical side of the epithelial cells faces the CSF and contains numerous villosities while the basal side faces the blood, lying in the stroma side in contact with a huge number of capillaries. In the central core of the connective tissue it is possible

to find, in addition to the fenestrated capillaries, dendritic cells, fibroblasts and macrophages. The basolateral side of the CP epithelial cells is, therefore, in contact with molecules and cells that freely pass through the brain capillaries, and also with the proteins and other molecules secreted by these other various cell types. Ultrastructurally, the epithelial cell contains numerous mitochondria, Golgi apparatus and a smooth endoplasmic reticulum which demonstrates that it is a structure with great synthetic capacity. Additionally, high number of vesicles with lisossomal characteristics is observed in the cytoplasm. In the apical side, lying over the microvillus layer, there are cells attached, designated as epiplexus cells or Kolmer cells (Pietzsch-Rohrschneider, 1980). In addition to these cells, free floating and supraependymal cells are observed in the lumen of the ventricles (Ling et al., 1998; Matyszak et al., 1992; Nataf et al., 2006; McMenamin et al., 2003). These cells, altogether referred as intraventricular macrophages, could represent, the first line of defence able to react against brain infections, after they transverse the CP.

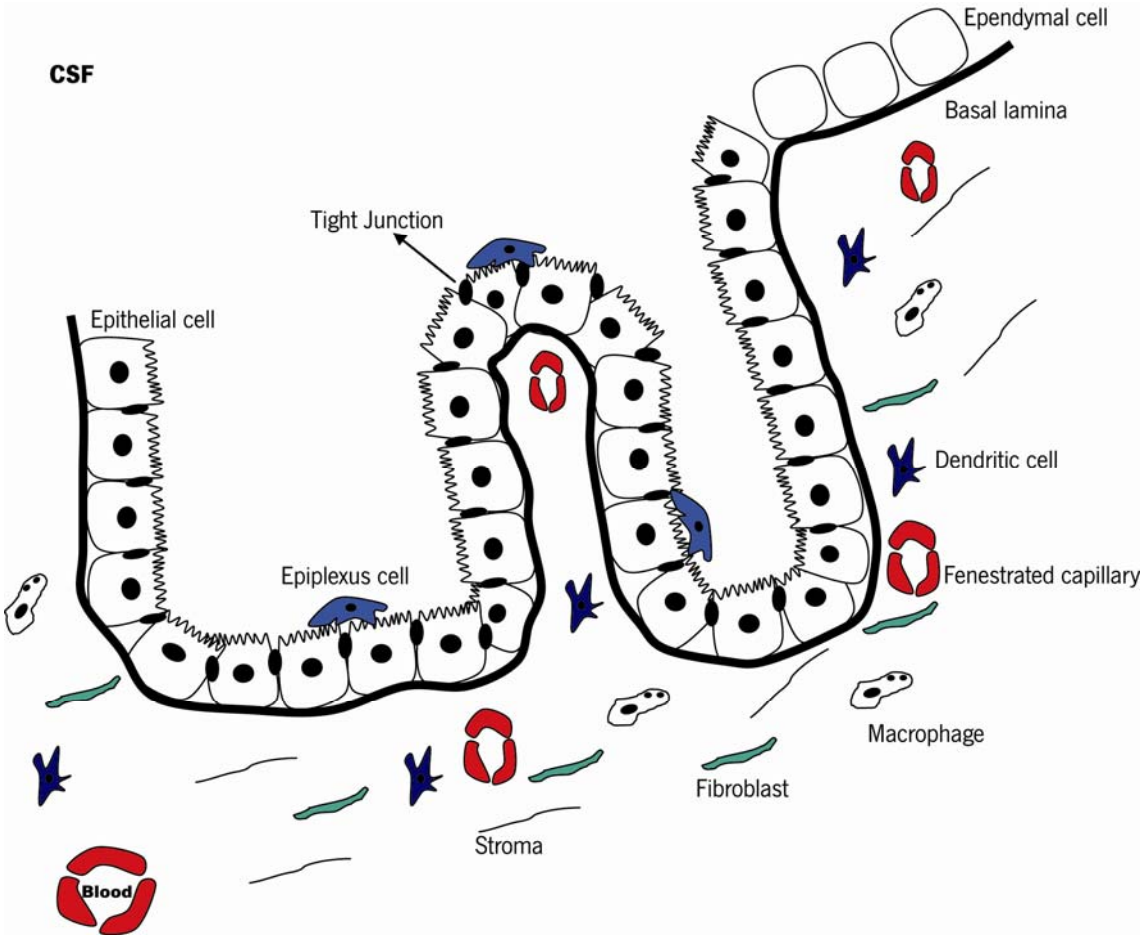

Figure 2. Morphological representation of the choroid plexus. The choroid plexus is formed by epithelial cells originated from the ependymal cell layer of the ventricules. Those epithelilal cells rest upon a basal lamina and inner core of highly vascularized connective tissue. The apical side of the epithelial cells faces the cerebrospinal fluid and contains villosities. Adjacent epithelial are bound together by tight junctions. Macrophages, dendritic cells, fibroblasts and fenestrated capillaries are present throughout the stroma. In the apical side, attached to the villosities are the epiplexus cells.

The intraventricular macrophages deserve some additional considerations given their role as scavenger cells and the participation in immunological responses within the ventricular system. Features of intraventricular macrophages are consistent with other mononuclear phagocytes, given their positive immunoreactivity for the presence of complement receptor 3, major histocompatibility complex class I and II antigens, leucocyte common antigen, and macrophage antigen (Ling et al., 1998). The expression of these antigens is noticeably enhanced following the intraperitoneally injection of lipopolysaccharide (LPS) in rats (Lu et al., 1994). Remarkably, the intraventricular macrophages are induced to increase the expression of major histocompatibility complex class II antigen after LPS or interferon-gamma injections. This suggests that these cells can function as antigen presenting cells in the brain ventricles (as macrophages do in the periphery) in situations of infection and injury. Several mechanisms might then contribute, within the brain, to respond to peripheral stimulus. Among these, the expression of transferrin receptor (TFR), as detected with OX-26 immunohistochemistry, is also upregulated after these treatments (Lu et al., 1995). These will conduct to a decrease of iron in the environment for the pathogens and, at the same time, the iron that is being endocytosed can be used in oxidative killing or immunological activation. Other characteristic in which intraventricular macrophages resemble peripheral macrophages is the fact that they are also elicited to display *a de novo* expression of nitric oxide synthase-like immunoreactivity following intracerebral injection of LPS (Ling et al., 1998). Whether this also occurs upon stimulus induced in the periphery is unknown but it could represent an additional mechanism of defence that can help in the protection of the brain against bacterial infections.

The number of intraventricular macrophages increases considerable with age in the rat (Ling et al., 1998). This increase seems to result partly from the proliferation of local cells but can also represent increased recruitment of blood monocytes that reach the ventricle through the CP epithelium. Whether this increase relates to decreased barrier function with age and how does it correlate with development of brain neurodegenerative disease remains to be studied.

1.2.2. Choroid plexus functions

1.2.2.1. Production of cerebrospinal fluid

The brain has two fluid environments: the brain interstitial fluid, which surrounds the neurons and glia, and the CSF, which fills the ventricles and the subarachnoid space. The

best recognized function of the CP is the production of CSF (Speake et al., 2001). Maintenance of the equilibrium in volume and composition of the CSF is vital for a normal brain function, ensuring an optimal environment for the neuronal cells. The CSF is a clear, slightly viscous liquid with few cells and a protein concentration about 10 times lowers then the one of the blood (Segal, 2001). Most of the CSF is generated by the CP with the remainder 10-30% being of extrachoroidal origin (reviwed by Chodobski and Szmydynger-Chodobska, 2001). The large secretory activity of the CP is in part provided by the high number of mitochondria. Another important feature of the CP is the high water permeability, made possible by the abundant expression of the water channel, Aquaporin-1, on the apical side of the membrane.

Since the CSF surrounds the CNS and exchanges with the brain interstitial fluid it clearly participates in the delivery of nutrients into the brain and also in the removal of waste products produced by the brain. For that, the CP is well equipped with transporters and receptors, both in the apical and basolateral membranes. Examples are the transport of vitamin C and the disposal of iodine. Such CP functions are responsible for the constant refreshment of CSF, essential for normal brain metabolism. In humans the CSF volume is 80-150mL and is estimated that 600mL of CSF are produced every 24h, which is enough to replace existing CSF three to four times per day (Wright, 1978).

1.2.2.2. Major proteins produced by the choroid plexus

The CP is ideally located to distribute molecules both locally and globally to the brain due to its secretory ability into the CSF. The CP possesses numerous specific transport systems, contains an array of receptors (Table 1) and also serves as a source of biologically active products. The CP epithelium is not only a target but also a source of neuropeptides, growth factores and cytokines for the CSF. Of notice, the proteins and molecules produced by the CP are well recognized as morphogens, mitogens and trophic factors important for the development of the CNS, namely by influencing axonal guidance and cell migration. In table 3 are listed some of the proteins already described to be secreted by the CP. Of notice, interleukin-1 beta (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor (TNF) have been described to be secreted by the CP in response to inflammation (Quan et al., 1998; Quan et al., 1999; Vallieres and Rivest, 1997).

Table 1. Major proteins synthesized by the choroid plexus	
Membrane receptors/transporters	Secreted polypeptides
Angiotensin II	Apolipoprotein J
Apolipoprotein E	Basic fibroblast growth factors 1 & 2
Apolipoprotein J	Brain-derived neurotrophic factor
Bradykinin	Cystatin C
Brain derived neurotrophic factor	Endothelin-1
Corticotropin-releasin factor	Hepatocyte growth factor
Digotoxin	Insulin-like growth factor binding protein 2–6
Endothelin	Insulin-like growth factor-II
Fibroblat growth factor	Interleukin-1β
Growth hormone	Interleukin-6
Insulin	Nerve growth factor
Insulin like growth factor	Neurotrophin-3 and 4
Interleukin1	Prostaglandin D synthase
Leptin	Transferrin
Nerve growth factor	Transforming growth factor α
Neurotrophin-4	Transthyretin
Prolactin	Tumor necrosis factor
Transforming growth factor β	Vascular endothelial growth factor
Vasopressin	Vasopressin
	β–amyloid precursor protein

Table modified from Chodobski, A. and Szmydynger-Chodobska, J., 2001.

One of the initial aims of the present study was to investigate how the levels of major proteins constitutively expressed by the CP are influenced by peripheral inflammation (chapter 2). Additionally, the CP secretome, by influencing the CSF composition, might be involved in protecting against or mediating deleterious signals originated in the periphery to the brain. This will be further discussed in chapter 5.

We will next briefly describe the function of major proteins constitutively expressed by the CP.

A. Transthyretin

Transthyretin (TTR), formerly designated as prealbumin due to its migration properties in native electrophoresis in relation to albumin (Ingbar, 1958), is a 55-kDa tetrameric protein synthesized mainly by the liver and by the CP (Dickson et al., 1985; Herbert et al., 1986) from where it is secreted into the blood and the CSF, respectively . TTR synthesis represents 20% of the total protein synthesized by the CP. The TTR expression is well conserved throughout evolution, starting in fish; and is an early event during mammalian embryogenesis, first in the thela choroidea (Murakami et al., 1987; Thomas et al., 1988) that will originate the CP, and later in the liver.

TTR is a plasma and CSF carrier for thyroxine (T4) and retinol (when bound to the retinol-binding protein) (Palha, 2002). Although TTR has been initially proposed to be essential to mediate thyroid hormone transfer into the tissues, particularly into the brain across the BCSFB, studies with a TTR-null mice strain have shown that TTR is not necessary for thyroid hormones entry into

and distribution within the brain and other tissues, or for the maintenance of an euthyroid status (Palha et al., 1994; 1997; 2000; Palha, 2002). Recently, TTR has been associated with other functions: TTR is able to bind Alzheimer Amyloid beta-peptide *in vitro* (Schwarzman et al., 1994) and, by sequestering it in CSF has been suggested to prevent amyloid formation (Golabek et al., 1995; Buxbaum et al., 2008). In accordance, decreased TTR levels in the CSF have been described in patients with Alzheimer disease (AD) (Serot et al., 1997). In agreement, experiments using TTR-null mice have shown that in the absence of TTR mice display spatial reference memory impairment compared to age-matched wild-type animals (Sousa et al., 2007). Of interest, in the absence of TTR, mice display decreased depression and anxiety-like behaviours, probably by modulation of the noradrenergic system (Sousa et al., 2004). One possible explanation for this phenotype could be hypothyroxinemia and hypovitaminosis A during embryonic development, since both thyroid hormone and retinol are requested for normal CNS formation and development. Of interest, certain mutations in the TTR gene have amyloidogenic potential that result in TTR protein deposition in peripheral nerves and cardiomyocytes in humans (Saraiva, 2002).

TTR has been implicated in various other functions. Among these, a single report implicates TTR as an endogenous anti-inflammatory mediator given its ability to inhibit, *in vitro*, monocyte and endothelial IL-1β production (Borish et al., 1992). While, in the liver, TTR is a negative acute-phase protein (Birch and Schreiber, 1986), subcutaneous injection of turpentine did not influence CP *Ttr* expression (Dickson et al., 1986). The differential response in the liver when compared to the CP possibly results from tissue specific transcription modulators (Yan et al., 1990; Costa et al., 1990).

It seems, therefore, that the precise functions of TTR, particulary within the brain, are far from being clearly understood.

B. Lipocalin-type prostaglandin D2 synthase

Lipocalin-type prostaglandin D2 synthase, also known as β-tracer, is a 30 kDa glycoprotein that catalyzes the formation of prostaglandin D2 (PGD2) from prostaglandin H2 (PGH2), the major prostanoid brain (Urade and Hayaishi, 2000). L-PTGDS is involved in the regulation of various physiological events such as pain and sleep (Qu et al., 2006; Urade, 2006; Hayaishi and Urade, 2002). L-PTGDS is constitutively expressed in the CNS, particularly in the leptomeninges, CP from where it is secreted into the CSF, and in oligodendrocytes (Urade et al., 1993; Nagasaka et al., 2004). In addition, although not detected normally in macrophages, L-PTGDS was found in

macrophages infiltrated in atherosclerotic plaques (Cipollone et al., 2004) and in macrophages stimulated with LPS or with *Pseudomonas aeruginosa* (Joo et al., 2007). However, the regulation and significance of L-PTGDS expression in macrophages are unknown.

In addition to its enzymatic function, L-PTGDS has also been described as an extracellular carrier for hydrophobic molecules given its ability to bind, *in vitro*, retinoic acid, thyroid hormones (Tanaka et al., 1997) and gangliosides (Mohri et al., 2006).

Various studies have investigated if CSF L-PTGDS levels could serve as a potential marker of brain disease. No differences were found in different pathological conditions of the CNS such as dementia, hydrocephalus, neuropathy, optic neuritis, multiple sclerosis (MS) and demyelinating syndrome, when compared to normal individuals; however, the level of L-PTGDS in the CSF obtained from patients with brain tumor, was reduced by as much as 2-fold when compared to control samples (Saso et al., 1998). Of interest, although the CSF level of L-PTGDS is not increased in MS patients, L-PTGDS is increased in the white matter of MS patients, especially in plaques (Kagitani-Shimono et al., 2006). In chapter 2 we will discuss the potential implications, for the brain, of the increased L-PTGDS we found in the CSF upon a peripheral inflammatory stimulus.

C. Transferrin

The transferrins are a family of nonheme iron-binding proteins that bind free iron in the blood and in other biological fluids. Two major types of transferrins have been described: serum TF and lactotransferrin. The principal biological function of transferrins is thought to be related to their iron binding properties. Serum TF is responsible for carrying iron from the sites of intake into the systemic circulation and then into tissues. Iron delivery to cells occurs via a receptor-mediated endocytotic process. TF is synthesized predominantly by hepatocytes, ependymal cells, oligodendrocytes and CP epithelial cells and it has been detected in various body fluids including plasma, CSF, lymph and breast milk (Zakin et al., 2002). Iron-loaded TF binds to the TFR on the cell surface. The TF-TFR complex is internalized and transported to endossomes. In the endossome iron is released from TF due to an ATP-dependent protein pump that lowers the pH of the endossome and thus facilitates release (Dautry-Varsat, 1986). Inside the cell, iron is delivered to various intracellular locations including mitochondria and ferritin (storage). The receptor bound apo-TF returns to the cell surface where the neutral pH promotes release of apo-TF into the circulation.

Iron uptake and storage is a well controlled system since while iron is necessary for various vital functions, in excess it has deleterious consequences for the cells. Of relevance in iron

homeostasis is the observation that free iron in the body promotes the growth of pathogens (Teehan et al., 2004). Most bacteria require iron for growth and survival and therefore possess mechanisms to acquire iron from the host by: (i) releasing sideropheres that sequester free iron, (ii) actively uptaking heme; or (iii) binding holo-transferrin via TFR-like receptors. In response to infection, the host up-regulates the synthesis of iron binding proteins, as it is the case for liver ferritin (Ceciliani et al., 2002) and macrophages/neutrophils lipocalin 2 (LCN2) (Flo et al., 2004) (an iron siderophore to which we will return in chapter 3). In accordance, LCN2 knockout mice have increased susceptibility to infections with *E. coli* and *S. aureus* (Flo et al., 2004).

All major proteins constitutively expressed by the CP have been somehow implicated in peripheral inflammation, which we will further discuss in chapter 2.

1.2.2.3. The choroid plexus as a first line of brain defence

The CP can be viewed as a first line in the brain defence against noxious stimulus, whether they are molecules, cells or pathogens. The presence of the CP tight junctions prevents the passage of several noxious compounds into the CSF. In addition, even for molecules that are taken up by the CP epithelial cells, most noxious substances are prevented from entering the CSF because the epithelial cells have metabolizing enzymes including Phase I – III enzymes for activation, conjugation and transport of drugs. In addition, the CP is able to protect free radical assess into the brain parenchyma, given the presence of enzymes such as superoxide dismutase, glutathione-s-transferase, and glutathione peroxidase and reductase that protect against free-radical oxidative stress.

The CP is also involved in the interaction between the peripheral immune system and the brain. Of notice, it has been shown that CP epithelial cells and the CP stroma allow the communication between a peripheric inflammatory stimulus and the initiation and development of an innate and adaptative response in the brain (Engelhardt et al., 2001). Following systemic activation of the immune system by the administration of LPS there is a rapid and transient induction, in the CP, of immune-modulators such as *Il-1β* and *Tnf* (Quan et al., 1999; Nadeau and Rivest, 1999; Marques et al., 2007). Activation of the CP may certainly spread throughout the brain indicating that the CP transmits information between the immune system and the brain. Choroidal epithelial cells also express major histocompatibility complex class I and class II molecules constitutively and inducible, respectively, (Irwin et al., 1999). This observation strongly suggests that the epithelial cells can present antigens to T cells in a major histocompatibility complex class II

context. Accessory molecules important for leukocyte adhesion such as selectin, lymphocyte (L-selectin), intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) are found expressed at low levels in CP epithelial cells but can also be up-regulated during inflammation (Endo et al., 1998; Wolburg et al., 1999). In a mouse model of infection with *Streptococcus suis* Serotype 2, early transcriptional activation of Toll-like receptor 2 (TLR2), CD14, and of inflammatory cytokines in the CP and cells lining the brain endothelium suggests that these structures are potential entry sites for bacteria into the CNS (Dominguez-Punaro et al., 2007). In addition, as the CP is an epithelium, microorganisms such as *Neisseria meningitides*, *Trypanosome brucei*, and *Sendai virus* could also have a tropism for CP epithelial cells.

Despite these various observations, the detailed involvement of the CP in signalling peripheral inflammatory stimulus to the brain remains to be elucidated. The work presented in chapters 5 and 6 intends to further contribute to clarify the CP response to inflammatory challenges induced outside the brain.

1.3. Systemic inflammation induced by lipopolysaccharide

1.3.1. Lipopolysaccharide

LPS is a molecular component of the outer membrane of Gram negative bacteria (Alexander and Rietschel, 2001; Raetz and Whitfield, 2002). The outer membrane functions as a physical barrier that protects bacteria from the surrounding environment and from antibacterial mechanisms. LPS is one of the most potent inducers of the immune system and is the principal initiator of the septic shock (Elin and Wolff, 1976).

The use of LPS mimics the inflammation with Gram negative bacteria and for that reason it is currently used in experimental models of inflammation. We have chosen this model to study the response of the CP to acute and sustained inflammatory challenges.

1.3.2. Immune response to lipopolysaccharide

The innate immune response is a universal mechanism of host defence against inflammation and infection. It functions on the basis of special receptors called pattern-recognition receptors which recognize conserved microbial structures called pathogen-associated molecular patterns. TLRs are a group of pattern-recognition receptors that play a crucial role in the recognition of

different PAMPs, such as LPS, and in the induction of the immune response (Akira and Hemmi, 2003; Netea et al., 2004). Cells of the immune system, epithelial cells and endothelium, recognize pathogens via TLRs. The signalling cascade downstream of TLR activates pathways that culminate with the production of inflammatory cytokines and the development of an immune response (Takeda and Akira, 2005). The TLR family comprises different members and it is known that TLR4 is responsible for LPS recognition and subsequent responses. However, TLR4 is not the sole receptor involved in LPS recognition. Transport of LPS molecules in the serum is mediated by LPS-binding protein (Ulevitch and Tobias, 1995). At the plasma membrane, LPS-binding protein is thought to transfer LPS monomers to CD14, a GPI-linked cell surface protein (Ulevitch and Tobias, 1995; Triantafilou and Triantafilou, 2002; Triantafilou and Triantafilou, 2005). Exactly how CD14 facilitates recognition of LPS by TLR4 is not clear, but its critical role is demonstrated by the LPS hyporesponsive phenotype of CD14-deficient mice (Moore et al., 2000; Haziot et al., 1996). CD14 is also a co-receptor for TLR2 (Rallabhandi et al., 2006; Remer et al., 2006; Viriyakosol et al., 2000). Of notice, apart from its well known function as co-receptor for TLR-dependent signalling in response to LPS, soluble CD14 can also act as a direct agonist for TLR2 (Bsibsi et al., 2007).

Although TLR2 is able to mediate the LPS response *in vitro* (Matsuguchi et al., 2000), its role as an LPS receptor *in vivo* has been questioned as a result of the recent findings that gene-disruption of TLR4 in mice, but not of TLR2, demonstrate phenotypes similar of LPS-hyporesponsive strains (Hoshino et al., 1999; Takeuchi et al., 1999).

Once this pathway is triggered it leads to the activation of the transcription factor, nuclear factor kappa B (NF-kβ) which regulates the transcription of pro-inflammatory cytokines such as TNF, IL-1β and IL-6. It can also activate members of the mitogen-activated proteins kinase family (MAPK) notably p38 and JUN N-terminal kinase (JNK). These kinases are involved in the transcription of genes and they also regulate mRNA stability.

A novel family of DNA binding proteins, referred to as interferon regulatory factors (IRFs) (Miyamoto et al., 1988) have been shown to have diverse roles in the regulation of the immune system. The involvement of these transcription factors in the innate and adaptative immune responses has been gaining more relevance principally because of the discovery that IRFs are downstream effectors of the TLR-mediated response (Colonna, 2007; Honda and Taniguchi, 2006a; Honda and Taniguchi, 2006b).

The mammalian IRF family comprises 9 members (Honda and Taniguchi, 2006a) and represents an additional molecular pathway in the complex response to LPS. All the members recognize sequences in DNA called the IFN-stimulated response element.

The present concept is that the IRF system governs a broad spectrum of cellular responses in immunity (Honda and Taniguchi, 2006a), participating in response to LPS (Barber et al., 1995) and as a key pathway in recognition and regulation of the TLR-dependent signalling (Honda and Taniguchi, 2006a; Honda and Taniguchi, 2006b; Negishi et al., 2006). The IRF pathway has been considered as MyD88 independent but it has already been shown that IRF5 and IRF7 directly interact with Myd88 and regulate the TLRs-mediated induction of pro-inflammatory cytokines (Honda et al., 2005; Uematsu et al., 2005). IRF-1 also interacts with and is activated by MyD88 upon TLR activation and when this interaction occurs the migration of IRF-1 into the nucleus is more efficiently than non-MyD88 associated IRF-1 (Negishi et al., 2006).

While LPS has been shown to induce, in the CP, the up-regulation of TLR down-stream genes such as IL-1β and TNF, it is still unknown the precise mechanism through which LPS mediates the overall CP response to inflammation, which will be further discussed in chapters 5 and 6.

1.3.3. Systemic inflammation and communication with the brain

During an immune response the brain and the immune system "talk to each other" and regulate each other to maintain body homeostasis. CNS signals the immune system via hormonal and neuronal pathways and the immune system signals the CNS mainly through cytokines. The precise mechanism by which cytokines act and signal the brain has been the subject of intense debate. Peripherally released cytokines can act on the brain via (i) fast transmission pathways involving primary afferent neurons of the vagus nerves innervating the site of inflammation; (ii) direct entry of cytokines, in small amounts, into the brain through the BBB or BCSFB via a saturable transport mechanism, (cytokines are large, hydrophilic proteins and are not expected to cross the BBB without a transport system); (iii) interactions within the circumventricular organs, for example at the organum vasculosum lamina terminalis or the median eminence and (reviewed by Hosoi et al., 2002); (iv) activation of second messengers, such as nitric oxide and prostaglandins after binding to receptors at the BBB (Gaillard, 1998) or BCSFB; (v) action of the cytokines present in the bloodstream that bind to receptors in endothelial cells of the BBB or the epithelial cells of the CP and activate the production of new cytokines.

Activation of afferent nerve fibers by peripherally released cytokines represents a fast pathway of

transmission of immune signals from the periphery to the brain. This pathway sensitizes the brain target areas to induce brain-produced cytokines that transmit and amplify the local action of peripheral cytokines (reviewed by Hosoi et al., 2002).

