INFLAMMATORY SIGNALLING IN POSTOPERATIVE COGNITIVE DYSFUNCTIONS

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Abstract

Major surgeries, such as cardiac or orthopaedic procedures in particular, expose the patient to extensive trauma, blood loss, and tissue injury; all of these factors effectively modulate the immune system to ultimately trigger an inflammatory response. Postoperative cognitive dysfunction (POCD), the condition being characterized by impairment of short and long-term memory, is one of common complicates following surgery. Recently, our data have demonstrated that neuroinflammation and microglia activation in the hippocampus following surgery are associated with cognitive decline.

The aim of this thesis is to investigate the inflammatory signaling pathways specifically involved with POCD, with a particular interest between systemic inflammation and local inflammation in the brain following surgery.

The data presented in this thesis introduce the general concepts and the involvement of inflammation in the etiology of cognitive dysfunctions using a mouse model of POCD. Upon the identification of specific pro-inflammatory markers both systemically and centrally and the delineation of the time course of events that characterize the inflammatory response following aseptic orthopaedic surgery, I describe how specific cellular signal pathways interact, mediate, and sustain this response. Following an initial non-specific approach using a general anti-inflammatory compound to identify whether inflammation plays a role in this scenario, I have exploited this model into a wide range of knockouts animals in the attempt of identifying specific signaling mechanisms and upstream receptors that mediate the behavioral abnormality following surgery. In order to achieve this, I have compared the inflammatory events after aseptic surgery with the response after a defined infectious stimulus, to ultimately joint the two in the context of a postoperative complication.

In conclusion, inflammation clearly plays a pivotal role in mediating physiological as well as behavioral changes after surgery and infection. This thesis has started to unmask the signaling pathways involved with surgery and how anti-cytokine therapy can potentially ameliorate the associated cognitive dysfunction.

Declaration

This thesis comprises results produced by me with the exception of two figures, where Mario Cibelli and Antonio Rei Fidalgo, colleagues in the laboratory, have generated the data. These figures are respectively acknowledged in this thesis. Antonio Rei Fidalgo has assisted me in the operational aspect of the initial acquisition of the behavioral data. This work has been carried out at Chelsea and Westminster Hospital, Anesthetics Departments, and at the Kennedy Institute of Rheumatology, Imperial College London.

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Writing this dissertation and investigating this area of research has been exceptionally challenging and fulfilling.

First and foremost I would like to thank my supervisor, Professor Mervyn Maze for his constant support and guidance; he has been an outstanding mentor and I feel privileged for having been trained under his supervision. I also wish to express my sincere appreciation for having had the pleasure of being supervised by Professor Brian Foxwell, who tragically passed away last December. Most of my research, in particular at the Kennedy Institute of Rheumatology, has been further inspired by his passion and interest for immunity and signaling mechanisms. I express my gratitude to Dr Claudia Monaco for her support, expertise and advices, as well as for the many *Nespresso* she offered me whilst discussing in her office. A special thank Dr Daqing Ma for his support and assistance in the lab. I also wish to thank Professor Marc Feldmann for sharing his wealth of knowledge with me and Professor Jagdeep Nanchahal for the useful conversation we engaged together. Last, but not least, I would like to acknowledge Professor Nick Franks for the opportunity he has offered me in joining Imperial College and his lab.

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Preface

Human nature has always been attracted by the need to know and discover. Since the ancient time, the human mind has been attracted by the complexity of phenomena. Often this interest has been described as an uncontrollable drive, which has been illustrated in many legends and tales. Greek mythology offered a great example of the concept of "insatiable curiosity" in the Pandora's box story, which exemplifies how inquisitiveness can lead to great sorrow to humankind. The endless human thirst for knowledge has been classically personified by Ulysses, initially by Homer in the Odyssey and then by Dante Alighieri in "La Divina Commedia" (The Divine Comedy). Ulysses, or Odysseus, has been described as a man with a strong personality, no limits or fear; with an irrepressible drive for discovery that led him to travel beyond the Pillars of Hercules, the limits of the known world.

> *"Considerate la vostra semenza: fatti non foste per viver come bruti, ma per seguir virtute e canoscenza"*
>
> *"Consider you origin: you were not born to live like brutes, but to follow virtue and knowledge"*
>
> Ulysses famous caption: Dante, Inferno, canto XXVI, lines 118-120

Ulysses, both in Homer and Dante, embodies the human dichotomy between fear and attraction to the unknown. With the establishment of the "scientific method" with Aristotle, innovative concepts based on empirical investigations were introduced in an ancient society. Philosophy, in particular epistemology (the theory of knowledge) has attempted to explain the source and limit of human knowledge, defeating old and preexisting assumptions. Science embodies this continuous effort to implement human knowledge using controlled and disciplined methods. Testing of hypothesis, data collection both with empirical and measurable corroborations provides an exquisite example of applied human reasoning and intellect.

N.T.

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Abbreviations and acronyms

AD:	Alzheimer's disease
AMPAR:	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ANOVA	Analysis of variance
AP-1:	activator protein 1
APC:	antigen-presenting cell
APO:	apolipoprotein
APP – FL:	β-amyloid precursor protein – full length
ATP:	adenosine triphosphate
BBB:	blood-brain barrier
cDNA	complementary DNA
CPB:	cardiopulmonary bypass
CNS:	central nervous system
CVOs:	circumventricular organs
DAB	3,3 'diaminobenzidine tetrahydrochloride
DAMP:	danger-associated molecular pattern
DD:	death domain
DNA	deoxyribonucleic acid
EDN:	eosinophil-derived neurotoxin
ELISA:	enzyme-linked immunosorbent assay
ERK:	extracellular-signal-regulated kinase
FiO_2:	fraction of inspired oxygen
GA:	general anesthesia
$GABA_A$:	gamma-aminobutyric acid $_A$

HMGB: high-mobility group box

HSP: heat-shock protein

IHC: immunohistochemistry

IKK: IkB kinase

IFN: interferon

IL: interleukin

IL-1R: interleukin-1 receptor

IL-1Ra: interleukin-1 receptor antagonist

iNOS: inducible nitric oxide synthases

i.p.: intra peritoneal

IRAK: IL-1R-associated kinase

IRF-3: interferon regulatory factor 3

ISPOCD: international Study of Postoperative Cognitive Dysfunction

JNK: JUN N-terminal kinase

KO: knockout

LPS: lipopolysaccharide

LRR: leucine-rich repeat

LTD: long-term depression

LTP: long-term potentiation

MAC: minimum alveolar concentration

MAPK: mitogen-activated protein kinase

MCI: mild cognitive impairment

MMP: matrix metalloproteinase

mRNA: messenger ribonucleic acid

MyD88: myeloid differentiation factor

NF-kB: nuclear factor-kappa B

NOD: nucleotide-binding oligomerization domain

P2X7: purinergic receptor, ligand-gated ion channel 7

PAMP: pathogen-associated molecular pattern

PBS:	phosphate buffered saline
POCD:	postoperative cognitive dysfunction
POD:	postoperative day
PRRs:	pattern recognition receptors
q-PCR:	quantitative real time polymerase chain reaction
RAGE:	receptor for advance glycation end-products
ROS:	reactive oxygen species
RT:	room temperature
s.c.:	subcutaneous
TBS:	tris buffered saline
TIRAP:	TIR-domain-containing adaptor protein
TFC:	trace fear conditioning
TJ:	tight junction
TLR:	toll-like receptor
TNF-α:	tumor necrosis factor-α
TRADD:	tumor necrosis factor receptor type 1-associated death domain
TRAF6:	tumor-necrosis-factor-receptor-associated factor 6
TRIF:	TIR-domain-containing adapter-inducing interferon β
WT:	wild type

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1 Chapter 1: General introduction

1.1 General considerations and immunity

Naturally, anything we are unable to explain raises some discomfort and simultaneously intrigues us to further understand it. Complex phenomena are commonly dissected into simple entities to be subsequently reassembled and fully appreciated into the "bigger picture". Especially in the field of biology and medicine this concept is widely applied and became essential for advancing knowledge. Besides experimentation, observations and serendipity also contribute to the progress of human knowledge. For instance the concept of immunity, derived from the Latin *immunis*, meaning "exempt" dates back to 430 BC with the historian Thucydides in his description of the war in the Peloponnesus (Thucydides). In the History of the Peloponnesian War, he exhaustively described the devastating plague in Athens giving an account on how people who survived the previous outbreak could look after sick people without contracting the disease.

