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Modulatory effects of the chemokine Fractalkine in a model of endotoxin-induced microglial activation

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Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, realizada sob a orientação científica do Doutor João Malva (Universidade de Coimbra, Portugal) e do Doutor Jorge Valero (Universidade de Coimbra, Portugal).

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"What makes it difficult is that research is immersion in the unknown. We just don't know what we're doing. We can't be sure whether we're asking the right question or doing the right experiment until we get the answer or the result."

Martin A. Schwartz, "The importance of stupidity in scientific research". *Journal of Cell Science* (2008) 121, 1771

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Abbreviations

ADAM – A disintegrin and metaloprotease
ATP – Adenosine triphosphate
BBB – Blood-brain barrier
BDNF – Brain-derived neurotrophic factor
bFGF – Fibroblast growth factor basic
CD11b – Cluster of differentiation molecule 11B, also integrin alpha-M beta-2 ($\alpha_M\beta_2$) or macrophage-1 antigen (MAC-1)
CD200 – Cluster of differentiation molecule 200
CD45 – Cluster of differentiation molecule 45, also Protein tyrosine phosphatase, receptor type, C (PTPRC) or leukocyte common antigen (LCA)
CNS – Central nervous system
CX_3CL1 – Chemokine (C-X3-C motif) receptor 1
DAMPs – Damage-associated molecular patterns
DNA – Deoxyribonucleic acid
ECM – Extracellular matrix
EGF – Epidermal growth factor
Fc – Fragment; crystallized
FcγR – Fc-gamma receptors
Fkn – Fractalkine, also Chemokine (C-X3-C motif) ligand 1 (CX_3CL1)
GFAP – Glial fibrillar acidic protein
GPCR – G protein-coupled receptor
GTPases – Guanosine triphosphatases
HIV – Human immunodeficiency virus
Iba1 – Ionized calcium binding adaptor molecule 1
IkB – NF-κB inhibitor
IkK – NF-κB inhibitor kinase
IL-1β – Interleukin-1 beta
IL-6 – Interleukin-6
JNK – c-Jun N-terminal kinase
LPS – Lipopolysaccharide
M-MLV RT – Moloney-murine leukaemia virus reverse transcriptase
MAPK – Mitogen-activated protein kinases
MCP-1 – Monocyte chemotactic protein-1
MGF-E8 – Milk-fat globule EGF factor-8
MHC – Major histocompatibility complex
MPTP – 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, neurotoxin precursor to MPP+
MyD88 – Myeloid differentiation primary response 88
NF-κB – Nuclear factor-kappa B
NGF – Neuronal growth factor
NO – Nitric oxide
OHSCs – Organotypic hippocampal slice cultures
PAMPs – Pathogen-associated molecular patterns
PI3K – Phosphatidylinositol 3-kinase
PRRs – Pattern-recognition receptors
PS – Phosphatidylserine
RNA – Ribonucleic acid
ROS – reactive oxygen species
TLR – Toll-like receptor
TNF-α – Tumour necrosis factor alpha

Resumo

A quimiocina fractalquina (Fkn) é uma molécula única, expressa constitutivamente por neurónios no sistema nervoso central (SNC), enquanto que neste tecido o seu receptor se encontra apenas em microglia. Um conjunto crescente de indícios sugere que a Fkn pode servir como modulador da interacção entre neurónios e microglia no SNC saudável e/ou em casos patológicos. Neste trabalho, investigámos os efeitos moduladores da Fkn no perfil inflamatório promovido pelo lipopolissacarídeo (LPS) na linha celular de microglia N9. Os nossos resultados mostram que a Fkn induz uma redução na expressão de mRNA para a interleucina 1-beta (IL-1β) e para o factor de necrose tumoral-alfa (TNF-α), aumentados pelo LPS; aumenta a actividade fagocítica e migratória das células N9 e induz uma reorganização do citoesqueleto, quer em condições basais quer na presença de LPS. Em suma, a Fkn modula a activação da microglia induzida por LPS, reduzindo o perfil inflamatório destas células, mas mantendo elevados níveis de actividade quer fagocítica, quer migratória. Os nossos dados confirmam que a Fkn pode ter um papel importante a desempenhar em patologias do SNC que sejam afectadas pela desregulação da actividade da microglia. O sistema da Fkn surge assim como um alvo importante no tratamento destas patologias.

Palavras-chave: fractalquina, microglia, lipopolissacarídeo, migração, fagocitose.

Abstract

Fractalkine (Fkn) is a unique chemokine, which is constitutively expressed by neurons in the central nervous system (CNS), while in this tissue its receptor is localized only in microglia. Increasing evidence suggests that Fkn may act as a key modulator of the cross-talk between neurons and microglia in health and in disease. In this work, we investigated the modulatory effects of Fkn on the inflammatory profile elicited by lipopolysaccharide (LPS) in the N9 microglial cell line. Our results show that Fkn reduces interleukin-1 beta (IL-1β) and tumour necrosis factor alpha (TNF-α) mRNA expression driven by LPS, increases the phagocytic and migratory activity of N9 cells and induces cytoskeleton reorganization both in basal conditions and under stimulation with LPS. In summary, Fkn modulates LPS-mediated microglial activation, reducing the inflammatory profile while maintaining high levels of migratory and phagocytic activity. Our data confirms that Fkn may play a major role in CNS pathologies affected by deregulation of microglial activity. Thus, Fkn system emerges as an important target for the treatment of such pathologies.

Keywords: fractalkine, microglia, lipopolysaccharide, migration, phagocytosis.

Chapter I – Introduction

1.1. On the origins of microglia

The publication of del Río-Hortega's seminal work in 1919 on the physiology and nature of what had been described until then as the 'third element of the nervous system' marked the beginning of a research interest on microglial cells, which has persisted until today (reviewed in (Rezaie & Male 2002)). What he observed and described in the early 20th century clearly defined the morphology and function of microglia, and the majority of his findings remain perfectly valid almost one century later. Broadly, microglial cells are critical in the protection of the central nervous system (CNS) against infection, trauma, and neurodegenerative processes. Indeed they are considered the resident macrophages of the CNS, the primary immune effector cells of the brain (see (Kettenmann *et al.* 2011) for a comprehensive review). Importantly, beyond these "classical" defence-oriented functions (e.g. starting inflammatory responses and clearing debris) they have also been linked to other, less canonical roles. Microglia have been associated with the development of the vascular and neuronal networks, axonal growth, synaptogenesis, neurogenesis, gliogenesis and developmental neuronal cell death (reviewed in (Pont-Lezica *et al.* 2011)). Such are the functions not of a strictly immune effector cell type, but of an all-purpose key player in the CNS environment (Barres 2008). This puts microglia in a privileged position, both within the immune as well as the nervous system, and the fact that these cells express molecules such as neuromodulators or receptors for neurotransmitters, and that neurons express chemokines and chemokine receptors, hints at a tight crosstalk between these systems (Rostène *et al.* 2011).

In their 2008 paper, Davoust *et al.* nicely review the heterogeneous population of CNS-associated macrophages, as well as their origins (Davoust *et al.* 2008). Adult microglia are thought to derive from two distinct waves of progenitors that invade the CNS. First, during embryonic and foetal development, microglia are derived from pial macrophages from the mesenchymal progenitor cells at the yolk sac (Alliot *et al.* 1999; Chan *et al.* 2007; Davoust *et al.* 2008). Later, before birth (in humans) or in the early postnatal period (in rodents), microglial cells originate from circulating monocytes that infiltrate the brain parenchyma through the blood-brain barrier (BBB) (Kaur *et al.* 2001; Davoust *et al.* 2008). To complicate matters further, there is a great difficulty to clearly discriminate parenchymal microglia from other macrophage-like cells residing in the CNS (Guillemin & Brew 2004).

These are essentially perivascular macrophages, meningeal macrophages and choroid plexus macrophages (Davoust *et al.* 2008). Finally, the replacement of microglia by circulating precursors in the adult CNS does not normally occur. This is probably due to the inability of myelomonocitic precursors with the capacity to generate microglia to reach the circulation (which happens in the case of irradiation/transplant) and the unpermissiveness of the BBB after the second wave of postnatal microglial production (Ajami *et al.* 2007). Thus, the resident microglia seem to exhibit enough proliferation potential to provide progeny, both with and without pathologies, for the lifetime of the organisms (Ajami *et al.* 2007).

1.2. General characteristics of microglia

1.2.1. *In vivo*

The morphology of microglial cells has been classically associated with their functional state. *In vivo*, microglia generally display a branched shape with a small soma and fine and long processes, very well depicted by del Río-Hortega in 1919 (Fig. 1.1.). This description defines the typical morphology of the so-called "resting" microglia, a widespread term for non-reactive microglial cells (Kettenmann *et al.* 2011). Microglial cells can be identified and their morphology revealed by a variety of cell surface-associated or cytosolic molecules. However, most of these molecules also have established functions and are present in macrophages (Kettenmann *et al.* 2011). While this might be a useful method to distinguish microglia from other brain cells (e.g. neurons or macroglia), a clear distinction between microglia and infiltrating monocytes/macrophages is difficult due to the overlap in the expression of the factors used in microglial staining (reviewed in (Guillemin & Brew 2004)). This typically happens with receptors or adhesion molecules. For example, the integrin alpha M (MAC-1, $\alpha_M\beta_2$ integrin or CD11b), which serves as complement 3b receptor, is involved in the clearance of opsonised material by phagocytosis, and is expressed both by macrophages invading the CNS as well as microglia (Guillemin & Brew 2004). Another widespread marker used to identify microglia morphology is ionized calcium (Ca^{2+}) binding adaptor molecule 1 (Iba-1). Iba-1 has been involved in the formation of membrane 'ruffles' on these cells, suggesting a major role in phagocytosis and

migration for this marker (Ohsawa *et al.* 2000). Other factors that are shared between microglia and macrophages are, for example, Fc (fragmented; crystallized)-gamma receptors (FcγR) (Peress *et al.* 1993), major histocompatibility complex (MHC) class II molecules (Bo *et al.* 1994), protein tyrosine phosphatase receptor type C (also leukocyte common antigen, LCA, PTPRC, CD45) (Lowe *et al.* 1989) and F4/80 (Perry *et al.* 1985).

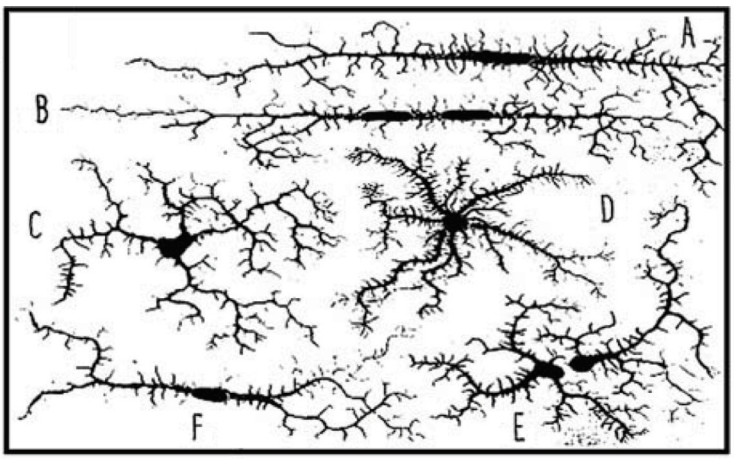

Figure 1.1. The different morphological states of human microglia in the normal state as illustrated by del Río-Hortega in 1919 (adapted from (Rezaie & Male 2002)). (A) Bipolar cell, (B) large cell with two nuclei, (C) multipolar cell with ramified expansions, (D) multipolar cell with spinal (thorny) appendages, (E) isogenic pair, (F) bipolar corpuscle with bifurcating appendices.

Microglia actively scour the brain parenchyma in the search for tissue debris, damaged cells, microbes, or other signals of homeostatic changes, remaining highly dynamic while apparently "resting" (Nimmerjahn *et al.* 2005). This behaviour ensures that signs of potential danger to the CNS can evoke rapid responses from this specialized line of defence. When presented with challenges or altered homeostasis, profound changes are elicited in the microglial cell shape, gene expression and functional behaviour. This process, commonly defined as "microglial activation", should be viewed as a shift from one functional phenotype to another rather than an "awakening" (Hanisch & Kettenmann 2007; Graeber & Streit 2010). Surprisingly, these adjustments can occur in minutes. Following activation, 'resting' microglia undergo a dramatic restructure of their cytoskeleton (Stence *et al.* 2001). This occurs through a multistep transformation involving the replacement of the ramified branches with an entirely new set of highly motile protrusions (Fig 1.2.).

Time-lapse imaging revealed that 30 min post-sacrifice, cells were already in the process of retracting branches ('withdrawal' or 'W-stage'), a process which lasts anywhere between a

few minutes to several hours, at an average retraction speed of 0.5–1.5 µm/min. Following this stage, microglial cells transition into a dynamic 'motility stage' (M-stage), which involves cycles of extension and retraction of new processes. Some cells begin shaping new protrusions only after a significant time delay (30 min or more) following W-stage, however, for other cells this occurred already during the final phase of the withdrawal process. These cells exhibited a 'transitional stage' (T-stage), in which retraction and emergence of cytoplasmic processes take place at the same time.