One of the first signals of the actions of cytokines in the brain response is the development of fever due to the action of IL-1β (Luheshi, 1998). Additionally, a number of physiological changes, known as cytokine-induced sickness behaviour, occur and include anorexia, depressed locomotor activity, decreased exploration of the physical and social environment, reduced food and water intake, and impaired learning and memory (Dantzer, 2001). Two major mechanisms have been evolved in the CNS regulation of the immune system: (a) the hormonal stress response and the production of glucocorticoids and (b) the autonomic nervous system with the release of noradrenaline. The cytokines in circulation lead to the activation of the hypothalamic-pituitary-adrenocortical axis ultimately inducing the expression and release of glucocorticoids by the adrenal glands. Glucocorticoids regulate the expression of a wide variety of immune molecules, including cytokines, adhesion molecules, chemoattractants and molecules involved in immune cell trafficking, maturation and differentiation.

The communication of the peripheral immune system with the brain will be analysed in more detail during this dissertation, with specific focus on the participation of the CP.

1.3.4. Acute-phase response

The first response of the body to an inflammatory stimulus is the activation of an innate immune non-specific response that precedes the specific and adaptative immune reaction. In the innate immune response (as is the case of the response to a single injection of LPS) the organism develops an acute-phase response and secretes pro-inflammatory cytokines such as IL-1β and TNF (Suffredini et al., 1999). The pro-inflammatory cytokines released activate the vascular system and inflammatory cells in the circulation. More inflammatory cytokines and other inflammatory mediators are produced by these cells and circulate in the blood.

The cytokines activate receptors on different target cells that respond by altering their metabolism and by synthesizing new molecules and proteins. The pattern of protein synthesis by the liver is drastically altered resulting in an increase of some blood proteins, globally denominated as positive acute-phase proteins, such as C-reactive protein, serum amyloid protein, haptoglobolin, and LCN2. The mRNA up-regulation of those proteins is associated with a decrease in the synthesis of other normally expressed blood proteins that include TTR, TF, retinol-binding protein,

and albumin, therefore designated as negative acute-phase proteins (Ceciliani et al., 2002; Gabay and Kushner, 1999; Birch and Schreiber, 1986).

Other protective mechanisms against invading microorganisms at the hepatic level are the induction of heat-shock proteins that function as chaperones for damaged cellular (molecules and metallothioneins that increase the hepatic resistance against metal toxicity and may enhance the intracellular metal ion binding capacity (Prohaszka, 2003; Borghesi and Lynes, 1996,.

In the context of response to inflammation, iron seems to be a key player. For that reason we will next briefly summarize iron metabolism.

1.4. Iron homeostasis

Iron is an essential element for the body homeostasis. Iron deficiencies or excesses can lead to pathological conditions such as anemia or hemochromatosis, respectively, and for this reason iron metabolism is tightly regulated. The main site of dietary iron absorption is the duodenum. Once within the absorptive enterocytes, iron enters a common intracellular pool and is subsequently transferred across the basolateral surface of the enterocytes into the bloodstream by the iron exporter ferroportin (FPN) (Chua et al., 2007). The released iron is oxidized to ferric ion by hephestin, a homolog of the serum ferroxidase ceruloplasmin, and binds to circulating plasma TF. TF bound iron is taken up by cells by transferrin receptor 1 (TFR1)-mediated endocytosis (Chua et al., 2007).

The liver, or more specifically the hepatocyte, is the main site of iron deposition and storage (Anderson and Frazer, 2005). When the body is iron-replete, macrophages of the liver, spleen, and bone marrow also store some of the iron recovered from the phagocytosis of senescent erythrocytes. When it is stored, iron is incorporated into ferritin.

In the bone marrow iron is used for the formation of haemoglobin for the erytrocytes (Figure 3.). Most of the body iron (70%) exists in circulation in the erytrocytes, that when these are old or damaged are removed from the circulation by macrophages of the reticuloendothelial system. Inside the macrophage iron is released from haemoglobin and can either be stored or be released back into circulation and bound to TF.

All this movement of iron, starting in the absorption, is highly regulated and modulated according to the iron body requirements and according to the pathological state of the organism.

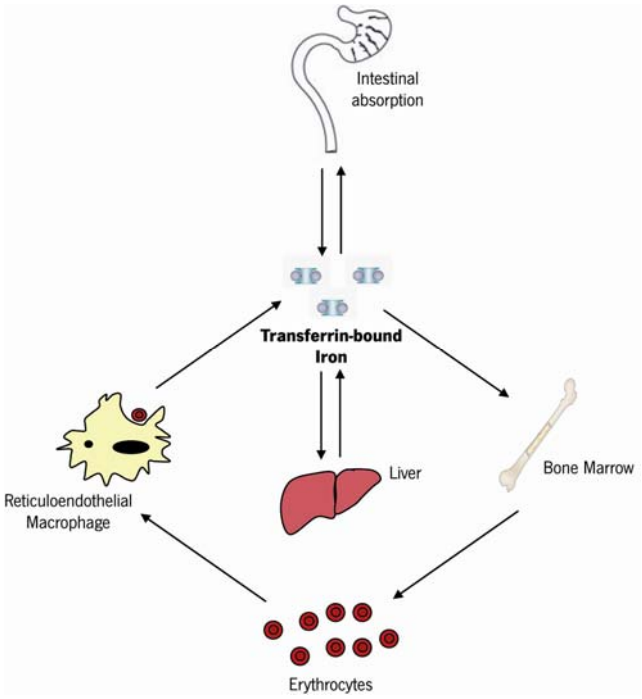

Figure 3. Iron metabolism in the human body. Iron absorption by the enterocyte, transport in the bloodstream bound to transferrin. The transferrin-iron complex is taken up by cells for usage or storage.

The uptake of iron is mostly regulated individually by each cell and the expression level of TFR1 is regulated by the intracellular level of iron. However, the efflux of iron from the cell seems to be systemically regulated by signals and represents the major regulatory point for the maintenance of the body iron homeostasis (Frazer and Anderson, 2003). Enterocytes, macrophages and hepatocytes are the major cell players in the regulation of iron levels since these are the cells that absorb and store higher quantities of iron.

As an iron exporter, FPN regulates the iron content in circulation; in consequence, the regulation of FPN has a central role in iron homeostasis. FPN is under the control of a newly described liver-derived peptide hepcidin (HAMP) (Krause et al., 2000). Circulating HAMP binds FPN on the surface of enterocytes, macrophages and other type of cells causing its internalization and degradation.

Under physiological conditions the level of HAMP is regulated by body iron requirements but recently hemochromatosis (HFE), transferrin receptor 2 (TFR2) and hemojuvelin have been shown to alter HAMP transcription (Schmidt et al., 2008). HAMP expression can be modulated by pro-inflammatory cytokines such as IL-6 and IL-1β during the inflammatory response. The up-regulation of HAMP was described to be an efficient way to limit iron concentration in circulation (Ganz, 2007). The complete mechanism beyond HAMP regulation is, however, still unknown.

1.4.1. Iron and innate immunity

The cells of the immune system are equipped with the machinery capable of regulating iron homeostasis through the regulation of several iron genes and proteins (Porto and De Sousa, 2007). In addition to immune cells, the epithelial cells of the CP, also express genes related to iron metabolism and homeostasis, and should therefore be considered in the brain immune response.

In mammalian host, most of the iron is normally bound to various proteins, the hemoproteins, ferritina and TF. During innate immune response additional proteins such as LCN2 and lactoferrin are increased in various body fluids (Liu and Nilsen-Hamilton, 1995; Sunil et al., 2007). During evolution pathogens developed highly selective skills for iron uptake. The microbes can obtain iron by direct mechanism that requires specific receptors for each iron source, TF, lactoferrin, ferritin (Gray-Owen and Schryvers, 1996). Indirect mechanisms are however more broadly distributed. Some Gram negative bacteria, for instance, secrete specialized proteins called hemophores to acquire heme from different sources (Wandersman and Stojiljkovic, 2000). The limitation of this hemophore system is the restriction to heme iron sources that could be problematic for pathogens in situations of low heme availability. Another indirect strategy is the secretion of other proteins that bind iron from others sources, in analogy to the hemophore system. A broad spectrum of prokaryotic and eukaryotic microbes uses small molecule compounds called siderophores as high-affinity ferric iron chelators (Miethke and Marahiel, 2007). These siderophores are secreted by cells during periods of iron starvation. Siderophores form extracellular Fe(III) complexes that are then taken up by the pathogen. Inside the cytoplasm iron is released from the complex by ferric-siderophore reductases or hydrolases (Miethke and Marahiel, 2007). The broad spectrum of microbial mechanisms to acquire iron is the result of several constraints made by the host to suppress pathogen multiplication.

During an acute-phase response the host creates a hypoferremic environment (Laftah et al., 2006; Fuchs et al., 1991). HAMP is the main mediator of hypoferremia associasted with inflammation (Ganz, 2006). Induction of hepcidin by IL-6 and other inflammatory cytokines (Nemeth et al., 2004, Kemna et al., 2005, Lee et al., 2005) decreasing the iron uptake in the enterocyte. In macrophages, IL-1β and TNF enhance iron storage bound to the ferritin pool (Pinero et al., 2000; Laftah et al., 2006). But beyond these basic strategies to create a hypoferremic environment, and in response to the mechanisms developed by the pathogens to acquire iron, the host has the innate ability to further respond by secreting siderophore-binding

proteins as is the case of LCN2 that is able to compete successfully with the high-affinity Fe-enterobacterin uptake system of enteric bacteria.

In this dissertation the role of LCN2 and iron in an inflammatory response will be further discussed in chapters 3 and 4.

1.5. Aims of the study

The overall objective of this thesis is the study of the CP response to peripheral inflammation. Specifically, we intended to:

- Determine whether the expression of major CP proteins (transthyretin, transferrin and L-PTGDS) is influenced by an acute inflammatory stimulus induced in the periphery.
- Elucidate how proteins related to iron metabolism, at the BCSFB, contribute to the innate immune response.
- Investigate whether the CP has an acute-phase response to peripheral inflammation.
- Establish how the CP responds to a continuous inflammatory stimulus induced in the periphery.

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The choroid plexus response to peripheral inflammatory stimulus

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THE CHOROID PLEXUS RESPONSE TO PERIPHERAL INFLAMMATORY STIMULUS*

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Abstract—Increased interest is being raised on the interaction between systemic inflammation and the brain. The choroid plexus (CP) constitutes a monolayer of epithelial cells located within the brain ventricles and is responsible for the production of cerebrospinal fluid (CSF). Despite the knowledge that the CP capillaries are fenestrated, allowing free passage of molecules and cells, the involvement of the vast blood-brain boundary represented by the CP/CSF barrier in brain inflammatory processes has seldom been considered. In the present study we investigate, in mice, how the expression of genes encoding major constitutively expressed CP proteins is influenced by a systemic inflammatory stimulus. Confirming that the CP responds to peripheral inflammation, the messenger RNA (mRNA) levels of the pro-inflammatory cytokines interleukin 1 beta and tumor necrosis factor alpha are rapidly induced. As for the constitutively expressed proteins, while the mRNA for genes encoding transthyretin and transferrin remain unaltered by the inflammatory challenge, that for prostaglandin D2 synthase (LPTGDS) is up-regulated at 6 h, and stays up-regulated up to 24 h after lipopolysaccharide administration. Accordingly, LPTGDS CSF levels are also augmented. LPTGDS catalyzes the synthesis of the major prostanoid of the CNS and, being increased in the CSF, might mediate immune signaling into the brain. These observations emphasize that the CP must be considered a relevant mediator of immune signals between the periphery and the brain.

Key words: inflammation, prostaglandin D2 synthase, transthyretin, cerebrospinal fluid, mice.

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Abbreviations: BBB, blood-brain barrier; CP, choroid plexus; CSF, cerebrospinal fluid; HPRT, hypoxanthine guanine phosphoribosyl transferase; IL-1β, interleukin 1 beta; LPS, lipopolysaccharide; LPTGDS, prostaglandin D2 synthase; mRNA, messenger RNA; PCR, polymerase chain reaction; PGD2, prostaglandin D2; PGH2, prostaglandin H2; TF, transferrin; TGF-β1, transforming growth factor beta 1; TNF-α, tumor necrosis factor alpha; TTR, transthyretin; VCAM-1, vascular cell adhesion molecule 1; 15d-PGJ_2, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2.

Although there is growing evidence of interactions between systemic inflammation, the brain and neurological disease (Cunningham et al., 2005), the precise mechanisms by which the peripheral immune system transmits signals to the brain are largely unknown. While most studies focus on the blood-brain barrier (BBB) (Banks and Kastin, 1991; Banks, 2005; Engelhardt and Ransohoff, 2005; Engelhardt, 2006), a less investigated pathway of communication involves the choroid plexus (CP)/cerebrospinal fluid (CSF) route.

The CP is an epithelial cell monolayer located within the brain ventricles, responsible for the production of at least two-thirds of the CSF (Speake et al., 2001). By filling all brain ventricles and the subarachnoid space, the CSF is in contact with a vast region of the brain parenchyma. Despite the knowledge that blood flow through the CP is up to 10 times higher than in other brain regions, and that the CP membrane surface area is of the same order of magnitude as that formed by the BBB (Keep and Jones, 1990), few studies address the CP participation in brain inflammatory processes. In addition to producing CSF, the CP is also an active site of protein synthesis and possesses various relevant receptors in the inflammatory process, including that for the bacterial lipopolysaccharide (LPS) (Lacroix et al., 1998) and several others for neurotransmitters, growth factors and hormones (Chodobski and Szmydynger-Chodobska, 2001). Therefore, the CP seems singularly well equipped to function in the surveillance and maintenance of the biochemical milieu of the CNS under both physiological and pathological conditions (Chodobski and Szmydynger-Chodobska, 2001) including inflammation. Furthermore, recent studies implicated CP proteins (transthyretin [TTR], the major protein synthesized and secreted by the CP, and the receptor megalin) as neuroprotective in Alzheimer's disease (Carro et al., 2005a; Carro et al., 2005b; Sousa et al., 2006).

One other finding indicating that the CP could have a critical role as a "mediator" of the interactions between the CNS and the peripheral immune system is the migration of lymphocytes from the blood to the apical side of the CP epithelia (Petito and Adkins, 2005), a process facilitated by the expression of adhesion molecules (Endo et al., 1998; Wolburg et al., 1999; Engelhardt et al., 2001). The CP has also been suggested as a route for virus (such as human immunodeficiency virus) access into the brain (Petito, 2004).

Collectively these observations strongly suggest that the CP could act as a brain "immune sensor" involved in the communication of the immune system with the CNS. Of relevance, it is unknown whether the inflammation often

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associated with neurological diseases could influence the CP/CSF barrier properties and/or affect decisively pharmacological treatment strategies (Johanson et al., 2005; Strazielle and Ghersi-Egea, 2005). In order to further study how the CP responds to a systemic inflammatory stimulus, the present study evaluates, over time, the expression of genes encoding major CP proteins.

EXPERIMENTAL PROCEDURES

Animals and LPS injection

All experiments were conducted using 8–9 week C57BL/6 male mice (Charles River, Barcelona, Spain), in accordance with the European Communities Council Directive 86/09/EEC guidelines for the care and handling of laboratory animals. All efforts were made to minimize animal suffering and reduce the number of animals used in these studies. Animals were maintained under a 12-h light/dark cycle at 22.5 °C and 55% humidity and fed with regular rodent chow (Mucedola, Milano, Italy) and tap water *ad libitum*. In order to reduce stress-induced changes in the hypothalamus-pituitary axis, animals were handled for 1 week prior to the beginning of the experiment. Animals were injected i.p. with 5 μg/g body weight of LPS (*Escherichia coli*, serotype O55:B5; Sigma, St. Louis, MO, USA) or vehicle alone (0.9% NaCl).

Animals were anesthetized with ketamine hydrochloride (150 mg/kg) plus medetomidine (0.3 mg/kg), transcardially perfused with cold saline and killed 1, 3, 6, 12, 24 or 72 h after LPS injection. For messenger RNA (mRNA) studies, liver and CP were rapidly removed, frozen in liquid nitrogen and stored at −80 °C. CP isolation was made under conventional light microscopy (Stemi DV4, Carl Zeiss, Oberkochen, Germany). At least five pools of CP (from four animals each) were prepared for each time point. The experiment was done twice. In another experiment, CSF was collected from the cisterna magna and pooled from several animals. An aliquot was kept for blood contamination analysis, and the remainder immediately frozen. No samples were contaminated, in accordance with the criteria previously described (Huang et al., 1995).

Gene expression analysis

Total RNA, isolated from liver using Trizol (Invitrogen, Carlsbad, CA, USA) and from CP using the RNAqueous-Micro Kit (Ambion, Austin, TX, USA), was reverse transcribed into first-strand complementary DNA using the superscript first-strand synthesis system for reverse-transcription polymerase chain reaction (PCR) (Invitrogen) according to the manufacturer's instructions. Semiquantitative multiplex PCRs were performed as previously described (Wong et al., 1994). Briefly, each PCR cycle was composed of the following steps: 94 °C for 30 s, 58 °C for 45 s, and 72 °C for 60 s. A sequential series of PCRs using each primer pair was performed initially to determine the number of cycles in which the amplification would reside within the exponential phase of the amplification curve both for the gene under study and for the housekeeping gene hypoxanthine guanine phosphoribosyl transferase (*Hprt*) (gene abbreviations and gene names are specified in accordance with the HUGO Gene Nomenclature Committee at http://www.gene.ucl.ac.uk/nomenclature/). The expression level of *Hprt* was used as internal standard, to which other PCR amplification products were normalized. Aliquots of the PCR products were separated by 2% agarose gel electrophoresis and stained with ethidium bromide. Gels were visualized with AlphaImager 2200 (AlphaInnotech, San Leandro, CA, USA) and analyzed densitometrically with the corresponding AlphaEase software. The oligonucleotide primers for (*Ttr*, transferrin (*Tf*), prostaglandin D2 synthase (*Lptgds*), transforming growth factor beta 1 (*Tgf-β1*), and *Hprt* were designed using the Primer3 software (Rozen and Skaletsky, 2000) on the basis of the GenBank sequences: NM013697; NM133977; NM008963; M13177; XM356404, respectively.

For interleukin 1 beta (*Il-1β)* and tumor necrosis factor alpha (*Tnf-α*), genes routinely studied in the laboratory, expression levels were studied by real-time PCR using the LightCycler instrument (Roche, Indianapolis, IN, USA). The LightCycler-FastStart DNA Master Hybridization Probes mixture was used according to the manufacturer's instructions. The cycling parameters were 1 cycle of 95 °C for 10 min, followed by 45 cycles of 94 °C for 10 s, annealing temperature (60 °C for *Hprt* and *Tnf-α* and 57 °C for *Il-1β*) for 10 s and 72 °C for 11 s. The specific probes for each cytokine were designed and synthesized by TIB MolBiol (Berlin, Germany). Single acquisition was done at the end of each annealing step. All primer sequences are available on request.

LPTGDS and TNF-α determinations

5 μl CSF or serum (negative control) were run on 15% SDS-polyacrylamide gel and transferred onto nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ, USA). LPTGDS was detected using a polyclonal antibody (Cayman Chemical, Ann Arbor, MI, USA) at 1:1000 dilution, followed by a secondary peroxidase-conjugated goat anti-rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:10000 dilution, and developed with the chemiluminescence system (Pierce Biotechnology, Rockford, IL, USA). Autoradiographs were scanned for densitometry analysis using a computerized image analysis system (AlphaInnotech, San Leandro, CA, USA; AlphaEase software). Data are reported as densitometry units.

TNF-α levels were determined in 50 μl serum and 10 μl pooled CSF samples using BD™ cytometric bead array (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions.

Statistical analysis

Values are reported as mean±S.E. Statistical significance was determined using Student's *t*-test and nonparametric Mann-Whitney test, with differences considered significant at $P<0.05$.

RESULTS

Choroid plexus and liver gene expression response to peripheral inflammation

To confirm that an acute-phase inflammatory response was properly elicited, we measured, in the liver, the expression levels of *Il-1β* and *Tnf-α*, two pro-inflammatory cytokines, at different time points. As expected, the expression of both genes is up-regulated by LPS. The expression of *Il-1β* and *Tnf-α* peaks at 1 h and remains significantly elevated at 12 h, returning to basal levels at 24 h (Fig. 1a and c, respectively) after LPS injection. Interestingly, liver *Tgf-β1* (an anti-inflammatory cytokine) expression levels are significantly higher at 3 h (when the pro-inflammatory response starts to decrease), peak at 24 h, and at 72 h are still higher compared to controls (Fig. 1e).

CP expression of *Il-1β* is not detected under basal conditions but is strongly elicited 1 h after injection of LPS, remaining detectable 72 h after (Fig. 1 b). Similarly, *Tnf-α* (Fig. 1d) is not detected in saline injected animals, peaks at 1h and at 72 h after LPS administration is still detectable. *Tgf-β1* up-regulation never reaches statistical significance when compared with the

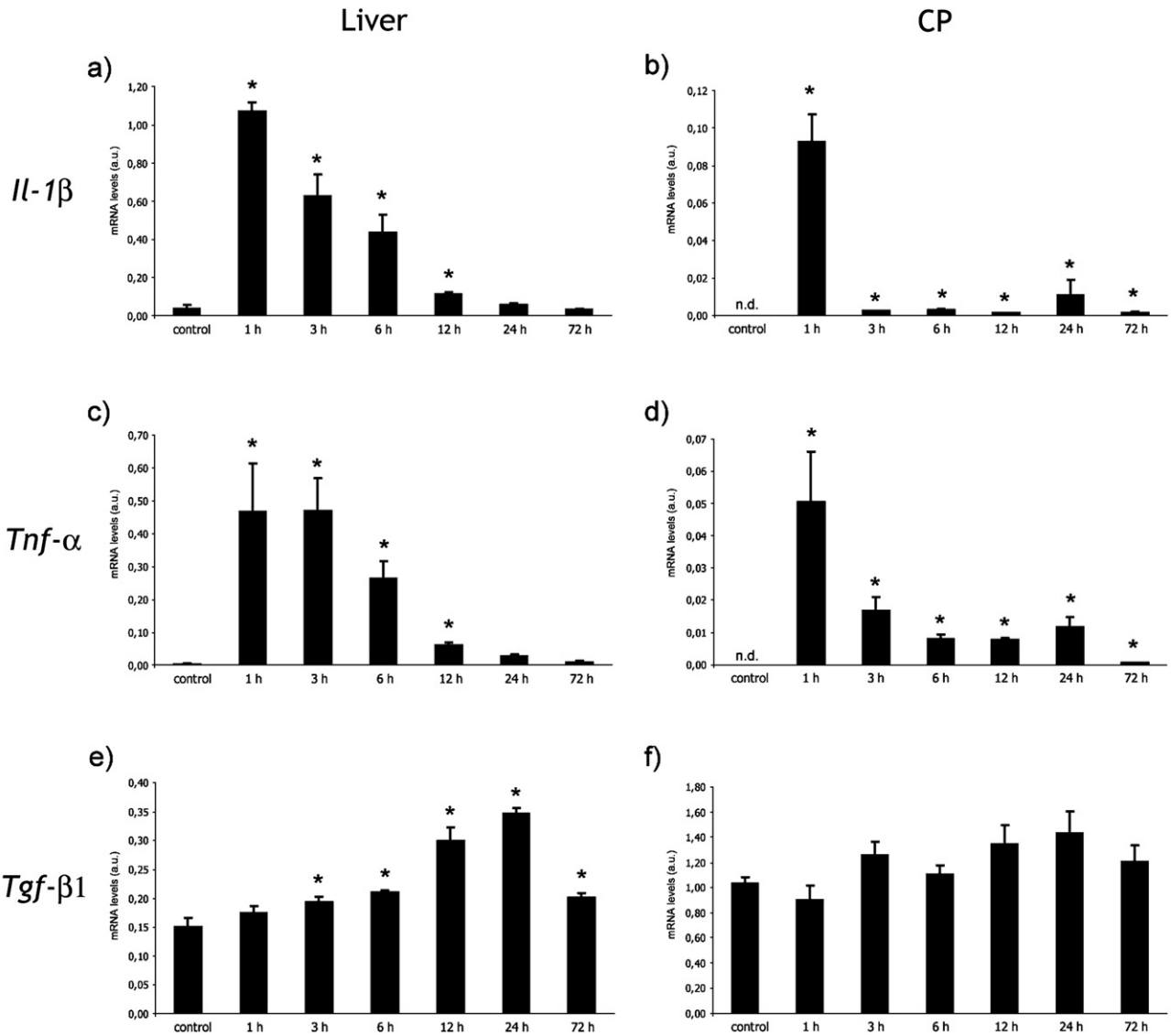

Fig. 1. mRNA expression kinetic profile of CP and liver cytokines upon LPS-induced systemic inflammation. Liver (a) and CP (b) *Il-1β* levels are up-regulated 1 h after LPS injection, and return gradually to the basal levels of saline-injected animals 72 h later. A similar kinetic profile is observed for *Tnf-α* expression (c, d). The expression of *Tgf-β1* is significantly up-regulated in the liver (e) from 3 h up to 72 h, while in the CP (f) it is only modestly up-regulated without reaching statistical significance.

basal level, but it is slightly and consistently up-regulated from 3 h up to 24 h (Fig. 1f).

We next investigated whether the expression of constitutive major CP proteins are influenced by an inflammatory stimulus and, when applicable, how this relates to the liver response. While the liver expression of *Ttr* is statistically significantly decreased from 6 h to 24 h (Fig. 2a), its levels in the CP decrease slightly and consistently without reaching statistical significance when compared with basal expression (Fig. 2 b). Similarly, *Tf* liver levels are decreased at 3 h (Fig. 2c) but unaltered in the CP for the time course of the experiment (Fig. 2d). In contrast, the expression of *Lptgds* is significantly increased from 6 h to 24 h and returns to basal levels at 72 h after LPS injection (Fig. 2e). *Lptgds* is not expressed in the liver.