> ἐπὶ πλέον δ' ὅμως οἱ διαπεφευγότες τόν τε θνῄσκοντα καὶ τὸν πονούμενον ᾠκτίζοντο διὰ τὸ προειδέναι τε καὶ αὐτοὶ ἤδη ἐν τῷ θαρσαλέῳ εἶναι· δὶς γὰρ τὸν αὐτόν, ὥστε καὶ κτείνειν, οὐκ ἐπελάμβανεν.
>
> *"Yet it was with those who had recovered from the disease that the sick and the dying found most compassion. These knew what it was from experience, and had now no fear for themselves; for the same man was never attacked twice- never at least fatally"*
>
> Thucydides on the plague, Second book, Chapter VII

The idea that people respond better to an illness after having contracted the disease was also previously observed in China. Back in the 15th century in Asia immunity was obtained by deliberately infecting individuals with pathogens. The technique was known as variolation and consisted of exposing healthy people with material from convalescing smallpox patients (Eyler 2003). Even if with risks, beneficial effects were achieved with a clear improvement in mortality rate. In 1719, Lady Mary Wortley Montagu, wife of the British ambassador to the Ottoman Empire, observed and witnessed the positive effects of variolation on her son whilst living in Constantinople. She brought this concept back into Europe and soon after in England, Edward Jenner significantly improved on this methodology and successfully provided immunity to smallpox after inoculating individuals with the fluid from a cowpox pustule (Belongia and Naleway 2003). In the 19th century Louis Pasteur developed the concept of vaccination by demonstrating, almost serendipitously, that an attenuated form of a pathogen could be administered to healthy individuals to protect against the disease (Parish 1965). This provided a major breakthrough in immunology and the current standard of many vaccines. The great achievements in vaccine therapies alongside the technological innovation allowed novel studies to evolve in better understanding the cellular components of the immune system and its mechanisms of action.

The interaction of multiple cellular entities and their function soon became the central theme of immunology. Over a century ago, in 1908, Elie Metchnikoff and Paul Ehrlich received the Nobel Prize for the identification of two distinct

aspects of the immune response: the innate and adaptive (acquired) immunity (Kaufmann 2008). Since the first description of immune cells and their mechanisms of action, significant advances in the field have been made. The past decade has entered a new era based on signaling mechanisms, transduction pathways, gene expression, and molecular interactions. Immunity is no longer only connected to infection and pathogens, but also tissue injury, auto-inflammation and autoimmunity became of paramount interest.

This thesis will focus on how the mechanisms of innate immunity affect a postoperative condition that may reach epidemic proportions in the future.

1.2 Cells and mechanisms of innate immunity

Each living organism has developed a system to counteract potential life threatening events such as infection and tissue injury. The innate immunity is the most conserved defense system shared throughout evolution from simple organisms including prokaryotes (bacteria), invertebrates (insects, worms), plants to human beings (Kimbrell and Beutler 2001). It is considered the basic, phyologenetically ancient mechanism of protection. Its molecules are shared between plants and animals and constitute the first line of defense juxtaposed to the more complex adaptive response, which only characterizes evolved systems (table 1.1) (Medzhitov and Janeway 2000; Matzinger 2002).

Table 1-1: Innate and adaptive immunity

Characteristics	*Cells*	*Molecules*
Innate immunity		
Immediate activation	Leukocytes	Cytokines (IL-1, IL6)
Limited specificity	Macrophages	Chemokines
Conserved molecular patterns	Dendritic	Complement
No memory	Natural killer	Acute-phase proteins
	Mast cells	
Adaptive immunity		
Slower activation	B and T	Antibodies
Highly specific		Cytokines (IL-2)
Details of molecular patterns		Effector cytokines
Memory		(IL-4, INFγ)

Abbreviations: IL: interleukin, INF: interferon

Aside the physical barriers, such as the skin, innate immunity evolved different mechanisms to fight infections. The initial observation by Metchnikoff that some white cells could phagocyte foreign materials established the concept of innate immunity. Neutrophils, monocytes and macrophages can directly engulf and destroy microorganisms as well as rapidly produce inflammatory mediators such as cytokines, reactive oxygen species (ROS), reactive nitrogen species (RNS), enzymes and lipid mediators. According to the phenotype (e.g. inflammatory, cytotoxic) and the nature of a threat, circulating monocytes can either differentiate into dendritic cells and function as antigen-presenting cells, or colonize organs such as liver (Kupffer cells), lungs (alveolar macrophages) and brain (microglia cells), becoming tissue

macrophages. Macrophages, literally meaning “big eaters” (from the Greek *macros*, meaning “large” and *phagein*, meaning “eat”) are classically associated with phagocytosis but also mediate protective actions by releasing trophic factors and anti-inflammatory molecules, making their function paradoxical and necessary for healing and restoring a correct homeostasis (Block and Hong 2005). These cells are also necessary to elicit an adequate immune response by directing and recruiting immune cells to specific sites in a process known as chemotaxis. Mechanical and molecular processes (adhesion molecules, chemokines and complement activation) are required to initiate this process that is fundamental in response to infection and, if this fails it results in poor prognosis and increase mortality rate for example in sepsis or multiple organ failure (Tavares-Murta et al. 2002). Neutrophils and monocytes are primarily associated with the margination process and movement across cellular compartments and sites (Wilson et al. 2009). However, it was also observed how these white cells could recruit lymphocytes and other immunocompetent cells at the site of infection to initiate an appropriate immune response, thus interfacing with the acquire component too (figure 1.1). It is important to note how the optimal host defense is achieved by synergism of innate and adaptive response, which shall not be seen as two separate entities.

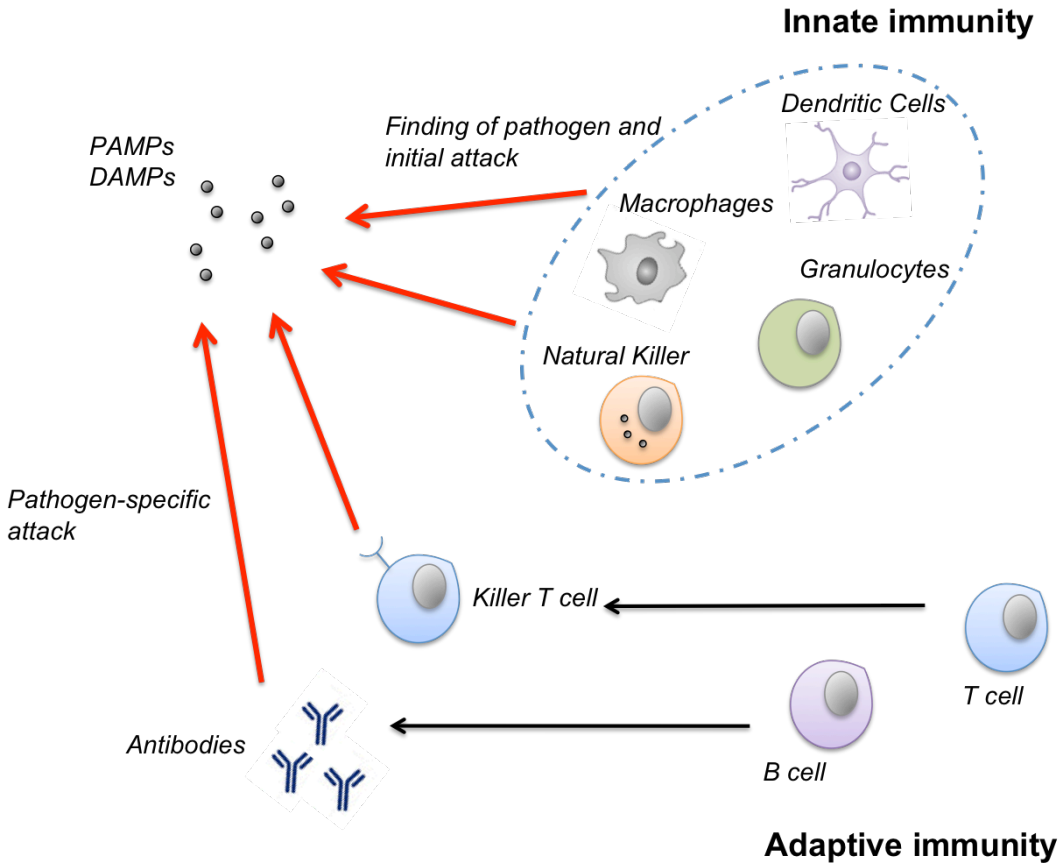


Figure 1-1: **Interaction between innate and adaptive immunity.**

Innate immunity (leukocytes, macrophages, and dendritic cells) rapidly recognizes and engulfs pathogens and microbes. Dendritic cells in particular have the ability to present antigents to T cells, thus actively interfacing with the adaptive component. This bidirectional communication allows both systems to cooperate and to activate mechanisms to protect and defend the organism against a threat. Adapted from (Akira 2009).