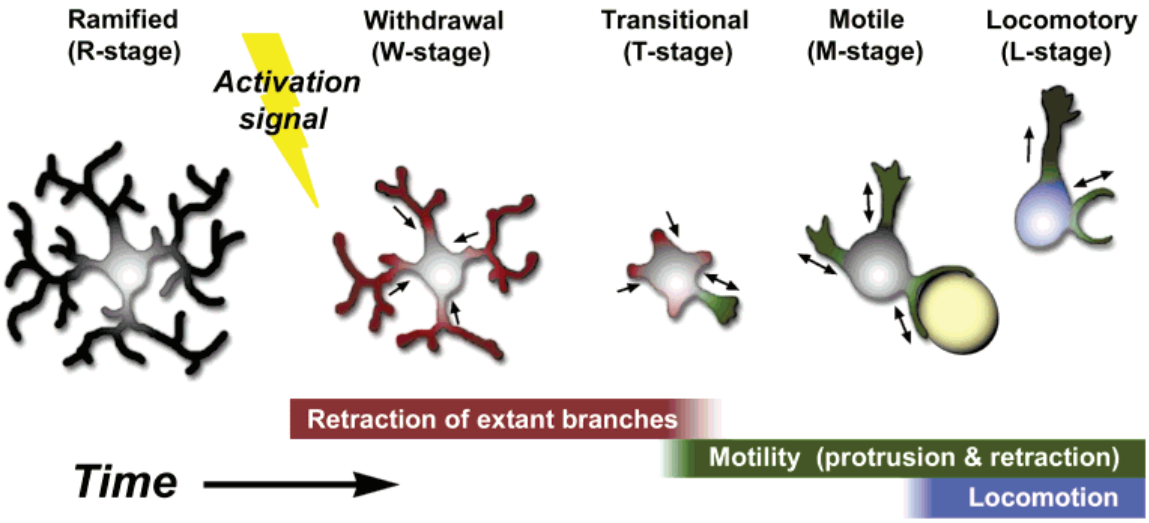

Figure 1.2. Multistep model of microglial activation dynamics. (Stence *et al.* 2001)

New processes were much more dynamic than the old ones: growth and retraction rate was three- to fourfold faster than the rate of retraction of the pre-existing branches. Cells then begin to move through the tissue, entering the last, so-called 'locomotory stage' (L-stage). Moving cells typically had a polarized morphology with one prominent leading process, and moved through the tissue at up to 118 µm/h. While moving, cells extended short, fleeting processes tangential to the primary direction of movement, which enabled direct physical contact with neighbour cells, establishing a support for tissue surveillance function (Stence *et al.* 2001).

Frequently, the expression levels of some of the aforementioned characteristic microglial/macrophage molecules are affected by the activation state of the cells, with an induced activation resulting in increased expression levels of CD11b, Iba-1 or MHC class II molecules, for example (Kreutzberg 1996; Ito *et al.* 2001; O'Keefe *et al.* 2002). Indeed, activated microglia express and release a variety of factors in order to control and minimize the impact of CNS injuries. Some of them are anti-inflammatory or neurotrophic, released

to promote cell survival and tissue recovery after injury. Others are inherently pro-inflammatory, ensuring restriction in the spread of the injury and rapid elimination of any possible insulting agent, but may possibly induce damage to the CNS by themselves (Czeh *et al.* 2011). Amongst the factors released by activated microglia are interleukin-1 beta (IL-1β), tumour necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), monocyte chemotactic protein-1 (MCP-1), neuronal growth factor (NGF) and brain-derived neurotrophic factor (BDNF), only to mention a few (Fig. 1.3.) (Garden & Möller 2006). These can further act upon microglial cells themselves, in autocrine or paracrine loops. The answer to whether the outcome of such feedback mechanisms is bad or good may lie in the balance between them (Pocock & Liddle 2001).

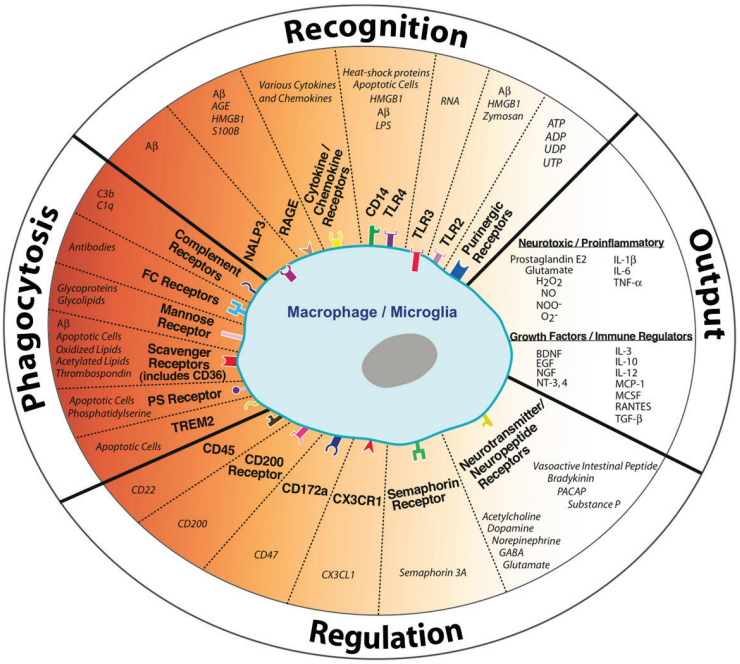

Figure 1.3. Receptors and effector molecules expressed by microglia. (Lucin & Wyss-Coray 2009).

1.2.2. *In vitro*

It is important to note that microglial cells cultured *in vitro* do not display the highly ramified structure that is typically observed in the normal, healthy CNS. Instead, they show heterogeneous shapes. Cells adopt a much less ramified, bipolar morphology, or amoeboid versions with short thick processes expanding as lamellipodia, depending on culture times and states of activation (Suzumura *et al.* 1991). Commonly, untreated cultured microglial cells exhibit a rounded, amoeboid morphology, and seem to be at least partially activated (Eder *et al.* 1999). Also, *ex vivo* microglial cells analysed immediately upon isolation

resemble the reactive amoeboid microglia normally found in inflamed or wounded CNS (Becher & Antel 1996). This activation seems to wane with time and passage of the cells, with an approximation to the typical "resting" in vivo morphology (Lauro *et al.* 1995), and it has also been reduced through the use of retinoic acid (Giulian & Baker 1986).

1.2.2.1. Organotypical cultures

Organotypic hippocampal slice cultures (OHSCs) are known to preserve physiological and morphological conditions of the *in vivo* situation regarding neuronal and some non-neuronal cells (Noraberg *et al.* 2005). However, explantation and *in vitro* cultivation of hippocampal slices were accompanied by a massive microglial reaction, taking place on slice surfaces and, to a lesser extent, inside the intermediate layers (Fig. 1.4.) (Hailer *et al.* 1996). This activation correlated with the distribution of dead or injured neuronal cells, either spontaneous or induced (Heppner *et al.* 1998). Since loss of neurons in OHSCs predominantly occurs right after explantation, significantly lower rate of spontaneous neuronal degeneration is reached after a few days *in vitro*, which enables microglial cells to gradually return to a 'resting' state (Hailer *et al.* 1996). Microglia in slice cultures respond to stimulation and retain their functional capabilities for producing superoxide anions or to engage in phagocytic activities (Czapiga & Colton 1999), and as such this model system can be useful to examine the interactions of microglia with neurons and other glia in the normal and injured brain. Of course, this comes at a high cost in both biological material as well as time, and alternative models to study microglial form and function have been optimized.

1.2.2.2. Primary cultures

Primary microglia cultures are most often derived from the cortex of a rat or mouse before or early after birth, generally through a protocol described by Giulian and Baker (Giulian & Baker 1986). The advantages of using amoeboid microglia directly from an animal are related to the functional characteristics that these cells are endowed with, such as secretory products, cell surface markers and phagocytic activity, which closely resemble those of cells in endogenous environment (Lauro *et al.* 1995). Indeed, morphological reorganization of

this isolated microglia can still be imposed by treatment with typical activating agents, such as bacterial lipopolysaccharide (LPS) (Abd-El-Basset & Fedoroff 1995), as can the release of cytokines and/or other factors related with activation (Colton & Gilbert 1987; Sebire *et al.* 1993; Romero *et al.* 1996).

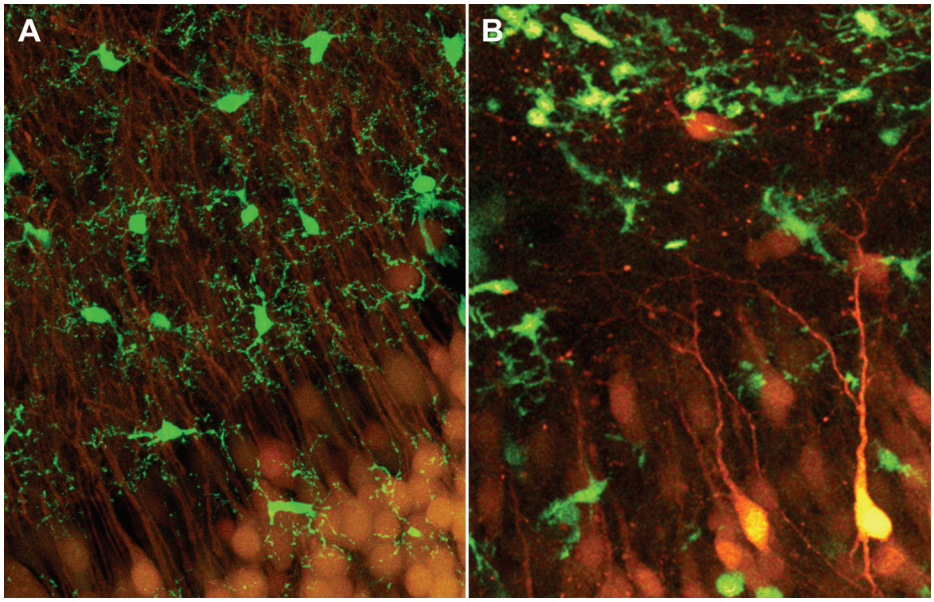

Figure 1.4. Classical "resting" and "activated" microglial phenotypes. (A) "Resting", green fluorescent protein-expressing microglial cells ($CX_3CR1^{GFP/+}$, green) and yellow fluorescent protein-expressing neurons (Thy1-YFP, orange) in a P12 mouse hippocampus. Notice the thin, ramified projections of microglia. (B) The same cells in an *in vitro* hippocampal slice culture. Notice how the projections in microglial cells are scarce as compared to the "resting" state, the result of the activation and subsequent phenotypical shift in microglia in a slice culture (Stence *et al.* 2001). Images from the laboratory of Michael E. Dailey (Dailey 2010).

Maintaining primary microglia in highly purified cultures turned out a great resource to study their behaviour and functions, but the fact that the yield of these cells *per* animal is low means that many animals are required to obtain a sufficient number of microglia (Imai *et al.* 2007). This makes this model more time consuming and perhaps less attractive as compared to other culture systems.

1.2.2.3. Microglial cell lines

Options were investigated to overcome the low-yield and time-consuming culture techniques presented thus far, in order to rapidly obtain large and homogeneous populations of microglial cells. Generating immortalized cell lines by infecting the cells with a retrovirus (Cepko 1989; Briers *et al.* 1994) offers a solution to this problem.

Two commonly used cell lines of this type are the BV2 and N9 microglia cell lines which

are derived from rat and mouse, respectively ((Righi *et al.* 1989; Blasi *et al.* 1990), reviewed in (Stansley *et al.* 2012)). Both cell lines were assessed for typical microglia cell markers and tested positive for FcγRs, CD11b, F4/80 and nonspecific esterase activity, while they were negative for glial fibrillar acidic protein (GFAP) and galactocerebroside (markers for astrocytes and oligodendrocytes, respectively) (Righi *et al.* 1989; Blasi *et al.* 1990). Apart from structural markers, these cells exhibit functional phenotypes of microglia as well, with the induction of cytokine release, migration and phagocytosis in the presence of endotoxins such as LPS (Horvath *et al.* 2008; Ferreira *et al.* 2011; Ferreira *et al.* 2012a).

These immortalized cell lines have been used to demonstrate the ability of microglia to change state and assume different roles in pathologies of the CNS. Despite the similarity to primary microglia, immortalization through transfection with oncogenes renders them in some ways different from primary microglia, in terms of variance of morphology or proliferation and adhesion (Horvath *et al.* 2008). But the fact that these cells have similar morphology and behaviour responses, and are more rapidly available and low-maintenance when compared with alternative culture systems, makes them valuable cell models to help answer very specific inflammatory research questions.

1.3. Microglial activation

Triggering microglial activation can be effected by an array of molecules, ranging from structural components of microorganisms (pathogen-associated molecular patterns – PAMPs), to abnormal endogenous proteins or signs of tissue damage (damage-associated molecular patterns – DAMPs), cytokines, chemokines, antibodies, and others (reviewed in (Hanisch & Kettenmann 2007)). To be able to discriminate all of these (and more) signals, microglial cells are equipped with an enormous variety of membrane receptors which are involved in different aspects of the microglial response (Fig. 1.3.) (Raivich *et al.* 1999; Nimmerjahn *et al.* 2005). Naturally, pattern-recognition receptors (PRRs), which recognize PAMPs and DAMPs, are among them (Ransohoff & Brown 2012). The most notorious and well-studied family of PRRs is the Toll-like receptor (TLR) family, which recognizes conserved structures in pathogens (Kawai & Akira 2010; Lehnardt 2010). A total of 13 TLRs have been described so far. However, TLR10 is not functional in mice, while TLRs 11 to 13 are not present in the human genome (Kawai & Akira 2010). TLRs are activated by

bacterial LPS, hypomethylated DNA, flagellin or double-stranded RNAs, for example. TLRs activation generally initiates signalling cascades that involve the recruitment of myeloid differentiation primary response 88 (MyD88) and generally result in mediation of transcription by nuclear factor-kappa B (NF-κB) (Fig. 1.5.) (Hanamsagar *et al.* 2012).