LPTGDS and TNF-α determination

The increased mRNA expression levels of cytokines and LPTGDS correlate with the increase in protein concentration. 1 h after LPS injection, TNF-α serum ($n=3$) and CSF ($n=3$) levels increase from 14.3 ± 0.1 to 2622 ± 121 and from below detection to 123 ± 12 pg/ml, respectively; and decay almost to normal levels after 12 h (Fig. 3a). As seen in Fig. 3b, CSF LPTGDS concentration increases about 50% when compared with basal conditions at 12 and 24 h after the inflammatory stimulus.

DISCUSSION

By measuring the expression levels of several CP genes, the present study clearly shows that the CP responds

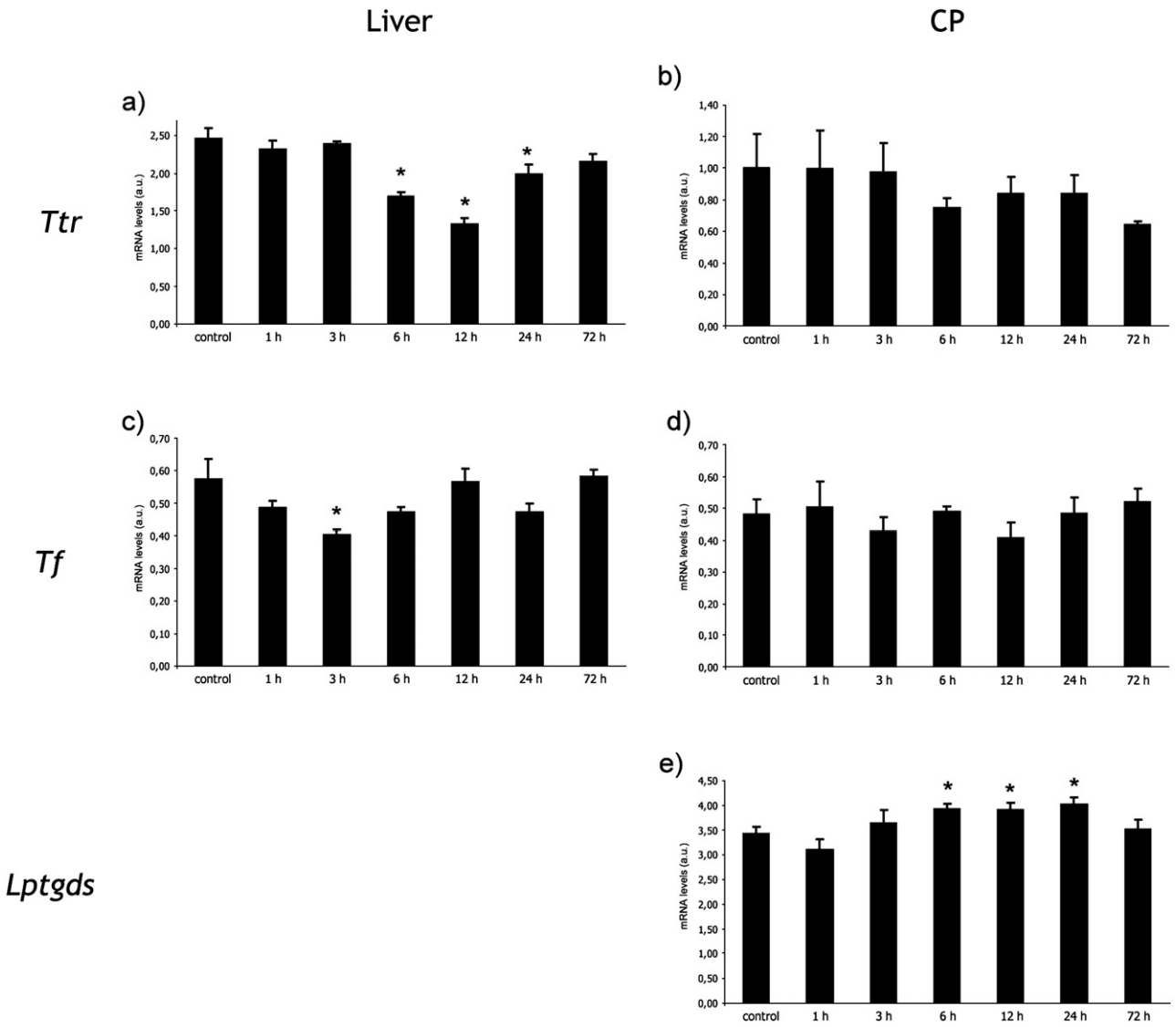

Fig. 2. CP and liver mRNA expression kinetic profile of proteins constitutively expressed by the CP. TTR mRNA levels in the liver (a) decrease from 6 to 24 h after LPS administration and those of TF (c) decrease at 6 h, while the expression of both genes is not influenced in the CP (b and d, respectively). The expression of *Lptgds,* which is absent in the liver, is significantly increased in CP (e) from 6 to 24 h.

promptly to a peripheral inflammatory stimulus. Such response includes not only genes encoding well-described immune modulators such as *Il-1*β and *Tnf*-α, but also genes encoding major constitutively expressed CP proteins such as LPTGDS. Of relevance, this effect on the CP is reflected in the CSF composition, as suggested by the increased CSF levels of TNF-α and LPTGDS.

We show here, for the first time, that the expression of *Lptgds* is up-regulated at 6, 12 and 24 h after the inflammatory stimulus, returning to basal levels at 72 h. Accordingly, CSF LPTGDS levels increase, which is in agreement with the kinetic profile of increased CSF concentration reported in rats after i.p. injection of LPS (Ishizaka et al., 2001). *Lptgds* is constitutively highly expressed by the CP (Blodorn et al., 1996), from which it is secreted into the CSF, and also by oligodendrocytes (Urade et al., 1993; Nagasaka et al., 2004). LPTGDS catalyzes the isomeration of prostaglandin H2 (PGH2) to produce prostaglandin D2 (PGD2), the major prostanoid in the CNS. The up-regulation of *Lptgds* might be secondary to the increase of IL-1β since the latter induces the expression of cyclooxygenase 2, an enzyme upstream in the prostaglandin pathway (Fujimori et al., 2003), as recently shown by the enhanced expression of spinal cord *Lptgds* and PGD2 biosynthesis during systemic inflammation (Grill et al., 2006). Whether directly or indirectly stimulated, a quick and transient increase in *Lptgds* expression in response to an inflammatory stimulus might be sufficient to induce second messenger signals to the brain parenchyma. PGD2, like other prostagladins, has been implicated as mediating both pro- and anti-inflammatory actions (Harris et al., 2002; Urade and Hayaishi, 2000). Recent studies showed, for instance, that PGD2 mediates neuroinflammation and demyelination in *twitcher* mice (Mohri et al., 2006). On the

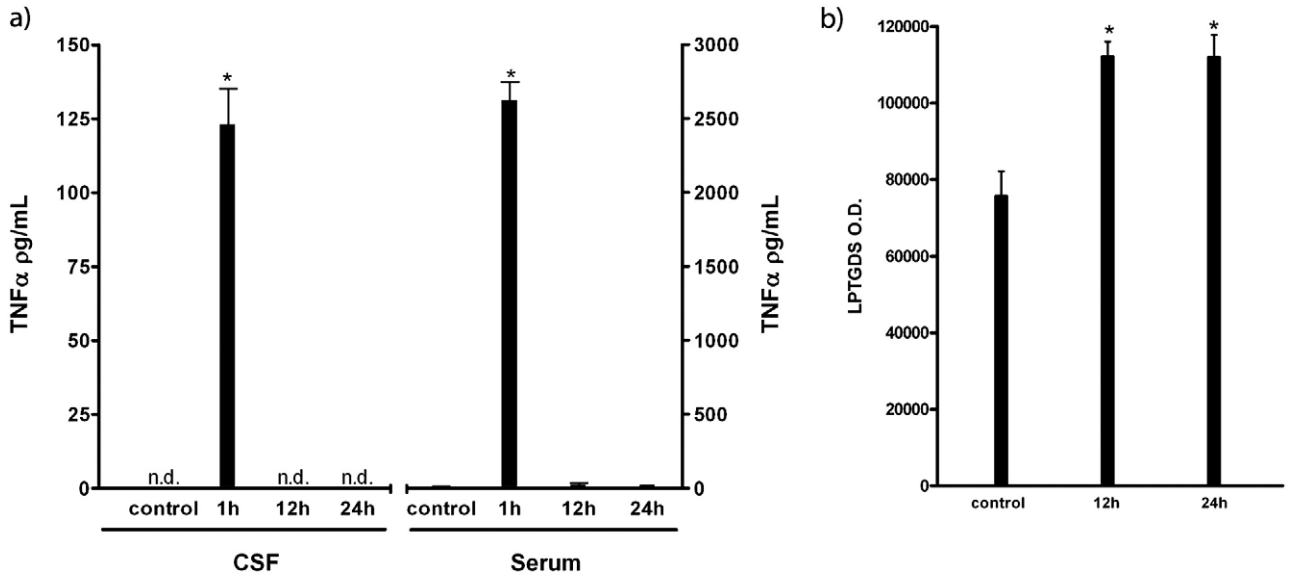

Fig. 3. TNF-α and LPTGDS levels. CSF and serum TNF-α levels drastically increase 1 h after LPS injection, rapidly returning to basal levels (a). Levels of CSF LPTGDS increase about 50% at 12 and 24 h after the peripheral inflammatory stimulus is imposed (b). OD, optical density.

other hand, endogenous PGD2 might display anti-inflammatory properties, given its ready conversion to bioactive cyclopentenone-type prostaglandins of the J2-series such as 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) (Armstrong, 1996; Straus and Glass, 2001; Mouihate et al., 2004). 15-dPGJ_2 displays anti-inflammatory properties by repressing the expression of genes encoding inflammatory molecules such as inducible nitric oxide synthase and *Tnf-α* (Jiang et al., 1998; Ricote et al., 1998; Rossi et al., 2000; Straus et al., 2000), attenuates immune activation of several pro-inflammatory molecules in various culture systems and has antipyretic properties (Mouihate et al., 2004). However, in specific circumstances, 15d-PGJ_2 also has pro-inflammatory properties (Harris et al., 2002). Of notice, increased immunoreactivity against vascular cell adhesion molecule 1 (VCAM-1) and major histocompatibility complex antigens in the apical site of the CP epithelium (Engelhardt et al., 2001) is observed in conditions of brain inflammation such as those in experimental autoimmune encephalitis, which might contribute to activation of intraventricular T lymphocytes. If so, the up-regulation of CP *Lptgds* we observe starting at 6 h after LPS stimulus could also contribute to the resolution of inflammation by down-regulating the expression of VCAM-1, as recently shown in endothelial cells with increased endogenous PGD2 levels (Negoro et al., 2005).

Since some of the CP major proteins are also synthesized by the liver, we could compare the liver and CP acute-phase responses to inflammation. Both the liver and the CP respond to LPS by quickly inducing the expression of the pro-inflammatory cytokine *Il-1β* and *Tnf-α* genes. Interestingly, the up-regulatory kinetic profile is similar in both tissues, peaking at 1 h and returning to basal levels (but still detectable in the case of the CP) at 72 h. Although CP *Il-1β* and *Tnf-α* expression has never been investigated or discussed, per se, in the context of inflammation, previous *in situ* hybridization studies have shown an identical kinetic profile for both genes after peripheral LPS administration (Quan et al., 1998; Nadeau and Rivest, 1999). Altered gene expression in the CP might ultimately influence CSF composition. While other sources may contribute to increased CSF cytokine concentration upon an inflammatory stimulus in the periphery, it is reasonable to suggest that the CP greatly contributes to it. Accordingly, the increase in TNF-α levels we find in the CSF correlates with the CP expression profile.

Up-regulation of anti-inflammatory cytokines in the liver, such as *Tgf-β1*, occurs by the time the inflammatory response starts to decrease, as we see here at 3 h after endotoxin administration. Interestingly, a similar increase is observed for the CP from 3 h up to 72 h, even though the difference from basal levels does not reach statistical significance. To our knowledge, no studies to date have addressed *Tgf-β1* expression in the CP under systemic inflammatory conditions. The slight but consistent increase in expression of CP *Tgf-β1* we show here might constitute an attempt to control the inflammatory response triggered in the CP. Indeed, TGF-β1 blocks proliferation and suppresses functions of activated microglia (Bottner et al., 1999). Notably, the CP also produces the receptors for TGF-β1 and IL-1β (Chodobski and Szmydynger-Chodobska, 2001). Therefore, even a slight change in IL-1β and TGF-β1 production might regulate the synthesis and secretion of other choroidal proteins via autocrine/paracrine signaling, a possibility that deserves further study.

In the present study we also analyzed the expression of two other major CP proteins: TTR (Aldred et al., 1995) and TF. As carriers for amyloid beta peptide and iron, respectively, both have recently been implicated in neurological diseases such as Alzheimer's disease (Palha, 2002; Zecca et al., 2004; Carro et al., 2005a; Carro et al., 2005b; Sousa et al., 2006). A single report implicates TTR

as an endogenous anti-inflammatory mediator given its ability, *in vitro*, to inhibit monocyte and endothelial IL-1β production (Borish et al., 1992). While TTR is a negative acute phase protein in the liver (Birch and Schreiber, 1986), s.c. injection of turpentine did not influence CP *Ttr* expression (Dickson et al., 1986). We also find that up to 72 h after i.p. injection, LPS administration does not influence CP *Ttr* expression; even though *Ttr* expression decreases in the liver from 6h up to 24h after injection. Similarly, the mRNA for TF, another liver negative acute phase protein, decreases after LPS administration; but no effect is observed in its CP expression. The differential response in the liver when compared with the CP possibly results from tissue-specific transcription factors influencing *Ttr* (Costa et al., 1990; Yan et al., 1990) and *Tf* (Zakin et al., 2002) transcription regulation, as previously suggested. The expression of cystatin C and insulin growth factor 2, two other proteins constitutively expressed by the CP, are not influenced by the inflammatory challenge used in this study (data not shown).

In summary, by influencing the expression of both pro- and anti-inflammatory molecules, the CP seems to behave as an immunosensor for the brain. Studying the detailed balance between both types of signals is certainly essential to understand the final response of the brain to an inflammatory insult, and the CP must be considered a relevant player in such processes.

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Lipocalin 2 is a choroid plexus acute-phase protein

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Brief Communication

Lipocalin 2 is a choroid plexus acute-phase protein

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Lipocalin 2 (LCN2) is able to sequester iron-loaded bacterial siderophores and, therefore, is known to participate in the mammalian innate immune response. Of notice, LCN2 was shown to display bacteriostatic effects both in *in vitro* and *in vivo*. To reach the brain, bacteria must cross the blood–brain or the choroid plexus (CP)/cerebrospinal fluid (CSF) barriers. Additionally, as the CP is responsible for the production of most of the CSF, responses of the CP mediate signaling into the brain. We show here that in conditions of peripheral inflammation, LCN2 behaves as an acute phase protein in the CP. As early as 1 h after lipopolysaccharide peripheral administration, *Lcn2* mRNA levels are upregulated, returning to basal levels after 72 h. Increased LCN2 protein is observed in choroidal epithelia and in endothelial cells of blood vessels in the brain parenchyma. Higher levels of LCN2 are also present in the CSF. These observations suggest that expression of LCN2 at the CP/CSF barrier might be bacteriostatic in the brain, avoiding bacteria dissemination within the CSF into the brain parenchyma. This study shows that the LCN2 is produced by the CP as a component of the innate immune response that protects the central nervous system from infection.
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Keywords: choroid plexus; inflammation; iron; lipocalin; lipopolysaccharide; siderophore

Introduction

The brain is protected from the periphery by the blood–brain and blood–cerebrospinal fluid (CSF) barriers. These barriers restrict the passage of several endogenous and xenobiotic compounds from the periphery to the central nervous system. Although most studies focus on the blood–brain barrier, evidence is increasing on the role of the choroid plexus (CP) in communication between the immune system and the central nervous system. The CP is located within the brain ventricles, and its epithelial cells are responsible for producing most of the CSF. In addition to producing CSF, the CP is also an active site of protein synthesis and possesses receptors for several immune mediators, hormones, and growth factors (Chodobski and Szmydynger-Chodobska, 2001). Therefore, anatomically and physiologically, the CP is well positioned to respond to stimulus induced in the periphery. Unraveling which are the molecular mediators of the CP response to inflammation and infection is, therefore, important to understand how the CP mediates or protects the brain from associated brain damage.

The Gram-negative bacteria lipopolysaccharide (LPS), a major molecular component of the cell wall, has been widely used as a model of inflammation and as a tool to study response to infection. Cells of the immune system are able to recognize LPS through the Toll-like receptors (TLRs), which induce signals responsible for the activation of the innate immune response. One of the proteins shown previously to participate in such a response is lipocalin 2 (LCN2). Lipocalin 2, a member of the lipocalin family of proteins, was initially found in neutrophil granules (Kjeldsen *et al*, 2000) and later described as an acute-phase protein in the liver (Liu and Nilsen-Hamilton, 1995; Sunil *et al*, 2007), in blood and peritoneal cells (Flo *et al*, 2004), and in several epithelial tissues (Cowland *et al*, 2003). Lipocalin 2 binds to the iron-loaded siderophores, iron chelators secreted by pathogens, and represents an effective defense strategy for the body to control the growth of pathogens (Neilands, 1995). By limiting iron availability, LCN2 exerts a bacteriostatic effect as shown both in *in vitro* (Goetz *et al*, 2002)

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and *in vivo* (Flo *et al*, 2004). Thus, limitation of iron availability is a very effective defense strategy for the body to control the growth of pathogens.

In this study, we intended to investigate whether the LCN2 was among the immune modulators induced in the CP in response to peripheral inflammation.

Materials and methods

Animals and Lipopolysaccharide Injection

All experiments were conducted using 8- to 9-week-old C57BL/6 male mice (Charles River, Barcelona, Spain), in accordance with the European Community Council Directive 86/09/EEC guidelines for the care and handling of laboratory animals. Animals were maintained under 12 h light/dark cycles at 22 to 24°C and 55% humidity and fed with regular rodent's chow and tap water *ad libitum*. To reduce stress-induced changes in hypothalamus–pituitary axis associated with the injection, animals were handled for 1 week before the beginning of the experiment. Animals were administered LPS intraperitoneally at a 5 μg/g body weight dose (*Escherichia coli*, serotype O26:B6; Sigma, St Louis, USA); a subset of animals was injected with vehicle (0.9% NaCl) alone.

Animals were anesthetized with ketamine hydrochloride (150 mg/kg) plus medetomidine (0.3 mg/kg), transcardially perfused with cold saline, and killed 1, 3, 6, 12, 24, or 72 h after LPS injection. For the mRNA studies, CP isolation was made under conventional light microscopy (SZX7; Olympus, Hamburg, Germany), and tissue was rapidly removed, frozen in dry ice, and stored at −80°C. At least five pools of CP (from three animals each) were prepared for each time point. The experiment was performed twice. Cerebrospinal fluid was collected from the cisterna magna and pooled from several animals. An aliquot of each pool was used to verify the absence of blood contamination and the remainder was immediately frozen.

Quantitative PCR Lipocalin 2 Gene Expression Measurements

Total RNA was isolated from CP using Trizol reagent (Invitrogen, Carlsbad, CA, USA). 500 ng of total RNA were amplified using the Superscript RNA amplification system (Invitrogen) according to the manufacturer's instructions. Choroid plexus RNA was reverse transcribed using random primers of the superscript first-strand synthesis system for reverse transcription PCR (Invitrogen).

Quantitative real-time PCR analysis was used to measure the expression levels of the *Lcn2* mRNA transcript. The reference gene, hypoxanthine guanine phosphoribosyl transferase (*Hprt*), was used as internal standard for normalization (gene abbreviations and gene names are specified in accordance with the HUGO Gene Nomenclature Committee at http://www.gene.ucl.ac.uk/nomenclature/), since we have first confirmed that its expression is not influenced by the experimental conditions.

The oligonucleotide primers for *Lcn2* and *Hprt* were designed using the Primer3 software on the basis of the GenBank sequences NM_008491 and NM_013556, respectively. The real-time PCR reactions, using equal amounts of total RNA from each sample, were performed on a LightCycler instrument (Roche Diagnostics, Basel, Switzerland) using QuantiTect SYBR Green RT-PCR reagent kit (Qiagen, Hamburg, Germany). Product fluorescence was detected at the end of the elongation cycle. All melting curves exhibited a single sharp peak at a temperature characteristic of the primer used.

Immunohistochemistry

Animals were transcardially perfused, under anesthesia, with 4% of paraformaldehyde in phosphate-buffered saline, 12 and 24 h after LPS or saline injections. After perfusion, brains were removed from the skull, left 24 h in the fixative solution, and then included in paraffin. Immunohistochemistry was performed on coronal sections (10 μm) with anti-mouse lipocalin-2/neutrophil gelatinase-associated lipocalin (R&D Systems, Minneapolis, MN, USA) at 1:500 dilution or anti-transthyretin (kindly provided by Dr MJ Saraiva, Institute for Molecular and Cell Biology, Porto, Portugal) at 1:1,000 dilution as primary antibodies. Secondary antibodies, biotinylated (Vector Laboratories Inc., Burlingame, CA, USA) or fluorescent (Molecular Probes, Carlsbad, CA, USA) were used at 1:200 and 1:500, respectively, following standard procedures. For this analysis, we used three animals per group. Samples were analyzed using optical (BX61; Olympus) or confocal (FV1000; Olympus) microscopes.

Protein Measurements

For the detection of LCN2 protein in the CSF, we performed a direct enzyme-linked immunosorbent assay. Two microliters of CSF was used to detect LCN2 levels at the different time points. Lipocalin 2 was detected using the same antibody mentioned above, at 1:300 dilution, followed by a secondary peroxidase-conjugated donkey anti-goat antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:500 dilution, and developed with 2-2′azinobis (3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt (ABTS) (Sigma). The reaction was stopped using 0.1 mol/L citric acid and read at an optical density of 405 nm. The standard curve was made with the recombinant mouse LCN2/neutrophil gelatinase-associated lipocalin (R&D Systems). The detection limit was 50 ng/mL.

Statistical Analysis

Values are reported as mean ± s.e. Statistical significance was determined using the non-parametric Mann–Whitney test, with differences considered significant at $P < 0.05$.

Results

Lipocalin 2 Expression Profile in Choroid Plexus and Cerebrospinal Fluid Levels after Lipopolysaccharide Injection

Choroid plexus *Lcn2* expression was very low in basal conditions (and identical at any time point after saline injection), but strongly upregulated from 1 to 72 h after LPS injection, with a peak at 12 h (Figure 1A). In the CSF, LCN2 was below detection level in basal conditions until 3 h after LPS injection (Figure 1B). A robust upregulation was observed at 6 h, remained for at least 24 h and returned to basal levels at 72 h after LPS administration. The same expression profile was observed in the liver (data not shown). Increased expression of *Lcn2* in the CP preceded the increase in the CSF protein, suggesting that LCN2 is produced and secreted by epithelial CP cells to the CSF.

Immunohistochemistry Analysis

We next studied, by immunohistochemistry, the LCN2 protein immunoreactivity in the CP. As shown in Figure 2, although no signal was observed in saline controls (Figures 2A, 2D and 2G) at 12 h (or at any time point after saline injection), at 12 h after LPS administration immuno-positive staining was observed in CP epithelial cells but also in other cells within the CP stroma (Figures 2B and 2E); staining was also observed in endothelial cells throughout the brain parenchyma (Figure 2H). At 24 h, staining was mostly observed in CP epithelial cells (Figures 2C and 2F). In the endothelial cells of the brain parenchyma, a weaker staining was still observed (Figure 2I). Of notice, not all CP epithelial cells showed labeling for LCN2. Double staining for LCN2 and transthyretin, a protein specifically synthesized by CP epithelial cells, confirmed that LCN2 is produced by epithelial cells of the CP (Figure 2J).

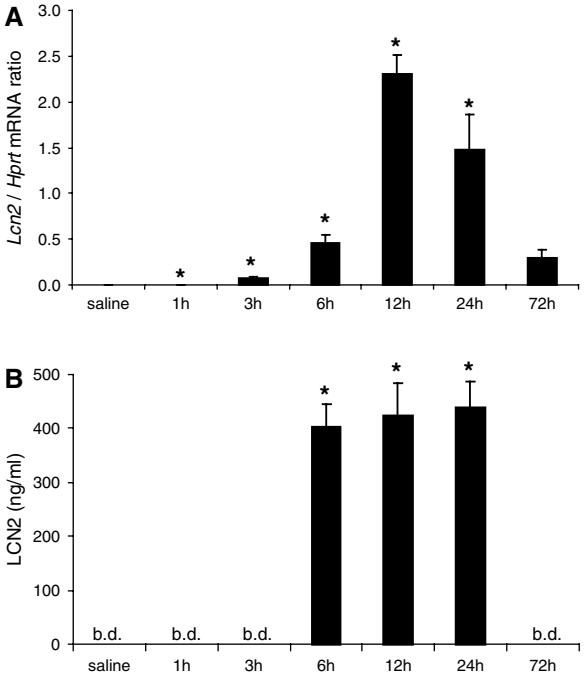

Figure 1 Lipocalin 2 (*Lcn2*) mRNA expression in the choroid plexus and LCN2 cerebrospinal fluid (CSF) levels are increased in mice injected with lipopolysaccharide (LPS). (**A**) *Lcn2* expression is strongly induced by peripheral administration of LPS, peaking at 12 h and returning to basal levels after 72 h. (**B**) This increase in *Lcn2* expression results in augmented circulating CSF LCN2 protein levels, observed from 6 until 24 h after LPS injection. b.d., below detection.