1.3 Cytokines and the acute-phase response

Besides the cellular component, innate immunity also relies on the release of soluble mediators (complement, cytokines, acute-phase proteins). These molecules are able to affect multiple biological processes both locally and in target organs. Due to the widespread expression of receptors, communication and amplification of the inflammatory response can be achieved by diverse mode of actions, usually in a short-range (paracrine – affecting other cells, or autocrine – affecting themselves) but sometimes over long distance (like hormones).

Cytokines (from the Greek *cyto*, meaning cell and *kinos*, movement) have a pivotal role in orchestrating the inflammatory response after viral or bacteria infection and are essential in restoring homeostasis after a challenge in the host organism (Matzinger 2002; Harris and Raucci 2006); they also affect behaviour, especially memory and cognition (Kelley et al. 1997; Pugh et al. 1998). Cytokines can be broadly categorized as interferons (anti-viral substances), interleukins (generated by leukocytes), monokines (generated by monocytes), chemokines (chemotactic factors), haematopoietic colony stimulating factors and connective tissue growth factors. In the past thirty years, cytokines and their cognate receptors have been extensively studied in many conditions from inflammatory diseases to cancer. Already found in invertebrates and evolved into the animal kingdom, they are very ancient molecules (Bird et al. 2002; Opp 2004). This large family of low-molecular weight protein mediators typically acts on a short-range distance and its role can be appreciated in almost every biological process ranging from inflammation, cell proliferation, fibrosis, healing, and angiogenesis (Feldmann

2008). Since the identification of these molecules in the pathogenesis of many disease states, cytokines became of great interest especially as plausible targets for specific therapeutics. Lipopolisaccharide (LPS), a predominant glycolipid in the outer membrane of Gram-negative bacteria, is a classic compound that stimulates monocytes, macrophages, and neutrophils to produce cytokines and a plethora of other pro-inflammatory mediators (Morrison and Ryan 1987). Although in the past decade far less effort has been devoted in understanding basic mechanisms of the innate immunity, recent work is revaluating its function. Innate immunity is essential in the process of counteracting possible threats without requiring clonal selection and expansion or receptor rearrangement, thus ensuring a rapid initiation of the immune system.

1.4 Signaling processing and inflammation

Innate immune response has been classically associated with the recognition of pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs). The mechanisms of PAMPs recognition exemplify the basic concept of self / non-self discrimination in activating a first line defense mechanism that alerts and protects the host organism against a possible threat. Microbes, bacteria and viruses all express highly conserved molecular patterns, for example peptidoglycan or LPS, which are distinctively recognized by immune cells. Recognition of these patterns is achieved via a set of structurally and functionally heterogeneous proteins known as pattern

recognition receptors (PRRs), the most important being the C-type lectin domain, scavenger receptor cysteine-rich domain and leucine-rich repeat (LRR) domain, which activate the innate immunity to release pro-inflammatory molecules such as cytokines (Medzhitov and Janeway 1997). Opsonization (activation of the complement system), coagulation, phagocytosis, activation of downstream signaling pathways, release of pro-inflammatory molecules, induction of transcription factors and apoptosis are all phenomena mediated by PRRs and all constitute crucial mechanisms of the innate immunity (Medzhitov and Janeway 2000; Janeway and Medzhitov 2002). PRRs are ubiquitously expressed in the body and highly conserved across species.

The phylogenetic conservation of these receptors throughout evolution has facilitated the understanding of inflammatory processes from simpler organisms to ultimately human complexity. Infection is a classic instigator of the inflammatory response and has been repeatedly used to better understand the inflammatory process. The pathological aspects of **inflammation**, from the Latin *inflamatio*, meaning “to set on fire” have been long known since the first description by Aulus Cornelius Celsus (25 B.C – 45 A.D *circa*). In his treatise *De Medicina*, a major source of information on early medical procedures, diseases and treatments, he described the four cardinal signs of inflammation: *rubor* (redness), *dolor* (pain), *calor* (heat), and *tumor* (swelling). Nevertheless the physiological role of inflammation yet remains elusive. Generally regarded as a necessary response to restore the correct homeostasis in the host organism after a challenge, inflammation can become uncontrolled and potentially fatal for instance during septic shock (Waage et al. 1989; Ackland et al. 2009). Acute inflammation in particular is correlated

with a rapid activation of the innate immunity and the production of a plethora of local inflammatory mediators. Toll-like receptors (TLRs) and NOD (nucleotide-binding oligomerization-domain protein)-like receptors (NLRs) are the two most important families of PRRs essential in counteracting a danger signal and initiating an inflammatory response (Castellheim et al. 2009).

1.5 Toll-like receptors

TLRs are molecules whereby innate immunity gets activated and communicates with the immune system. The discovery of TLRs started in the *Drosophila* fruit fly with the identification of Toll. Initially associated with dorsoventral polarity during embryonic development, in adults it was later described to protect flies against fungal infections (Lemaitre et al. 1996). Loss-of-function mutations in the Toll gene prevent the synthesis of a major antifungal peptide, drosomycin, making *Drosophila* extremely vulnerable to fungal infection and leading to a rapid death. Mutations on other members of the TLR subfamily such as the 18 wheeler have also been demonstrated to be key participants in the antimicrobial response in *Drosophila* (Williams et al. 1997). These findings really illustrated the involvement of these receptors in recognizing pathogens and specifically activating the immune response, theory that Charles Janeway postulated almost thirty years ago in his significant prediction of the link between innate and adaptive immunity (Rudensky and Chervonsky 2003).

At present, twelve members of TLRs have been found in mammals and they share different degrees of homology with another membrane-bound class of receptors of the innate immunity as part of a larger superfamily that includes the interleukin-1 receptor (IL-1R). TLR1 – 9 are shared between humans and mice. Most of what is known about their ligands and signaling is coming from mice lacking in the expression of a particular receptor. TLRs are type I integral membrane glycoproteins; the cytoplasmic region shares a significant homology with the IL-1R, thus it is referred as the Toll/IL-1R (TIR) domain (Medzhitov 2001; Takeda and Akira 2004; Frantz et al. 2007). By contrast, the extracellular domain largely differs between TLRs and IL-1Rs since the former has 19-25 tandem copies of the LRR motif and the latter contains three immunoglobulin-like domains (Akira and Takeda 2004). Classification of TLRs has been conveniently made according to their cellular localization, which correlates to some extent to the ability of recognizing specific ligands. TLR1, 2, 4, 5, and 6 are expressed on the cell surface and generally recognize microbial component such as lipids, lipoproteins, and peptides. By contrast, TLR3, 7, 8 and 9 are involved in the recognition of nucleic-acid-like structures and are expressed in intracellular vesicles such as the endoplasmic reticulum or lysosomal compartments (Zhang and Ghosh 2001; Yang et al. 2008; Kawai and Akira 2009). Even if TLRs share different degrees of homology and structural conservation, several unique molecular patterns originated both by pathogens and endogenous ligands are recognized by each receptor (table 1.2).