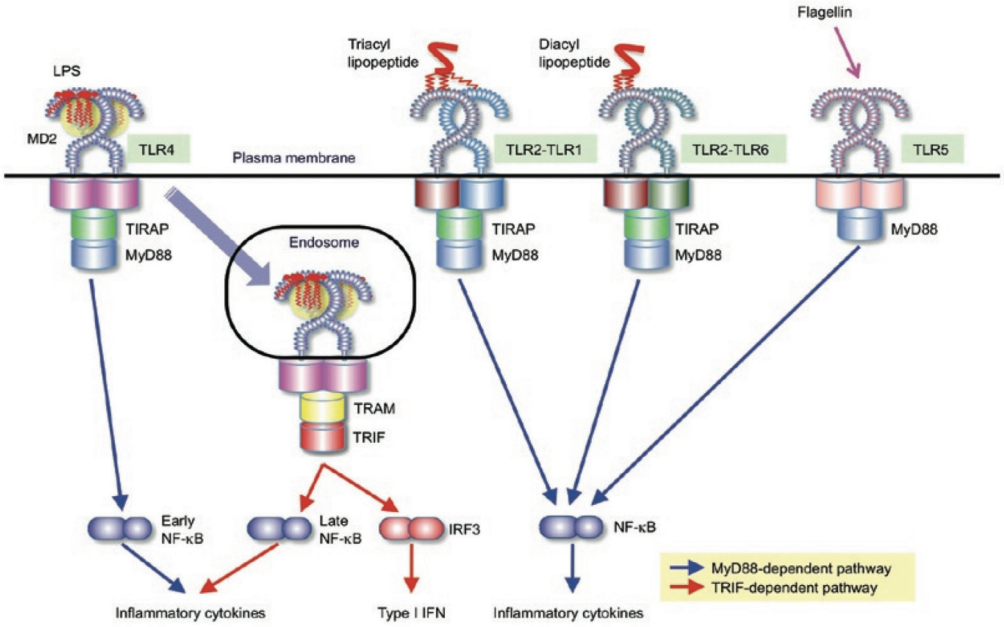

Figure 1.5. PAMP recognition by TLRs and subsequently activated signalling cascades. (Kawai & Akira 2010).

In addition, microglial activity can also be controlled by signals from neurons themselves. Neurons have classically been placed on the 'receiving' end of microglia's actions, either endangered or protected by microglial activation (Butovsky *et al.* 2005). But it is now established that this is not the case. Neurons express their own set of agents to act upon microglia, which can either elicit activation or "quiescence": an 'on and off' system (Biber *et al.* 2007). 'Off' signals are constitutively expressed in the brain, and loss of 'off' signalling is enough to drive microglia into a different functional state. Several molecules have been identified as "off" signals, namely CD200 and fractalkine (Fkn, CX_3CL1) constitutively expressed by neurons, and most neurotransmitters (except for glutamate) keep microglia in a non-activated state. On the other hand, "on" signals like purines, glutamate or matrix metalloproteinases can elicit microglial activation (Biber *et al.* 2007). Such a system is designed to tightly control microglial activity in a healthy CNS (van Rossum & Hanisch 2004).

Of course, not all activation signals bring about the same result in microglia (Butovsky *et al.* 2005). Depending on the state of the brain prior to the stimulus, the stimulus itself and

subsequent secondary events, microglia can adopt one from a spectrum of phenotypes that might not be readily distinguishable through morphological observation only (Perry *et al.* 2010). Indeed, microglial activation has been associated with either neurodegeneration or neuroprotection, in a sort of Dr. Jekyll and Mr. Hyde duality (Czeh *et al.* 2011). Answering the question of whether microglial activation is detrimental or neuroprotective is not trivial as the line separating neuroprotective from neurotoxic responses may be very thin.

1.4. Mechanisms of microglial migration

Moving microglial cells follow the same basic mechanisms of migration common to various other cell types (fibroblasts, neuroblasts, leukocytes, etc.), in a tightly regulated process driven by dynamic cellular cytoskeletons (reviewed in (Ridley *et al.* 2003)). Migration is a multistep, cyclic event, in which cells acquire a spatial asymmetry, enabling them to turn intracellularly generated forces into net cell body translocation. Typically, cells become polarized within the plane of movement, upon detection of a migration-promoting agent, extend membrane protrusions in the sought direction, and finally retract the cell body in the direction of the extension (Schwab 2001). Front and rear end can be easily distinguished. The former is a flat, organelle-free process, the so-called lamellipodium, while the latter is formed by the prominent cell body that extends into a uropod (Fig 1.6.) (Schwab 2001).

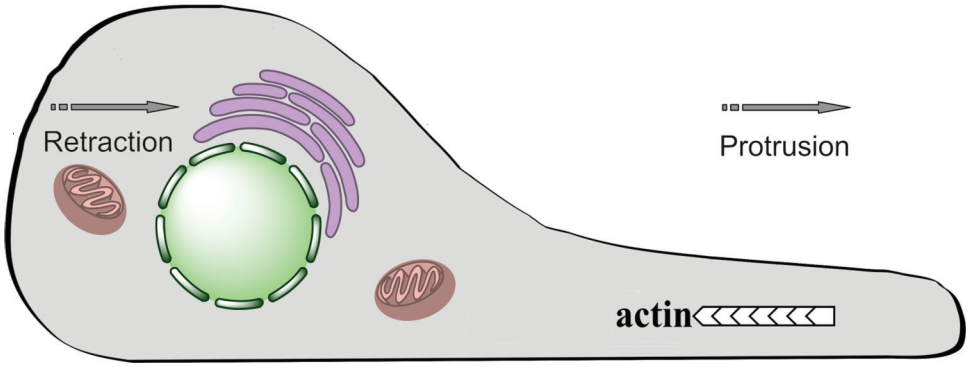

Figure 1.6. Spatial asymmetry during microglial migration. Lamellipodium (right, thin) protrudes into the direction of movement while the uropod (left, thick) retracts into the direction of movement. Adapted from(Kettenmann *et al.* 2011)

Composed by a dense meshwork of actin filaments that project from the cell surface, the lamellipodium is driven by actin polymerization that occurs in the direction of locomotion, at the leading edge of the cell. It is stabilized by the establishment of focal complexes with

the extracellular matrix (ECM) or adjacent cells via transmembrane receptors linked to the actin cytoskeleton (Ridley *et al.* 2003), thus providing the traction force necessary for the cell to move. But for migrating cells to form new contacts at its front, old contacts must be released at the rear, which implies that the interaction between extracellular matrix and cell adhesion receptors has to be a highly coordinated process (Schwab 2001). Actin-binding proteins play major roles in this process. (Ridley *et al.* 2003).

The rates at which cells can migrate depend on cell type as well as on the communication with the ECM or substrate. When cells cannot form contacts with the matrix (too little friction), locomotion is impaired. Similarly, locomotion is also impaired when the matrix is too "sticky" and cells cannot release their focal complexes (Schwab 2001). The formation of these complexes requires integrin clustering, a process activated by talin and mediated by PKC, Rap1 and PI3K signalling pathways (Ridley *et al.* 2003), while disassembly requires Rho kinase and is a myosin-dependent process. Also, the rise of intra-cellular Ca^{2+} levels have been implicated in the disassembly of adhesions at the rear, through Ca^{2+}-activated protease calpain, which has the potential to cleave several focal adhesion proteins (Ridley *et al.* 2003).

Migration by chemotaxis depends on the establishment of membrane protrusions following a chemotactic gradient, mediated by membrane receptors of the chemotactic agents, which activate signal transduction pathways and mould the cytoskeleton locally in the direction of the gradient (Van Haastert & Devreotes 2004). In a uniform concentration of chemoattractant, cells move in a persistent but random direction. Differences as small as 2–10% in the concentration of chemoattractants between the front and the back of the cell are enough to elicit a directed migration, whether the average level of receptor occupancy is small or large (Van Haastert & Devreotes 2004). Microglia have been shown to migrate in the presence of ATP released from damaged cells, glutamate, (endo)cannabinoids, microbial signals, various growth factors, β–amyloid, various cytokines and chemokines, among others (Kettenmann *et al.* 2011).

1.5. Microglial phagocytosis

Microglial cells are the resident professional phagocytes of the CNS, critical in its

protection against damage in pathology, but equally important during brain development. Microglial cells actively scan tissue under both normal and pathologic contexts. They play a specific role in removing apoptotic cells, but are also considered to be involved in synapse removal during development (Pont-Lezica *et al.* 2011). Phagocytosis is a key function for microglial cells, and as such, microglia display a wide range of receptors that mount a concerted response leading to phagocytosis and removal of cell debris (Walter & Neumann 2009).

Phagocytosis is the process by which the phagocyte surrounds the particle or cell to be engulfed with extensions of its plasma membrane; this process results in the formation of an intracellular vesicle called a phagosome, which contains the ingested particle (Abbas *et al.* 2012). Clearance of the phagocyted particles is achieved by fusing phagosomes with lysosomes, which contain several hydrolytic enzymes, thus digesting the contents of the new phagolysosome. Remnants are later exocyted out of the cells. Recognition of pathogens or cell damage-related antigens activates phagocytes, which migrate to the lesion site, release a wide range of soluble factors that include cytotoxins, neurotrophins and/or immunomodulatory factors and clear cellular debris by phagocytosis (Neumann *et al.* 2009). Phagocytosis is a highly regulated process that requires high cytoskeleton dynamics. Phagocytic processes are driven by a controlled rearrangement of the actin cytoskeleton, in which actin fibers are continually assembled and disassembled in response to the local activity of signalling transduction systems. Networks of highly branched filaments are created, generating the forces involved in changing cell shape (Fletcher & Mullins 2010).

Microglia, as well as other phagocytes, express more than one kind of phagocytic receptor. There are two distinct functional types of phagocytic receptors related to two different types of phagocytic behaviour. Stimulation of TLRs and phagocytosis of pathogens induce a pro-inflammatory cascade, while recognition of phosphatidylserine (PS) residues and clearance of apoptotic cell membranes induces an anti-inflammatory response (Ravichandran 2003). Removal of apoptotic material without inflammation is an essential and beneficial function of microglia. (Fig. 1.7.).

Broadly, phagocytosis can occur in an opsonin-dependent or –independent manner (Choucair *et al.* 2006). Opsonin-dependent receptors, such as FcγRs and complement binding receptors, depend on the previous signalization of the phagocytic target by soluble

receptors (*opsonins*) for a proper phagocytic induction. Opsonisation is achieved through the binding of immunoglobulins (in the case of FcγRs), or complement components (in the case of complement receptors) to the surface of the pathogen or apoptotic structure targeted for phagocytosis. Opsonin-independent phagocytosis occurs for example through scavenger receptors, which can recognize a variety of ligands, namely acetylated or oxidised low-density lipoprotein, bacterial endotoxins such as lipoteichoic acid or LPS, or fibrillar β-amyloid (Choucair *et al.* 2006). Upon ligand binding, scavenger receptors cluster on the membrane surface and induce rearrangement of the actin cytoskeleton, thereby inducing phagocytosis. The family of Rho guanosine triphosphatases (GTPases), which includes Cdc42, Rac, and Rho, link membrane receptors to the actin cytoskeleton, mediating phagocytosis (Choucair *et al.* 2006).

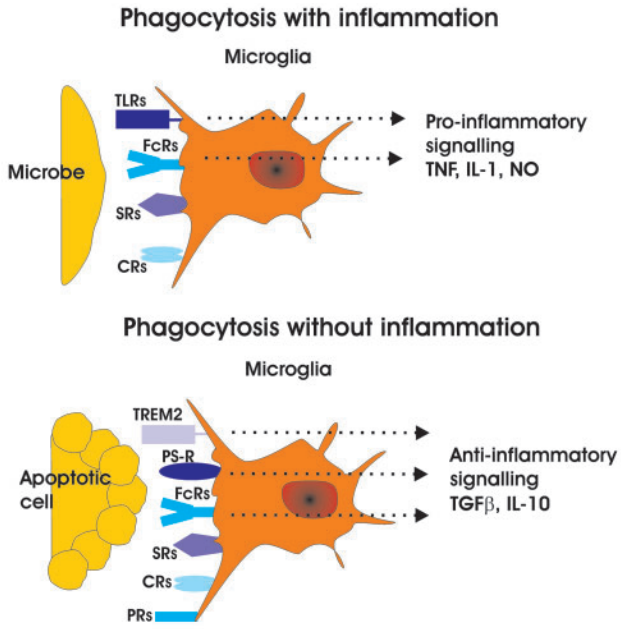

Figure 1.7. Microglial phagocytic receptors. (Neumann *et al.* 2009)

1.6. LPS induced activation of microglia

The activation of TLR4 via LPS is perhaps the most used model to study microglial activation. LPS is a component of the outer cell membrane of Gram-negative bacteria, and it is by itself able to trigger chemical and functional responses in microglia, such as the release of cytokines, the induction of cytoskeleton reorganization, as well as cellular migration (Lee *et al.* 1993; Abd-El-Basset & Fedoroff 1995). This endotoxin has been demonstrated to activate microglia to such extent as to cause neurodegeneration in vitro and in vivo, without being neurotoxic by itself (Block *et al.* 2007). For these reasons, LPS

has become a gold standard to study the activation of microglia (Rivest 2003).

LPS binding to TLR4 leads to the recruitment of MyD88, and consequently the activation of the downstream c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinases (MAPK) signalling pathways. Also, signalling through TLR4 with LPS recruits NF-κB inhibitor kinase (IκK), which phosphorylates the NF-κB inhibitor (IκB). This results in the release of NF-κB, and its translocation into the nucleus (Kawai & Akira 2010). Both of these signalling cascades result in the transcription of pro-inflammatory regulators, leading to the activation profile exhibited by microglia in the presence of LPS: enhanced pro-inflammatory cytokine production, cytoskeleton reorganization and enhanced mobility.

LPS was shown to stimulate the release of IL-1β in N9 cells, which could in turn increase the expression of iNOS and thus the production of NO in an autocrine / paracrine fashion (Ferreira *et al.* 2010). The same LPS / IL-1β signalling was also shown to mediate the increase in phagocytic activity in these cells, through activation of the p38 MAPK pathway; inhibition of this pathway as well as of IL-1β signalling reduced the observed phagocytic behaviour (Ferreira *et al.* 2011). Finally, this same signalling cascade has also been involved in the increase in cell motility and in the reorganization of the cytoskeleton in N9 microglia stimulated with LPS (Ferreira *et al.* 2012a).

1.7. Fractalkine-mediated regulation of microglial activity

The main objective of this study is to evaluate the modulatory effect of the chemokine fractalkine (Fkn) on microglial activation elicited by the endotoxin LPS. Fkn, as well as its G protein-coupled receptor (GPCR) CX_3CR1, are constitutively expressed in various non-hematopoietic tissues, including the brain (Heinisch & Kirby 2009). It is the only member of the CX_3C family (with cysteines separated by three amino acids) and can exist as either a membrane-anchored protein or a soluble glycoprotein (Kettenmann *et al.* 2011). Also differing from other chemokines, which can interact with multiple GPCRs, Fkn has only one cognate receptor, CX_3CR1 (Imai *et al.* 1997). Furthermore, the two structural forms of Fkn, membrane-bound and soluble, enable it to act as an adhesion molecule as well as a chemoattractant (Bazan *et al.* 1997).