Discussion

Although an acute-phase response is well described for the liver, increased evidence is supporting a similar response at the blood–brain barriers (Marques *et al*, 2007; Quan *et al*, 1998). This study shows, *in vivo*, that the gene encoding for LCN2 is quickly upregulated in the CP in response to a peripheral immune challenge. As a result, increased levels of LCN2 are secreted into the CSF. Similarly, increased LCN2 is also observed in blood vessels of the brain parenchyma. Because LCN2 binds to various iron-loaded bacterial siderophores, this increase in LCN2 levels is expected to decrease iron availability for bacterial growth and, therefore, may constitute an important mechanism of neuroprotection against bacteria accessing and disseminating within the brain.

While reporting the blood–brain and blood–CSF barriers as novel sites of LCN2 synthesis, we confirm liver upregulation of *Lcn2* mRNA after an LPS inflammatory challenge with a profile similar to that just recently reported (Sunil *et al*, 2007).

Within the CP, immunohistochemistry reveals positive labeling for LCN2 in epithelial cells only in LPS-treated animals. Interestingly, not all CP epithelial cells are immunoreactive to LCN2 antibodies, suggesting that only certain cells express LCN2 in detectable amounts, at least at a given time. Staining is also observed in blood vessels in the brain parenchyma. It is likely that the other cells of the CP stroma immuno-positive for LCN2 are also endothelial cells of the CP vasculature. These observations together with the fact that endothelial cells of the CP capillaries are fenestrated, and that LCN2 also increases in the blood upon an inflammatory stimulus (Flo *et al*, 2004) might be particularly relevant in impeding pathogen access into the brain. On one hand, LCN2 might reduce iron access to the bacteria in the blood vessels and, in the case of bacteria entering into the CSF, CSF-borne LCN2 can sequester siderophore-bound iron and prevent bacteria dissemination within the ventricular brain system and into the brain parenchyma. Of notice, it has been recently reported that human neutrophil gelatinase-associated lipocalin, the human homolog

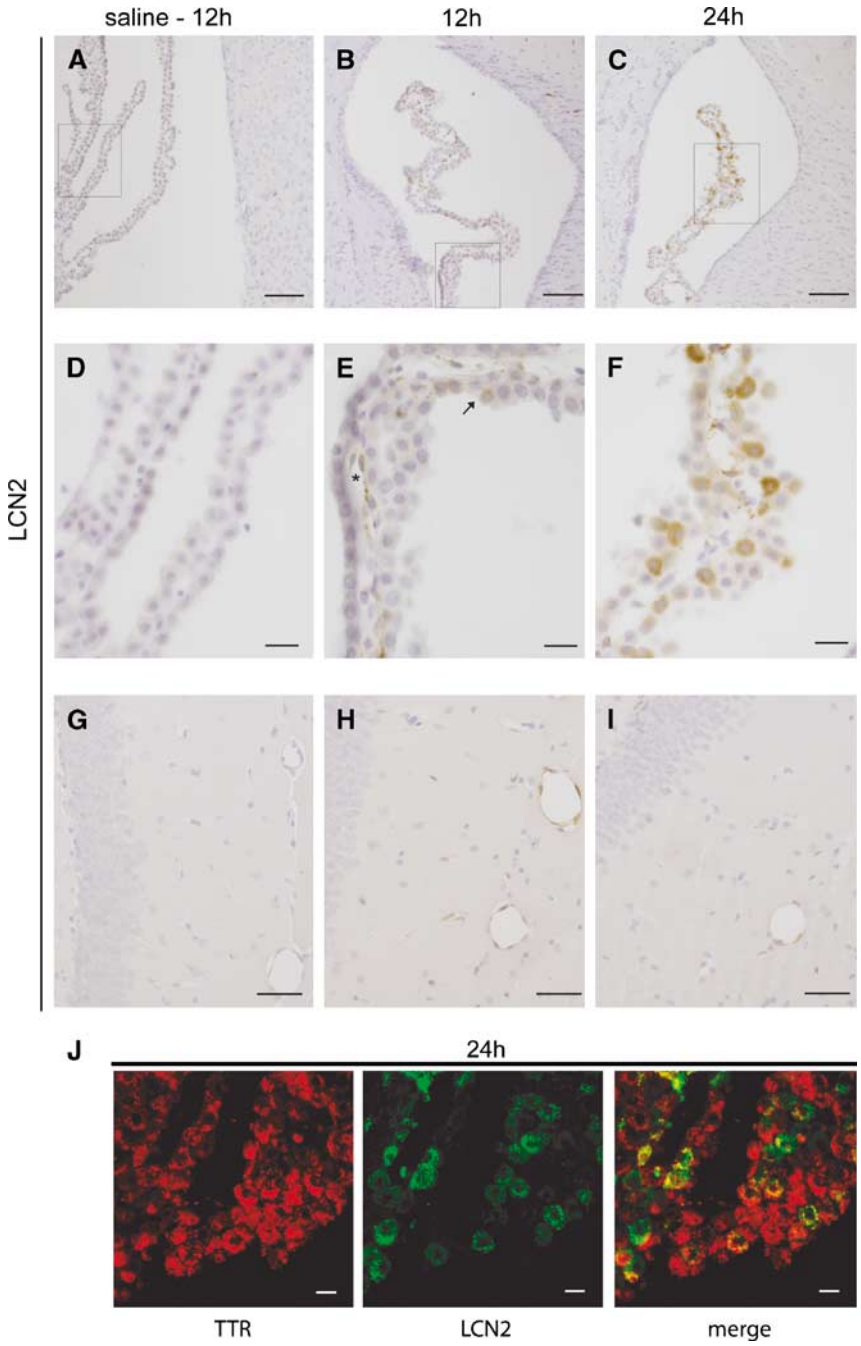

Figure 2 Immunohistochemistry for lipocalin 2 (LCN2) in the choroid plexus (CP) and in blood vessels of the brain parenchyma of mice injected with lipopolysaccharide (LPS). Although no staining is present in (**A**, **D**, and **G**) saline controls, LCN2-positive cells are observed (**B**, **E**, and **H**) 12 h and (**C**, **F**, and **I**) 24 h after LPS injection. At 12 h, positive staining is observed not only in epithelial cells (arrow) but also in other cells of the (**B** and **E**) CP stroma (star), as well as in endothelial cells of blood vessels in the (**H**) brain parenchyma. In the CP, at 24 h staining seems restricted to (**C** and **F**) epithelial cells, and a weaker staining is still present in the (**I**) blood vessels. Staining for (**J**) transthyretin, a marker of CP epithelial cells, confirms the presence of LCN2 in epithelial cells. Scale bar = (**A**–**C**) 100; (**D**–**F**) 20; (**G**–**I**) 50, and (**J**) 10 μm.

of LCN2, is taken up by cells through the receptor megalin (Hvidberg *et al*, 2005). Since megalin is a scavenger receptor present in the CP and in the endothelial cells of blood vessels, it is possible that neutrophil gelatinase-associated lipocalin bound to iron-loaded siderophore can be taken up by these cells, further preventing iron availability for bacterial growth within the CSF and the brain parenchyma.

This is, to the best of our knowledge, the first demonstration of LCN2 synthesis at the blood–brain barriers *in vivo*. Previous studies failed to show *Lcn2* mRNA in whole brain extracts or several regions of the brain parenchyma (Cowland *et al*, 2003) under

normal physiological conditions. On the contrary, two other studies have previously reported upregulation of LCN2 expression in whole brain extracts by turpentine (Liu and Nilsen-Hamilton, 1995) and in the basal forebrain of mice intranasally infected with influenza virus (Ding and Toth, 2006). Given this data, it is likely that *Lcn2* expression in the CP and in blood vessels is contributing to the overall upregulation of *Lcn2* expression observed in whole brain homogenates, particularly since some of these brain regions are often contaminated by CP tissue (Sousa *et al*, 2007). Of notice, a recent report using LPS stimulation of primary cultures enriched in CP epithelial cells showed induction of LCN2 secretion, which is in agreement with the *in vivo* data we report here (Thouvenot *et al*, 2006).

Still to investigate is the molecular pathway that triggers LCN2 expression at these sites. *In vitro* studies in a lung epithelial cell line stimulated by interleukin-1β (IL-1β) showed upregulation of LCN2, whereas LPS did not (Cowland *et al*, 2006). It is possible that IL-1β produced in the periphery in response to LPS induces a response in the CP and in blood vessels through IL-1β receptors (Cunningham *et al*, 1992). This would induce the synthesis of additional IL-1β and other immune mediators, such as LCN2. Alternatively, or in addition, the response might be directly linked to activation of receptors to bacterial endotoxins located at the barriers, as has been shown *in vitro* for LPS activation of LCN2 expression in macrophages (Meheus *et al*, 1993) and in lung epithelial cell lines transfected with TLR4 (Cowland *et al*, 2003). In accordance, several TLRs, including TLR4 and TLR2, are constitutively expressed in the CP and in cells lining the blood vessels (Laflamme *et al*, 2003).

Irrespective of the molecular pathway that triggers their expression, proteins secreted by the CP toward the CSF may participate in the host's defense against CSF bacterial infections. Interestingly, in the presence of IFN-γ, the supernatant of porcine CP epithelial cells displayed a bacteriostatic effect against *Streptococcus suis*. This was at least partially attributed to the induction of indoleamine 2,3-dioxygenase activity and the resulting depletion of L-tryptophan (Adam *et al*, 2004). Given the data we now present, it is also possible that the secretion of LCN2 into the media also contributes to this bacteriostatic effect. Recent studies clearly show that LCN2-null mice exhibit increased susceptibility to infection by bacteria that depend on entherocalin-like siderophore-dependent iron uptake (Berger *et al*, 2006; Flo *et al*, 2004). It will be interesting to study the brain response to infection in these mice.

In summary, we describe here a novel acute-phase response protein produced at the barriers between the blood and the brain. By increasing the expression and secretion of LCN2, these barriers contribute to restrict access of bacteria into the brain. This adds CP and endothelial cells of blood vessels as sites in which LCN2 expression is induced by inflammation, and further supports a role for the blood–brain and blood–CSF barriers as part of the innate immune response against central nervous system infection.

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Disclosure

The authors declare no conflict of interest.

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4

Altered iron metabolism is part of the choroid plexus response to peripheral inflammation

Altered iron metabolism is part of the choroid plexus response to peripheral inflammation.

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Abstract

Iron is essential for normal mammalian cellular homeostasis but, in excess, promotes free radical formation and is associated with neurodegenerative disorders such as Alzheimer's disease. Iron is also required for microbial growth and in case of infection the host activates several mechanisms to prevent iron availability for bacteria. We have recently shown that by synthesizing and secreting lipocalin 2 into the cerebrospinal fluid (CSF), the choroid plexus can contribute to the brain innate immune response. Here we show that other iron-related genes participate in brain iron homeostasis. As early as 3h after intraperitoneal administration of lipopolysaccharide (LPS), hepcidin (HAMP) mRNA levels are up-regulated in the choroid plexus, returning close to basal levels at 24h. HAMP has been described as a liver-derived hormone that regulates iron availability by decreasing ferroportin (FPN)-mediated iron exportation from the enterocyte into the bloodstream. The expression of genes encoding for other proteins, also involved in the regulation of iron metabolism including ceruloplasmin, transferrin receptor type 2 (TFR2), interleukin-6 (IL-6) and transcription factors such as signal transducer and activator of transcription 3 (STAT3), are also up-regulated by the peripheral inflammatory stimulus. We propose that upon an acute peripheral inflammatory stimulus HAMP decreases FPN-iron delivery into the CSF and increases iron uptake from the CSF, therefore decreasing iron availability for bacterial growth and dissemination within the brain.

Keywords: Choroid plexus; inflammation; iron metabolism; lipopolysaccharide; hepcidin.

Introduction

Iron is a required element for normal cellular homeostasis, participating in processes that range from cell proliferation, oxygen transport and energy metabolism (Aisen et al., 2001; Papanikolaou and Pantopoulos, 2005). While iron deficiency leads to conditions such as anemia, in excess and by promoting free radical formation and oxidative stress, it is deleterious for the organism. Iron homeostasis is, therefore, tightly regulated. Hepcidin (HAMP), a liver-derived peptide hormone, has been recently described as the main iron regulator, by modulating iron release from the intestine into the plasma (Nemeth and Ganz, 2006; Darshan and Anderson, 2007), the rate limiting process in iron homeostasis (De Domenico et al., 2007). Iron is taken up from the diet by enterocytes, and it is released into the bloodstream through the iron exporter ferroportin (FPN). In conditions of iron sufficiency, HAMP secreted by the liver into the bloodstream reaches the enterocyte and binds to FPN which is then internalized and degraded (Nemeth et al., 2004). As a consequence iron is kept within the cell which results in lower iron release into the circulation. It has been shown that increased HAMP levels result in anemia (Means, 2004; De Domenico et al., 2007) while decreased levels are associated with iron-overload diseases (De Domenico et al., 2007). Once in the blood iron is mostly bound to plasma proteins, particularly to transferrin (TF), and is delivered to cells through TF receptor (TFR)-mediated endocytosis (Moos and Morgan, 2000; Ponka and Lok, 1999). Within the cells, iron can be stored bound to ferritin (Harrison and Arosio, 1996). Iron homeostasis is challenged in several conditions such as infection by most microorganisms. As any other cell, microorganisms need iron for survival and proliferation and, upon infection, the host promptly responds by increasing the levels of several iron-binding proteins. Among these, ferritin and ceruloplasmin are well known liver positive acute-phase proteins (Ceciliani et al., 2002). However, most organisms secrete siderophores, molecules with higher affinity for iron than the major plasma iron-carrier proteins (Miethke and Marahiel, 2007). In response, the host, mostly circulating neutrophils but also hepatocytes, secretes lipocalin 2 (LCN2) (Borregaard et al., 2007; Jayaraman et al., 2005), a protein that has the ability to sequester iron-loaded siderophores (Flo et al., 2004).

Several studies have addressed how the innate immune system influences iron metabolism, but few focused on the brain. Understanding brain iron metabolism might be of particular interest since in aging mammals iron accumulation is believed to contribute to neurodegeneration (Zecca et al., 2004). It has been proposed that iron uptake by the brain depends on TF-mediated endocytosis by endothelial cells of the blood-brain barrier (Moos and Morgan, 2000; Moos et al.,

2006). Iron would then be likely released through FPN (Rouault and Cooperman, 2006) into the interstitial fluid of the brain parenchyma and then taken up by oligodendrocytes again through TFR mediated endocytosis.

Despite the observation that TF (Dickson et al., 1985), TFR (Giometto et al., 1990) and FPN (Wu et al., 2004) are synthesized by the choroid plexus, little attention has been paid to these structure in iron access into the brain. Of relevance, it has been recently shown that LCN2 is quickly synthesized and secreted by the choroid plexus into the cerebrospinal fluid (CSF) upon an inflammatory stimulus induced in the periphery (Marques et al., 2008). This observation suggests that limiting iron availability to microorganisms is part of the strategy set by the choroid plexus to fight potential infections of the central nervous system. Taken together, these observations prompted us to further investigate the choroid plexus iron homeostasis upon an inflammatory stimulus induced in the periphery. The data suggest that the choroid plexus has, for the brain, a similar role in iron metabolism of that played by both the liver and intestine for the rest of the body.

Materials and Methods

Animals and LPS injection

All experiments were conducted using 8-9 week-old C57BL/6 male mice, and Wistar neonates (postnatal day 1 or 2) rats (Charles River, Barcelona, Spain), in accordance with the European Community Council Directive 86/09/EEC guidelines for the care and handling of laboratory animals. Animals were maintained under 12h light/dark cycles at 22-24°C and 55% humidity, and fed with regular rodent's chow and tap water *ad libitum*. In order to reduce the stress-induced changes in the hypothalamus-pituitary axis associated with the injection, animals were handled for 1 week prior to the beginning of the experiment. Animals were given LPS (*Escherichia coli*, serotype O26:B6; Sigma, St. Louis, USA) (5μg/g body weight) intraperitoneally (i.p.); control animals were injected with vehicle (0.9% NaCl) alone.

Mice were anesthetized with ketamine hydrochloride (150mg/Kg) plus medetomidine (0.3mg/Kg), transcardially perfused with cold saline and sacrificed 1, 3, 6, 12, 24 or 72h after LPS injection. For the mRNA studies, choroid plexus was rapidly removed under conventional light microscopy (SZX7, Olympus, Hamburg, Germany), frozen in dry ice and stored at -80°C. At least 5 pools of choroid plexus (from 3 animals each) were prepared for each time point. Adult rats similarly treated and sacrificed 6h after the LPS injection. Blood was collected, centrifuged and serum kept at -80°C until use in cell culture stimulation (see below).

qRT-PCR gene expression measurements

RNA preparation and PCR from mice choroid plexus

Total RNA was isolated from choroid plexus using Trizol reagent (Invitrogen, Carlsbad, CA, USA). 500 ng of total RNA were amplified using the Superscript RNA amplification system (Invitrogen) according to the manufacturer's instructions. RNA was reverse transcribed using random primers of the Superscript First-strand Synthesis System for RT-PCR (Invitrogen).

qRT-PCR analysis was used to measure the expression levels of ceruloplasmin (*Cp*), ferritin light chain 1, (*Ftl1*) ferroportin (*Slc40a1*), hepcidin (*Hamp*), interleukin-6 (*Il-6*), the signal transducer and activator of transcription 3 (*Stat3*), transferrin receptor type 2 (*Tfr2*) and transferrin receptor (*Tfr1*), mRNA transcript. The reference gene, hypoxanthine guanine phosphoribosyl transferase (*Hprt*), was used as internal standard for normalization (gene abbreviations and gene names are specified in accordance with the HUGO Gene Nomenclature Committee available at http://www.genenames.org), since we have previously shown that its expression is not

influenced by the experimental conditions (Marques et al., 2008). The oligonucleotide primers for the transcripts analyzed were designed using the Primer3 software on the basis of the GenBank sequences: NM_001042611; NM_010240.1; NM_016917; NM_032541; NM_031168.1; NM_011486.2; NM_015799.2; NM_011638; NM_013556. The real-time PCR reactions, using equal amounts of total RNA from each sample, were performed on a LightCycler instrument (Roche Diagnostics, Basel, Switzerland) using QuantiTect SYBR Green RT-PCR reagent kit (Qiagen, Hamburg, Germany). Product fluorescence was detected at the end of the elongation cycle. All melting curves exhibited a single sharp peak at a temperature characteristic of the primers used.

RNA preparation and PCR from rat choroid plexus

Total RNA was collected after 6h of stimulation, and extracted using a Micro Scale RNA Isolation Kit (Ambion, Austin, TX, USA). The oligonucleotide primers for the transcripts analyzed were designed using the Primer3 software on the basis of the GenBank sequences: NM_053469 for *Hamp*; NM_031512), interleukin-1β (*Il-1β*); NM_012589 for *Il6* and NM_012583 for *Hprt* that was used as internal standard for normalization. qRT-PCR was performed as described above.

Primary cultures of rat choroid plexus epithelial cells

Epithelial cells from rat choroid plexus were prepared as described previously by Strazielle and Ghersi-Egea, (1999) with minor modifications. Briefly, neonates (postnatal day 3 or 4) were sacrificed and choroid plexuses were dissected under conventional light microscopy. The tissue was rinsed twice in phosphate buffered saline (PBS) (without Ca^{2+} and Mg^{2+}) followed by a 25 min digestion with 0.1mg/ml pronase (Sigma) at 37°C. Predigested tissue was recovered by sedimentation and briefly shaken in 0.025% of trypsin (Invitrogen) containing 12.5µg/ml DNAseI (Roche). The supernant was then withdrawn and kept on ice with 10% fetal bovine serum (FBS) (Invitrogen). This step was repeated 5 five times. Cells were pelleted by centrifugation and ressupended in culture media consisting of Ham's F-12 and DMEM (1:1) (Invitrogen) supplemented with 10% FBS, 2mM glutamine (Invitrogen), 50µg/ml gentamycin (Sigma), 5µg/ml insulin, 5µg/ml transferrin, 5ng/ml sodium selenite (ITS, Sigma), 10ng/ml epidermal growth factor (Sigma), 2µg/ml hydrocortisone (Sigma), 5ng/ml basic fibroblast growth factor (Invitrogen). For further enrichment, cells were incubated on platic dishes for 2h at 37°C. A differential attachment on plastic dish occurred, and supernant containing mostly epithelial cells was collected and placed for seeding on laminin (Boehringer Ingelheim, GmbH, Germany) coated

transwells (Corning, Lowell, MA, USA).

Experiments were performed after the formation of confluent cell monolayers, approximately after 7 days in culture. Choroid plexuses epithelial cells were stimulated in the basolateral side with various stimuli: serum collected from LPS-treated rats 6h after the LPS administration; LPS (200ng/ml), IL-6 (2.8ng/ml) or IL-1β (0.5ng/ml), in choroid plexus epithelial cells medium.

Total RNA from choroid plexuses epithelial cells cultures was obtained at 6h after stimulation, and extracted using a Micro Scale RNA Isolation Kit (Ambion, Austin, TX, USA).

Statistical analysis

Values are reported as mean±SE. Statistical significance was determined using the non parametric Mann-Whitney test, with differences considered significant at $p<0.05$.

Results

The choroid plexus alters, *in vivo*, iron metabolism in response to the peripheral administration of LPS

Hamp expression is rarely detectable in the basal conditions by qRT-PCR, but it is up-regulated from 1 to 12h after LPS injection, reaching the maximum expression at 3h (Figure 1a), and returning almost to control levels at 24h. We next studied the expression of genes encoding proteins known to modulate *Hamp* expression in the liver. Expression of *Tfr2* is strongly up-regulated during the first 24h after induction of the peripheral inflammatory response (Figure 1b). Of notice the up-regulation of *Il-6* is very rapid and transient, peaking at 3h after LPS injection, and returning almost to basal levels at 6h (Figure 1c). Response to IL-6 is known to activate STAT3; the expression of *Stat3* increases up to 3h, and returns to basal levels at 24h after LPS injection (Figure 1d).

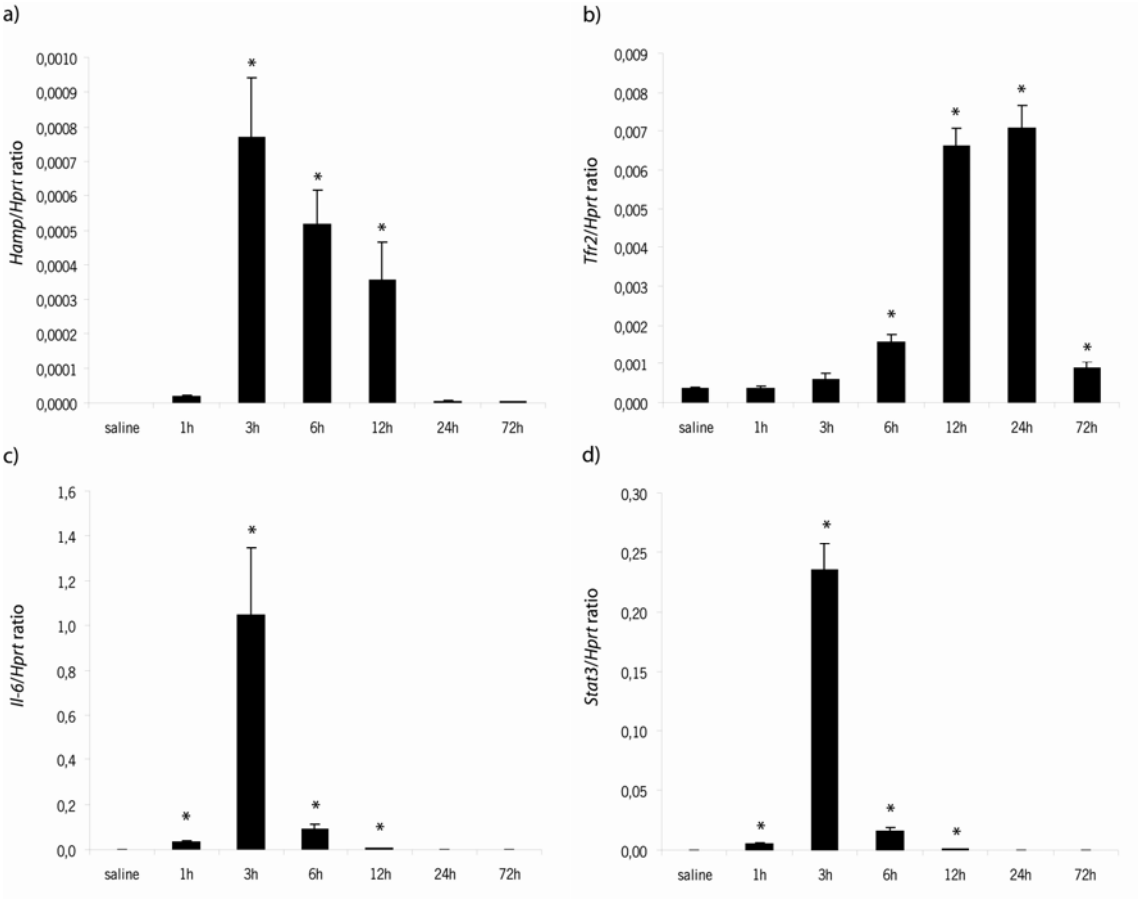

Figure 1. Hepcidin (*Hamp*) mRNA expression is strongly induced by peripheral LPS peaking at 3h and returning to basal levels after 24h (a). The possible upstream regulators of *Hamp* gene are also induced in the choroid plexus after peripheral LPS: Transferrin receptor type 2 (*Tfr2*) (b) and interleukin-6 (*Il-6*) (c). The signal transducer and activator of transcription 3 (*Stat3*) is also up-regulated in the choroid plexus in response to LPS (d).