Table 1-2: **List of TLR2 and TLR4 ligands**

Modified from (Kanzler et al. 2007; Miyake 2007)

DAMPs	Source	Receptor	PAMPs
HSP60	Necrosis	TLR 2 / 4	Tryacil lipopeptide (Pam2CSK4), lipoteichoic acid, zymosan, bacterial peptidoglycan, LPS, phospholipids, envelope proteins
HSP70	Necrosis	TLR 2 / 4	
gp96	Necrosis	TLR 2 / 4	
HSP22		TLR 4	
Biglycan	ECM	TLR 2 / 4	
Hyaluronan	ECM	TLR 2 / 4	
Fibronectin ectodomain A	Tissue injury	TLR 4	
Surfactant protein A	ECM	TLR 4	
HMGB-1	Nucleus	TLR 2 / 4	
LDL		TLR 4	
β-defensin		TLR 2 / 4	

Abbreviations: HSP: heath shock protein, HMGB-1: high-mobility group box-1, LDL: low density lipoprotein, ECM: extracellular matrix, TLR: toll-like receptor, LPS: lipopolysaccharide

1.6 TLR signaling

Amongst all TLRs, TLR4 can be considered the human homologue of the *Drosophila* Toll and is the most active interface to bridge microbial infection with inflammation (Levy et al. 2006; Mollen et al. 2006; Kigerl et al. 2007). Its role in mediating inflammation after LPS-endotoxemia has been extensively studied (Hoshino et al. 1999), thus I will use it to exemplify the signaling pathway and the molecules which are involved in this process. Initiation of the innate effector response and activation of transcription factors to induce

synthesis of pro-inflammatory mediators strongly relies on conformational changes and dimerization of the TIR domain after ligand binding to the receptor. Key molecules in this downstream signaling include the myeloid differentiation factor (MyD88), IL-1R-associated kinase (IRAK), tumour-necrosis-factor-receptor-associated factor 6 (TRAF6) and nuclear factor-kappa B (NF-κB).

MyD88 was initially found in response to IL-6 stimulation, but only in 1997 was discovered in mediating signaling in the IL-1R, and soon thereafter in TLRs (Burns et al. 1998). This small protein has a modular structure composed of a C-terminal TIR domain and an N-terminal death-domain (D-D). This enables interaction both with the receptor (TIR domain) and in mediating phosphorylation of IRAK (D-D), providing significant signaling capabilities (Wesche et al. 1997; Janssens et al. 2002). Largely utilized by all TLRs, with the exception of TLR3, MyD88 is an essential component in activating transcription factors including NFκB and two mitogen-activated protein kinases (MAPK), p38 and JUN N-terminal kinase (JNK). Signaling specificity of individual TLRs is achieved by selective recruitment of signaling adaptors. Four main positive regulators have been described in TLRs signaling: MyD88 (all except TLR3), Mal (MyD88-adaptor like – in TLR2 and TLR4), TRAM (TRIF-related adaptor molecule – only in TLR4), and TRIF (TIR-domain-containing adaptor protein inducing IFN-β – in TLR4).

MyD88 signaling is essential in orchestrating several inflammatory pathway; MyD88-deficinet mice have reduced cytokines and appear protected in sepsis and infection (Kawai et al. 1999; Takeuchi et al. 2000; Weighardt et al. 2002). Recruitment of MyD88 leads to activation of IRAK4 through D-D interactions,

which subsequently phosphorylate IRAK1 (Janssens and Beyaert 2003). It has been also observed that also IRAK1-deficient mice have impaired, but not abolished, production of cytokines in response to both LPS and IL-1; however no response is found in IRAK4-deficient mice, thus making IRAK1 a direct substrate of IRAK4 and not *vice versa* (Thomas et al. 1999; Suzuki et al. 2002). Interestingly, also human patients with mutations in IRAK4 show unresponsiveness to IL-1, IL-18 and failed to respond to five TLRs ligands (at TLR2, TLR3, TLR4, TLR5 and TLR9) (Picard et al. 2003). Downstream signaling from IRAK converges to TRAF6, an evolutionary conserved ubiquitin protein ligase. TRAF6 activates TGF-β-activated kinase 1 (TAK1), leading to activation of the IKK complex and MAPK pathway, which activates NFκB and JNK/ p38 MAPKs, respectively. This signaling pathway is illustrated in figure 1.2.

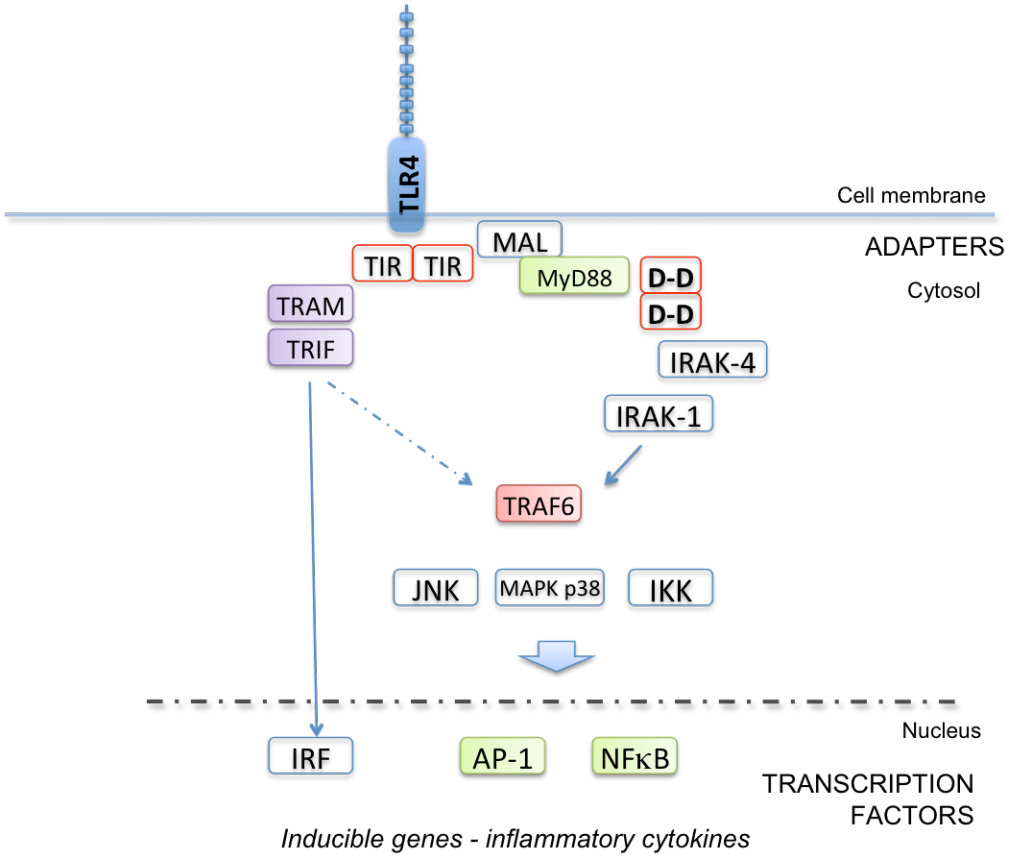


Figure 1-2: **TLR4 signaling**.

TLR4 signaling shares many homologies both with the IL-1R and other TLRs. Activation of MyD88-dependent signaling relies on recruitment of Mal (MyD88-adaptor like), which is an intermediate membrane proximal adaptor in the TLR4 signaling. This leads to the recruitment of the IL-1R-associated kinase (IRAK). Phosphorylation of IRAK signals to tumor-necrosis-factor-receptor-associated factor 6 (TRAF6), which affects two distinct downstream pathways: NFkB and activator protein 1 (AP-1). p38 MAPK and JUN N-terminal kinase (JNK) mainly signal to AP-1 promoting a rapid activation of pro-inflammatory genes (Lotze and Tracey 2005). Nuclear translocation of NFkB and translation of inflammatory cytokines is driven by phosphorylation of the IkB kinase (IKK) complex and also depends on the activation of TRAF6 (O'Neill 2000).

MyD88-independent signaling via TRAM (TRIF-related adaptor molecule), and TRIF (TIR-domain-containing adaptor protein inducing IFN-β) can also activate NFkB. However, this signaling also activates interferon regulatory factor 3 (IRF-3) to induce interferon β (INF-β) (Takeda et al. 2003). It is suggested that MyD88 mediates the early phase activation of NFkB, whereas TRAM perpetuates the signaling to a deferred stage (Takeda and Akira 2005).

Recently a MyD88 independent pathway has been proposed both in TLR4 and IL-1R signaling (Watters et al. 2007; Kenny and O'Neill 2008). Signaling via TRAM and TRIF bypasses MyD88 in TLR4, leading to alternative activation of NFkB and IRF (IFN regulatory factors). Despite the existence of Myd88-independent pathways, this adaptor molecule maintains a pivotal role in promoting transcription of many pro-inflammatory genes in different disease states.