What really makes this chemokine/receptor pair almost unique in the brain is their role in

the neuron/microglia communication. In the CNS, neurons are the primary source of Fkn, while CX_3CR1 has been almost exclusively detected on microglial cells (Berangere Re & Przedborski 2006; Cardona *et al.* 2006). Chemokines were mostly regarded as glia-expressed molecules in the CNS, but their expression in neurons and the expression of the respective receptors in microglia can contribute to neuron-microglia interactions, both under physiological and pathological conditions (reviewed in (de Haas *et al.* 2007)). The involvement of the Fkn–CX_3CR1 axis in fundamental processes of communication between neurons and microglia has been proposed a long time ago (Pan *et al.* 1997), given the expression pattern of the chemokine and the receptor.

Signalling differences were immediately noticeable between soluble and membrane-bound forms of Fkn, as both forms were able to elicit intracellular Ca^{2+} mobilization, yet this potential was greater for membrane-anchored Fkn, as compared to soluble forms. This implied a differential regulation of intracellular levels of calcium within microglia by neurons (Harrison *et al.* 1998). Shedding of membrane-anchored Fkn is regulated by proteases from A Disintegrin And Metaloprotease (ADAM) family, namely ADAM-10 and ADAM-17 (Garton *et al.* 2001; Tsou *et al.* 2001; Hundhausen *et al.* 2003). The former is responsible for the constitutive cleavage of Fkn, while ADAM-17 has been shown to mediate inducible cleavage of Fkn from the cell membrane. Cleavage of Fkn from cultured neurons is rapidly induced in response to an excitotoxic stimulus, preceding neuronal death (Chapman *et al.* 2000). Inducible shedding by neurons and differences in signalling activity on microglia might suggest that Fkn differentially regulates microglial activity in the CNS, depending on the existing homeostatic balance. Indeed, Fkn was shown to modulate signs of microglial activation elicited through stimulation with LPS. Microglia increase production of pro-inflammatory cytokines, such as TNF-α, IL-1β or IL-6, and reactive oxygen species (ROS), such as NO, in the presence of this endotoxin. Release of all of these factors was dose-dependently reduced by the stimulation with Fkn (Zujovic *et al.* 2000; Mizuno *et al.* 2003). Notably, Fkn binding to CX_3CR1 reduces proinflammatory cytokine expression through the induction of an increase in Akt phosphorylation, leading to the activation of the downstream PI3K pathway (Meucci *et al.* 2000; Lyons *et al.* 2009). Soluble and membrane-bound forms of Fkn might, nevertheless, exert slightly different effects on these intracellular signalling mechanisms (Berangere Re & Przedborski 2006). Signalling through CX_3CR1 plays a role in shifting or diminishing microglial activation,

thus making it an excellent target to try and damper neurodegeneration caused by reactive microglia (Fig. 1.8.).

Despite of seemingly reducing microglial activation, Fkn has been shown to induce cell migration in leukocytes or monocyte-derived cells (Imai *et al.* 1997; Ruitenberg *et al.* 2008). In microglia, Fkn induces actin cytoskeleton reorganization and cell migration, in a mechanism dependent on the increase in intracellular Ca^{2+} mobilization and MAPK phosphorylation (Maciejewski-Lenoir *et al.* 1999). While increases in mobility of microglia have been correlated with activation, Fkn apparently shifts microglia to a state where cells reduce pro-inflammatory signalling but exhibit enhanced mobility.

Phagocytic behaviour of microglia is also affected by stimulation with Fkn. In the presence of this chemokine, microglia up-regulate the expression of milk-fat globule EGF factor-8 (MFG-E8), a tethering molecule that recognizes PS exposed on the surface of apoptotic cells (Miksa *et al.* 2007). Opsonizing MFG-E8 then serves as a bridging adaptor, attaching to integrin receptors on the surface of microglia and eliciting phagocytosis (Akakura *et al.* 2004; Fuller & Van Eldik 2008). In a model of excitotoxicity caused by glutamate, the release of Fkn from neurons induced an increase in the expression of MFG-E8 by microglia, leading to enhanced phagocytic activity (Noda *et al.* 2011).

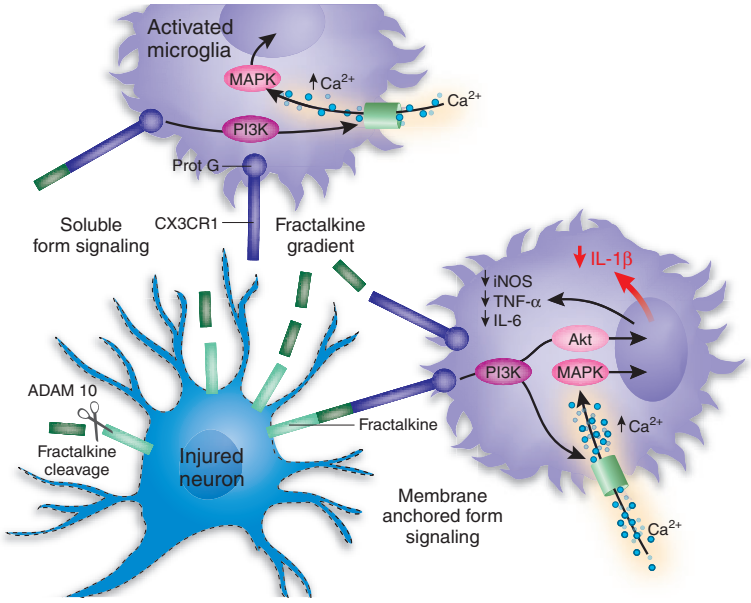

Figure 1.7. Fkn mediated neuron-microglia crosstalk. Fkn activates the PI3K pathway, leading to a decrease in the release of cytokines and ROS associated to inflammation. (Berangere Re & Przedborski 2006)

In vivo, deficient signalling through the Fkn/CX_3CR1 system due to a knock-out of CX_3CR1 deregulated microglial responses and aggravated inflammation in an 1-methyl-4-

phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced Parkinson's model, resulting in neurotoxicity (Cardona *et al.* 2006). The same study also revealed increased microgliosis in CX_3CR1-ko mice challenged with systemic inflammation induced by intraperitoneal injections of LPS. Moreover, CX3CR1-deficient mice also show deficits in the resolution of microglial activation after an LPS injection, when compared with heterozygote mice (Corona *et al.* 2010). In a model of Alzheimer's, CX_3CR1 deficiency was shown to worsen neuronal and behavioural deficits by enhancing Tau pathology and calbindin depletion, resulting in an increase in TNF-α and IL-6 expression by over-activated microglia (Cho *et al.* 2011). These reports suggest that Fkn is of great importance in the control of microglial activity in the case of altered brain homeostasis.

The influence of Fkn on microglia extends beyond the regulation of these defence mechanisms. Microglia can easily be located in neurogenic niches, and have thus become interesting candidates for modulating neurogenesis in both the healthy and injured brains (reviewed in (Ekdahl 2012)). Signalling through CX_3CR1 reportedly modulates hippocampal neurogenesis by regulating the levels of IL-1β. Disruption of the Fkn/CX3CR1 axis diminishes proliferation of neural progenitor cells in young rodents, while the age-related decrease in hippocampal neurogenesis due to lower levels of Fkn in old rats could be restored by the delivery of exogenous Fkn (Bachstetter *et al.* 2011).

Considering the previously exposed considerations, we decided to study the modulatory effects of Fkn on microglia, using the cell line N9 as a biological model. Our hypothesis is that soluble Fkn is able to change the pro-inflammatory profile elicited by LPS in N9 microglia ((Ferreira *et al.* 2010; Ferreira *et al.* 2011; Ferreira *et al.* 2012a; Ferreira *et al.* 2012b)) to a less aggressive one, but maintaining increased migratory and phagocytic activities. Therefore, and given the evidence that support the modulatory role of Fkn, we aim to analyse the effect of this chemokine in N9 microglial cells under basal and LPS-stimulated conditions in classical signs of microglial activation, such as:

- Cytokine expression;
- Morphological characteristics;
- Phagocytic activity;
- Migratory ability.

Through this study, we set out to understand whether and how Fkn could be used to down-

regulate negative aspects of microglial activation, which induce or exacerbate neurodegeneration, in order to establish a working model where Fkn can be used to protect CNS from over-activated microglia.

Chapter II - Methodology

2.1. Cell line culture

Murine N9 microglia cell line (kind gift from Prof. Claudia Verderio, CNR Institute of Neuroscience, Cellular and Molecular Pharmacology, Milan, Italy) was maintained in Roswell Park Memorial Institute (RPMI) 1640 medium with GlutaMAX™ (#61870-010 gibco®, Life Technologies, Carlsbad, California, USA), supplemented with 5% heat-inactivated foetal bovine serum (#10106-169, gibco®), D-Glucose to a final concentration of 30 mM (#G7021, Sigma-Aldrich, St. Louis, Missouri, USA), and penicillin-streptomycin (P/S) (100U/ml and 100µg/ml, respectively, #15140-122, gibco®). Cells were grown at 37°C in a 95% atmospheric air and 5% CO_2 humidified atmosphere. For immunocytochemistry studies, cells were plated at a density of 1×10^4 cells *per* cover slip (Ø=10mm). In remaining experiments, cells were plated at a density of 2×10^5 cells *per* well in 12-well trays or 5×10^5 cells *per* 6-well trays. The number of viable cells was evaluated by counting trypan blue-excluding cells.

Prior to treatment incubations, cell medium was changed from the above to Dulbecco's Modified Eagle Medium:Nutrient Mixture F-12 (DMEM/F-12) with GlutaMAX™ (#31331-028, gibco®), supplemented with B-27® serum-free supplement (#17504-044, gibco®, 1:100), P/S (100U/ml and 100µg/ml, respectively, #15140-122, gibco®), natural mouse epidermal growth factor (EGF, 5µg/ml, #53003-018, Invitrogen™®, Life Technologies, Carlsbad, California, USA) and recombinant human fibroblast growth factor basic (bFGF, 2,5µg/ml, #13256-029, Invitrogen™). Cells were maintained in DMEM/F-12 for 4 hrs prior to the beginning of any experimental protocol to minimize unspecific stress. Cell treatment included the following incubation setups: LPS from *Escherichia coli* (055:B5, 100 ng/ml, #L2880, Sigma-Aldrich), Fkn chemokine domain (200ng/ml, #F2303, Sigma-Aldrich), or a combination of the aforementioned for 4 hrs in the case of RNA isolation, 6 hrs for western blotting, immunocytochemistry analysis and phagocytosis assays or 12 hrs for the motility assay.

2.2. RNA isolation from N9 microglial cells

Total RNA was isolated with Aurum™ Total RNA Mini Kit (#732-6820, Bio-Rad, Hercules, California, USA), using an adapted protocol based on manufacturer's instructions. Briefly,

cells were lysed with the provided lysis buffer supplemented with 1% β-mercaptoethanol (#63689, Sigma-Aldrich) by thoroughly scraping the wells. The lysate was frozen and stored at -20ºC. After thawing, ethanol was added to the sample tubes, followed by vortexing and up-and-down pipetting to ensure proper mixing. Homogenized lysates were then transferred to RNA binding columns and centrifuged for 1 min at $1{,}48\times10^4$g, the speed used for all centrifugations in this protocol. Samples were washed through centrifugation with low stringency wash solution from the kit, followed by a 15 min incubation with 5 µl of provided DNAse I, diluted with 75 µl of DNAse dilution solution, in order to remove genomic DNA contamination. Further purification and removal of organic contaminants was achieved through two centrifugation steps, with provided high stringency and low stringency wash solutions, respectively. Purified RNA samples were obtained through centrifugation for 2 min with the elution solution from the kit. Diluted RNA was stored at -80ºC prior to quantification and purity analysis for further use. Quantification was performed by optical density (OD) measurement at 260 nm, whereas sample purity was analysed using the ratios OD_{260}/OD_{280} and OD_{260}/OD_{230} (only samples whose ratios were between 1.9 and 2.1 were transcribed), using a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

2.3. Reverse transcription (RT) and quantitative polymerase chain reaction (qPCR)

The extracted RNA (400 ng) of was reverse transcribed on a UnoCycler (VWR International, Radnor, Pennsylvania, USA) using iScript™ cDNA Synthesis Kit (#170-8890, Bio-Rad,) following manufacturer's instructions. In short, reaction mix containing oligo(dT)s, random hexamers, dNTPs and a modified Moloney-murine leukaemia virus reverse transcriptase (M-MLV RT) pre-blended with RNAse inhibitor were added to the extracted RNA, as well as nuclease-free water to top up the volume to 20 µl. Some samples were submitted to the same protocol without reverse transcriptase (RT- sample) to be used as non-template negative controls for qPCR. Complete reaction mixes were incubated for 5 min at 25ºC for annealing, followed by 30 min at 42ºC for extension and 5 min at 85ºC for melting. Generated cDNAs were stored at -20ºC until further use.

qPCR analysis of the reverse transcription products was performed on a iQ™5 Multicolor

Real-Time PCR Detection System (Bio-Rad) using iQ™ SYBR® Green supermix reaction kit (#170-8882, Bio-Rad) following the manufacturer's protocol. Briefly, amplification reactions were performed in 10 µl reaction medium containing 2.5 µl of template cDNA, 5 µl of 2x iQ™ SYBR® Green supermix (50U/ml iTaq™ DNA polymerase, 0,4 mM deoxynucleotide mix and 6 mM $MgCl_2$), forward (F) and reverse (R) primers (Table 1) and nuclease-free water to complete the reaction volume. Some wells contained nuclease-free water or RT- samples instead of cDNA as non-template samples. These non-template controls served as negative controls to detect possible contaminations and/or the amplification of genomic DNA.