In order to better characterize the overall alterations in the choroid plexus iron metabolism, the genes encoding other key proteins in the process were studied. Among these, the expression of the gene encoding for ceruloplasmin is increased at 3h and remains up-regulated until 24h after LPS injection (Figure 2a); while that of genes encoding for TFR1, FTL1, and FPN are not clearly modified in response to the inflammatory stimulus (Figure 2b, c and d).

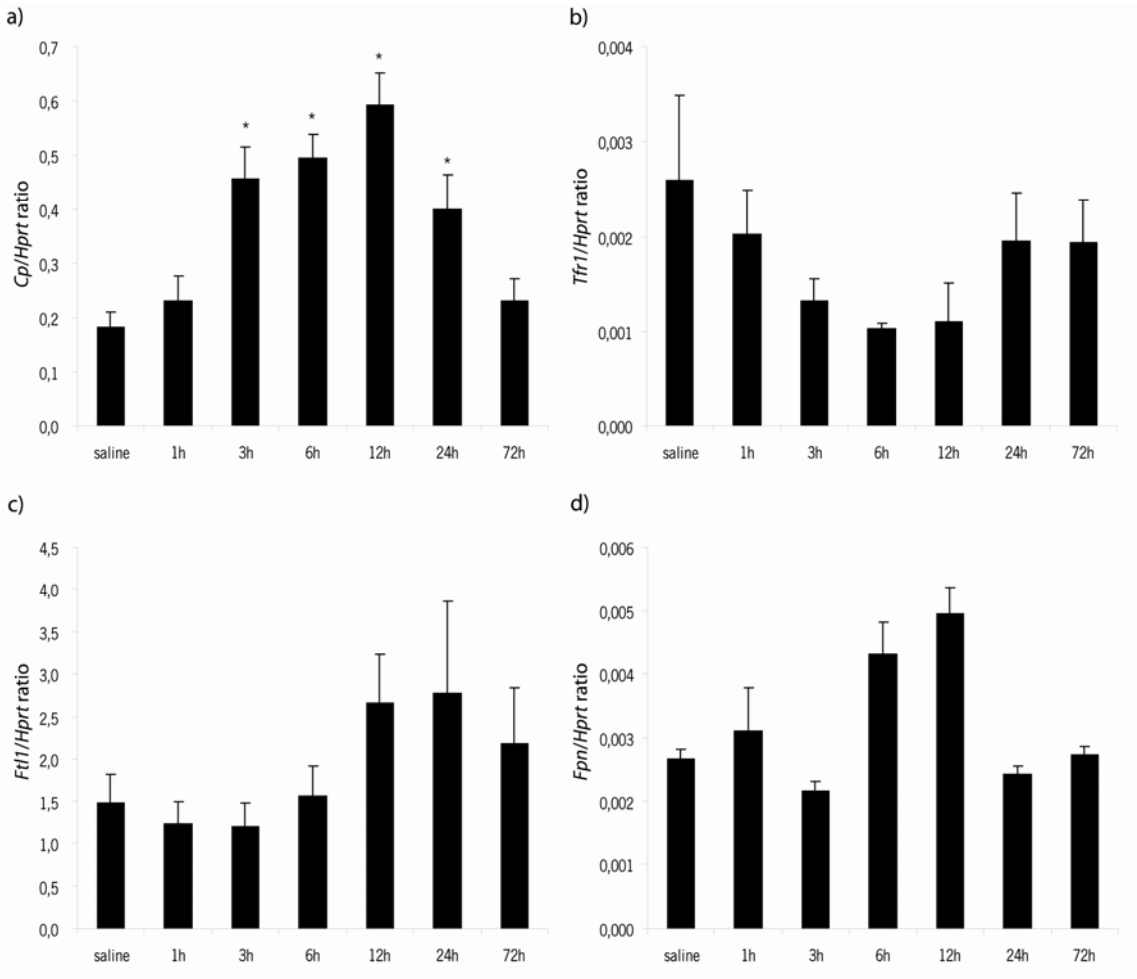

Figure 2. Expression levels of iron related protein in the choroid plexus after *i.p.* injection of LPS. Ceruloplasmin (*Cp*) mRNA expression is increased in response to LPS (a). No alterations were observed in the expression levels of transferrin receptor type 1 (*Tfr1*) (b), ferritin (*Ftl1*) (c) and ferroportin (*Fpn*) (d).

Triggering effectors of the choroid plexus response

The choroid plexus extracted for the *in vivo* analysis is composed not only by the choroid plexus epithelial cells, but also by cells that constitute the choroid plexus stroma, including essentially endothelial cells of the blood vessels but also eventually cells of the immune system like macrophages and dendritic cells. Thus, changes in the choroid plexus mRNA composition might result from altered gene expression of the choroid plexus epithelial cells and/or of stroma cells.

Therefore, and as an attempt to specifically identify the contribution of the choroid plexus epithelial cells to the choroid plexus response to peripheral inflammation, we used *in vitro* cultures of rat newborn choroid plexus epithelial cells. When exposed to serum collected from rats 6h after LPS administration, the epithelial cells response is similar to that observed *in vivo*. There is up-regulation of *Hamp* gene (Figure 3a) and also up-regulation of IL-6 and IL-1β (Figure 3b and c, respectively).

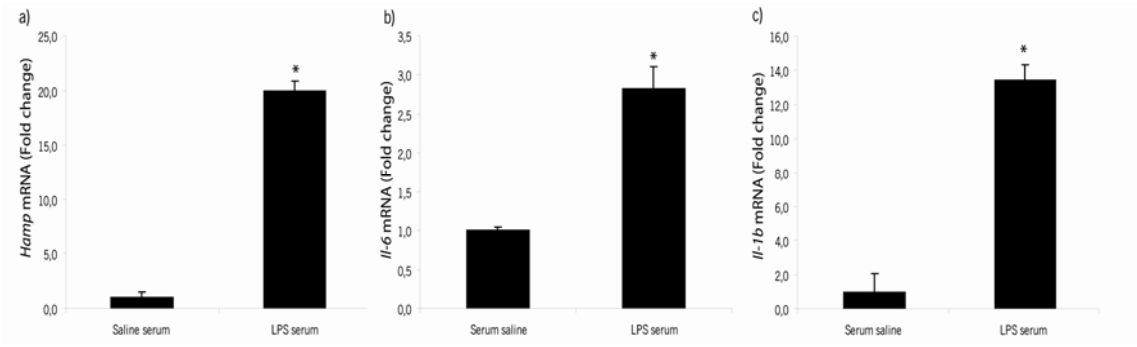

Figure 3. Serum collected from rats 6h after LPS injection is able to induce the expression of hepcidin (*Hamp*) (a), IL-6 (b) and IL-1β (c).

It is acknowledged that the choroid plexus expresses receptors not just for LPS (the TLR4) but also for several immune molecules whose expression increases in the blood during inflammation. Thus, the altered gene expression associated with iron metabolism might result from a response to LPS alone and/or to immune modulators present in the blood.

In another set of experiments, cells were exposed to various individual stimuli. As an attempt to discriminate among the various blood-born constituents which one(s) modulate iron metabolism in the choroid plexus. Preliminary data show that LPS or IL-6 alone do not seem able to induce alterations in the *Hamp* expression levels (Figure 4).

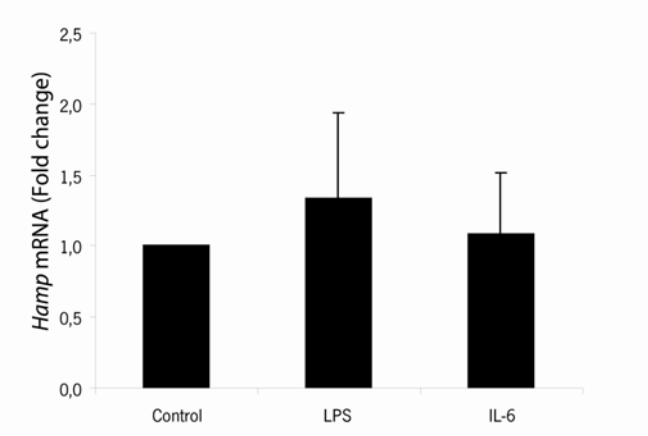

Figure 4. LPS or IL-6 alone do not seem able to influence the expression of hepcidin (*Hamp*) in rat primary choroid plexus epithelial cell culture.

Discussion

This study shows that the choroid plexus contributes to the brain iron metabolism reaction in response to peripheral inflammation. This regulation seems to be quick and transient, and highlights the choroid plexus as a prompt conveyor of signals from the periphery into the brain. Of notice, it shows that the gene encoding HAMP, rarely detected in basal conditions, is strongly up-regulated soon after LPS administration. HAMP is recognised as an endocrine hormone secreted by the liver and that, in the intestine, inhibits FPN-mediated iron release into the bloodstream, therefore reducing iron absorption. We suggest that HAMP in the choroid plexus may have an autocrine mode of action, similarly reducing FPN-mediated iron exportation into the CSF. On the other hand, by secreting TF and ceruloplasmin into the CSF, the choroid plexus might facilitate iron uptake by cells in contact with the CSF. In this way, HAMP in the choroid plexus could participate in the regulation of brain iron "absorption". Iron entry into the brain has been characterized mainly through the endothelial cells that constitute the blood-brain barrier. Iron is believed to be taken up through TFR-mediated endocytosis (Moos and Morgan, 2000). Release of iron into the brain parenchyma is suggested to occur also through FPN, and less is known about iron access and distribution within the brain. The presence of TFR in neurons (Moos, 1995) and glia cells (Orita et al., 1990; Kaur and Ling, 1995) suggests that the process of iron uptake by cells is quite uniform. The data now presented provides evidence that, at least, in conditions of acute peripheral inflammation, the choroid plexus contributes to brain iron content. The quick regulation of iron metabolism in the choroid plexus might be particularly relevant in preventing dissemination of bacteria that reach the CSF and eventually the brain parenchyma. We have previously shown that LCN2, a sequester for bacterial iron-loaded siderophores is up-regulated in the choroid plexus, CSF and endothelial cells of the blood-brain barrier (Marques et al., 2008) after peripheral administration of LPS. Studies in *Lcn2*-null mice have shown increased rate of mortality and bacterial load in several organs (Flo et al., 2004). It would be interesting to investigate whether the same holds true for bacteria that normally infect the brain. The data of the present study extends further our knowledge on how the choroid plexus contributes to deplete circulating iron, which may prevent bacteria proliferation in the CSF and, consequently, dissemination within the brain.

The trigger of the choroid plexus response to peripheral inflammation remains to be clarified. LPS is known to induce an acute-phase response in the liver and in other organs (Kramer et al., 2008), and these react by secreting proteins such as interleukins. Among these, IL-6 has been

shown, in the liver, to regulate *Hamp* expression, upon interaction with the cognate cellular receptor, and through the STAT3 signalling transduction pathway (Kemna et al., 2005; Nemeth et al., 2004; Nemeth et al., 2003). However, the exact mechanism by which the body senses the iron status and regulates *Hamp* expression is not completely understood. The discovery of different forms of hereditary hemochromatosis (disorders of iron overload) caused by mutations in genes involved in iron homeostasis suggests some possible answers for this question. Patients with mutations in hemacromatosis (*Hfe*), *Tfr2*, hemojuvelin (*Hjv*) or *Hamp* genes, all show iron deposition that is similar and consistent with elevated iron absorption. HFE and TFR2 are two upstream regulators of *Hamp* expression. In the present study we did not find alteration in the expression levels of *Hfe*, but a strong increase in the *Tfr2* levels were detected in the choroid plexus after LPS stimulation. The mechanism by which TFR2 regulates HAMP is not clear but the level of differic TF has emerged as a potential regulator (Frazer and Anderson, 2003; Wilkins et al., 2006). *Tfr2* encodes a protein that shares 45% of identity with TFR1, but the expression is more restricted to the liver, spleen, brain and heart (Kawabata et al., 1999) and, as we now show, also the choroid plexus. The mechanism through which TFR2 regulates *Hamp* expression is unknown, but *Hamp* expression is down-regulated in *Tfr2*-mutant mice (Kawabata et al., 2005). The affinity of TFR2 for TF is about 25 times smaller than that of TFR1 (West et al., 2000), and although the transferrin is described as a negative acute-phase protein it is possible that iron saturation of the circulating serum TF in response to peripheral inflammation stimulates TFR2 occupancy and downstream activated pathways. We used *in vitro* cultures of rat choroid plexus epithelial cells as an attempt to discriminate among the potential modulators of the choroid plexus response to inflammation, with respect to iron homeostasis. The data clearly shows that LPS alone is able to induce the expression of *Il6*, *Il-1β* and *Lcn2* (data not shown) but not several other genes implicated in iron metabolism. Preliminary results suggest that IL6, alone, is not able to induce the expression of any of the genes, including *Hamp*. It is therefore possible that the regulation of Hamp is mediated by TFR2 given the increase in blood of the basal TF as holo-TF. We are currently investigating this possibility.

Irrespectively from the precise mechanisms, the overall response of the choroid plexus to peripheral inflammation includes changes in iron homeostasis. These modifications seem concordant with a reduction in iron availability in the CSF, as depicted in Figure 5, and consequently, as an important intervenient in the all innate immune response. Up-regulation of *Hamp* by the inflammatory stimulus may decrease FPN-mediated iron release into the CSF. In

addition, increased secretion of ceruloplasmin may facilitate iron binding to TF and internalization by the cells of the brain parenchyma. Finally, and in the case bacteria invade the CSF, lipocalin 2 is promptly ready to sequester bacteria siderophores. Therefore, all together the response seems to restrict iron availability for bacteria. The precise modulator of such regulation remains however to be clarified.

The ability of the choroid plexus to regulate iron metabolism in conditions of peripheral inflammation might impact on diseases such as Alzheimer and multiple sclerosis. The observations that an inflammatory component may underlie such diseases, that iron accumulates with aging, and that, ceruloplasmin-deficient mice exhibit increased iron accumulation and demyelination when exposed to LPS (Glezer et al., 2007), warrant that additional studies on choroid plexus iron metabolism in neurological diseases are necessary.

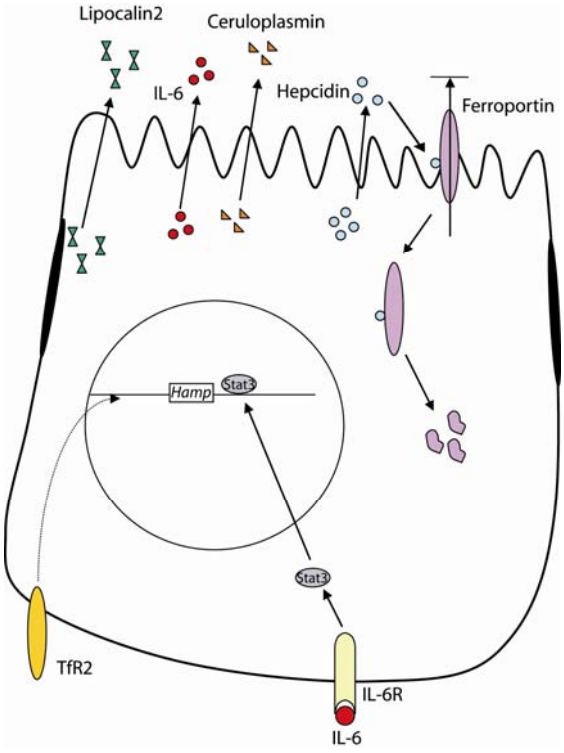

Figure 5. Iron metabolism in the choroid plexus after LPS injection.

Acknowledgements

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5

The choroid plexus displays an acute-phase response to peripheral inflammation

The choroid plexus displays an acute-phase response to peripheral inflammation

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Abstract

The choroid plexus (CP) is responsible for the production of cerebrospinal fluid (CSF) and is part of the blood-brain barriers. In addition, it has receptors and transporters for molecules such as neurotransmitters, immunomodulators, carrier proteins and nutrients, in both the apical and basolateral membranes. Therefore, it is ideally positioned to transmit signals from and into the brain. As part of the brain-barrier system, changes in the expression of CP proteins will ultimately influence the composition of the CSF and the information conveyed into the brain. Using microarray analysis the present study shows a specific acute-phase response of the mouse CP transcriptome after an inflammatory stimulus induced in the periphery by lipopolysaccharide (LPS). Remarkably, the response seems specific to a restricted number of genes; from a total of 24,000 analysed genes, 252 were found up-regulated and 173 down-regulated, during the time course of the experiment. When clustered into families, the genes up-regulated are mostly implicated in immune-mediated cascades and in extracellular matrix remodelling, while those down-regulated encode for proteins involved in the maintenance of the CP barrier function. Of relevance, the transcriptome profile returns almost completely to basal levels 72h upon the inflammatory stimulus is triggered. It is therefore clear that the CP quickly and actively responds to peripheral inflammation and that the balance of such response should be considered in the final homeostasis of the brain in health and disease.

Keywords: Choroid plexus; inflammation; lipopolysaccharide; pathway.

Introduction

The precise mechanisms through which peripheral inflammatory stimuli trigger brain inflammation are poorly understood. Interest on the subject is increasing given the suggestion that an inflammatory state of the central nervous system (CNS) is implicated in neurodegenerative diseases such as parkinson´s (PD) and Alzheimer's diseases (AD) and that is well recognized in multiple sclerosis (MS). While several studies have addressed the communication between the periphery and the CNS by looking at the blood-brain barrier (BBB), less have specifically addressed the participation of the blood choroid plexus (CP)-cerebrospinal fluid barrier (BCSFB). The CP, located within the brain ventricles, is composed by a vascularized stroma surrounded by a tight line of epithelial cells that control the cellular and molecular traffic between the blood and the cerebrospinal fluid (CSF) (Redzic and Segal, 2004). Its better known function is the production of at least 2/3 of the CSF (Speake et al., 2001). The CP is therefore, ideally located to transmit signals into and out of the brain (Emerich et al., 2005). Following systemic administration of lipopolysaccharide (LPS), there is a rapid and transient CP induction of immune mediators such as interleukin IL-1β and TNF (Konsman et al., 2004; Marques et al., 2007; Nadeau and Rivest, 1999; Quan et al., 1998; Quan et al., 1999,), enzymes such as prostaglandin D2 synthase and bacteriostatic proteins such as lipocalin 2 (LCN2) (Marques et al., 2007; Marques et al., 2008). Accessory molecules important for leukocyte adhesion such as selectin, lymphocyte (L-selectin), intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) are expressed in basal conditions at low levels but are up-regulated on CP epithelial cells during inflammation (Endo et al., 1998; Engelhardt et al., 2001; Wolburg et al., 1999), eventually facilitating access of immune cells into the brain. Of notice, it has been recently suggested in the experimental allergic encephalomyelitis model of MS that the CP may be the primary route of immune cells entry into the CSF (Brown and Sawchenko, 2007). Similarly, the CP may facilitate bacteria entry, as indicated in a mouse model of infection with *Streptococcus suis* (Dominguez-Punaro et al., 2007). The CP activation initiates a process that ultimately spreads throughout the brain suggesting that the CP transmits information between the immune system and the brain. The studies published so far on the CP mostly focus on single proteins or group of proteins. The full elucidation on how the CP stroma and epithelial cells respond to inflammatory stimulus and contribute to the brain innate and adaptative immune responses is far from understood. In the present study we evaluate the kinetic overall response of the CP to an acute inflammatory stimulus induced by the peripheral administration of LPS. We show that the CP displays a rapid transient acute-phase response to LPS. A model of signalling in the CP is proposed, and the major pathways influenced by the acute peripheral stimulus are highlighted.

Experimental procedures

Animals and LPS injection

All experiments were conducted using 8-9 weeks old C57BL/6 male mice (Charles River, Barcelona, Spain), in accordance with the European Community Council Directive 86/09/EEC guidelines for the care and handling of laboratory animals. Animals were maintained under 12h light/dark cycle at 22.5°C and 55% humidity and fed with regular rodent's chow and tap water *ad libitum*. In order to reduce the stress-induced changes in the hypothalamus-pituitary axis, animals were handled for 1 week prior to the beginning of the experiment. Animals were injected intraperitoneally (*i.p.*) with LPS (5μg/g of body weight; *Escherichia coli*, serotype O26:B6; Sigma, St. Louis, USA) or vehicle alone (0,9% NaCl). Animals were anesthetized with ketamine hydrochloride (150mg/Kg) plus medetomidine (0.3mg/Kg), transcardially perfused with cold saline and sacrificed 1, 3, 6, 12, 24 or 72 hours after LPS injection. For mRNA studies, the CP was rapidly removed, stored in RNA later (Ambion, Austin, TX, USA) and kept at -80°C. CP isolation was made under conventional light microscopy (SZX7, Olympus, Hamburg, Germany). At least 3 pools of CP (from 3 animals each) were prepared for each time point. This experiment was performed twice: CP samples from one experiment were used for microarray analysis; CPs collected in independent experiment were used to confirm the results, by quantitative RT-PCR (qRT-PCR), obtained in the array study. In this second experiment 5 pools of CP were prepared.

Microarray experimental design and data analysis

Total RNA was isolated with Trizol (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. After quality assessment using the Agilent Bioanalyzer (Santa Clara, CA, USA), 100ng of total RNA from 3 pooled controls and 2 pooled samples of each time point were amplified and labelled with Illumina TotalPrep RNA Amplification Kit. The labelled cRNA was then hybridized using the recommended protocol in a total of two Illumina Whole-genome Mouseref-8 expression Beadchips (San Diego, CA, USA). This mouse beadchip contains eight arrays, each comprising a total of 24,000 well-annotated RefSeq transcripts.

After scanning, raw data from BeadStudio software (San Diego, CA, USA) was read into R/Bioconductor and normalized using quantile normalization. A linear model was applied to the

normalized data using Limma package in R/Bioconductor (Gentleman et al., 2004). A contrast analysis was applied and differentially expressed genes were selected using a Bayesian approach with a false discovery rate of 0.5%.

The differentially expressed genes were categorized using Gene Ontology from Biomart (http://www.biomart.org/) or Ingenuity tools (Redwood City, CA, USA). Enrichment analysis was performed using the DAVID (http://david.niaid.nih.gov/david/ease.htm) and the Ingenuity softwares.

Gene expression measurements by qRT-PCR

500ng of total RNA isolated as described above were amplified using a SuperScript RNA Amplification System (Invitrogen) according to the manufacturer's instructions. After amplification, RNA was reverse transcribed into first strand cDNA using random hexamers of the Superscript First-strand Synthesis System for RT-PCR (Invitrogen) accordingly to the manufacturer's instructions.

qRT-PCR analysis was used to measure the expression levels of selected mRNA transcripts. Primers were designed using the Primer3 software (Rozen and Skaletsky, 2000) on the basis of the respective GenBank sequences. The expression level of the reference gene hypoxanthine guanine phosphoribosyl transferase (*Hprt*) (accession number from GenBank: NM_013556) was used as internal standard for normalization, since we have first confirmed that its expression is not influenced by the experimental conditions.

All the other accession numbers are available upon request. Reactions using equal amounts of total RNA from each sample were carried out on a LightCycler instrument (Roche Diagnostics, Basel, Switzerland) with the QuantiTect SYBR Green RT-PCR reagent kit (Qiagen, Hamburg, Germany) according to the manufacturer's instructions. Product fluorescence was detected at the end of the elongation cycle. All melting curves exhibited a single sharp peak at the expected temperature.

Statistical analysis

Values are reported as mean±SE. Statistical significance was determined using the non parametric Mann-Whitney test, with differences considered significant at $p<0.05$.

Results

Reproducibility of the gene array data

As described above, 2 pooled CP samples for each LPS time point were compared with 3 pooled CP samples from saline-injected animals. Data was analyzed in the Bioconductor. Quality control using inter-array Pearson correlation and clustering based on variance allowed us to ensure the reproducibility between the replicates. After data normalization, the differentially expressed genes were selected based on a false discovery rate (FDR) of 0.5%. We obtained a final list of 324 differentially expressed genes considering all the time points.

Gene expression kinetic profile of the choroid plexus after and inflammatory peripheral stimulus

As shown in Figure 1, that depicts the number of genes up- and down-regulated throughout the experimental period, the CP displays an acute-phase response to LPS.

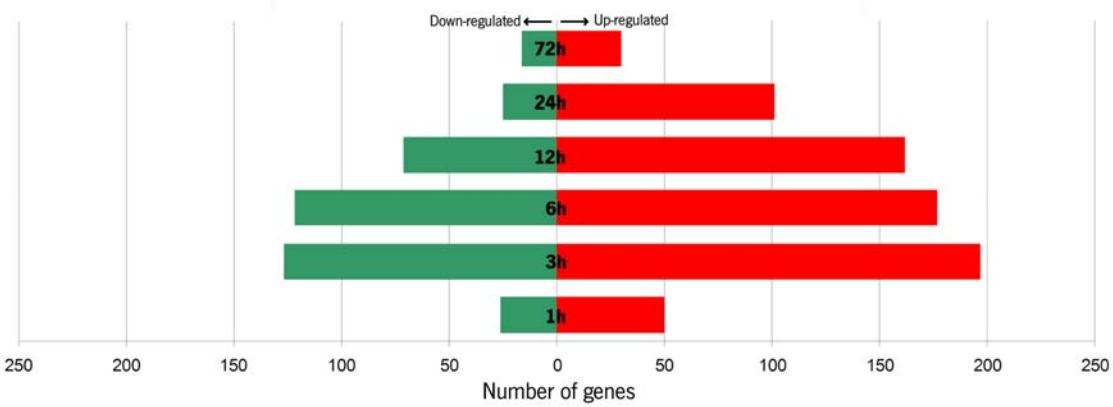

Figure 1. Number of genes whose expression is found altered after a peripheral injection of LPS. The CP gene expression profile was analysed from mice 1, 3, 6, 12, 24 and 72 hours after LPS administration, and compared with that from saline-injected mice. In red are represented the up-regulated and in green the down-regulated genes.