1.7 Interleukin 1 signaling

Since the first description of IL-1 as the endogenous pyrogen, numerous functions of this molecule have been described in controlling endocrine, immune, and nervous functions (Saper and Breder 1992). IL-1 is a classic mediator of the body's response to inflammation and has a pivotal role in immune-to-brain signaling and in modulating many innate immune processes (Dinarello and Savage 1989). The IL-1 family is composed of 11 members: IL-1α, IL-1β, IL-1 receptor antagonist (IL-1 Ra) and IL-18 have been extensively studied cell cultures, animal models, and humans. IL-1β can be considered the prototypic multifunctional and pleiotropic cytokine especially due to its widespread effects on immunity and diseases, appetite regulation, synaptic plasticity, sleep modulation, endocrine functions, and memory functions (Dinarello 1996; Murray and Lynch 1998; Luheshi et al. 1999; Rachal Pugh et al. 2001; Ikegaya et al. 2003; Spencer et al. 2004; Yasuda et al. 2005; Lai et al. 2006). Blood monocytes, tissue macrophages and dendritic cells primarily

account for the production of this cytokine, however a self-induction mechanisms has also been demonstrated both *in vitro* and *in vivo* (Dinarello et al. 1987).

Production of both IL-1β and IL-18 is initially achieved by an inactive cytoplasmic precursors (i.e. pro-IL-1β and pro-IL-18), which require enzymatic cleavage by caspase-1 in order to become active (Dinarello 1998). This process usually occurs in specialized secretory lysosomes or in the cytoplasm but a number of pathways seem to mediate processing and exiting the cell (exocytosis, microvesicles shedding, and transporters) (Andrei et al. 1999; Andrei et al. 2004; Qu et al. 2007). Several intracellular proteins constitute the molecular platform known as the NALP3 inflammasome that regulates the cleavage and release of IL-1β into the mature and active form. Activation of caspase-1 is regulated by the NALP3 gene, or cryopyrin/CIAS1/PYPAF1, which is a member of the aforementioned NLR family (Ogura et al. 2006). Multiple pathways lead to activation of caspase-1 and are summarized in figure 1.3.

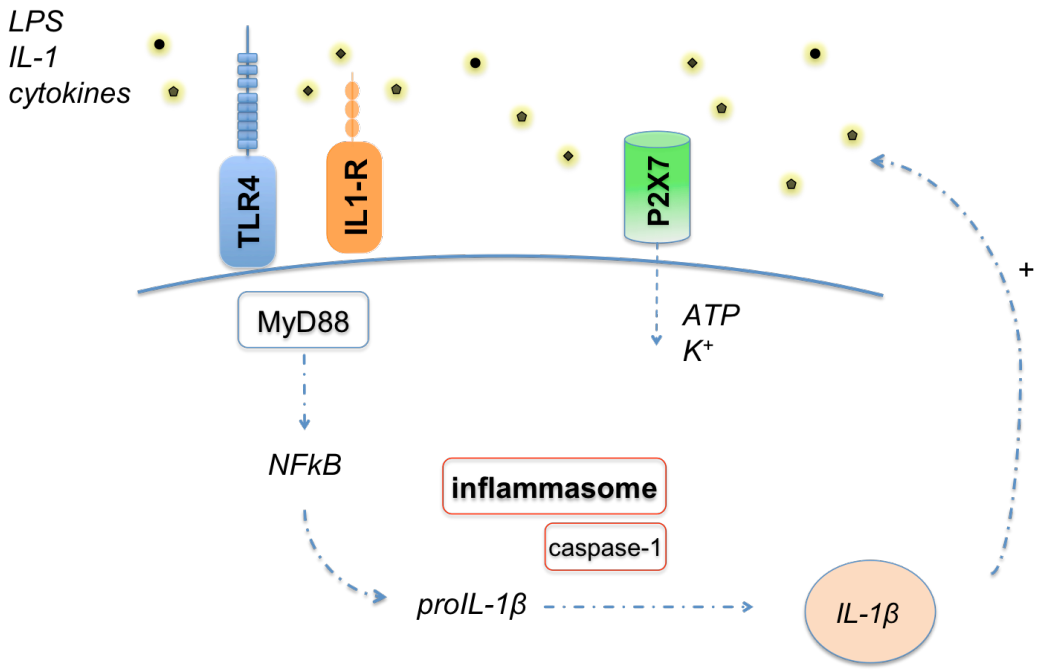


Figure 1-3: **Signaling and regulation of IL-1β**

Production and regulation of IL-1β is controlled by several mechanisms. Direct stimulation of the IL-1 R, via IL-1, or activation of TLR4, via PAMPs or DAMPs signaling, leads to *de novo* production of IL-1 and other pro-inflammatory cytokines. Activation of NFkB allows transcription of IL-1β mRNA. Translation to the functional protein occurs by enzymatic cleavage of the 31 kDa precursor molecule, which gets translated to the bioactive 17 kDa form. This process is regulated by the inflammasome and caspase 1, which is affected by cytokines as well as other factors including ATP and increased K^+ release. Synthesis of new IL-1 engages in a positive feedback loop with the receptors; endogenous regulators like the IL-1 receptor antagonist interfere with this process blocking the interaction of the bioactive form with the type I IL-1 receptor. Modified from (Netea et al. 2008).

Activation of caspase-1 inflammasome complex relies also on cytosolic receptors, NLRs, which are mainly involved with the immune regulation and apoptosis (Kawai and Akira 2009). Tightly controlled and well-regulated mechanism, apoptosis requires efficient signaling to induce blebbing and structural shrinkage of the cell combined with DNA fragmentation, chromatin condensation and formation of the membrane bound vesicles (apoptotic bodies). This process is energy adenosine triphosphate (ATP)-dependent and engages in a complex signaling pathway requiring both external and internal signals ("death activators", cytochrome c, caspases); apoptosis is a normal and essential process that elicits a controlled response or "quiet" death of the cells, without incurring in an inflammatory response. Tissue injury (mechanical stimulation) or toxic compounds can however directly disrupt cells leading to inflammation of the surrounding tissues in an antithetical process referred as necrotic cell death.

IL-1 has been chiefly correlated with both sickness behaviour and cognitive impairments (Takao et al. 1990; Gibertini et al. 1995; Gibertini et al. 1995; Nguyen et al. 1998; Pugh et al. 1999; Rachal Pugh et al. 2001), however is still unknown known how IL-1β in particular leads to cognitive dysfunction. Concentration of cytokines in the normal brain is usually negligible and the main source of brain IL-1β is from activated microglia (Pinteaux et al. 2002; Caza et al. 2008). Additional physiological (pyrexia), endocrinological, especially the resetting of the hypothalamic-pituitary-adrenal (HPA) axis, and metabolic (synthesis of acute phase response proteins) changes fully complete this adaptive behaviour (Dantzer 2004). Several downstream effectors have been implicated with this process, including stress-related

MAPK, transcription factors and microglial activation (Ma et al. 2005), alongside the elaboration of further molecular markers including TNF-α, metalloproteinases, cyclo-oxygenase 2, and corticotrophin releasing factor (Roe et al. 1998; Serou et al. 1999). Complementary interactions and synergism with other pro-inflammatory cytokines such as TNF-α and IL-6 enhance the behavioural depression, providing great complexity in dissecting the signaling pathways and roles of each mediator (Dantzer et al. 1998; Pugh et al. 1999; Konsman et al. 2002; Allan et al. 2005; Godbout et al. 2005). In particular, IL-1 and TNF-α can act directly but also synergize with each other (Dinarello et al, 1986).

1.8 TNF signaling

Many cell types can produce TNF, however monocytes / macrophages are considered to be the major producers. LPS can be considered the prototypic and best characterized inducer of TNF, making it an essential molecule in many inflammatory processes as well as disease pathogenesis. With a defined physiological and homeostatic role in protecting against infections or in the healing process (review Aggarwal & Natarajan, 1996), excessive production or chronic expression make TNF a key target for many diseases including rheumatoid arthritis. Since its first identification over 10 years ago for the ability of killing tumors *in vitro*, the TNF superfamily has been extensively studies especially in relationship to infection and injury. Due to the ability of this molecule to rapidly recruit and induce adhesion molecules as

well as producing a myriad of chemoattractant molecules, is considered a putative target for many therapeutic avenues.

Two different receptors mediate downstream signaling: p55 (TNF-R1) and p75 (TNF-R2). The p55 TNF-R is widely expressed on almost every cell, p75 TNF-R is found on cells of the immune system and primarily on endothelial cells. Proteolytic cleavage of these receptors results in the production of soluble TNFRs, which can antagonize the biological role of TNF (Gray et al, 1990). Downstream signaling also activates NFkB and MAP kinases (ERK, p38 and JNK) leading to cytokines up regulation, and regulation of apoptotic and cell-death signaling (figure 1.4) (Liu 2005).