The primers used for qPCR analysis were designed using the open-source software tool PerlPrimer (Marshall 2004) (v. 1.1.20), except for the primers for reference genes. Primer pairs were designed to be exclusively complementary to the cDNA sequence of the individual detection target transcripts. The primers were selected according to the following criteria: a) melting temperature between 58 and 62ºC, with a maximum difference of 2ºC between forward and reverse primers; b) no more than 3 identical nucleotides in a row; c) GC content about 40-60%; d) a GC clamp in the 3' end (at least 2 of the last 3 bases should be G or C); e) span intron-exon boundary and overlap the boundary by 7 bps; f) final product length between 50-200 bps and g) primer length between 18-24 bps.

Table 1 – Primer sequences (F and R) used for qPCR analysis.

Gene	Tm (ºC)	Amplicon size (bps)	Primer sequence (5'-3')
IL-1β	**F** - 60 **R** - 59	125	**F** - ATT AGA CAA CTG CAC TAC AGG **R** - CAT GGA GAA TAT CAC TTG TTG G
TNF-α	**F** - 58 **R** - 58	252	**F** - CTG TAG CCC ACG TCG TAG CA **R** - CGG CAG AGA GGA GGT TGA CT
CX_3CR_1	**F** - 61 **R** - 60	100	**F** - ATC TGC TCA GGA CCT CAC **R** - CAA TGT CGC CCA AAT AAC AG
MFG-E8	**F** - 62 **R** - 63	71	**F** - GAG TTG CAC GGA TGT TCT GAG **R** - GGA GGC TGA CAT CTG GCT G
HPRT1	N/A	168	N/A
GAPDH	N/A	144	N/A

The specificity of the sequence of the designed primers was checked using BLAST and was further investigated for the formation of primer-dimers and secondary structures (e.g.

hairpins). Targeted amplicons were checked for the presence of single-nucleotide polymorphisms with the online software tool RTPrimerDB (Pattyn *et al.* 2006; Lefever *et al.* 2009). Hypoxanthine-guanine phosphoribosyltransferase 1 (HPRT1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as reference genes, and their respective primer sets were obtained from selected QuantiTect Primer Assays (#QT00166768 and #QT01658692, respectively, Qiagen, Austin, Texas, USA). qPCR protocol comprised two phases, an initial template denaturation/enzyme activation step at 95°C for 3 min, followed by 40 cycles of denaturation (95°C, 10 s) and annealing (60°C, 30 s) steps. Fluorescence was determined at the end of the annealing step. Finally, melting curves were obtained for all samples, between 55 and 95°C, with a temperature increase of 0.5°C every 10 s, with continuous fluorescence measurement. All reactions were performed in duplicate for each of the samples. Primer concentrations and qPCR conditions were optimized to obtain the minimum non-specific amplification for all primers. The specificity of amplification for each primer pair was confirmed by determining that the melting curve showed a single dissociation peak, corresponding to a single amplification product. Furthermore, the determined melting temperature of the amplified product should lie in the vicinity of the predicted melting temperature for the selected amplicon.

2.4. qPCR analysis

Real-time qPCR results were analysed with Relative Expression Software Tool - Multiple Condition Solver (REST-MCS©) version 2, which is based on the mathematical model proposed by Pfaffl (Pfaffl, 2001). Like other qPCR analysis methods, such as the "double-delta C_t" method, this model takes into account the differences of variation of threshold cycle (C_t) values between one (or more) reference genes and genes of interest, in control (baseline) versus experimental (treated) samples. Importantly, this method further takes into account the mean amplification efficiencies of the qPCR reactions for the selected amplicon/primer pair as well, in order to calculate the fold variation in expression ratios of genes of interest as compared to reference genes. By factoring in the amplification efficiency instead of assuming that the theoretical 100% efficiency holds true, this method yields more consistent results than comparative C_t or double-delta C_t methods. Amplification efficiencies for each amplicon and C_t values for each particular reaction were

determined with LinRegPCR (Ramakers *et al.*, 2003; Ruijter *et al.*, 2009). LinRegPCR uses an unbiased linear regression algorithm to analyse the raw (i.e. non-baseline corrected) data generated with the thermal cycler for each sample and to determine optimal baselines, C_t values and estimate amplification efficiencies based on the log-linear phase of the amplification reactions. REST-MCS© was then used to analyse differences between groups, with a non-parametric randomisation test (Pair Wise Fixed Reallocation Randomisation Test©). The advantage of this test lies in its independence of assumptions about the distributions of experimental data, while remaining as powerful as more standard statistical tests, such as analysis of variance tests (Pfaffl *et al.*, 2002). 2000 iterations of the Pair Wise Fixed Reallocation Randomisation Test© were run for each condition pair to determine how likely the differences between group means were to be greater in the case of a random allocation than those observed. Results were considered statistically significant for p values < 0.05. Data were presented as percentage of control ± standard error of mean (SEM).

2.5. Western blotting

Protocol for western blotting was used as previously described (Ferreira 2012). Briefly, to obtain total protein extracts, cells were incubated with lysis cocktail solution (75 mM NaCl, #31434, Sigma-Aldrich, 25 mM Tris, #T1378, Sigma-Aldrich, 2.5 mM EGTA, #E4378, Sigma-Aldrich, 0,5% Triton X-100, #BP151-500 Thermo Scientific, 6.03 mM DOC, #D6750, Sigma-Aldrich, 1.73 mM SDS, #161-0302, Bio-Rad, 0.05 mM DTT, D9779, Sigma-Aldrich and cOmplete, Mini Protease Inhibitor Cocktail, #11836153001, Roche Applied Science, Penzberg, Germany) (pH 7.5), and gently homogenized. Samples were then centrifuged for 20 min at 4300×g and 4°C, the supernatant collected, and the total amount of protein was quantified using the BCA assay (#23225, Thermo Scientific). 50 µg per sample were then loaded onto 5% stacking / 10% resolving acrylamide/bisacrilamide gels (#161-0158, Bio-Rad) and separated by SDS-PAGE using a bicine (#B3876, Sigma) / SDS, Bio-Rad) electrophoresis buffer (pH 8.3), at constant voltage (120 V) for 2 hrs at room temperature. After separation, proteins were transferred to PVDF methanol-activated membranes (#IPVH00010, Millipore Corporation, Billerica, Massachusetts) with 0,45 µm pore size under the following conditions: constant amperage (300 mA), 90 min at 4°C in a solution containing 10 mM CAPS (#C2632, Sigma-Aldrich) and 20% methanol

(#20903.368, VWR International) at pH 11.0. Membranes were blocked in Tris-buffer saline (TBS, 15 mM Tris, #T1378, Sigma, 150 mM NaCl, Sigma, pH 7.6) containing 5% w/v bovine serum albumin (BSA, #SC-2323, Santa Cruz Biotechnology, Santa Cruz, California, USA) and 0.1% v/v Tween® 20 (#P5927, Sigma-Aldrich) for 1 hr, at room temperature, and then incubated overnight at 4°C with the primary antibody solution (rabbit polyclonal anti-CX_3CR1 #14-6093-81, eBioscience, San Diego, California, USA) diluted in TBS-T (TBS, 1% v/v Tween® 20, Sigma-Aldrich), 5% BSA. After rinsing three times with TBS-T, membranes were incubated for 1 hr at room temperature with an alkaline phosphatase-linked goat anti-rabbit IgG (#65-6122, novex®, Life Technologies) 1:10,000, in TBS-T with 5% w/v BSA. Protein immunoreactive bands were visualized in a Versa-Doc 3000 Imaging System (Bio-Rad), after incubation of the membrane with ECF chemifluorescent reagent (#RPN5785, GE Healthcare Life Sciences, Uppsala, Sweden) for 5 min.

2.6. Immunocytochemistry

Cells were washed with phosphate-buffered saline pH 7.4 (PBS) (137 mM NaCl, Sigma-Aldrich, 2.7 mM KCl, #1.04936, Merck KGaA, Darmstadt, Germany, 10 mM Na_2HPO_4, Panreac Química S.L.U., Barcelona, Spain, 2 mM KH_2PO_4, Merck) (3 × 5 min), fixated for 30 min with 4% buffered paraformaldehyde (PFA) (#P6148, Sigma-Aldrich), rinsed again in PBS (3 × 5 min) and then placed for 30 min at room temperature in a blocking solution containing 3% bovine serum albumin (BSA, #SC-2323, Santa Cruz Biotechnology, Santa Cruz, California, USA) and 0.2% Triton X-100 (Thermo Fisher Scientific) diluted in PBS. Incubation with primary antibodies in blocking solution was performed overnight at 4°C in a humid chamber. Cells were washed with PBS (3 × 5 min) and incubated for 1 hr at room temperature with the corresponding secondary antibodies in PBS, as well as Hoechst 33342 (2 µg/ml, #H3570 Molecular Probes®, Life Technologies, Carlsbad, California, USA) for nuclear labelling. Finally, coverslips were mounted in Fluoroshield mounting medium (#ab104135, Abcam, Cambridge, UK).

Fluorescent images were acquired using a confocal LSM 510 Meta microscope (Carl Zeiss, Göttingen, Germany). For every immunocytochemistry analysis, at least 6 independent, randomly chosen microscopy fields were acquired, *per* coverslip, with a 40× magnification objective. Controls without primary antibodies were performed for each experiment, to

exclude non-specific labelling. Primary antibodies were used as listed: rat monoclonal anti-CD11b (1:500) (#MCA711, AbD Serotec, Oxford, UK) and rabbit polyclonal anti-CX_3CR1 (eBioscience) in blocking solution. The presence of primary antibodies was revealed by using the following fluorochrome-conjugated antibodies diluted at 1:200 in PBS: Alexa Fluor 488 donkey anti-rat IgG (#A21208, Molecular Probes®) and Alexa Fluor 594 donkey anti-rabbit IgG (#A21207, Molecular Probes®). Membrane ruffling was visualized using a fluorochrome-conjugated phalloidin, which specifically binds to filamentous actin (F-actin). Cells were incubated for 2 hrs in phalloidin-Alexa Fluor 594 conjugate (1 U/ml) (#A12381, Molecular Probes®) in PBS, at room temperature.

2.7. Bead phagocytosis assay

Latex beads (Ø=3µm, 2.08×10^{10} beads/ml, #LB30, Sigma-Aldrich) were opsonised with IgG from rabbit serum (10 ng/ml, #I8140, Sigma-Aldrich), under constant agitation at 4 rpm overnight at 4°C. Beads were then resuspended in previously heated DMEM/F-12 and distributed by each well at a density of 2×10^6 beads *per* well. After 1 hr of incubation, cells were washed with PBS, fixed with buffered 4% PFA for 20 min at room temperature and rinsed again in PBS. Beads were labelled with secondary antibody Alexa Fluor 594 donkey anti-rabbit IgG (#A21207, Molecular Probes®) 1:200, in PBS). For nuclear labelling, cell preparations were stained with Hoechst 33342 (2 µg/ml) in 0.3% BSA, 0.1% Triton-X100 solution, for 5 min at room temperature (RT) and mounted in Fluoroshield mounting medium.

Fluorescent images were acquired using an Axio Observer.Z1 microscope (Carl Zeiss). Each experimental condition was performed in triplicate and at least 12 images were acquired for each replicate. Photomicrographs were analysed with NIH ImageJ Software (Schneider *et al.* 2012), and for each image four parameters were determined: the total cell number, the total bead number, the total number of phagocyting cells and the total number of internalised beads. Only negative-labelled beads were considered as internalised, as assumed that secondary antibody could not reach intracellular bead Analysis was carried out with a designed, particle-counting macro, ensuring that only sphere-shaped signals which displayed a fluorescence intensity moderately higher than background were taken into account. Visual confirmation was used whenever the automated determination yielded

abnormal results. Protocol adapted from (Ferreira *et al.* 2011)

2.8. Motility assay (Scratch wound assay)

Before cell seeding, two parallel lines were carved on the underside of each well with a scalpel. These lines, together with the line given by the scratch wound, served as a guidance axis for image acquisition. Cell monolayer was approximately 95% confluent before the motility assay took place. The performed wound was a scratch in the monolayer, perpendicular to the lines carved on the underside, made with a P10 pipette tip. Immediately after performing the scratch, wells were slowly and carefully washed twice with sterile PBS, in order to remove cells released by the scratching procedure, and DMEM/F-12 with LPS, Fkn or both (see cell line section) was added to the cells. Two regions were photographed *per* well with an inverted Axiovert 200 microscope (Carl Zeiss), using a 5× objective and a CoolSNAP digital camera (Roper Scientific, Tucson, Arizona, USA). Cells were allowed to migrate into the scratch wound for 12 hrs and image acquisition was repeated in the same two areas per well. Differential interference contrast (DIC) images were acquired using MetaFluor Software (Universal Imaging, Downingtown, Pennsylvania, USA) and analysed with NIH ImageJ Software (Schneider *et al.* 2012). Cell migration was determined by counting the number of cells that migrated towards the middle of the wound during the period of treatment. Each experimental condition was performed in triplicate and 4 images (2 at t_0 and 2 at t_{12}) were acquired for every replicate. Protocol as described in (Ferreira *et al.* 2012a).

2.9. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0a (GraphPad Software, San Diego, California, USA) except for qPCR (see qPCR analysis). Statistical significance was determined by two way analysis of variance (ANOVA) followed by Bonferroni *post hoc* test for comparison among experimental settings. Statistical significance was considered relevant for p values < 0.05. Data were presented as means ± standard error of the mean (SEM).

Chapter III – Results

3.1. Analysis of the fractalkine receptor (CX3CR1) expression and regulation in the murine N9 microglial cell line

The N9 microglial cell line has been used as an experimental model to study the molecular biology and function of microglia. Thus, we carried out our work with this cell line to explore the modulatory effect of the chemokine Fkn in a model of endotoxin-induced microglial activation. Considering the lack of reports analysing the expression of Fkn receptor CX_3CR1 by this cell line, we decided to study the presence of CX_3CR1 mRNA and protein in our N9 microglial cell cultures.

First, CX_3CR1 mRNA expression was evaluated with a qPCR protocol. Samples from negative reverse transcription reactions (no amplification controls, NACs) and no template controls (NTCs) were used as negative controls. Evaluation of the melting curves generated after the amplification reactions (Fig. 3.1. A) revealed a single peak at 81,5ºC. No significant variations in the fluorescence were observed in the reactions of negative controls. This data, while taking into account the specificity of the amplicon selected for *cx3cr1*, strongly suggests that the *cx3cr1* gene is transcribed by N9 microglia. Furthermore, we evaluated whether LPS or Fkn induced changes in the expression levels of CX_3CR1 mRNA in N9 cells. Our results indicate that CX_3CR1 mRNA levels did not show significant differences throughout all experimental conditions 4 hrs after treatment with Fkn, LPS or both together (Fig. 3.2.).