The CP response to the LPS stimulus peaks at 3-6h. Of notice, at 1h, only 50 and 26 genes are up-regulated and down-regulated, respectively, but 2h later (time point 3h) 197 and 127 genes are up- and down-regulated, respectively. The CP response starts to slow down at 6h after the LPS stimulation and returns to basal levels, for most of the genes, at 72h. Table 1 lists the genes for which the expression is most altered, for each time point after the LPS injection.

Table 1. Genes whose expression is most altered at each time point after LPS

1h			3h			6h		
Up-regulated			**Up-regulated**			**Up-regulated**		
Accession	**Gene**	**Fold change**	**Accession**	**Gene**	**Fold change**	**Accession**	**Gene**	**Fold change**
NM_008176	Chemokine (C-X-C motif) ligand 1 (Cxcl1)	14,9	NM_008176	Chemokine (C-X-C motif) ligand 1 (Cxcl1)	50,9	NM_011315	Serum amyloid A 3 (Saa3)	31,1
NM_007707	Suppressor of cytokine signaling 3 (Socs3)	5,4	NM_011315	Serum amyloid A 3 (Saa3)	28,8	NM_008491	Lipocalin 2 (Lcn2)	26,7
NM_013652	Chemokine (C-C motif) ligand 4 (Ccl4)	5,0	NM_008491	Lipocalin 2 (Lcn2)	19,8	NM_008176	Chemokine (C-X-C motif) ligand 1 (Cxcl1)	11,6
NM_007913	Early growth response 1 (Egr1)	4,4	NM_013652	Chemokine (C-C motif) ligand 4 (Ccl4)	18,4	NM_009252	Serine (or cysteine) proteinase inhibitor, clade A, member 3N (Serpina3n)	9,8
NM_013654	Chemokine (C-C motif) ligand 7 (Ccl7)	4,1	NM_013654	Chemokine (C-C motif) ligand 7 (Ccl7)	15,1	NM_011593	Tissue inhibitor of metalloproteinase 1 (Timp1)	7,3
NM_008361	Interleukin 1 beta (Il1b)	4,0	NM_007707	Suppressor of cytokine signaling 3 (Socs3)	14,9	NM_015783	Interferon, alpha-inducible protein (G1p2)	7,3
NM_009841	CD14 antigen (Cd14)	3,2	NM_010493	Intercellular adhesion molecule (Icam1)	13,5	NM_010493	Intercellular adhesion molecule (Icam1)	6,8
NM_008416	Jun-B oncogene (Junb)	3,1	NM_015783	Interferon, alpha-inducible protein (G1p2)	11,3	NM_008161	Glutathione peroxidase 3 (Gpx3)	5,8
NM_133662	Immediate early response 3 (Ier3)	3,0	NM_011593	Tissue inhibitor of metalloproteinase 1 (Timp1)	11,1	NM_008620	Guanylate nucleotide binding protein 4 (Gbp4)	5,5
NM_010234	FBJ osteosarcoma oncogene (Fos)	2,9	NM_009252	Serine (or cysteine) proteinase inhibitor, clade A, member 3N (Serpina3n)	10,6	NM_013653	Chemokine (C-C motif) ligand 5 (Ccl5)	4,9
Down-regulated			**Down-regulated**			**Down-regulated**		
Accession	**Gene**	**Fold change**	**Accession**	**Gene**	**Fold change**	**Accession**	**Gene**	**Fold change**
NM_029928	Protein tyrosine phosphatase, receptor type, B (Ptprb)	-1,7	NM_013723	Podocalyxin-like (Podxl)	-3,9	NM_013723	Podocalyxin-like (Podxl)	-2,6
NM_023612	Endothelial cell-specific molecule 1 (Esm1)	-1,7	NM_023055.1	Solute carrier family 9 (sodium/hydrogen exchanger), isoform 3 regulator 2 (Slc9a3r2)	-3,0	NM_010686	Lysosomal-associated protein transmembrane 5 (Laptm5)	-2,6
NM_009987	Chemokine (C-X3-C) receptor 1 (Cx3cr1)	-1,6	NM_029928	Protein tyrosine phosphatase, receptor type, B (Ptprb)	-2,8	NM_029928	Protein tyrosine phosphatase, receptor type, B (Ptprb)	-2,5
NM_172411	RIKEN cDNA 2310007B03 gene (2310007B03Rik)	-1,5	NM_023612	Endothelial cell-specific molecule 1 (Esm1)	-2,4	NM_023612	Endothelial cell-specific molecule 1 (Esm1)	-2,3
NM_010171	Coagulation factor III (F3)	-1,5	NM_010171	Coagulation factor III (F3)	-2,4	NM_008128.3	Gap junction membrane channel protein beta 6 (Gjb6)	-2,2
NM_026524.2	Mid1 interacting protein 1 (gastrulation specific G12-like (zebrafish)) (Mid1ip1)	-1,5	NM_007409	Alcohol dehydrogenase 1 (class I) (Adh1)	-2,3	NM_009320	Solute carrier family 6 (neurotransmitter transporter, taurine), member 6 (Slc6a6)	-2,1
NM_010496	Inhibitor of DNA binding 2 (Id2)	-1,5	NM_009987	Chemokine (C-X3-C) receptor 1 (Cx3cr1)	-2,2	NM_032398	Plasmalemma vesicle associated protein (Plvap)	-2,0
NM_001029934	Ubiquitin specific peptidase 32 (Usp32)	-1,5	NM_133249	Peroxisome proliferative activated receptor, gamma, coactivator 1 beta (Ppargc1b)	-2,1	NM_001001309	Integrin alpha 8 (Itga8)	-1,9
NM_152804	Polo-like kinase 2 (Drosophila) (Plk2)	-1,4	NM_010686	Lysosomal-associated protein transmembrane 5 (Laptm5)	-2,1	NM_009349	Thioether S-methyltransferase (Temt)	-1,9
NM_020278	Leucine-rich repeat LGI family, member 1 (Lgi1)	-1,4	NM_013805	Claudin 5 (Cldn5)	-2,1	NM_020278	Leucine-rich repeat LGI family, member 1 (Lgi1)	-1,8

12h			24h			72h		
Up-regulated			**Up-regulated**			**Up-regulated**		
Accession	**Gene**	**Fold change**	**Accession**	**Gene**	**Fold change**	**Accession**	**Gene**	**Fold change**
NM_008491	Lipocalin 2 (Lcn2)	52,7	NM_008491	Lipocalin 2 (Lcn2)	19,2	NM_008161	Glutathione peroxidase 3 (Gpx3)	3,3
NM_011315	Serum amyloid A 3 (Saa3)	46,9	NM_011315	Serum amyloid A 3 (Saa3)	14,8	NM_133903	Spondin 2, extracellular matrix protein (Spon2)	2,4
NM_009252	Serine (or cysteine) proteinase inhibitor, clade A, member 3N (Serpina3n)	18,1	NM_009252	Serine (or cysteine) proteinase inhibitor, clade A, member 3N (Serpina3n)	7,6	NM_017373	Nuclear factor, interleukin 3, regulated (Nfil3)	1,9
NM_008176	Chemokine (C-X-C motif) ligand 1 (Cxcl1)	11,8	NM_009778	Complement component 3 (C3)	6,8	NM_029803.1	Interferon, alpha-inducible protein 27 (Ifi27)	1,8
NM_008161	Glutathione peroxidase 3 (Gpx3)	10,5	NM_008161	Glutathione peroxidase 3 (Gpx3)	6,8	NM_017372	Lysozyme (Lyzs)	1,5
NM_009778	Complement component 3 (C3)	8,6	NM_029803.1	Interferon, alpha-inducible protein 27 (Ifi27)	4,7	NM_007572	Complement component 1, q subcomponent, alpha polypeptide (C1qa)	1,4
NM_008620	Guanylate nucleotide binding protein 4 (Gbp4)	7,5	NM_013654	Chemokine (C-C motif) ligand 7 (Ccl7)	4,4	NM_199024	Nucleolar protein 4 (Nol4)	1,4
NM_013653	Chemokine (C-C motif) ligand 5 (Ccl5)	6,5	NM_008620	Guanylate nucleotide binding protein 4 (Gbp4)	3,9	NM_007440	Arachidonate 12-lipoxygenase (Aka12)	1,4
NM_011593	Tissue inhibitor of metalloproteinase 1 (Timp1)	6,1	NM_008176	Chemokine (C-X-C motif) ligand 1 (Cxcl1)	3,8	NM_007574	Complement component 1, q subcomponent, gamma polypeptide (C1qg)	1,4
XM_355243	Proteoglycan 4 (megakaryocyte stimulating factor, articular superficial zone protein) (Prg4)	5,9	NM_025378	Interferon induced transmembrane protein 3 (Ifitm3)	3,7	NM_008625	Mannose receptor, C type 1 (Mrc1)	1,4
Down-regulated			**Down-regulated**			**Down-regulated**		
Accession	**Gene**	**Fold change**	**Accession**	**Gene**	**Fold change**	**Accession**	**Gene**	**Fold change**
NM_023612	Endothelial cell-specific molecule 1 (Esm1)	-2,6	NM_010382	Histocompatibility 2, class II antigen E beta (H2-Eb1)	-2,0	NM_011066	Period homolog 2 (Drosophila) (Per2)	-1,6
NM_008973	Pleiotrophin (Ptn)	-2,5	NM_207105	Response to metastatic cancers 1 (Rmcs1)	-1,8	NM_010902	Nuclear factor, erythroid derived 2, like 2 (Nfe2l2)	-1,4
NM_177470	Acetyl-Coenzyme A acyltransferase 2 (mitochondrial 3-oxoacyl-Coenzyme A thiolase) (Acaa2)	-2,3	NM_146257	Solute carrier family 29 (nucleoside transporters), member 4 (Slc29a4)	-1,6	NM_009427	Transducer of ErbB-2,1 (Tob1)	-1,4
NM_010686	Lysosomal-associated protein transmembrane 5 (Laptm5)	-1,9	NM_172411.2	RIKEN cDNA 2310007B03 gene (2310007B03Rik)	-1,5	NM_177470	Acetyl-Coenzyme A acyltransferase 2 (mitochondrial 3-oxoacyl-Coenzyme A thiolase) (Acaa2)	-1,4
NM_145434	Nuclear receptor subfamily 1, group D, member 1 (Nr1d1)	-1,9	NM_008128.3	Gap junction membrane channel protein beta 6 (Gjb6)	-1,4	NM_207105	Response to metastatic cancers 1 (Rmcs1)	-1,3
NM_007472	Aquaporin 1 (Acp1)	-1,9	NM_145434	Nuclear receptor subfamily 1, group D, member 1 (Nr1d1)	-1,4	NM_023671	Chloride channel, nucleotide-sensitive, 1A (Clns1a)	-1,3
NM_008481	Laminin, alpha 2 (Lama2)	-1,9	NM_172802	Fascin homolog 2, actin-bundling protein, retinal (Strongylocentrotus purpuratus) (Fscn2)	-1,4	NM_009855	Cytokine inducible SH2-containing protein (Cish)	-1,3
NM_146257	Solute carrier family 29 (nucleoside transporters), member 4 (Slc29a4)	-1,8	NM_008973	Pleiotrophin (Ptn)	-1,4	NM_009948	Carnitine palmitoyltransferase 1b, muscle (Cpt1b)	-1,3
NM_007833	Decorin (Dcn)	-1,8	NM_201639	Desmuslin (Dmn), transcript variant 1	-1,4	NM_013807	Polo-like kinase 3 (Drosophila) (Plk3)	-1,3
NM_025953.1	Potassium channel, subfamily K, member 4 (Kcnk4)	-1,8	NM_080575	Acetyl-Coenzyme A synthetase 2 (AMP forming)-like (Acas2l)	-1,4	NM_011404	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 5 (Slc7a5)	-1,3

Identification of altered gene pathways

Gene ontology and biological pathway analysis of differentially expressed genes was performed using the ingenuity software and the DAVID program. This analysis shows that the major altered biological pathways are related with innate immune recognition, as depicted in Table 2.

Table 2. Clustering of the genes whose expression is altered in the choroid plexus upon peripheral LPS injection.

Immune molecules	chemokines 15↑1↓
	Interleukines 3↑
	Other molecules with cytokine activity 3↑
Antigen presentation pathway	Antigen presentation pathway 5↑ 1↓
Signalling pathways	TLR and co-estimulatory molecules 2 ↑
	JAK/STAT signalling pathway 6↑ 1↓
	MAPK signalling pathway 6↑ 1↓
	NF-Kb signalling pathway 15↑ 1↓
	complement signalling 6↑
	interefon signalling 9↑
	IL-10 signalling 10↑
	IL-6 signalling 11↑
Acute phase response signalling	Acute phase response: 22↑
Glucocorticoid receptor signalling	Glucocorticoid receptor signalling 23↑ 1↓
Cell adhesion molecules	Tight junctions 3↓
	Gap junctions 1↓
	Leukocyte transendothelial migration 4↑ 1↓
	Extracellular matrix 7↓
Proteins that contribute to integrity of ECM	Proteases 12↑
Transporters	Solute carrier 6↑9↓
	ABC transpoter 1↑

Confirmation of array results by qRT-PCR on a set of relevant genes

Within each pathway, and using RNA extracted from CP pools of an independent experiment, a number of genes were chosen to confirm the array data by qPCR: *Lcn2*, *Il6*, *Stat3*, *Saa3*, *Cxcl1*, *Timp1*, *Tap2*, *Tlr2*, *Icam1*, *Cd14*, *Socs3*, *Stat1*, *Irf1* and *Irf7* as up-regulated genes and *Cldn5*, *Cldn11*, *Lama2* and *Gjb6* as down-regulated genes (Figure. 2).

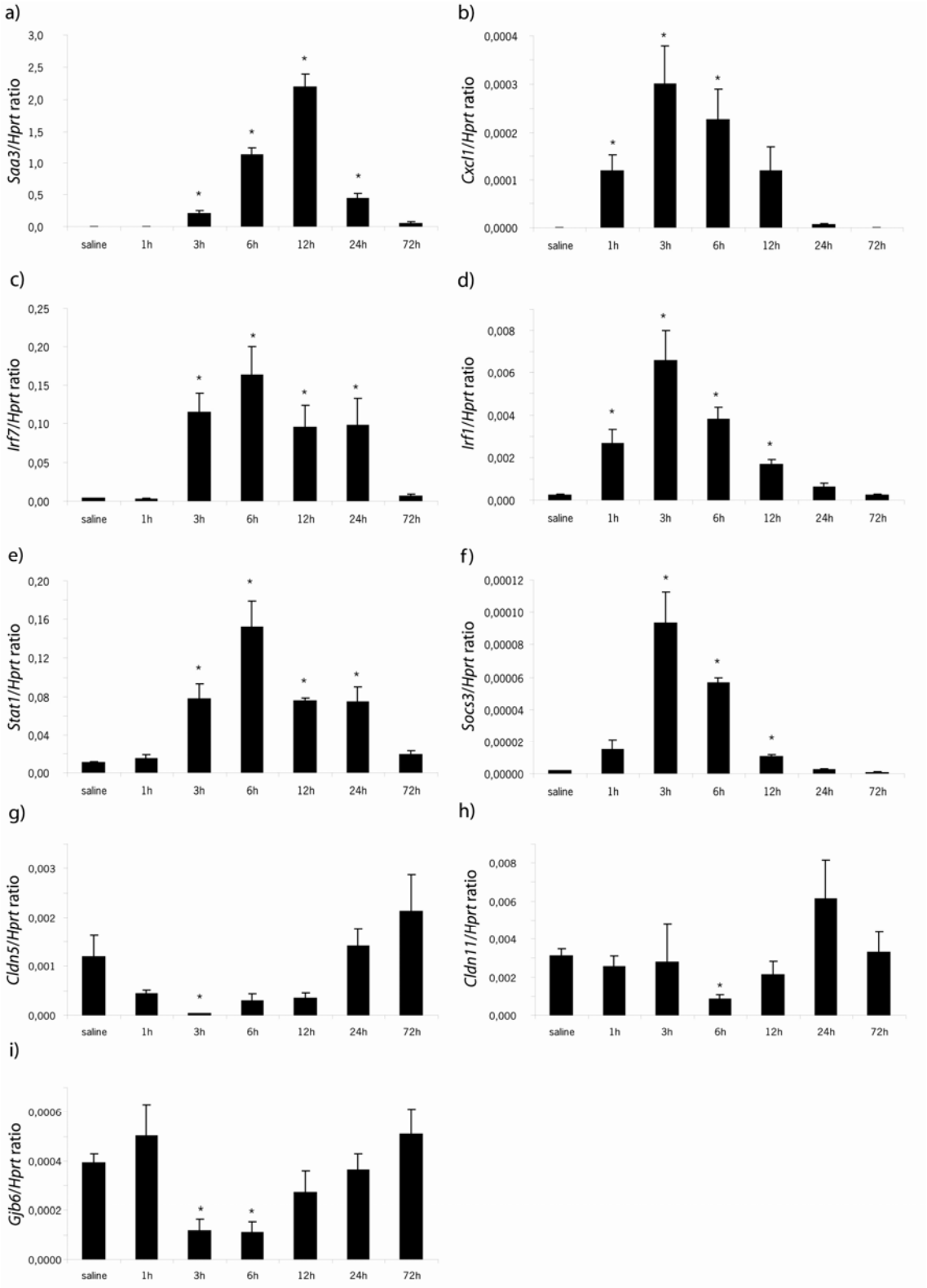

Figure 2. Gene expression analysis, by qRT-PCR, from CP of saline and LPS injected mice. Confirming the array results, we show that the expression of *Saa3*, *Cxcl1*, *Irf7*, *Irf1*, *Stat1* and *Socs3* (a-f) are up-regulated and that of *Cldn5*, *Cldn11* and *Gjb6* (g-i) are down-regulated.

Proteins involved in barrier function and in cell adhesion during the choroid plexus acute-phase response to inflammation

Most of the genes whose expression is found altered are up-regulated, but those encoding proteins that are involved in the formation of the tight junctions are down-regulated after the LPS injection (Table 2). Among those down-modulated are claudin 3 (*Cldn3*), claudin 11 (*Cldn11*) and claudin 5 (*Cldn5*). Also down-regulated are proteins of the extracellular matrix that contribute to cell-to-cell interactions such as endothelial cell-specific molecule 1 (*Esm1*), integrin alpha 8 (*Itga8*), nidogen 1 and 2 (*Nid1/2*), protocadherin 7 (*Pcdh7*), laminin alpha 2 (*Lama2*) and decorin (*Dcn*).

On the contrary, the *Icam1* and the mucosal vascular addressin cell adhesion molecule 1 (*Madcam1*) are up-regulated after the LPS treatment. In addition, proteins involved in leukocyte transendothelial migration, such as selectin, platelet (P-selectin) (*Selp*) and selectin, endothelial cell (E-selectin) (*Sele*) are also up-regulated. Degradation of extracellular matrix is achieved through the actions of proteases and the role of matrix metalloproteinases (MMPs) has been extensively investigated in this context (Flannery, 2006). In this study we observe an up-regulation of the genes encoding proteases such as collagenase 13 (*Mmp13*) and the disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) 1, 4 and 7 (*Adamts1*, *Adamts4* and *Adam7*) which may contribute to the leakage of the BCSFB. Of notice, the expression of the gene encoding the tissue inhibitors of metalloproteinase *Timp-1* is also up-regulated already at 3h after the injection, in what might be perceived as a response mechanism to avoid extracellular matrix degradation. Taken together these observations suggest that cell migration might be facilitated in transient conditions of a compromised BCSFB.

Acute-phase proteins during the choroid plexus response to lipopolysaccharide

One of the most investigated, but still not well understood, characteristics of the acute-phase response is the up-regulation, or down-regulation, of many plasma proteins, known as positive and negative acute-phase proteins, respectively. The altered concentration of these proteins results from changes in the synthetic and secretory liver machineries as an adaptation to the stimulus. Several well described liver acute-phase proteins (Ceciliani et al., 2002; Gabay and Kushner, 1999) are also up-regulated in the CP soon after the LPS injection. For instances, a peak in the expression of serum amyloid A 1 (*Saa1*) and serum amyloid A 3 (*Saa3*) in the CP is

observed at 3h and 12h, respectively, after LPS injection. The purpose of the acute-phase reaction in the periphery is to counteract the underlying challenge and to restore homeostasis. This result is accomplished by isolating and destroying the infective organisms, or removing the harmful molecules, and activating the repair process. Acute-phase reaction includes a wide range of neuroendocrine, hematopoietic, metabolic and hepatic changes (Ceciliani et al., 2002). The production of some of the peripheral acute-phase proteins by the CP could represent a mechanism of defence within the brain similar to that observed in the periphery. Among these is *Lcn2* (Sunil et al., 2007), an iron sequestering protein relevant to fight bacterial infection (Flo et al., 2004), and previously shown to be induced in the CP by the LPS stimulus (Marques et al., 2008).

Signalling transduction pathways involved in the choroid plexus response to lipopolysaccharide

We find no alterations in the expression of Toll-like receptor 4 gene (*Tlr4*), the best well known and recognized target for LPS, but rather an increase in that encoding TLR2 in accordance with previous findings by Laflamme et al., (2001). The expression of the gene encoding CD14, a down-stream modulator in the TLR4- and TLR2-mediated cascades is, however, increased. This may indicate that the TLR2- and TLR4-mediated signal transduction pathways are induced by LPS.

The expression of genes belonging to various well described signalling transduction pathways [Janus kinase/signal transducer and activator of transcription (JAK-STAT), mitogen-activated protein kinase (MAPK), nuclear factor kappa B (Nf-Kβ) and interferon regulatory factors (IRFs)] are altered by the LPS stimulus. These include several of the intermediate modulators and transcription factors, such as the Nf-kβ, IRF 1, 2, 7 and 9 (*Irf1*, *Irf2*, *Irf7*, *Irf9*), activated protein-1 (AP-1), *Stat1* and *Stat3*, which are all up-regulated. Many of these genes have, as downstream targets, genes encoding cytokines such as *Il-1β*, *Il6*, and chemokines such as (C-C motif) ligand 3 (*Ccl3*), chemokine (C-C motif) ligand 4 (*Ccl4*). Other known target genes are those encoding for ICAM1 and MMP13 which are also induced as mentioned before.

Of notice, the CP is not only able to activate these pathways but also to synthesize molecules responsible for the induction of inhibitory mechanisms as is the case of the proteins that belong to the SOCS family. In our array the suppressor of cytokine signalling 2 (*Socs2*) and suppressor of cytokine signalling 3 (*Socs3*) appear up-regulated.

Discussion

The present study reveals that the CP displays an acute-phase response to an inflammatory stimulus induced in the periphery. As part of the blood-brain barriers, and because it influences the composition of the CSF, changes in the CP gene expression profile might influence the brain response to inflammation. Of notice, the kinetic profile presented by the CP is similar to that of the liver (Sunil et al., 2007; Vemula et al., 2004), with respect not only to the onset but also to the time in which the expression profile returns to the basal state. In addition, the acute responses both in the liver and in the CP share common pathways such as the Nf-Kβ, JAK/STAT and MAPK pathways. On the other hand, the nature of the CP epithelium reveals particular genes, especially those related with the barrier function, that are specifically implicated in intercellular junctions, both the tight junctions and the adherens junction, and that are altered by the inflammatory stimulus. Previous studies have shown induction of cytokines (Marques et al., 2007; Thibeault et al., 2001; Nadeau and Rivest, 1999; Brochu et al., 1999; Tonelli et al., 2003), acute-phase proteins (Schreiber et al., 1989; Marques et al., 2008) and major constitutively expressed proteins (Marques et al., 2007; Dickson et al., 1985) in the CP in response to acute peripheral inflammation. However, with the present data, the recognition that the CP, as a whole, mounts a specific and transient acute phase response becomes evident.

It is well known that many disorders change the functionality and integrity of the BBB. BBB breakdown or alterations in transport systems play an important role in the pathogenesis of many CNS diseases (HIV-1 encephalitis, AD, MS, PD, ischemia and tumors) (Persidsky et al., 2006; Avison et al., 2004). Although the response of endothelial cells of the blood vessels that form the BBB to an inflammatory stimulus is not completely known, it is reasonable to expect that the CP response shares many of the mechanisms already described for the BBB. Together with studies implying particular CP proteins in diseases such as AD (Carro et al., 2005; Sousa et al., 2007) the present study warrants the need for further addressing the CP transcriptome in neurological and psychiatric diseases. Pro-inflammatory substances produced by microglia and specific disease-associated proteins (β-amyloid in AD and reactive T cells in MS) often mediate BBB dysfunction. These pro-inflammatory substances include cytokines, chemokines, reactive oxygen species, glutamate, and MMPs that may alter the integrity of the tight junctions. As we now show here, the CP may well be, as well, the source of many of these deleterious substances. In fact the integrity of the BBB is compromised in diseases with an important inflammatory component, for which there is leukocytes entry into the brain (Wong et al., 2007; Archambault et al., 2005). A

number of studies support the idea that changes in BBB endothelial cell tight junctions and cytoskeletal organization facilitates leukocyte migration. Adhesion molecules, such as E-selectin, VCAM-1, and ICAM-1, were proposed as key molecules in the interaction with leukocytes and in inducing functional changes in the BBB endothelial cells (Engelhardt, 2006). The data we now present on the CP show decreased expression of tight junction proteins and increased expression of proteins that facilitate migration. This may underlie the preferential migration of T lymphocyte across the BCSFB observed in animal models of MS (Brown and Sawchenko, 2007) and supports a relevant role of the CP in cell entry into the CSF and ultimately into the brain parenchyma.