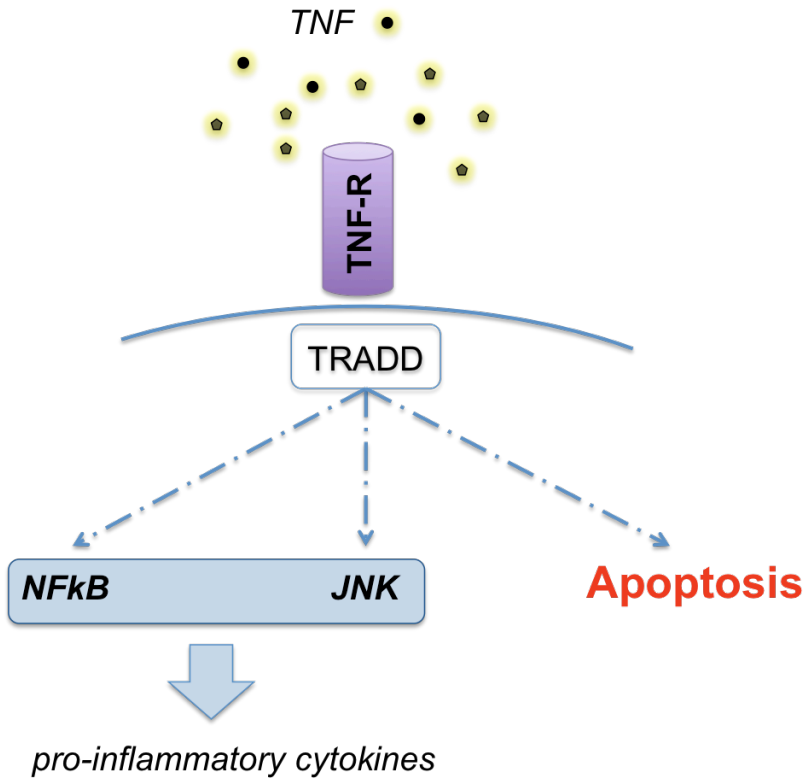


Figure 1-4: **TNF-α signaling**

TNF has a crucial role in regulating both pro- and anti-inflammatory cytokines, including IL-1, IL-6, IL-10, IL-1 Ra, sTNFR. Upon stimulation of the receptor, plasma membrane-associated TNF-receptor I trimerize and recruits TRADD (tumor necrosis factor receptor type 1-associated death domain protein) via D-D interactions. Downstream of TRADD, two main pathways are activated: apoptosis via the caspases cascade, and inflammation via NFkB and JNK regulation (Verstrepen et al. 2008).

1.9 Sickness behavior

Systemic infection and inflammation are often associated with physiological and behavioral changes in humans and animals alike (Hart 1988). The body's response to infection can be ancestrally correlated to a protective mechanism that limits and prevents an animal to become exposed in a potentially dangerous context. The ensuing "sickness behaviour" is classically characterized by a decline in cognitive function, fever, decreased food intake, somnolence, hyperalgesia, and general fatigue (Konsman et al. 2002; Dantzer 2004). These symptoms usually associate with a systemic release of cytokines and neuroinflammation in different brain regions including the hippocampus, amygdala and limbic system (Annane 2009). For peripheral immune messages to reach the brain and cause neuroinflammation, two main pathways are activated. Nerve conduction, especially from the primary afferent neurons that innervate the site of injury, or via afferent vagal projections to the brainstem act as a fast processor that directly conveys to the brain (Wan et al. 1994). The secondary pathway is slower and relies on the production of cytokines to ultimately reach the central nervous system (CNS) (Konsman et al. 2002; Quan and Banks 2007). Diffusion of cytokines into the brain occurs either via specific transporters and in the circumventricular organs (CVOs), resulting in a direct activation of brain resident immune cells (microglia) (Banks et al. 1995; Plotkin et al. 1996; Pan and Kastin 2002; Pittman 2003; Quan 2008). Furthermore, inflammatory mediators including cytokines (Wan et al. 2007; Kobbe et al. 2008), chemokines (Gao et al. 2005), and matrix metalloproteinases (Rosenberg

2002; Colnot et al. 2003) are likely to compromise the stability of the blood-brain barrier (BBB) itself, resulting in an increased permeability by a direct proteolytic action on the tight junctions, thus allowing the aforementioned molecules and potential pathogens in the brain (Huang et al. 2006; Zhao et al. 2007; Rudolph et al. 2008; Wang et al. 2008). Thus sickness behaviour becomes a tangible consequence of neuroinflammation (Dantzer 2004).

The transduction of peripheral inflammation into the brain results in the activation of microglia, the intrinsic macrophages of the CNS (Aloisi 2001) (figure 1.5). Upon sensing changes in brain homeostasis, for instance in the presence of inflammatory molecules, microglia shift from the normal resting state becoming *reactive* and mount macrophage-type innate immunity within the CNS (Raivich et al. 1999; Schubert et al. 2000; van Rossum and Hanisch 2004; Bianco et al. 2005; Bernardino et al. 2008). During this process the cells undergo stereotypical conformational changes both in morphology, acquiring an amoeboid shape, and function (Kim and de Vellis 2005). They also release cytotoxic factors and cytokines, but they also mediate protective actions by releasing neurotrophic and anti-inflammatory molecules, making their function paradoxical and yet not fully understood (Block and Hong 2005). Activated microglia secrete pro-inflammatory cytokines (including IL-1 and TNF-α) (Aloisi 2001), ROS, excitotoxins (such as glutamate and ATP) (van Rossum and Hanisch 2004), putative neurotoxins such as amyloid precursor protein and as well as blood-derived factors, serum factors, chemokines and pathological proteins (Farber et al. 1995; Garden and Moller 2006). Activated microglia can inhibit neurogenesis in the hippocampus following LPS, thereby impairing synaptic plasticity and hippocampal-

dependent memory processes (Barrientos et al. 2002; Ekdahl et al. 2003; Monje et al. 2003). Interestingly, activation of microglia is also causally related to impairment of long-term potentiation (LTP) in advanced age (Griffin et al. 2006). Activation of microglia appears necessary for cognitive dysfunction associated with several neurological insults including sickness behaviour and hypoxia-ischaemia and associates with the neuroinflammation in many neurodegenerative processes ranging from Alzheimer's disease (AD), to surgery-related complications (postoperative cognitive dysfunction [POCD]) (Fan et al. 2006) (Kim and de Vellis 2005; Tanaka et al. 2006; Wan et al. 2007). Nonetheless the putative association between cytokine release, microglial activation, and memory dysfunction remains elusive.

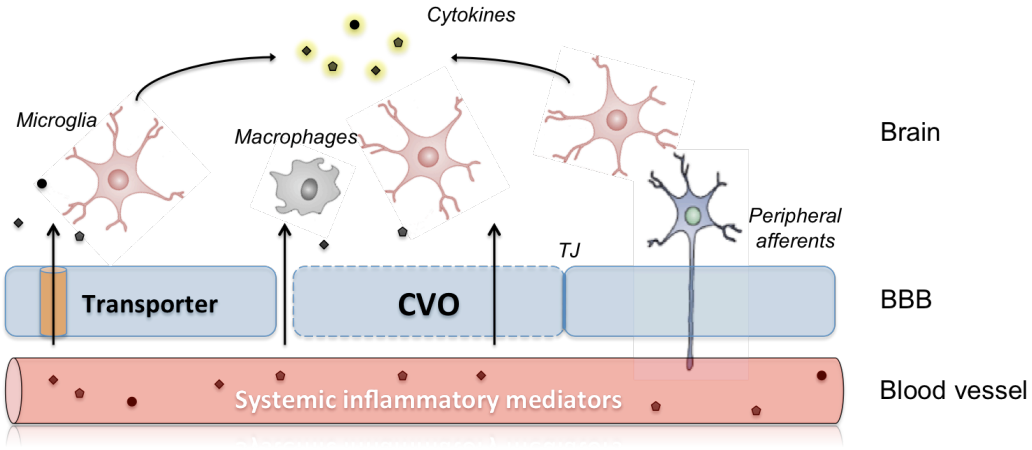


Figure 1-5: **Immune-to-brain communication pathways.**

1) Cytokines penetrate the BBB via selective transporters that actively pump cytokines in the brain.
2) CVOs and the choroid plexus lack a patent BBB and cytokines directly leak into the brain. CVOs act as a crucial interface in transducing peripheral inflammation to the brain and sensing inflammatory mediators thanks to the high vascularization and expression of PRRs.
3) Peripheral afferents arising from the site of injury mediate immune-to-brain signaling and activate microglia in the CNS. Inflammatory mediators from the blood vessels, especially MMP-9 and chemokines, directly damage the BBB by compromising the tight junction (TJ) integrity. This process produces new fenestrations in the BBB allowing cytokines as well as immunocompetent cells (macrophages, monocytes) from the periphery into the brain. Overall, entrance of pro-inflammatory mediators into the brain is sensed by PRRs on microglia cells. Their activation will trigger release of new cytokines in the brain producing neuroinflammation and eventually affecting higher cognitive functions.