We also assessed CX_3CR1 protein expression through western blotting and immunocytochemistry techniques. Our western blotting analysis showed three specific bands for CX_3CR1, with MWs of 40, 50 and 55 kDa respectively (Fig. 3.1. B). Such pattern of CX_3CR1 expression has been previously described (Yang *et al.* 2007; Donnelly *et al.* 2011). Immunofluorescence staining for CX_3CR1 also demonstrated the expression of Fkn receptor under basal conditions in N9 microglia. CD11b, a well-established microglial and mononuclear phagocyte marker, was used to reveal cell morphology (Fig. 3.1. C, D). Almost all cells in our basal culture conditions showed expression of CX_3CR1, while negative controls showed just a weak background staining.

Our data indicates that there is a constitutive expression of CX_3CR1 at mRNA and protein levels, suggesting that this cell line should respond to Fkn treatment. Therefore, we considered that N9 microglial cell culture is an excellent *in vitro* model to analyse the

modulatory effect of Fkn in the activation profile of microglial cells under inflammatory conditions.

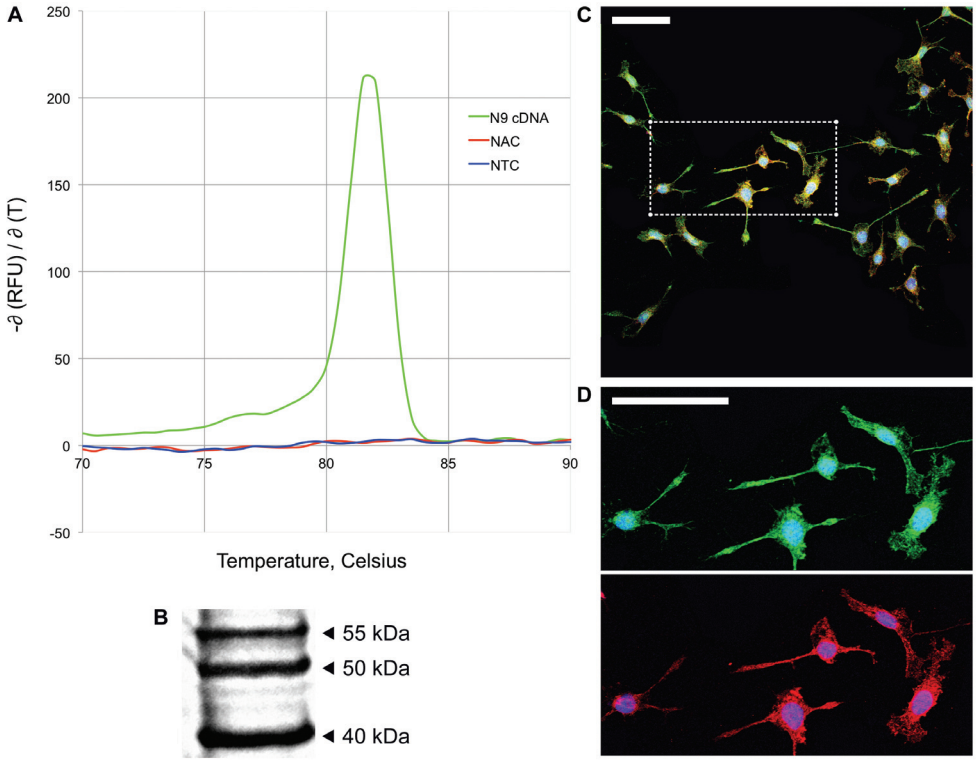

Figure 3.1. Analysis of CX_3CR1 expression in the N9 microglial cell line. (A) Representative melting curves obtained from N9 microglial cells cDNA (green) or negative template samples (red and blue). (B) Western blot of 50 µg whole cell extracts from N9 cells resolved on a 10% SDS-PAGE gel revealed with anti-CX_3CR1 (1:1000). Three bands were observed, at 40, 50 and 55 kDa. (C) Representative confocal microscopy photomicrograph of N9 microglial cells stained for CX_3CR1 (green) and CD11b (red) under basal (control) conditions. Cell nuclei were stained with Hoechst 33342 (blue) (D) Magnification of the highlighted area in (C). Scale bar: 50 µm.

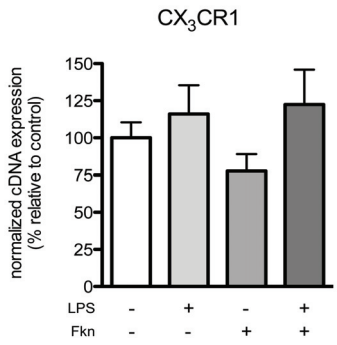

Figure 3.2. Quantification of CX_3CR1 mRNA expression changes induced by LPS (100 ng/ml) and Fkn (200 ng/ml). qPCR analysis revealed no significant variations in CX_3CR1 mRNA levels throughout all experimental conditions. Data are expressed as mean ± SEM (n=3) and as a percentage of control. Results were analysed using Pair Wise Fixed Reallocation Randomisation Test©.

3.2. Fkn decreases mRNA levels of the pro-inflammatory cytokines IL-1β and TNF-α in LPS stimulated N9 microglial cells

Fkn has been shown to lower microglial activation and pro-inflammatory cytokine expression [(Zujovic *et al.* 2000; Mizuno *et al.* 2003; Lyons *et al.* 2009)]. Considering this data we analysed the effect of Fkn on the expression levels of IL-1β and TNF-α mRNA in N9 microglial cells at a dilution that has been previously demonstrated to be effective (200 ng/ml) (Lyons *et al.* 2009).

Our results showed that stimulation with LPS triggered a great increase in IL-1β and TNF-α mRNA expression, though more noticeable in the case of the former ($IL\text{-}1\beta_{CTRL}$=100.00 ± 12.17%, $IL\text{-}1\beta_{LPS}$=2079.14 ± 431.76%, $p \leq 0.001$; $TNF\text{-}\alpha_{CTRL}$=100.00 ± 22.18%, $TNF\text{-}\alpha_{LPS}$=402.92 ± 110.78%, $p \leq 0.01$; n=3, Fig. 3.3). Fkn treatment did not significantly change mRNA levels for both cytokines ($IL\text{-}1\beta_{Fkn}$=83.61 ± 14.79%; $TNF\text{-}\alpha_{Fkn}$=90.02 ± 20.79%, $p > 0.05$) under basal conditions. However, Fkn induced a 50% decrease on the mRNA levels of both cytokines upon stimulation of N9 cells with LPS ($IL\text{-}1\beta_{LPS+Fkn}$=993.75 ± 116.15%, $p \leq 0.001$; $TNF\text{-}\alpha_{LPS+Fkn}$=216.18 ± 45.12%, $p \leq 0.05$). Our results are in agreement with previous reports demonstrating that Fkn downregulates the increase on the expression of IL-1β and TNF-α mRNAs induced by LPS.

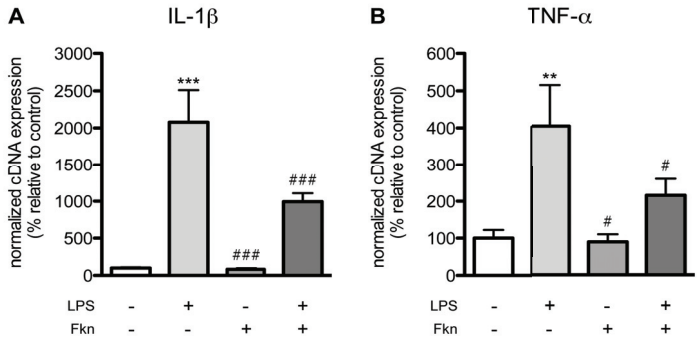

Figure 3.3. Fractalkine lowers LPS-induced cytokine expression. (A) IL-1β and (B) TNF-α mRNA expression was induced in the presence of LPS (100 ng/ml), while it did not vary with Fkn (200 ng/ml) alone. Co-incubation with LPS and Fkn resulted in a significant decrease in mRNA levels when compared to LPS. Data are expressed as mean ± SEM (n=3) and as a percentage referred to control levels. $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ vs control; $^{\#}p \leq 0.05$, $^{\#\#\#}p \leq 0.001$ vs LPS, using Pair Wise Fixed Reallocation Randomisation Test©.

3.3. Fkn modulates Fc receptor-mediated phagocytosis by microglial cells

Fkn has been described to induce opsonin-dependent phagocytosis via MFG-E8 expression (Noda *et al.* 2011). Nevertheless, and to the best of our knowledge, there is no data

available about the effect of Fkn in FcγR-mediated phagocytosis, while LPS is known to increase this type of phagocytic activity (Ferreira *et al.* 2011). Thus, we analysed FcγR-mediated phagocytic activity of N9 microglia after LPS and/or Fkn treatment, using a well-established essay in our laboratory based on IgG-coated latex beads (Ferreira *et al.* 2011).

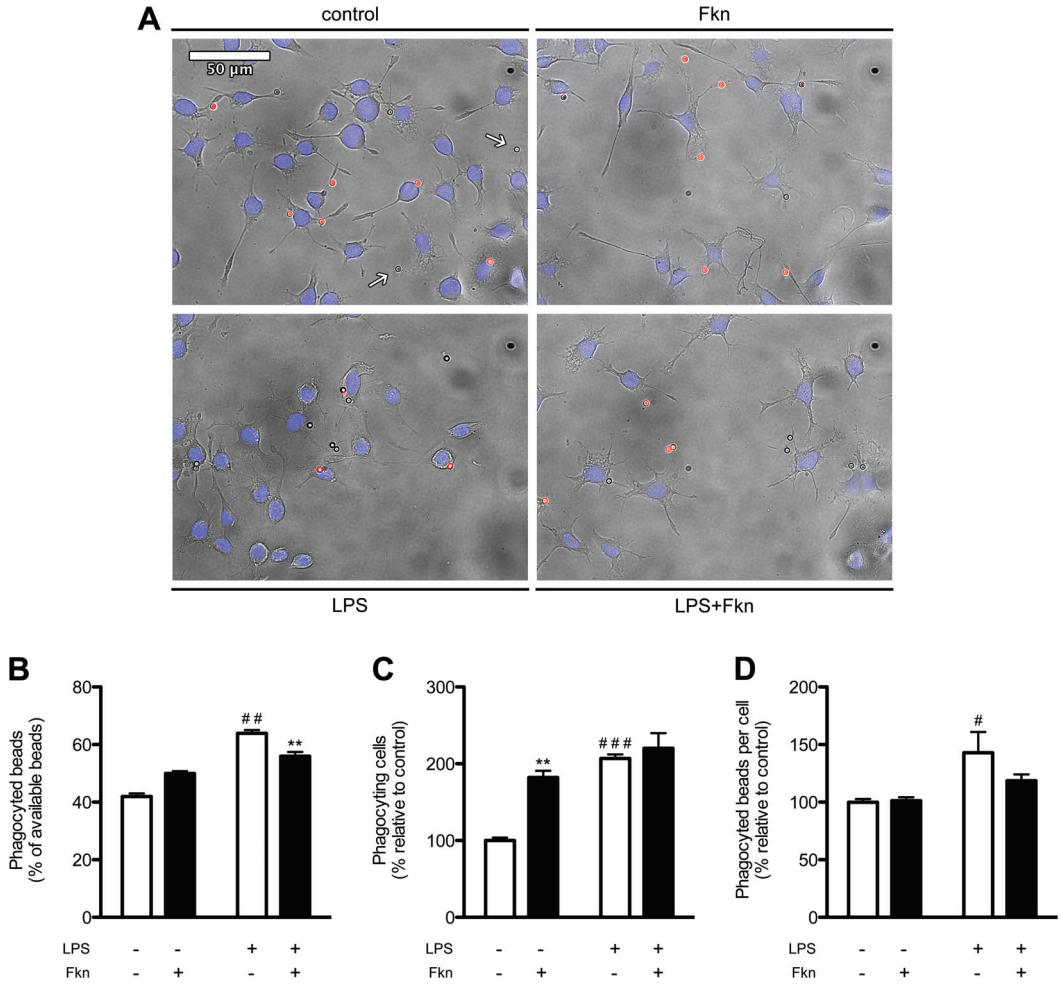

Figure 3.4. Fkn modulates bead phagocytosis by microglial cells. (A) Representative photomicrographs from every experimental condition. Phagocyted beads are not stained (arrows) while non-phagocyted beads show a fluorescent red staining (B) LPS (100 ng/ml) led to an increase in the total number of phagocyted beads, as compared to control. Fkn (200 ng/ml) did not show any overall effect but reduced the percentage of beads phagocyted on LPS treated cells. (C) LPS and Fkn increased the proportion of phagocyting cells but did not show any additive effect. (D) LPS induced a greater phagocytic activity per cell, whereas Fkn presumably reduced this effect. Data are expressed as mean ± SEM (n=3). ** $p \leq 0.01$, vs respective condition without Fkn, $^{\#}$ $p \leq 0.05$ $^{\#\#}$ $p \leq 0.01$ and $^{\#\#\#}$ $p \leq 0.001$ vs basal conditions (–LPS –Fkn), using two-way analysis of variance (ANOVA) with Bonferroni's multiple comparison *post hoc* test.