Apart from the role of the CP as a potential site of cell entry into the brain, due to leakage of its barrier function, the CP is also an active site of protein synthesis. Several inflammatory markers have been described in the CSF of individuals with CNS diseases (Andreasen and Blennow, 2005; Giovannoni, 2006). The precise origin of these molecules is still a matter of debate, but the CP could greatly contribute for their presence in the CSF. In disease, or in response to various stimuli, the nature of the proteins secreted by the CP may change, as has been already shown for various cytokines, carrier proteins and iron-related proteins (Marques et al., 2007, 2008; Hughes et al., 2002; Thibeault et al., 2001). The inflammatory chemokines, CCL5, CXCL10 and CCL2, whose genes have expression altered in the CP after peripheral LPS injection, are increased in the CSF of MS subjects (Trebst and Ransohoff, 2001). CCL2 up-regulation in the CP has been previously shown by *in situ* hybridization (Thibeault et al., 2001), and here in the CSF (data not shown) after a single systemic bolus of LPS. While these may originate in the BBB or in the brain parenchyma, as suggested, it is likely that the CP, at least in part, also contributes. In addition, secondary lymphoid chemokines such as CXCL12, CXCL13 detected in the CSF of MS patients (Corcione et al., 2004) are equally up-regulated in the CP.

Other proteins such as MMP-9 and TIMP-3 have been described in the CSF of patients with meningitis (Kolb et al., 1998). The origin of such proteins may also likely be the CP. It is interesting that the balance between matrix metalloproteinases and their tissue specific inhibitors is crucial for extracellular matrix degradation. It is still unclear whether, as a whole, the CP response to peripheral inflammation favours one or the other, since we also find *Timp1* up-regulated at the same time points in which MMPs are induced. Similarly, the balance between pro- and anti-inflammatory molecules and how this relates to the final message transmitted into the brain is far from being understood. The CP synthesises and secretes into the CSF both pro-

inflammatory (IL-1β, IL-6 and TNF) and anti-inflammatory (TGF-β) cytokines (Chodobski and Szmydynger-Chodobska, 2001). Anti-inflammatory, neuroprotective and antioxidant proteins such as metallothioneins -I and –II are increased and suggested as protective in neurodegenerative diseases such MS (Penkowa et al., 2001 Espejo et al., 2005), and AD (Carrasco et al., 2006). Under basal conditions, Espejo et al., (2005) have described low expression of MT-I and -II in the ependyma, CP, and meninges; and their expression is increased in the recovery phase of MS models being therefore suggested as protective. Of interest, LPS has been shown to induce the expression of metallothioneins in whole brain extracts (Zheng et al., 1995); and the present data shows that the CP certainly contributes to such increase.

Other recent observations add to the neuroprotective role of the CP, since in response to LPS the CP regulates iron metabolism by inducing a decrease in iron availability for microorganism proliferation within the CNS (Marques et al., 2008, Chapter 4).

Irrespectively from the final balance, the present data give us insight on the signalling transduction pathways that ultimately result in the observed altered gene expression profile (Figure 4). In response to the peripheral administration of LPS, the serum concentration of several cytokines, such as IL1β and IL6 is increased (Ramadori and Christ, 1999). Since receptors for these cytokines have been described in the epithelial cells of the CP (Chodobski and Szmydynger-Chodobska, 2001), and also found in basal conditions in our array, both LPS- and cytokine-mediated signalling transduction pathways may be operating simultaneously in response to peripheral inflammation. They may in fact be more relevant than LPS for the final response since the transcriptome of CP cells cultured *in vitro* in the presence of LPS display a much reduced response with only a few proteins up-regulated (Thouvenot et al., 2006).

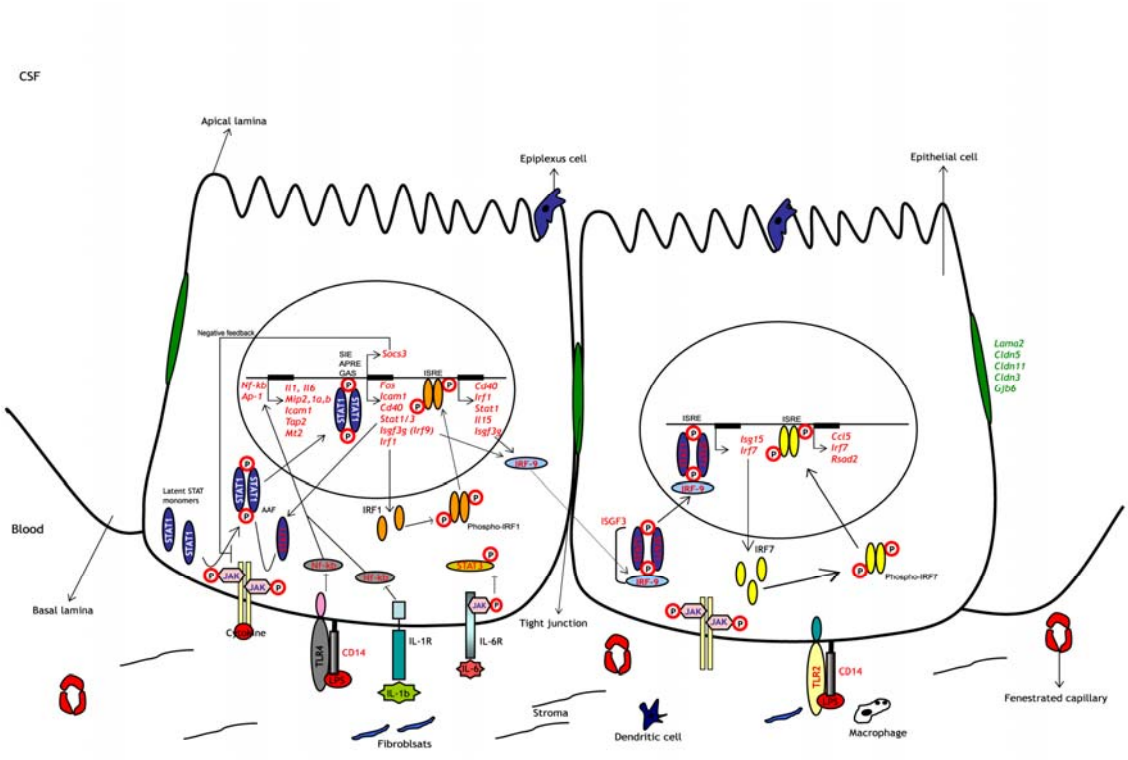

Figure. 4. Suggested pathway of the CP epithelial cells response to peripheral injected LPS. In green are the genes that are, in one or more time points, down-regulated, in red are the genes that appear up-regulated during one or more time points of the kinetic study.

LPS is the natural ligand of TLR4, which is expressed in the CP and in the circuventricular organs within the brain (Laflamme and Rivest, 2001). While we find no effect of LPS on the levels of TLR4, the expression levels of the gene encoding CD14 are strongly induced, and increased levels of CD14 may well contribute to the activation of TLR4. Of notice, and similarly to what has been described previously (Laflamme et al., 2001), we observed an increase in the expression of TLR2. Identical observations were described in splenic macrophages (Matsuguchi et al., 2000). While LPS is not described as a ligand for TLR2, it is possible that molecules such as CD14, whose expression is quickly induced, contribute to its activation. In the TLR-mediated signalling pathways, CD14 is known as a co-receptor for TLR2 and TLR4 (Rallabhandi et al., 2006; Remer et al., 2006; Viriyakosol et al., 2000) but it may act as a direct agonist for TLR2 when other ligands are absent (Bsibsi et al., 2007). Alternatively, signalling through TLR4 may ultimately lead to TLR2 activation (Matsuguchi et al., 2000). Activation of Toll-like receptors and of receptors for IL-1β, IL-6 and TNF induces several transcription factors, including interferon regulatory factor 3 (IRF3), activator protein -1 (AP-1) and the NF-kβ (Colonna, 2007; Honda and Taniguchi, 2006a; Honda and Taniguchi, 2006b). We show here that several genes encoding proteins belonging to the NF-Kβ, the MAPK, STAT-JAK and IRFs

signalling pathways are induced during the CP acute-phase response (Table 2) and these are described to contribute in numerous ways to the development and regulation of innate and adaptative immune responses (Ihle, 2001; Rawlings et al., 2004; Wesoly et al., 2007; Decker et al., 2002). It is therefore noticeable that the CP, as has also been demonstrated for the BBB endothelium (Laflamme et al., 1999; Laflamme and Rivest, 1999), responds to peripheral LPS through activation of all these pathways. This activation may also result in posttranslational modifications of transcription factors such as the phosphorylation of STATs, which should next be investigated.

Termination of the CP acute-phase response is observed at 72h after stimulation. This might be the consequence of the negative feedback inhibition of STAT signalling by SOCS/CIS (Cooney, 2002; Naka et al., 2005; Nicola and Greenhalgh, 2000), whose expression we also found increased after LPS administration; this seems to occur as well in the BBB (Lebel et al., 2000).

In summary, the present study shows that the CP displays an acute-phase response similar to the one observed in the liver. This ultimately results in the release of immune modulators, both pro- and anti-inflammatory, into the CSF; and in the loosening of the barrier properties of the CP. The final balance and consequence for an immune response within the brain parenchyma remains to be elucidated, but it may contribute to the inflammatory component underlying diseases such as AD and MS. Highlighting the CP as a relevant mediator in the crosstalk between the immune and the CNS, the present study strongly suggests that the CP should be thoroughly considered in the final brain homeostasis in health and disease.

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6

The choroid plexus response to a sustained peripheral inflammatory stimulus

The choroid plexus response to a sustained peripheral inflammatory stimulus

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Abstract

It is recognized that a chronic systemic inflammation might trigger alterations in the central nervous system (CNS). Several illnesses of the CNS, including multiple sclerosis and Alzheimer's disease have an underlying chronic inflammatory component. Whether and how peripheral inflammation contributes in triggering the brain response in the course of such illnesses is far from understood. As part of the barriers that separate the blood from the brain, the choroid plexus (CP) may convey immune signaling into the brain; particularly given its function as the major producer of the cerebrospinal fluid (CSF). The composition of the CSF is altered in various CNS diseases, for which the CP secretome certainly contributes. In the present study we investigated the CP gene expression profile in response to a chronic inflammatory stimulus, induced by the intraperitoneal administration of lipopolysaccharide every two weeks for a period of three months. The data shows that the CP displays a sustained response to the chronic inflammatory stimulus by altering the expression of several genes. Major altered pathways include those facilitating cells entry into the CSF, and others that participate in the innate immune response to infection by microorganisms. Whether the final balance of the CP response is protective or deleterious for the CNS remains to be clarified and should be considered in the context of the brain response to inflammation.

Keywords: choroid plexus; lipopolysaccharide; gene expression.

Introduction

Inflammation is implicated in the appearance and in the progression of central nervous system (CNS) diseases such as multiple sclerosis (MS) and Alzheimer's diseases (AD), although the mechanisms are poorly understood (Cunningham et al., 2005; Chen et al., 2008; Tan et al., 2007; Qin et al., 2007). It is well accepted that the inflammation observed in some of these diseases may originate in the periphery (Bar-Or, 2008; O'Connor et al., 2008; Dunn, 2006), particularly when the inflammatory stimulus persists as is the case in chronic inflammation. Persistent or chronic inflammatory signals can result in the inappropriate recruitment of leukocytes and cause localized or disseminated tissue dysfunction and damage, ultimately resulting in brain pathology.

The natural mediators of the communication between the periphery and the brain are the blood-brain barriers, constituted by the endothelial cells of the blood capillaries (blood-brain barrier-BBB) and by the epithelial cells of the choroid plexus (CP) that separate the blood from the cerebrospinal fluid (BCSFB). Most studies published to date address the BBB interactions in response to acute peripheral stimulus or in the context of CNS diseases (Banks, 2005; Engelhardt and Ransohoff, 2005; Engelhardt, 2006). However, several reports have identified genes whose expression is altered in the CP mostly by acute peripheral inflammation. Among these are proinflammatory cytokines such as tumor necrosis factor (TNF) (Nadeau and Rivest, 1999; Brochu et al., 1999), interleukin-1β (IL-1β) (Tonelli et al., 2003) and interleukin-6 (IL-6) (Brochu et al., 1999); adhesion molecules such as vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1), P- and E-selectins (Carrithers et al., 2000; Lopez et al., 1999). We have previously shown that the CP mounts an acute-phase response to a single bolus injection of lipopolysaccharide (LPS), triggering molecular pathways that are both neuroprotective and deleterious for the brain. Deleterious because it results in the secretion of proinflammatory cytokines into the CSF, in the decreased expression of proteins that form the epithelial cells tight junctions and in the increased expression of proteins that may facilitate leukocyte trafficking into the brain. Protective since it modulates iron metabolism in a way that may prevent microorganism entry into and dissemination within the brain. While these observations relate to the CP response to acute inflammatory stimulus, they also suggest that the CP may as well be equipped to maintain a sustained response to peripheral inflammation.

Of notice, several scattered but relevant reports have shown that the CP may contribute to the aetiology of CNS diseases in which persistent rather than acute inflammation is more likely to

persist. In MS the CP has been proposed as the main route of leukocyte entry into the brain (Brown and Sawchenko, 2007). In AD, the CP has been proposed to participate in amyloid β peptide clearance out of the brain, through CSF carrier proteins (e.g. transthyretin and apolipoprotein J) (Golabek et al., 1995) that bind to receptors (e.g. megalin) (Hammad et al., 1997; Sagare et al., 2007; Zlokovic et al., 1996; Carro et al., 2005) in the apical membrane of the CP epithelial cells. Again, the CP seems able to mediate both neuro-deleterious and neuro-protective pathways.

These observations prompted us to investigate further how the CP transmits immune signals into the brain, in response to peripheral chronic inflammation.

Materials and Methods

Animals and LPS injection

All experiments were conducted using 8-9 week-old C57BL/6 male mice (Charles River, Barcelona, Spain), in accordance with the European Community Council Directive 86/09/EEC guidelines for the care and handling of laboratory animals. Animals were maintained in 12 h light/dark cycles at 22-24°C and 55% humidity and fed with regular rodent's chow and tap water *ad libitum*. In order to reduce stress-induced changes in the hypothalamus-pituitary axis associated with the injection, animals were handled for 1 week prior to the beginning of the experiment. Animals were given LPS (*Escherichia coli*, serotype O26:B6; Sigma, St. Louis, USA) (5μg/g body weight) intraperitoneally (*i.p.*); control animals were injected with vehicle (0.9% NaCl) alone.

Mice received i.p. LPS or vehicle injections once every 2 weeks for 3 months.

Animals were sacrificed 3 or 15 days after the last LPS or saline injection, under anesthesia with ketamine hydrochloride (150mg/Kg) plus medetomidine (0.3mg/Kg), and transcardially perfused with cold saline. For the mRNA studies, CP isolation was made under conventional light microscopy (SZX7, Olympus, Hamburg, Germany) and tissue was rapidly removed, frozen in dry ice and stored at -80°C. At least 3 pools of CP (from 3 animals each) were prepared for each time point and for each treatment.

Microarray experimental design and data analysis

Total RNA was isolated using Trizol reagent (Invitrogen, Calrsbad, CA, USA). Total RNA quality was assessed using the Agilent Bioanalyzer (Santa Clara, CA, USA). After quality assessment, 100ng of total RNA from 3 pooled controls and 3 pooled samples for each time point were amplified and labelled with Illumina TotalPrep RNA Amplification Kit according to manufacturer instructions. The labelled cRNA was then hybridized using Illumina recommended protocol in a total of two Illumina Whole-genome Mouseref-8 expression Beadchips (San Diego, CA, USA). This mouse beadchip contains eight arrays, each comprising a total of 24,000 well-annotated RefSeq transcripts.

After scanning, raw data from BeadStudio software (San Diego, CA, USA) was read into R/Bioconductor and normalized using quantile normalization. A linear model was applied to the normalized data using Limma package in R/Bioconductor (Gentleman et al., 2004). A contrast analysis was applied and differentially expressed genes were selected using a Bayesian approach

with a false discovery rate (FDR) of 5%.

Three pools of LPS injected animals were analysed and compared with control animals. Data was analyzed in the Bioconductor. Quality control using inter-array Pearson correlation and clustering based on variance allowed us to ensure that there was reproducibility between the replicates (data not shown). After data normalization, the differentially expressed genes were selected based on FDR of 5%.

The differentially expressed genes were categorized using Gene Ontology from Biomart (http://www.biomart.org/) or Ingenuity tools (Redwood City, CA, USA). Enrichment analysis was performed using the DAVID (http://david.niaid.nih.gov/david/ease.htm) and the Ingenuity softwares.

Results:

Chronic peripheral inflammation induces a manifest alteration in the choroid plexus gene expression profile

Mice were administered with LPS once every 2 weeks for 3 months and were sacrificed 3 and 15 days after the last LPS injection. These time-points were chosen since we have previously shown (chapter 5) that 3 days after a single LPS injection almost no genes had an altered expression. Moreover, as LPS was administered every 15 days, the analysis at 2 weeks after the last injection would allow us to evaluate if the gene profile was continuously altered during chronic inflammation. Figure 1 shows that 3 days after the last LPS injection more than 500 genes (536 genes) have an altered expression when compared to saline injected animals. Since at the same time-point, after a single LPS injection, only 46 genes were found to have an altered expression, the response we now find might be attributed to the chronicity of the stimuli. A response of the CP to the continuous peripheral stimuli is still present 15 days after the last injection; suggesting that contrary to what is observed upon a single acute stimulus, repetitive LPS injections do not allow the CP gene expression profile to recover as quickly.

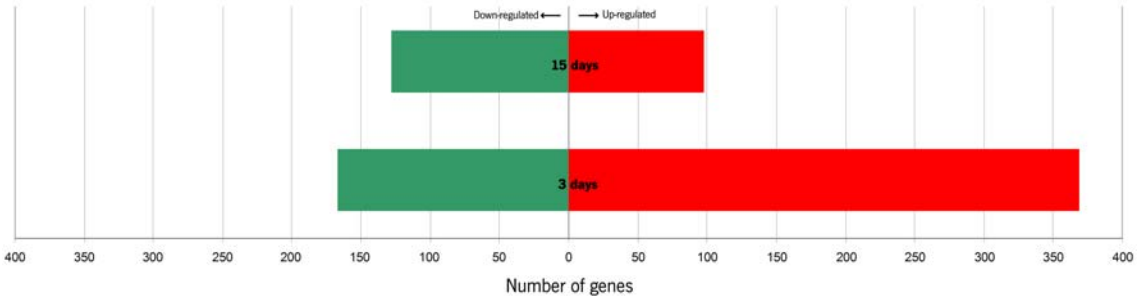

Figure 1. Chronic inflammation induces an altered CP gene expression profile. Number of genes up-regulated (red) and down-regulated (green) in the CP 3 and 15 days after the last injection of the chronic peripheral treatment with LPS. All genes with a variation in expression of at least 20% ($p<0,05$) were considered.

Despite the number of genes whose expression is found statistically altered, not many display a sustained fold change higher than 50% (Figure 2). In fact, as can be observed in Figure 2a, 3 days after the last LPS injection, from the 369 genes whose expression is up-regulated, only for 25 the fold change is ≥2,0. In addition, from the 167 genes whose expression is down-modulated, none shows a strong down-modulation and only 7 display a decrease ≥1,7 (Figure 2a). The fold changes are even smaller (but still statistically significant) at 15 days after the last LPS injection (Figure 2b); only 2 genes present a fold increase ≥2,0.

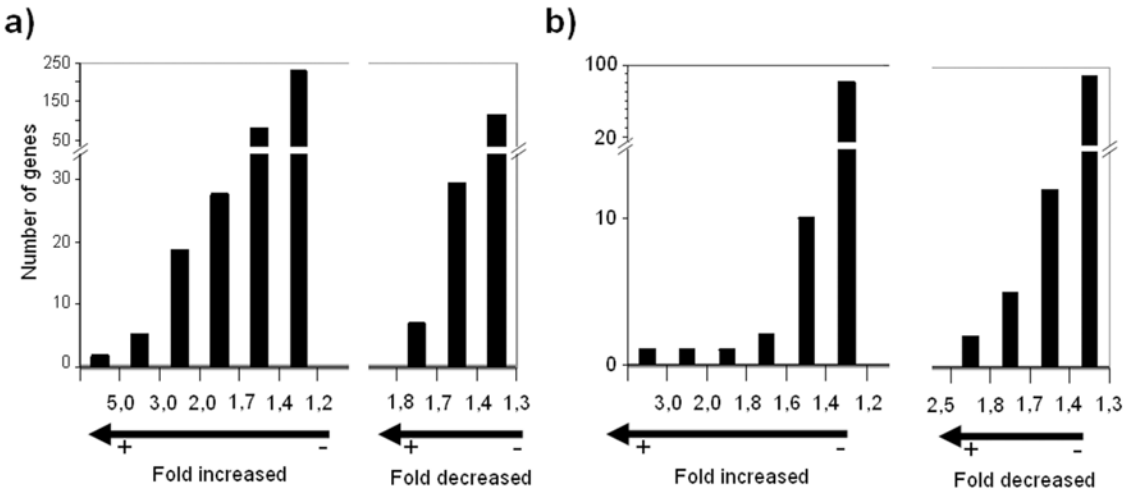

Figure 2. The fold change induced in most genes by the chronic stimulus is below 50%, both 3 days (a) and 15 days (b) after the last lipopolysaccharide administration.

Table 1 lists the 25 genes that showed at least a 2-fold up-regulation at 3 days. For the genes whose expression was down-regulated at 3 days, or up- and down-regulated at 15 days, we focused our attention on those whose expression showed at least a 40% change (Tables 2, 3 and 4).

Table 1. Genes whose expression is induced more that 2-fold 3 days after the last LPS injection

3 days		
Up-regulated		
Accession	**Gene**	**Fold change**
NM_011315	Serum amyloid A 3 (Saa3) mRNA	12,1
NM_008161.1	Glutathione peroxidase 3 (Gpx3) mRNA	6,7
NM_017372.2	Lysozyme (Lyzs) mRNA	4,2
NM_009252.1	Serine (or cysteine) proteinase inhibitor clade A member 3N (Serpina3n) mRNA	3,8
NM_008134.1	Glycosylation dependent cell adhesion molecule 1 (Glycam1) mRNA	3,7
NM_030707	Macrophage scavenger receptor 2 (Msr2) mRNA	3,2
NM_013653.1	Chemokine (C-C motif) ligand 5 (Ccl5) mRNA	3,1
NM_010185.2	Fc receptor IgE high affinity I gamma polypeptide (Fcer1g) mRNA	2,9
NM_007574.1	Complement component 1 q subcomponent gamma polypeptide (C1qg) mRNA	2,8
NM_008491.1	Lipocalin 2 (Lcn2) mRNA	2,7
NM_009777.1	Complement component 1 q subcomponent beta polypeptide (C1qb) mRNA	2,7
NM_009778.1	Complement component 3 (C3) mRNA	2,6
NM_026835.1	Membrane-spanning 4-domains subfamily A member 11 (Ms4a11) mRNA	2,4
NM_010130.1	EGF-like module containing mucin-like hormone receptor-like sequence 1 (Emr1) mRNA	2,4
NM_007572	Complement component 1 q subcomponent alpha polypeptide (C1qa) mRNA	2,4
NM_011662.2	TYRO protein tyrosine kinase binding protein (Tyrobp) mRNA	2,3
NM_010188.2	Fc receptor IgG low affinity III (Fcgr3) mRNA	2,3
NM_010686.2	Lysosomal-associated protein transmembrane 5 (Laptm5) mRNA	2,1
NM_009663	Arachidonate 5-lipoxygenase activating protein (Alox5ap) mRNA	2,1
NM_178911.2	Expressed sequence AI132321 (AI132321) mRNA	2,1
NM_013650.1	S100 calcium binding protein A8 (calgranulin A) (S100a8) mRNA	2,1
XM_359281.1	Leukocyte specific transcript 1 (Lst1) mRNA	2,1
NM_029803.1	Interferon, alpha-inducible protein 27 (Ifi27), mRNA	2,0
NM_013706	CD52 antigen (Cd52) mRNA	2,0
NM_008176.1	Chemokine (C-X-C motif) ligand 1 (Cxcl1) mRNA	2,0

Table 2. Genes whose expression is at least 40% down-regulated 3 days after the last LPS injection.

3 days		
Down-regulated		
Accession	**Gene**	**Fold change**
XM_355574.1	Guanine nucleotide binding protein alpha inhibiting 1 (Gnai1) mRNA.	-1,7
NM_177644.2	Hypothetical protein 6530401P13 (6530401P13) mRNA.	-1,7
NM_178404.2	Zinc finger CCCH type containing 6 (Zc3h6), mRNA	-1,7
NM_011638.3	Transferrin receptor (Tfrc) mRNA.	-1,7
NM_018824.2	Solute carrier family 23 (nucleobase transporters) member 2 (Slc23a2) mRNA.	-1,7

Table 3. Genes whose expression is at least 40% up-regulated 15 days after the last LPS injection.