1.10 Learning and behaviour

Virtually every organism, from simple animals to the complexity of human beings, has developed mechanisms to learn from the environment and acquire knowledge about the world. Both innate and learned responses play a pivotal role in determining the personality of each individual. Innate responses are inherited processes that have evolved to protect and help organisms towards primary necessities and survival (food, reproduction, avoidance of dangerous situations). These stereotypical responses are often inflexible and hard to modify but provide a basic tool to respond and adapt to unpredictable events in a given environment. Learning and memory are basic mechanisms in adaptation and evolution, influencing the nervous system at multiple levels (morphology, biochemistry, functions). Whereas learning is the process whereby the knowledge and new information are acquired, memory is defined as the ability of retaining and storing this information.

In the mid-twentieth century, the field of learning and memory was influenced by a great uncertainty whether memory was a discrete function localizable to specific brain regions. The discoveries of Broca in defining specific language deficits restricted to the posterior portion of the frontal lobe on the left side of the brain posed the question whether memory functions could also be localized to specific brain areas. In the 1950s Wilder Penfield begun to explore and map motor, sensory, and language functions in human patients undergoing neurosurgery, introducing the concept that memory may rely and use multiple systems. Soon after, Brenda Milner studied the effects of temporal lobe removal and bilateral hippocampal ablation in patients suffering from epilepsy. The fascinating story of the patient Henry Molaison, perhaps

better known by neuroscientists and psychologists as H.M., revealed the crucial role of the hippocampus in forming new memories. After bilateral removal of the medial portion of the temporal lobes due to untreatable epilepsy, the seizures that daily affected H.M. were greatly reduced. However, as a consequence of this procedure, H.M. lost the ability of producing new long-term memories, what in clinical terms is referred as anterograde amnesia. Despite a good use of language and vocabulary, the ability of retaining past memories prior to the operation and the maintenance of short-term memories, H.M. was unable to retain new information after few minutes. After the hippocampal ablation, the transfer of information from the short to long-term storage was impaired. This demonstrated that the hippocampus is necessary for forming new long-term memories but is not required for maintaining or retrieving them. Furthermore, H.M. maintained a relatively intact learning of motor skills. This led Milner to differentiate between procedural memory (memory for a skill) and declarative memory (conscious recall of having used that skill, memory for facts) (Bear 2001).

1.11 Classical conditioning

Human studies asides, memory and ensuing behavioural changes have been thoroughly studied also in animals. The serendipitous discovery by the Russian physiologist Ivan Pavlov introduced the basic concept of classical conditioning, or Pavlovian conditioning. Interested in understanding neuronal mechanisms of glandular secretion during digestion, Pavlov studied salivation

in dogs by administering food powder in the animal's mouth and measuring the amount of secreted saliva. He found that food elicited salivation in dogs, but more remarkably, once that dogs became experienced after several trials, they started salivating as soon as they saw the laboratory assistant coming in to feed the animals. Pavlov designed a more controlled experiment in which before offering food powder a bell or another auditory stimulus was played. Similarly, after a number of pairings, the dogs learnt to salivate when the bell rang in the absence of food, which was no longer needed to trigger salivation. Thus Pavlov concluded that a similar response to the original reflex (salivation) could be elicited if a neutral stimulus predicts the occurrence of a significant incentive, such as food (Carlson 2003). Food, a natural reward that can elicit an innate response is known as the unconditional stimulus (US) and produces an unconditional response (UR), the salivation. When a previously neutral stimulus (a tone) is paired with the US and produces a similar effect (salivation), it is referred as a conditional stimulus (CS), and the following behavioural response is known as conditional response (CR). After the association, the former neutral stimulus (US) acquires a positive value and can act as a predictor of a pleasant event, establishing memory. The significance of classical conditioning can be appreciated in accomplishing two main functions:

1) to allow an organism to respond faster and more effectively to a stimulus that predicts an important event (this requires the subject to learn to recognize the stimuli and remember the association of events)

2) this behavioural modification is a product of a previously neutral, perhaps futile, stimulus that acquired importance and properties to be subsequently remembered

The essence of classical conditioning is based on the association of two stimuli, the CS and US. Classical conditioning relies on different phases and several learning principles. Indeed the cause – effect relationship occurs only when the rewarding (or warning) stimulus is carefully sequenced and timed in order for an initially neutral stimulus to elicit reflexive behaviour. Establishment of learning and memory hardly relies on a single event; after different trials an innate reflexive behaviour can be eventually elicited. Only with repeated CS – US pairings the conditional response gradually appears. Both stimuli have to be presented simultaneously, or with a small delay in which the CS always precedes the US, and no other confounding cues should be provided during this phase.

1.12 Fear conditioning

The principles of classical conditioning have been exploited in many behavioural models, and this thesis will focus on the use of fear conditioning. Innate responses, both in animals and humans, are able to trigger strong outcomes; fear learned response requires rapid associations and reliable actions during learning and memory processes. Primordially correlated with

survival, learning of fearful events can be extremely rapid (even limited to a single association) and long lasting, making fear a suitable tool for many experimental settings. In the model presented in this thesis mice learnt a conditioned fear response in which a neutral stimulus (tone – CS) is temporally associated with a noxious stimulus (foot shock – US). Intensity of the US and timing of the CS greatly affect the speed and rate of learning, making it faster if both stimuli are intense and carefully timed; this phase is known as acquisition. After pairing the neutral and aversive stimuli, the neutral cue will itself be able to elicit an aversive response from the animal. The innate reaction to fear in rodents is immobility (freezing), an element that can be scored as an index of memory (Quinn et al. 2005). Freezing is defined as the complete absence of mobility with the exception of respiratory movements (Kim and Fanselow 1992). Because of the type of conditioning used in these experiments that does not purely rely on a unique sensorial modality, different brain areas can be assessed, including the hippocampus (Fanselow 1980; Phillips and LeDoux 1992). Contextual fear conditioning relies on the same tone – shock principle but the subject also learns to associate the environment with the US. Contextual fear conditioning is modulated by two key elements: forming a memory of the context and associating that memory with the shock. Learning, memory consolidation and reproduction of the context require higher faculties that strongly rely on the hippocampus. There are substantial evidence to support the role of this structure in spatial learning and contextual fear memory through the use of other techniques like surgical lesions, pharmacological manipulations, or genetic manipulations (McEchron et al. 1998; Quinn et al. 2002; Koh et al. 2009). Using this paradigm,

hippocampal functioning is pivotal during the acquisition phase when CS – US stimuli become associated but also, once this association is made, the hippocampus is required to create a uniform reconstruction of the context. The contextual retrieval test (context task) aims to target and primarily assess this hippocampal-dependent memory. Hippocampal damage following fear conditioning reflects many parallels to clinical cases of amnesia following hippocampal damage (Kim and Fanselow 1992). Trace fear conditioning also allows assessment of non-hippocampal pathways, in particular the prefrontal cortex and the amygdala (Han et al. 2003; Runyan et al. 2004; Misane et al. 2005). In particular, monosynaptic projections from the geniculate nucleus and neocortical pathways present auditory information to the lateral amygdala, which encodes emotional memories (LeDoux 2000). By testing in a novel environment (tone task), the assessment is not tainted by the fear instilled by the acquisition environment and this can be relates to hippocampal functioning (Romanski and LeDoux 1993). This thesis will primarily focus on the hippocampal-dependent component of the behavioral dysfunction, other brain regions are currently under investigation.