Stimulation with LPS induced a 1.5 fold increase in the fraction of phagocyted beads (CTRL=41.94 ± 1.54%; LPS=63.90 ± 1.03%; $p < 0.001$, Bonferroni *post hoc* test ; n=3; Fig. 3.4. B), doubled the percentage of phagocyting cells (CTRL=100 ± 3.93%; LPS=206.78 ± 5.96%; $p < 0.001$; Fig. 3.4. C), and also augmented the number of beads phagocyted *per* cell

(CTRL=100 ± 2.79%; LPS=142.85 ± 18.04%; $p < 0.05$, Fig. 3.4. D), when compared to control conditions. Interestingly, Fkn alone stimulated FcγR-mediated phagocytosis as demonstrated by the increase in the fraction of phagocyting cells (FKN=182.08 ± 8.82%; $p < 0.01$; Fig. 3.4. C). However, no significant overall effect due to Fkn was detected on the general percentage of phagocyted beads or the mean number of phagocyted beads *per* cell. Nevertheless, Fkn leads to a reduction of the percentage of phagocyted beads in LPS-stimulated cells (interaction LPS*Fkn, $p = 0.0001$, two-way ANOVA). Furthermore, the LPS-mediated increase in the mean number of beads phagocyted *per* cell is apparently diminished by Fkn treatment (Fig. 3.4. D). This data indicates that Fkn is able to increase FcγR-mediated phagocytosis mainly by increasing the proportion of phagocyting cells. Moreover, despite further studies being needed to completely address the specific effect of Fkn on FcγR-mediated phagocytosis, our data suggests that this chemokine modulates LPS-induced phagocytosis on N9 microglial cells.

3.4. Fkn induces cytoskeleton reorganization in microglia

Although *in vitro* "resting" microglia does not show the typical ramified morphology of CNS microglial cells, classical activating agents like LPS induce their morphological reorganization (Abd-El-Basset & Fedoroff 1995). Indeed, pro-inflammatory cytokines, such as IL-1β and TNF-α, can promote F-actin rearrangement and "membrane ruffling" (Abd-El-Basset *et al.* 2004). Considering our previous data, we investigated the effects of Fkn on the morphological signs of activation in N9 microglial cells.

In basal (control) conditions, N9 microglial cells showed a slim cell body mainly characterized by a single (unipolar) or a pair of processes (bipolar or spindle-like), projecting from the opposing poles of the cell body. We also observed also some cells with a more ramified morphology displaying radial but long and slim processes (Fig. 3.6., top panel). As expected, LPS induced swelling and loss of ramified morphology in N9 microglia. Membrane "ruffles", thick protrusions of the cell membrane formed due to F-actin polymerization, were frequent in LPS stimulated cells, usually a sign of microglia activation (Fig. 3.6., second panel). Interestingly, microglia displayed a ramified morphology in the presence of Fkn, with numerous filopodia-like structures protruding

from the surface, which could loosely be described as a dendritic-like morphology (Butovsky *et al.* 2006) (Fig. 3.6., third panel). In the presence of both LPS and Fkn, N9 cells displayed an intermediate morphology characterized by the presence of thick membrane "ruffles", but also have many thin, finger-like protrusions which extend from the primary branches (Fig. 3.6., bottom panel).

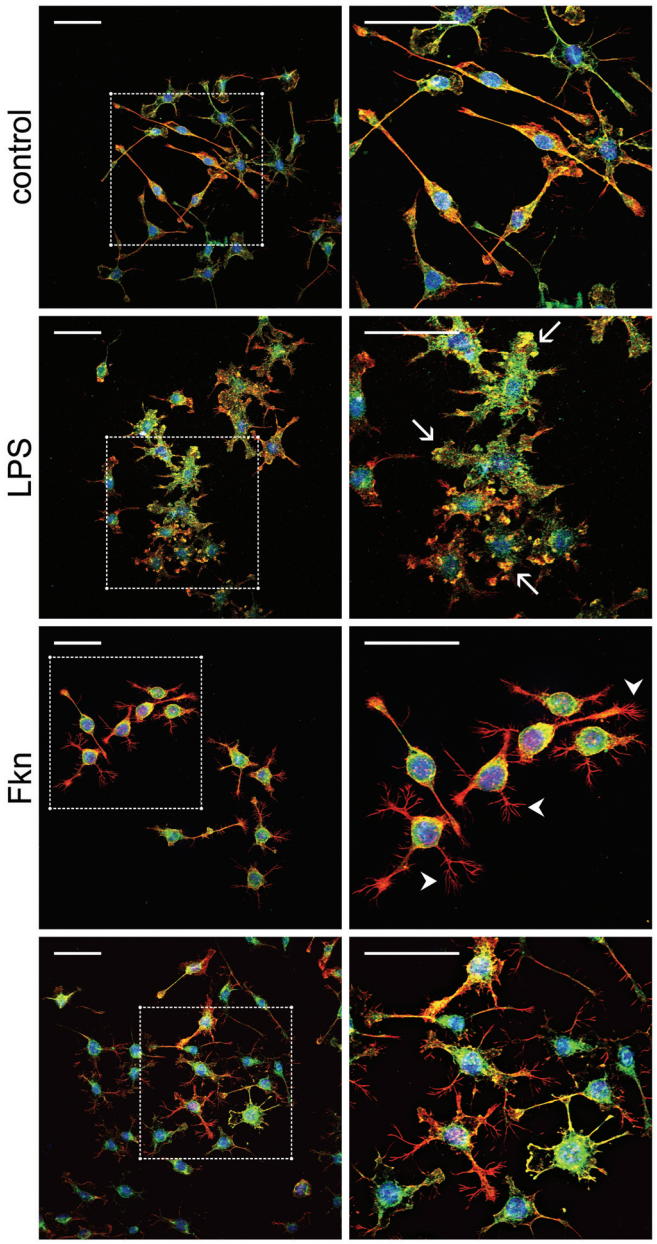

Figure 3.5. Fkn induced cytoskeleton reorganization in microglia. Representative confocal photomicrographs were taken to assess the role of Fkn on the rearrangement of the actin cytoskeleton. Microglial cells treated with LPS (100 ng/ml) showed significant membrane ruffling (arrows), while Fkn (200 ng/ml) induced a prominent ramified morphology characterized by thin filopodia-like structures (arrowheads). The simultaneous presence of Fkn and LPS resulted in an intermediate morphology. Right column displays magnifications of the highlighted areas in the images on the left. Cells were stained for actin (in red), CD11b (in green) and Hoechst 33342 (nuclei in blue). Scale bars 50 μm.

This data demonstrates that Fkn has an important regulatory effect on the profile of microglial activation, which is clearly manifested at the morphological level.

3.5. Fkn promotes microglial motility

The increase in IL-1β and TNF-α release, mediated by LPS, has been described to enhance microglial motility in an autocrine/paracrine way (Bellavance & Rivest 2012; Ferreira *et al.* 2012a). Curiously, Fkn is a chemoattractant (Bazan *et al.* 1997; Pan *et al.* 1997) but reduces the expression of IL-1β and TNF-α. We, therefore, studied the effect of Fkn on the migratory activity of N9 cells under basal conditions and LPS stimulation. A scratch wound assay was used to analyse cell motility (Ferreira *et al.* 2012a), determined according to the mean number of cells that migrated into the wound in a 12 hr period (Fig. 3.6.).

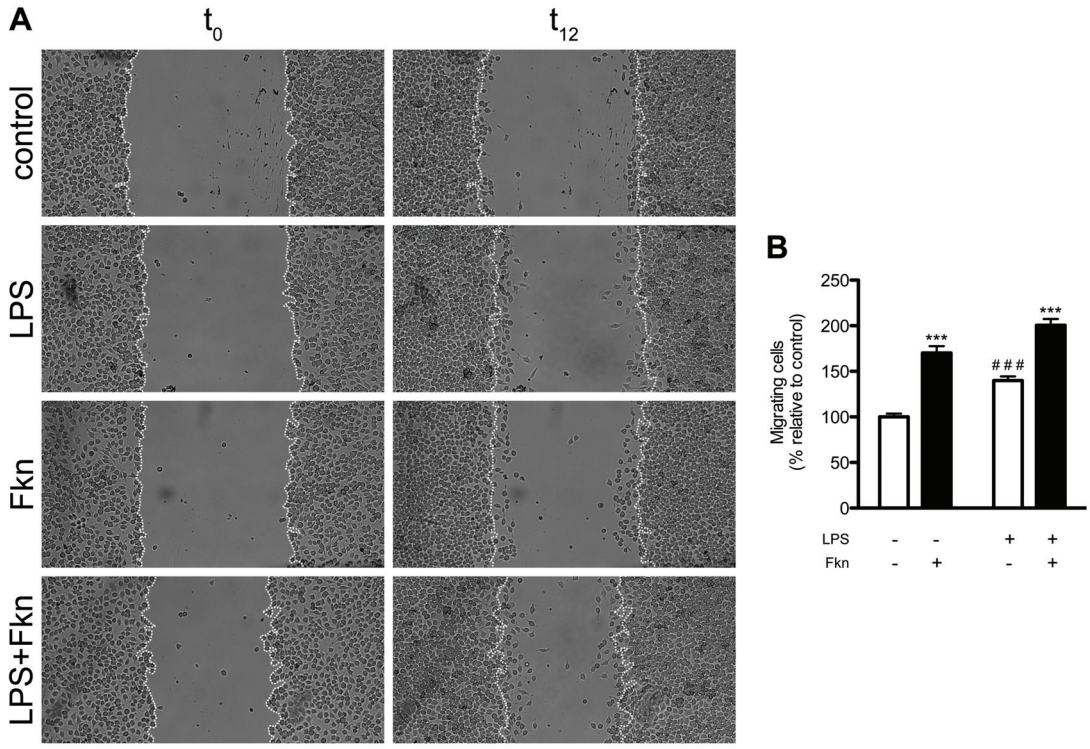

Figure 3.6. Fkn promotes microglial motility. (A) Representative photomicrographs illustrate the migratory stimulus induced by LPS (100 ng/ml) and Fkn (200 ng/ml). (B) LPS induced cell motility, while Fkn had a greater effect than LPS. Co-incubation with LPS and Fkn resulted in a greater migratory induction than that observed with LPS or Fkn alone. Data are expressed as mean ± SEM (n=6) and as a percentage of control. *** $p \leq 0.001$, vs respective condition without Fkn, ### $p \leq 0.001$ vs basal conditions (–LPS –Fkn), using two-way analysis of variance (ANOVA) and Bonferroni's multiple comparison *post hoc* test.

Both LPS and Fkn produced extremely significant overall effects on the migratory activity of N9 cells ($p < 0.0001$ in both cases, two-way ANOVA). As expected, the presence of LPS

in the medium induced a greater mobilization of cells towards the gap (CTRL=100 ± 5.84%, LPS=140.51 ± 9.37%, $p < 0.001$, Bonferroni *post hoc* test, n=6). Interestingly, the number of migrating cells was also significantly increased by the presence of Fkn (Fkn=170.10 ± 10.48%, $p < 0.001$, n=6), when compared to control. Finally, Fkn further induced an increase in mobility in LPS-stimulated cells (LPS+Fkn=200.11 ± 10.03%, $p < 0.001$ n=6), a merely additive effect due to the presence of both stimuli (interaction LPS*Fkn, p=0.4238, two-way ANOVA). These results demonstrate that Fkn increased migration of N9 microglia in basal conditions and also after LPS challenge.

Chapter IV – Discussion

Microglial cells respond to altered homeostasis in the CNS by switching their functional phenotype to 'activated', migrating to the origin of the insult, eliminating pathogens or debris and releasing neurotoxic and/or neurotrophic factors to return the brain parenchyma back to a steady state (Block *et al.* 2007). Failed regulation of microglial activity can result in an over-reactive microgliosis, which can in turn be detrimental to the physiology and function of the brain (Block *et al.* 2007). In this context, the chemokine Fkn emerged as a highly attractive molecule with the ability to modulate microglial response, avoiding or ameliorating their possible negative effects.

In the present work, we sought to determine the effects of Fkn on different characteristics of N9 microglial cells, in basal conditions and after activation induced by LPS. Thus, we analysed several parameters, by means of qPCR, protein detection, immunofluorescence staining and functional assays to establish the role of Fkn. Our first results showed that N9 microglial cells express the Fkn receptor CX_3CR1, thus indicating that these cells are able to respond to Fkn stimulus. This was confirmed in following experiments, which demonstrate that Fkn reduces mRNA expression levels of the pro-inflammatory cytokines IL-1β and TNF-α in LPS-treated N9 microglial cells. Furthermore, we also observed that Fkn promotes morphological changes, migration and phagocytic activity in N9 cells. Altogether, our data indicates that Fkn induces a change in the activation profile of microglia, modulating the pro-inflammatory effect of LPS challenge while maintaining desired effects like increased migratory and phagocytic capacities.

4.1. Expression of CX_3CR1 in the murine N9 microglial cell line

Since no data on the expression of CX_3CR1 by the N9 cell line was available, we first demonstrated the expression of this receptor in this cell line using qPCR, Western blotting and immunofluorescence staining (Fig. 3.1.). While the theoretical molecular weight (Mw) for CX_3CR1 is of 40.26 kDa (as calculated with ExPASy Compute pI/Mw tool (Bjellqvist *et al.* 1993; Bjellqvist *et al.* 1994), using the UniProtKB entry Q9Z0D9 for the murine CX_3CR1 complete protein sequence), our western blotting analysis showed three specific bands for CX_3CR1, with Mws of 40, 50 and 55 kDa respectively (Fig. 3.1. B). Such pattern of CX_3CR1 expression has been demonstrated previously (Yang *et al.* 2007; Donnelly *et al.* 2011), although there is not any definitive explanation for it. Hypothetical justifications for this

include the expression of multiple isoforms of CX_3CR1 in mice, as described for its human counterpart (Garin *et al.* 2002; Garin *et al.* 2003), and/or post-translational modifications. Expression of CX_3CR1 indicates that N9 microglia should respond to Fkn treatment, as clearly demonstrated in further experiments.

Contrary to what has been previously reported, we did not observe any significant change in CX_3CR1 mRNA expression levels in the presence of LPS or Fkn. Indeed, CX_3CR1 mRNA levels have been reported to be reduced (Wynne *et al.* 2010), unaltered (Mizuno *et al.* 2003), or increased (Chu *et al.* 2009), depending on the experimental paradigm of microglial activation or the time post treatment at which the analysis is conducted. Despite no changes in mRNA levels of CX_3CR1 were observed, the expression levels of the protein seemed to be enough to elicit a response by the binding of Fkn, as further demonstrated. Thus, we considered N9 cells as a good model to dissect the direct effects exerted by soluble Fkn in microglial cells, whether maintained in a "non-activated" basal state or after stimulation with LPS. This system allows us to analyse direct Fkn effects on microglia, without the interference of other cellular or molecular elements that could complicate the interpretation of the results.