15 days		
Up-regulated		
Accession	**Gene**	**Fold change**
NM_016974.1	D site albumin promoter binding protein (Dbp) mRNA.	3,3
NM_145434.1	Nuclear receptor subfamily 1 group D member 1 (Nr1d1) mRNA.	2,5
NM_008176.1	Chemokine (C-X-C motif) ligand 1 (Cxcl1) mRNA.	1,9
NM_017372.2	Lysozyme (Lyzs) mRNA.	1,7
NM_011328.1	Secretin (Sct) mRNA.	1,6
NM_008161.1	Glutathione peroxidase 3 (Gpx3) mRNA.	1,5
NM_175030.1	Tctex1 domain containing 4 (Tctex1d4), mRNA	1,5
NM_010493.2	Intercellular adhesion molecule (Icam1) mRNA.	1,5
NM_009778.1	Complement component 3 (C3) mRNA.	1,4
NM_030707	Macrophage scavenger receptor 2 (Msr2) mRNA.	1,4
NM_008898.1	P450 (cytochrome) oxidoreductase (Por) mRNA.	1,4
NM_008223.1	Serine (or cysteine) proteinase inhibitor clade D member 1 (Serpind1) mRNA.	1,4
NM_008630.1	Metallothionein 2 (Mt2) mRNA.	1,4
NM_026436.1	Transmembrane protein 86A (Tmem86a), mRNA	1,4
NM_172976.1	6-phosphofructo-2-kinase/fructose-26-biphosphatase 3 (Pfkfb3) mRNA.	1,4

Table 4. Genes whose expression is at least 40% down-regulated 15 days after the last LPS injection.

15 days		
Down-regulated		
Accession	**Gene**	**Fold change**
NM_007489.1	Aryl hydrocarbon receptor nuclear translocator-like (Arntl) mRNA.	-2,8
NM_175475.2	Cytochrome P450 family 26 subfamily b polypeptide 1 (Cyp26b1) mRNA.	-2,3
NM_133903.2	Spondin 2 extracellular matrix protein (Spon2) mRNA.	-1,7

To understand the genes that are continuously altered during a chronic process of peripheral inflammation we looked for the genes whose expression was altered, at least 40%, both at 3 and at 15 days after the last LPS stimulus. Only 7 fulfilled such criteria (Table 5). No down-regulated genes are common at 3 and 15 days.

Table 5. Genes whose expression is at least 40% altered both at 3 and at 15 days after the last LPS injection.

Genes altered 3 and 15 days after last LPS injection			
		Fold change	
Accession	Gene	3 days	15 days
NM_030707	Macrophage scavenger receptor 2 (Msr2) mRNA.	3,2	1,4
NM_009778.1	Complement component 3 (C3) mRNA.	2,6	1,4
NM_010493.2	Intercellular adhesion molecule (Icam1) mRNA.	1,5	1,5
NM_008161.1	Glutathione peroxidase 3 (Gpx3) mRNA.	6,7	1,5
NM_011328.1	Secretin (Sct) mRNA.	1,6	1,6
NM_017372.2	Lysozyme (Lyzs) mRNA.	4,2	1,7
NM_008176.1	Chemokine (C-X-C motif) ligand 1 (Cxcl1) mRNA.	2,0	1,9

We next analysed, using the Ingenuity software, the pathways to which the genes altered at 3 and 15 days after the chronic LPS treatment belonged. From Figure 3 it is clear that 15 days after the last LPS injection only a few genes are still altered within each biological pathway.

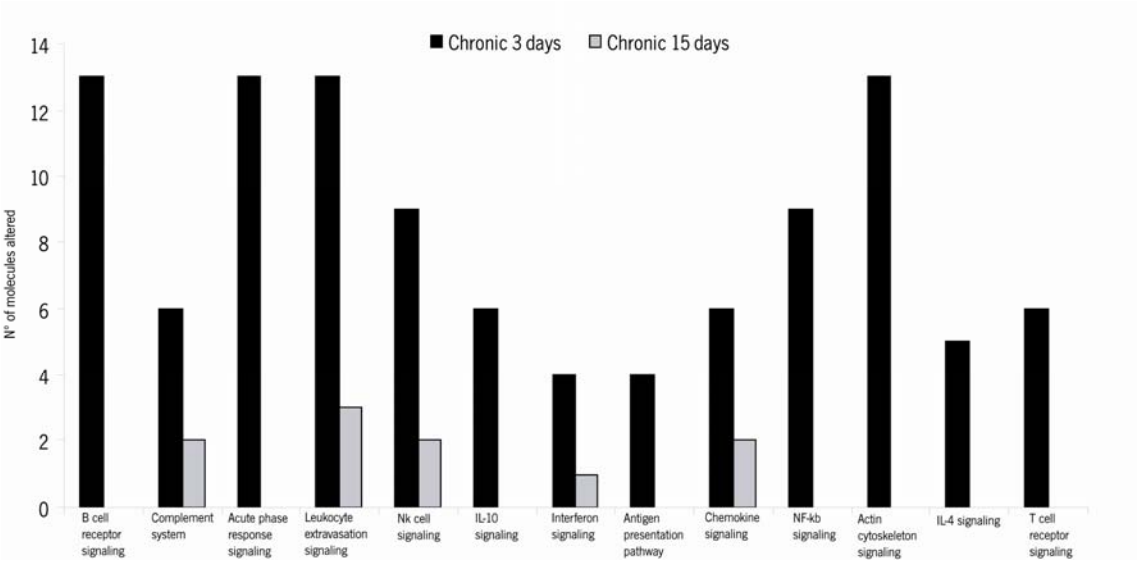

Figure 3. Annotation-based functional analysis of gene expression changes after sustained injection of lipopolysaccharide – comparative analysis between the two time points analysed.

When compared to the acute response presented in chapter 5, it should be notice that: only one gene (rather then 13) of the acute phase response signalling, one gene (rather then 6) of the chemokine signalling, 2 genes of the complement system (rather then 6) and 3 (rather then 4) from the antigen presentation pathway was found altered 3 days after an acute stimuli, showing that these alterations are certainly associated with the chronicity of the model used here.

Discussion

A repetitive inflammatory peripheral stimulus induces an altered gene expression pattern that is still present 3 and even 15 days after the last LPS injection. We have previously shown that 3 days after an acute LPS injection only 46 genes were altered and with a tendency to normalization. Of these, only 12 coincide with genes we now found altered at 3 days after the last injection of the continuous LPS treatment. Therefore, we can conclude that most of the alterations we now observe result from the chronicity of the inflammatory stimuli. However, it seems that the repeated injection of LPS induces in the CP the sustained transcription of specific molecules already found altered upon a single LPS injection. Namely, molecules known to kill or neutralize microorganisms such as the enzyme lysozyme (LYZS) or the bacteria siderophore-iron sequester protein, lipocalin 2 (LCN2) and elements of the complement (e.g. complement component 2, component 3, component 1 q subcomponent alpha polypeptide).

When the overall CP response is evaluated in terms of the major biological pathways, 3 days after the last LPS administration, the CP response is mainly characterized by the increased synthesis of chemokines, complement system activation, activation of the leukocyte extravasation signalling and activation of the NK and B cell signalling. Of notice, it is well known that the signals of a chronic inflammation can result in the inappropriate recruitment of leukocytes and cause localized or disseminated tissue dysfunction and damage. The "leukocyte extravasation signaling" pathway seems, in fact, the most sustained both at 3 and 15 days. We show here that a considerable number of molecules involved in this pathway are altered, at least 3 days after the last LPS injection. Among these is the increased expression of the genes encoding for ICAM-1, selectin P ligand (*Selpl*), glycosylation dependent cell adhesion molecule 1 (*Glycam1*), mucosal vascular addressin cell adhesion molecule 1 (*Madcam1*), matrix metalloproteinase 23 (*Mmp23*). Other molecules such as chemokines are essential for the trafficking of inflammatory cells from the blood into tissues. Chemokine ligand 7 (*Ccl7*) and chemokine (C-C motif) ligand 13 (*Ccl13*) are up-regulated both at 3 and 15 days. Other relevant gene found up-regulated and that is involved in the migration of immune cells is interleukine-16 (IL-16). Of notice, we did not find IL-16 influenced by acute inflammation. IL-16 is a pleiotropic cytokine that is a natural ligand of CD4 (Berman et al., 1985; Cruikshank and Center, 1982) and has been identified at sites of allergic inflammation in both the murine and the human airway epithelium (Laberge et al., 2000; De Bie et al., 2002). It is known as a chemoattractant for $CD4^+$ T cells, monocytes, eosinophils and dendritic cells, with preferential chemoattractant activity for the Th1 subset of $CD4^+$ T cells

(Kaser et al., 1999; Lynch et al., 2003; McFadden et al., 2007). Despite the increase in molecules that participate in leukocyte recruitment, no changes were observed in the expression levels of genes that encode tight junction proteins. Therefore, and contrary to what we previously observed in the acute response, the CP seems to more easily assure the integrity of the barrier in response to a sustained inflammatory stimulus.

The CP response seems to share some mechanisms previously described at the BBB, which include the activation of adhesion and chemoattraction signals in endothelial cells in CNS diseases such as MS (Bullard et al., 2007; Engelhardt and Ransohoff, 2005). Therefore, both the blood-CSF barrier and the BBB seem equipped to convey signals to the brain parenchyma in response to both acute and chronic inflammation.

When compared to the data obtained after a single bolus injection of LPS, the present data allow us to address the phenomenon of endotoxin tolerance described for bacterial LPS. It seems that the fold change is considerably smaller for genes whose expression is found altered both in response to acute and chronic LPS administration. While this could be related with the tolerance to LPS, future experiments should further investigate this possibility by analyzing the gene expression profile soon (e.g. 3h) after the last LPS injection.

Despite the apparent tolerance to LPS, the expression of some CP genes seems solely altered after the chronic stimulation. Among these are genes encoding proteins of the S100 family. The S100 family of calcium-binding proteins comprises a new group of pro-inflammatory molecules that have been discussed in the context of MS (Giovannoni, 2006) and in AD (Geroldi et al., 2006). Here we show increased levels of S100a8 and S100a9. It would next be interesting to investigate precisely the cell types expressing these proteins within the CP, and whether its levels are altered in the CSF of patients with MS and AD. Another molecule exclusively expressed during the chronic treatment is the macrophage scavenger receptor 2 (*Msr2*). Scavenger receptors (SRs), initially described as high-affinity receptors on macrophages for acetylated low-density lipoproteins, comprise several receptor classes (Husemann et al., 2002; Murphy et al., 2005) and are expressed in various cell types. SRs have a role in the binding and internalization of many unrelated ligands, such as fibrillar β-amyloid, lipids, glycated collagen and apoptotic cells, and, therefore, are important for tissue homeostasis. The up-regulated expression of this gene in the CP could indicate the possible protective role of the CP in the progression of diseases such as AD. As referred to above, clearance of amyloid β peptide out of the brain is reported to occur through the megalin and low-density lipoprotein receptor-related protein receptors that are SR (Zlokovic, 2004).

In summary, we describe here that the CP displays a sustained response to a continuous inflammatory stimulus induced in the periphery. The altered gene expression profile includes the up-regulation of genes encoding for both neuroprotective and deleterious molecules. Therefore, future studies should further investigate the final balance of the CP response in the context of diseases of the CNS.

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7

Discussion and future perspectives

General discussion

The work developed during this thesis shows that the CP mounts a complex response to an inflammatory peripheral stimulus. The response of these cells alters the composition of the CSF and, consequently, might influence several regions of the brain parenchyma. Moreover, the pattern of genes up- and/or down-regulated in response to a single stimulus (acute inflammation) or repeated stimuli (sustained inflammation) differs greatly. The potential consequences of these differences will be discussed below.

Most studies addressing the interactions between the periphery and the CNS focus on the BBB (Banks and Kastin, 1991; Banks, 2005; Engelhardt and Ransohoff, 2005; Engelhardt, 2006). The BCSFB represents also an important route between the periphery and the CNS, but has been poorly investigated. The CP is ideally located to respond to peripheral stimulus since it is well vascularised by fenestrated capillaries and because it produces most of the CSF. An altered profile of proteins secreted into the CSF may affect CNS function in health and in disease, and the composition of the CSF has been found altered in several neurological and psychiatric diseases. Of relevance, many of such disorders, including AD and MS are known to have as underlying inflammatory component. Thus, a more specific study on the profile of CP secreted proteins during inflammation may provide key information on physiological and pathological conditions.

Several studies have previously identified CP receptors and immune modulators whose expression is found altered upon various systemic or intra cerebral inflammatory stimuli (Xia et al., 2006; Thibeault et al., 2001; Nadeau and Rivest, 1999; Brochu et al., 1999; Tonelli et al., 2003; Wolburg et al., 1999; Engelhardt et al., 2001; Lopez et al., 1999; Carrithers et al., 2000). Most of these studies did not address specifically the CP, but rather several regions of the CNS focusing the attention on particular proteins.

In the present study we considered the CP as a whole as an attempt to identify the molecular pathways involved in the response to inflammation and the impact on the CSF composition.

The main achievements of the work developed in this thesis are that:

1. The CP mounts an acute response to a single peripheral inflammation stimulus. This response induces alteration in the expression profile of a set of genes, occurs rapidly (can be detected as soon as 1h after LPS injection) and is transient (the profile of gene expression is almost back to normal 72h after the stimulus);

2. The CP response differs greatly if the stimulus is administered repeatedly.

3. The CP seems to participate in modulating iron availability for the brain in conditions of inflammation.

The overall choroid plexus response to peripheral lipopolysaccharide administration

We first studied how the expression of major CP proteins responds to LPS. We chose to analyse the expression of TTR and TF because they are two liver negative acute-phase proteins (Ceciliani et al., 2002). In addition we decided to study lipocalin-type prostaglandin D2 synthase (L-PTGDS) because it is the second major protein synthesised by the CP and is responsible for the production of the major prostanoid of the CNS. We showed that the expression of L-PTGDS is increased upon LPS administration. Because prostaglandin D2 (PGD2) like other prostagladins has been implicated as a mediator for both pro- and anti-inflammatory actions it is difficult to conclude about the beneficial/detrimental effects of this increase (Harris et al., 2002; Urade and Hayaishi, 2000). Recently it has been suggested that endogenous PGD2 might display anti-inflammatory properties, given its readily conversion to bioactive cyclopentenone-type prostaglandins of the J2-series such as 15-deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2) (Mouihate et al., 2004; Straus and Glass, 2001). It has been shown that 15d-PGJ2 regulates important biological responses, including protection against oxidative stress (Oh et al., 2008; Napimoga et al., 2008). However, in specific circumstances, 15d-PGJ2 also has pro-inflammatory properties (Harris et al., 2002); so the final relevance of its increased levels remains to be clarified. Because within the brain L-PTGDS is also synthesized by oligodendrocytes it will next be interesting to investigate whether the LPS stimulus also induces L-PTGDS expression in other brain regions. This might be particularly relevant in light of the recent description of L-PTGDS immunoreactivity in AD and in MS patients (Kagitani-Shimono et al., 2006; Kanekiyo et al., 2007). Additional studies on this subject should be performed in animal models of both diseases.

These initial observations prompted us to investigate the overall response of the CP to LPS. Microarray analysis of the kinetic response to a single LPS injection revels that several major biological pathways are quickly and transiently altered. These pathways include a decrease in the expression of genes encoding tight junction proteins, which might compromise the CP barrier function; and the up-regulation of genes encoding proteins that promote cell migration, which may facilitate leukocyte entry into the brain.

Of interest the CP response includes the up-regulation of genes encoding pro- and anti-inflammatory molecules. The levels of several of those were also increased in the CSF. Weather the final balance is protective or deleterious for the brain, specifically to neurons and glial cells, requires further investigation. Future studies should address the precise fate of these proteins secreted into the CSF in response to inflammation; both through the intrathecal administration of radiolabeled proteins and through studies on their effects on neuronal and glial cultures *in vitro*.

In response to LPS, the levels of several cytokines increases in the bloodstream. Therefore, the CP response certainly reflects the influence not only of LPS but also of cytokines such as IL-1β, and/or IL-6, for which the receptors have been described in the basolateral membrane of the CP epithelial cells. In accordance, the expression of several genes belonging to intracellular pathways that respond to these molecules, and their downstream target genes were found altered in response to the LPS injection. Among theses genes are the JAK-STAT, MAPK, Nf-Kβ and IRFs signalling transduction pathways.

Among the genes mostly altered in the acute-phase response is that encoding for LCN2. LCN2 binds to bacterial siderophores, iron-chelators produced by bacteria as an attempt to acquire iron from the host. Since iron is requested for bacterial survival and replication, the host mounts an innate response in which several iron-binding proteins participate. The expression of LCN2 had been previously shown to be up-regulated in neutrophils, macrophages, adipocytes and in the liver, in response to peripheral infection and inflammation (Zhang et al., 2008; Borregaard and Cowland, 2006; Borregaard et al., 2007; Sunil et al., 2007). Of notice, LCN2-null mice exhibit increased susceptibility to infection by bacteria that depend on entherocalin-like siderophore-dependent iron uptake (Berger et al., 2006; Flo et al., 2004). However, none of the studies done previously addressed the role of LCN2 in the brain. The fact that we now observed a strong LCN2 expression in the CP, which results in increased CSF LCN2 levels, suggests that the CP participates in the innate response to infection. Whether the lower susceptibility of the brain to various agents depends on LCN2 is an interesting question to investigate. Future studies should address, *in vivo*, how LCN2 protects the brain from infection by bacteria. In addition, it will also be useful to investigate whether the ability of bacteria to access and colonize the brain depend on the amount of LCN2 produced by the host. Meningitis are among the most serious and deadly infections. Major bacterial meningitis pathogens are *Neisseria meningitidis*, *Haemophilus influenzae* and *Streptococcus pneumoniae*. As neither *S. pneumoniae* nor *H. influenzae* are known to produce or utilize siderophores, they may have developed siderophore-independent

mechanisms to acquire iron. The specific expression of iron-related genes in the CP (please see below) is now warranted both for bacteria that normally colonize or are prevented from colonizing the brain. It will also be interesting to study how administration of LCN2 interferes with the course of infection by *Mycobacterium tuberculosis*, a microorganism also known to be able to cause meningitis and that produces siderophores (Miethke and Marahiel, 2007). It should lastely be mentioned that while no mammalian siderophores have ever been identified, other functions have been suggested for LCN2 in iron homeostasis, particularly in iron delivery to cells. Whether this may be relevant during development, before the onset of TF expression, or in specific conditions such as inflammation is certainly worth exploiting further. This may be particularly relevant since up-regulated LCN2 expression remains when LPS is administered repeatedly.

These observations prompted us to characterize, in the CP, other molecules involved in iron homeostasis.

Iron metabolism in the brain after peripheral inflammation

In the context of response to inflammation, iron metabolism seems an interesting issue. Iron is essential for the basic cellular functions but it can also have deleterious effects essentially because its accumulation can contribute to production of damaging reactive oxygen species (ROS) (Brewer, 2007). ROS formation leads to oxidative stress when there is an imbalance in the redox status of a cell. This is described to occur in various neurodegenerative and neuroinflammatory disorders such as AD and PD (Zecca et al., 2004; Qian and Wang, 1998). Furthermore, there is evidence suggesting that iron deficiencies, during pregnancy or early during postnatal development, can cause mental disorders and severe motor impairments (Lozoff et al., 1991). Therefore, by modulating iron availability for the brain, specifically in the CSF, the CP may have a relevant function in response to a potential brain infection but also in neurodegeneration of the CNS.

Understanding the timing of iron mismanagement in relation to various pathological conditions may provide information on pathogenesis and on susceptibility to conditions such as infection by pathogens. This may be particularly relevant if these changes are a consequence of breakdown of the BBB and BCSFB that allows more iron to access the brain.

Surprisingly our observations suggest that the CP rapidly responds in order to prevent iron release into the CSF upon a peripheral inflammatory stimulus. This response includes not only the expression of hepcidin, a key modulator of iron homeostasis, but also ceruloplasmin and transferrin

receptor type 2 (TFR2). Preliminary data suggest that the molecular pathway triggering hepcidin expression *in vivo* may be mediated by the TFR2 since IL-6 fails, *in vitro*, to stimulate its expression. These observations encourage us to propose additional studies on iron metabolism, both in chronic inflammation but also in models of neurodegenerative disease such as MS and AD.

New insights from the sustained inflammation

It is known that in response to a peripheral inflammation or infection, innate immune cells produce pro-inflammatory cytokines that act on the brain to cause sickness behaviour (Konsman et al., 2002). When activation of the peripheral immune system continues, such as during systemic infections, cancer or autoimmune diseases, the resulting immune signalling to the brain can lead to an exacerbation of sickness and the development of symptoms of various CNS disease including depression (Dantzer et al., 2008) and exacerbation of motor neuron disease (Nguyen et al., 2004). In addition, several evidences suggest that a chronic inflammatory component is implicated in the appearance and in the progression of neurodegenerative diseases such as MS and AD. Here we used a model of multiple injections of LPS, every two weeks, for three months, as an attempt to mimic a sustained inflammatory response. We chose to sacrifice the animals at two time points: 72h after the last LPS injection, in order to distinguish between the genes whose expression corresponded to the acute effect of the last LPS injection (studied 72h after a single injection of LPS in the acute response) from those that represent a sustained response; and 15 days after the last LPS treatment, to address the genes whose expression remains continuously altered. When we compared with the acute-response the expression of much less genes was found altered, and the fold changes observed were also smaller. However, since the altered expression of some genes seems to be sustained, the effect in the overall expression, and the impact on the CSF composition, may be equally relevant. Among the pathways mostly up-regulated is that of adhesion molecules that might promote migration of immune cells into the brain. This observation reinforces the idea of the CP as a possible place of entry of the immune cells, in addition to the better studied BBB route. This may be particularly relevant for diseases such as MS. In fact, the pathogenesis of MS is also characterized by a massive entry of inflammatory auto-reactive T cells in the parenchyma of the CNS in which the CP has been proposed to participate (Strazielle and Ghersi-Egea, 2000; Brown and Sawchenko, 2007). Of notice the demyelination plaques in MS are frequently located in the periventricular area, wich is easily influenced by molecules and cells reaching the CSF through the CP. In this

work we show that in response to sustained inflammation the CP synthesizes molecules (chemokines and adhesion molecules) that are strong candidates to promote leukocytes adhesion and extravasations from the periphery into the brain. Ongoing studies are addressing whether the CP expression profile in MS shares similarities with that observed during response to peripheral inflammation. Preliminary results have shown LCN2 among the highly expressed proteins, further suggesting that LCN2 may have another function in addition to the known ability to bind bacteria siderophores.

The relevance of the CP response to sustained inflammation is not limited to the fact of being a place of immune cells entry but also as a site for microorganism invasion into the brain. Some pathogen-induced inflammation occurs within the CP given the tropism to epithelial cells that bacteria, parasites and viruses such as *Neisseria meningitidis*, *Trypanosoma brucei*, *Sendai virus*, *mumps virus* and perhaps even HIV-1 and human T cell leukemia virus-1 (HTLV-1) present. In fact, they have been shown to accumulate preferentially into the CP (Petito and Adkins, 2005). The data we present here highlight certain antimicrobial proteins such as lysozyme and LCN2 that may be important to fight brain infection. Whether these, or others, may become interesting targets for novel therapeutic approaches should certainly be further investigated.

Choroid plexus as a component of the neuroimmune system - possible contribution of stromal cells

It will be next important to discuss the involvement of other cell types present in the CP stroma, namely those of the lymphoid lineage, in the overall CP response to inflammatory stimuli. The CP should be viewed, in our perspective, as a small and complex organ. For that contributes the fact that the CP is formed by cuboidal epithelial cells resting upon a basal lamina and central core of connective and highly vascularised tissue. Globular macrophages, dendritic cells (Ling et al., 1998; McMenamin et al., 2003; Hanly and Petito, 1998), and fibroblasts are found throughout the stroma; while epiplexus cells are located on the apical surface of the epithelial cells (Emerich et al., 2005; Ling et al., 1998). Therefore, when CP is removed from the brain ventricles, it certainly counts with the contribution of proteins expressed by all the different cell types. This may be particularly relevant in situations of infection or inflammation because of the immune cells that are travelling in the bloodstream in such conditions. It is therefore necessary to discriminate, by immunohistochemistry, *in situ* hybridization or laser microdissection of particular

cells types and/or by using in vitro models of CP epithelial cells, in which cell types, each individual proteins is expressed. As shown in chapter 3 for LCN2, this protein is expressed both by the CP epithelial cells and also by endothelial cells of the blood vessels. Other acute-phase proteins, known to be produced in response to inflammation by neutrophils and macrophages, may also contribute to the overall CP expression profile shown here. Another aspect that we should always consider is that when LPS is injected in the periphery, it also elicits a peripheral immune response. This results in the expression of several immune mediators by several blood circulating cells, and by those present in the CP stroma. These molecules reach and activate receptors in the basal membrane of the CP epithelial cells. Therefore, and as mentioned before, all these other cell types ultimately contribute to the overall CP response to inflammation.

The question on how the CP responds to inflammation induced within the brain has not been addressed in the present study, but it is certainly of relevance, keeping in mind that some inflammatory response may be elicited within the brain by, for instances, neuronal degeneration. This may as well be relevant in the neurodegenerative disorders mentioned before. Of notice, the CP has been proposed as a site of exit of proteins such as the AD amyloid β peptide (Carro et al., 2005; Zlokovic et al., 1996). This is proposed to occur through receptors (e.g. megalin) present in the CP apical membrane, and mediated by proteins the CP secretes into the CSF (e.g. TTR, cystatin C, apolipoprotein J). Future studies should, therefore, not only study the CP expression profile in diseases such as AD, but also the overall CP response to inflammatory stimulus elicited both in the circumventricular space and within the brain parenchyma. In such an approach, it will also be interesting to evaluate the epithelial interaction with dendritic cells in regulating antigen sensitization to the periphery. It has been shown in the intestine and in the airways epithelia that intraepithelial dendritic cells extend processes into the intestinal or airway lumen, respectively, to collect antigenic material from the mucosa surface (Schleimer et al., 2007; Shaykhiev and Bals, 2007, Kato and Schleimer, 2007). Whether such interaction also occurs between the dendritic cells within the CP epithelium and the ventricular space of the brain can eventually be visualized by electronic microscopy.

In summary, the work presented in this thesis highlights the CP as a relevant mediator in the interaction between the immune system and the CNS, raising numerous exciting questions for studies to be launched in the near future.

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