4.2. Fkn induced down-regulation of pro-inflammatory cytokine expression

Two main pro-inflammatory cytokines, IL-lβ and TNF-α, have been involved in the induction of neuronal loss (Gayle *et al.* 2002) and are reportedly elevated in models of neurodegenerative diseases ((Patel *et al.* 2005; Cunningham *et al.* 2009), reviewed in (Viviani *et al.* 2004; Sugama *et al.* 2009)). LPS induced large increases in the expression of the mRNA for both of these molecules, as expected. Co-incubation with LPS and Fkn resulted in a significant decrease in mRNA levels when compared to LPS, which is in accordance with published results (Zujovic *et al.* 2000; Mizuno *et al.* 2003; Lyons *et al.* 2009).

Signalling through Fkn leads to a reduction in the expression of pro-inflammatory factors, thus, it stands to reason that Fkn exerts neuroprotective effects through the regulation of microglial production of cytokines. This has been proposed before (Mizuno *et al.* 2003), and has been observed in different models of neurodegenerative diseases *in vivo* ((Cardona

et al. 2006; Bhaskar *et al.* 2010; Pabon *et al.* 2011), reviewed in (Khandelwal *et al.* 2011)). Despite of just having analysed the effect of Fkn on mRNA expression levels, our data indicates that Fkn induces an anti-inflammatory effect on N9 microglial cells by moderating the LPS-mediated increase of pro-inflammatory cytokines. We will confirm our ideas in future essays aimed to analyse protein levels of IL-lβ and TNF-α and their receptors, as well as a larger panel of other molecules related with inflammatory responses (e.g. IL-6, IL-10, iNOS, etc.).

4.3. Fkn modulates Fc receptor-mediated phagocytosis

Many, if not all, neurodegenerative diseases show neuroinflammatory changes. Indeed, these disorders are associated with marked alterations in microglial distribution, numbers and activation state (El Khoury 2010). Neuronal death prompts reactive microgliosis, which can in turn drive progressive neurotoxicity (Block *et al.* 2007). The removal of apoptotic material without further inducing inflammation is then essential for the outcome of these disorders. Fkn has been shown to induce opsonin-dependent phagocytosis through the increased expression of MFG-E8, alongside releasing neuroprotective signals and reducing inflammation (Noda *et al.* 2011). Here, we investigated phagocytosis mediated by a different opsonin-dependent mechanism, namely through FcγRs. LPS treatment is known to induce FcγR-mediated phagocytosis of IgG-coated beads in N9 cells (Ferreira *et al.* 2011), but the effect of Fkn on this kind of phagocytosis by microglia had not yet been addressed.

Interestingly, we observed that the presence of Fkn increased the number of cells engaging in FcγRs-mediated phagocytic activity, but not the predisposition of individual cells to phagocyte more IgG-coated beads (however, these data should be confirmed by further analysis in the future). Taking into account the reduction in the expression of inflammatory cytokines, as well as the morphological restructuring (which will be discussed further on) elicited by Fkn, it is plausible to suggest that Fkn induces a less reactive, heightened-awareness state in microglia. In this state, microglial cells are prone to engage in defensive actions such as phagocyting opsonized particles, while moderating inflammatory signalling (Neumann *et al.* 2009).

4.4. Fkn induces cytoskeleton reorganization in microglia

Cytoskeleton reorganization is crucial for cell shape alterations, on which activities such as lamellipodial and filopodial protusion, vesicle and organelle motility, chemotaxis and phagocytosis all intrinsically depend (Fletcher & Mullins 2010). Activating agents, such as LPS, but also inflammatory cytokines such as TNF-α or IL-1β have been shown to induce reorganization in the actin cytoskeleton in microglia (Abd-El-Basset & Fedoroff 1995; Kloss *et al.* 2001; Abd-El-Basset *et al.* 2004). This can, at least in part, contribute to the increased rate of phagocytosis observed by activated microglia (Ferreira *et al.* 2011). Fkn has been shown to elicit actin polymerization and cytoskeletal rearrangement in dendritic cells (Dichmann *et al.* 2001) and in microglia (Maciejewski-Lenoir *et al.* 1999), but the effect of Fkn on the cytoskeleton of endotoxin-activated microglial cells has not been fully addressed so far.

We observed that N9 microglia exhibited significant membrane 'ruffling' in the presence of LPS, as was expected (Ferreira *et al.* 2011). Curiously, the presence of Fkn induced a densely ramified, dendritic-like morphology. Such a phenotypical manifestation has been shown before in a primary culture of mouse microglia, although through the use of anti-inflammatory cytokine IL-4 (Butovsky *et al.* 2006). As is the case with Fkn-treated N9 cells, IL-4 stimulated microglia maintains phagocytic activity (Butovsky *et al.* 2006), and its appearance more closely resembles the ramified morphology displayed by 'surveying' or 'resting' microglia *in vivo* (Kettenmann *et al.* 2011). In our case, the change in morphology might correlate with the higher frequency of phagocyting cells exhibited in the presence of Fkn. The increase in ramified protrusions can reflect a higher 'reconnaissance' activity by these cells, which might increase the probability of an encounter with a phagocytic target, yet keeping the bias to engulf opsonized particles at the same level of unstimulated controls. Thus, Fkn plays a major role in regulating the activity profile of N9 microglia, which is clearly manifested at the morphological level.

4.5. Fkn promotes microglial motility

When activated, microglial cells display enhanced mobility (Abd-El-Basset & Fedoroff 1995; Horvath *et al.* 2008). This capacity is essential to deal with and resolve insults to the

brain parenchyma (Noda & Suzumura 2012). Enhanced migratory activity can be due to the autocrine or paracrine effect of pro-inflammatory mediators, e.g. cyto- and chemokines, released by microglia in response to such challenges (Bellavance & Rivest 2012; Ferreira *et al.* 2012a). In contrast, Fkn reduces the expression of these pro-inflammatory mediators, which could result in reduced cell migration. This is however not the case, as Fkn was shown to stimulate migration in monocyte-derived cells (Imai *et al.* 1997; Ruitenberg *et al.* 2008) and in microglia (Maciejewski-Lenoir *et al.* 1999; Carter & Dick 2004).

Our results confirm that Fkn induces a great mobilization in microglia, independently of the presence of LPS (which also stimulated migration, as expected). The simultaneous effects of both stimuli do not show any signs of synergy nor destructive interference, portraying merely the addition of the isolated effect of each stimulus. This hints at probably separate signal transduction mechanisms at the intracellular level, with the signalling pathway activated by Fkn displaying a relative degree of independence from the p38 MAPK pathway activated by LPS and IL-1β to elicit migration (Ferreira *et al.* 2012a). In the light of the reduction of TNF-α and IL-1β expression by N9 cells in the presence of Fkn, the increased capacity for mobilization in the presence of both pro-inflammatory stimuli and Fkn is of even greater relevance to the surveillance activity discussed above.

4.6. Functional implications of Fkn effects in N9 cells

The differences between the basal state of any type of microglial cell used as *in vitro* model to study neuroinflammation and the regular, 'quiescent', phenotype displayed by microglia *in vitro* are evident and have been thoroughly laid out before. But taking together data from the literature and provided from the observations analysed in this work, it becomes clear that Fkn serves, at least in part, to shorten the gap between the typical morphology and behaviour of microglia *in* vivo and that displayed by most *in vitro* models. Fkn has a clear role in reducing or shifting the activation of microglial cells, evident at the molecular, morphological and functional levels, thus the lack of Fkn in isolated microglial cultures is a possible reason for the lack of 'surveying'-like microglia observed in these systems. This role could perfectly be carried out by membrane bound or even the constitutively cleaved Fkn *in vivo*, keeping the cells in an alerted but relatively latent state. However, microglial

cells stimulated with Fkn exhibit a state which could also be considered as activated, since they display a reorganized cytoskeleton coupled with increased phagocytic and mobility behaviour. But, as previously mentioned, this is not an 'activated' state in the classically attributed meaning. In the presence of Fkn, cells seem to exhibit an 'alternative' activation behaviour. Stimulation of phagocytosis and migration while keeping a low inflammatory profile is a characteristic of alternatively (M2) activated macrophages, which allow for normal tissue maintenance to be performed without setting off or worsening an inflammatory response (Cameron & Landreth 2010). Apparently, exposure to Fkn induces such a state in microglia. Curiously, this phenotype switch in microglia has very recently been proposed to ameliorate the disease progression in a model of Alzheimer's disease, as well as to have direct influence in the capacity for neurogenesis displayed in healthy and diseased CNS (Varnum & Ikezu 2012). Fkn can thus assume a major role as a valuable tool in the study and treatment of diseases of the CNS.

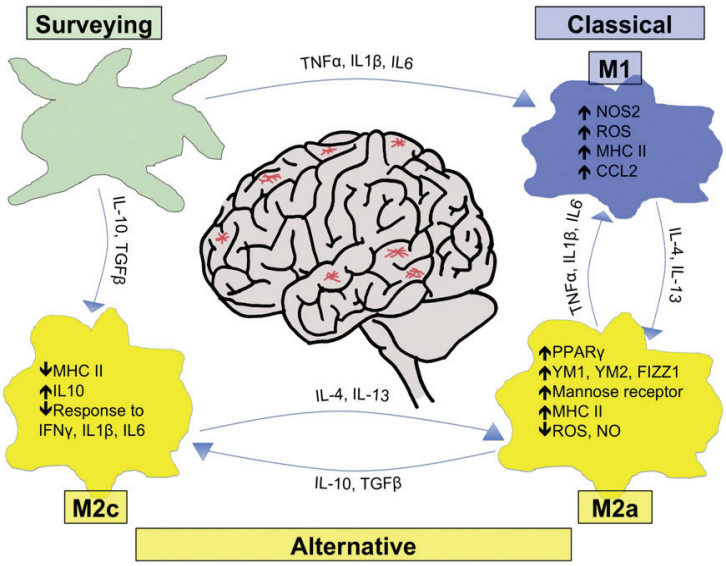

Figure 4.1. Different activation states of microglia. Microglia exist in different phenotypic states with differing physiological characterizations, ranging from surveying to classical to various alternatively activated states. (from (Cameron & Landreth 2010))

Fkn could thus serve to keep reactive microgliosis at a minimum, a 'buffer system' to control microglial overreaction in the case of localized, slightly altered brain homeostasis. In contrast, major insults or imbalances in the CNS could promote a major shedding of Fkn from neuronal cells, thus eliciting another type of response in microglia. This differentiated signalling potential can be inferred from the distinct types of Ca^{2+} transients that cleaved and membrane-bound Fkn elicit in these cells (Harrison *et al.* 1998). But the

underlying mechanisms of signal transduction are still in the dark and a great deal of work is still required to shed some light on this particular issue. For that purpose, N9 cells could be a valuable tool allowing an initial study of different signalling pathways activated by soluble and (when used in co-cultures with neurons) membrane attached Fkn.

That Fkn can be used *in vitro* to reduce or shift the activation of microglia is definitively clear, both from data reviewed or presented here for the first time. Thus, it seems that Fkn has a great but as of yet untapped potential as a mechanism to control microglial behaviour *in vitro*, to study the effects of microglia on CNS disease progression *in vivo*, and quite possibly to be used as a means or a target in therapeutic strategies to improve the conditions of patients suffering from CNS disorders.

Chapter V – Conclusion

Considering our work hypothesis, the main aims of this study and the results obtained, we conclude that:

1.- Fkn reduces mRNA levels of the pro-inflammatory cytokines IL-1β and TNF-α in N9 cells treated with LPS.

2.- Fkn induces an increase in the phagocytic activity of N9 cells.

3.- N9 cells morphology is affected by their exposure to Fkn in basal conditions, which leads to a more ramified shape of these cells.

4.- Drastic morphological changes observed in N9 cells after LPS exposition are ameliorated by co-treatment with Fkn.

5.- Fkn increases motility of N9 cells in basal and also LPS stimulated conditions.

6.- N9 cell culture is a good *in vitro* model for the study of Fkn effects on microglial cells.

Summarizing, we conclude that Fkn modulates LPS-mediated activation of N9 microglial cells, reducing their production of pro-inflammatory cytokines while maintaining high levels of migratory and phagocytic activity.

Acknowledgements

First, I would like to thank my supervisor, Doutor João Malva, for the unique opportunity of allowing me to carry out my work at the Neuroprotection and Neurogenesis in Brain Repair Group at the CNC. Thank you so much.

Also, I want to thank Doutor Jorge Valero, my co-supervisor, for being more than my co-supervisor. In the past few months you have been both a teacher and a friend, who time and again went out of his own path to course-correct mine. I cannot put into words how much this means to me, so I'll just discreetly say "Thank you very, very much".

From all the people in the group, I would like to give my warmest thanks to Raquel Ferreira, for having put up with my constant questions and doubts about pretty much everything I needed to learn during the course of my work. I hope that what you see here somehow makes you proud of having shown me the ropes. Thank you.

Naturally, I would also like to thank all the other great people in the group: Tiago, Elizabete, Francisca, Sofia. Thank you for the fun moments, and for your support in the less fun moments.

I would also like to extend my thanks to those without whose technical support this work would not have been what it is, Isabel Nunes and Luísa Cortes, thank you very much.

All of the other students who make the CNC one of the best places to work in, Pedro, Diogo, Maria Joana, Dominique, thank you all for having been friends instead of just co-workers. That is one of the things that make this whole experience so valuable.

To friends, old and new, too many to number but never forgotten, thank you for being there when I needed you the most. Especially Sérgio, Rita, Quintas, Roldão, Daniela, Sónia, Sabrina, Catarina - this trip could not have been the same without you guys. I'm thankful for having each and every one of you as a dear friend.

To my mother and father, who hardly could have been more supportive during the last two years, and the rest of my family, I cannot stress how thankful I am for all they have done for me. Thank you.

And finally, to my girlfriend, thank you, Maria, for having helped putting this work on track, for having helped putting my life on track, and for being even greater than the most extraordinary gift I could have thought of wanting for my life. Even abroad, you were always there, and for that I have to thank you so much.

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