1.13 Memory and neuroinflammation

The hippocampus (from the Greek *hippos*, “horse” and *kampos*, “sea monster”) is a primitive *C*-shaped structure in the limbic system composed of three distinct regions known as the dentate gyrus (DG), the hippocampus proper (CA1, CA2 and CA3) and the subiculum. Situated in the deep caudal

part of the brain, it plays a pivotal role in learning, processing and storing long-term memories (only declarative memories), navigation and cognitive maps. Learning and memory processes largely rely on the hippocampus and this brain region expresses the highest density of IL-1 binding sites, making it particularly vulnerable to the adverse consequences of neuroinflammation (Kim and Fanselow 1992; Ban 1994; Parnet et al. 1994; Gemma et al. 2005). Although IL-1β is required for normal learning and memory processes, exogenous administration or excessive endogenous levels produce detrimental cognitive behavioral effects (Oitzl et al. 1993; Gibertini 1998; Barrientos et al. 2008; Chen et al. 2008). The neurobiological correlate of memory consolidation involves LTP in several hippocampal pathways. In mammals, an increase in synaptic strength, or LTP, is considered to constitute the cellular mechanism for encoding memories (Bliss and Lomo 1973). This phenomenon is observed in brain areas, such as the hippocampus, amygdala, cerebellum and cerebral cortex. LTP improves the ability of two neurons, one presynaptic, and the other, postsynaptic, to communicate with each other across a synapse. IL-1β directly inhibits LTP by modulating surface expression and phosphorylation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs), an ionotropic transmembrane receptor for glutamate fundamental in mediating fast synaptic transmission in the CNS (Lai et al. 2006). Synergistic interaction between IL-1β and other cytokines, such as TNF-α and IL-6, enhances this cognitive dysfunction (Dantzer et al. 1998; Pugh et al. 1999; Konsman et al. 2002; Allan et al. 2005; Godbout et al. 2005). TNF-α also affects LTP by changing the balance between AMPARs and gamma-aminobutyric acid $_A$ ($GABA_A$) in the

hippocampus and IL-6 can enhance glial activation and inflammation, directly affecting working memory (Stellwagen et al. 2005; Sparkman et al. 2006).

1.14 Surgery-induced memory dysfunctions

Systemic and central release of cytokine after a surgical procedure has become a noteworthy event associated with cognitive dysfunction (Moller et al. 1998; Gao et al. 2005; Kalman et al. 2006; Beloosesky et al. 2007). The observation that after an operation some patients are more likely to develop cognitive deterioration and changes in personality has also been long known. Hippocrates introduced the concept of mental confusion when describing the symptoms of "phrenitis", which was characterized by fluctuating unpredictability and associated with acute illness and fever (Morandi et al. 2008). Aulus Cornelius Celsus in *De Medicina* further described the milieu of symptoms including sudden confusion, cognitive decline, inertia, impairment in attention and circadian rhythm that defined as delirium (from the Latin *de* – *lira*, in agricultural terminology meaning "to be away from plowing in a straight line", thus be deranged). Diagnosis was generally associated with head trauma, fever or mental disorders, mainly involving the elderly, and the terminology of confusion, delirium and dementia was often interchanged and used inappropriately, as synonyms. In 1887 George Savage associate the delirium with surgical procedures and how surgery and anaesthesia may contribute to the development of "insanity".

> "*One or two practical questions arise for the surgeon, one of the most important being whether neurotic inheritance or neurosis in the individual, as proved by previous attacks of insanity, should in any way affect the prognosis in operations, and to what degree it should interfere with operations of convenience not essential for the prolonging or saving life*."
>
> George Savage, British Medical Journal, December 1887

This observation failed to make POCD a plausible hazard of surgery until more recent reports revamped the concept and focused in particular on the role of anaesthetics as possible cause of cognitive dysfunction. Especially in the context of cognitive dysfunctions anesthesia has been, and currently is, studied as a likely contributor in the genesis of this phenomenon. Thanks to the inestimable value of anesthetic agents in providing a reversible, drug-induced loss of consciousness and a balanced amnesia, immobility, muscle relaxation and analgesia (Franks 2006), their association with impairment in cognitive performance became utterly probable. Mortality and morbidity after surgery in the past has been chiefly associated with anesthesia. Nowadays critical incidents rarely happen during the deep sleep; anaesthesia is innovative, safer, and better tolerated, but the question "*is anesthesia safe enough?*" still remains unanswered. Until quite recently, the prevailing wisdom was that the CNS effects of general anesthesia are transient and completely reversible; general anesthesia is known though to affect the brain at many

different molecular targets (Franks 2008) and there are evidence for several general anesthetic agents to produce neurotoxicity and detrimental effects in synaptic circuitry and behavior (Slikker et al. 2007). Animal studies suggest that anesthetics may be responsible for modifications in the brain that outlast their elimination from the body (Futterer et al. 2004). Developmental neurotoxicity in rodents by exposure to ketamine, nitrous oxide, propofol, midazolam, barbiturates, and isoflurane, alone or in anesthetic "cocktails", is classically associated with apoptosis and delayed behavioral abnormalities (Culley et al. 2007; Sanders et al. 2008; Sanders et al. 2009).

Aside developmental toxicity in the newborn, also the elderly patients (>65 year-old) appear to be more vulnerable to the deleterious effects of anesthetics. Co-morbidities and physiological changes induced by the aging process generally reflect to higher vulnerability and greater risks *per se*. With the fastest growing segment of the population in the industrialized world and the highest probability of requiring anesthesia and surgical resources, this age group is becoming the largest segment of surgical patients by 2020 (Etzioni et al. 2003). The number of major surgical interventions requiring anesthesia already exceeds 230 million world-wide (Weiser et al. 2008), making surgery a putative instigator of poor prognosis, increased likelihood of complications and development of neurodegenerative conditions and permanent dementia such as Alzheimer's disease. Animal studies in aged rats suggest that cognitive dysfunction persists several weeks after exposure to a commonly used anesthetic regimen (Culley et al. 2003) and this is associated with change in gene expression in the hippocampus (Culley et al. 2006). Yet, some anesthetics do not produce any disturbance in cognition post-exposure

even in aged rats (Lee et al. 2008) and clinically-evident POCD also occurs after regional anesthesia (Rasmussen et al. 2003).

A relationship between anesthesia, β-amyloid and tau phosphorylation metabolism in particular has been suggested, however mechanisms are still unclear and a number of limitations need to be addressed in these studies (Planel et al. 2007; Dong et al. 2009). Despite the evidence from cell culture data or animal models, randomized clinical trials in older patients undergoing major non-cardiac surgery have reported similar incidence in POCD after regional or general anesthesia, thus making the role of anesthesia yet controversial (Williams-Russo et al. 1995; Rasmussen et al. 2003).

1.15 Postoperative cognitive dysfunction (POCD)

Recently, we explored the role of the neuroinflammatory response to surgery as a possible cause for cognitive dysfunction. POCD is now an emblem in the field of surgery-induced complications, with a significant burden on health care management. Postoperative dysfunctions have been generally classified according to the duration of the behavioral abnormality after surgery and can follow two patterns: early POCD or interval delirium and late POCD (Cohendy et al. 2005). Delirium is commonly seen after surgery, usually soon after surgery (hours to days) and fluctuating over time. Perturbed behavior is characterized by impairments in consciousness, cognition, emotions and stereotypical psychosis may vary between patients ranging from hyperalert to hypoalert, or mixed. Although the dysfunction lasts over a short period of

time, delirium is associated with increased mortality (Ely et al. 2004), greater care dependency, costs (Milbrandt et al. 2004) and prolonged hospitalization (Ely et al. 2001). Due to the dynamic and complex processes that underlie this phenomenon and the significant impact on the intensive care unit, many strategies are looking into prevention and treatment of delirium, especially in older patients that are at higher risk (Ely et al. 2002; McNicoll et al. 2003).

It is possible to diagnose POCD a considerable time after surgery and is defined as a subtle dysfunction in one or more cognitive domains also characterized by impairment of short and long-term memory (i.e. reduced ability to learn or recall information), mood, consciousness and circadian rhythm (Bekker and Weeks 2003; Caza et al. 2008). POCD has been thoroughly studies in the first prospective multicenter study, International Study of Postoperative Cognitive Dysfunction (ISPOCD), involving more than 1200 patients over the age of 60 undergoing major non-cardiac surgery with general anesthesia (Moller et al. 1998). Neuropsychological tests showed that 25.8% of the patients had POCD one week after surgery, and 9.9% showed persistent cognitive deficit 3 months postoperative. This investigation first reported that advanced age, duration of surgery, additional operations, postoperative respiratory and/or infectious complications were significant risk factors in the development of cognitive abnormalities. By deliberately using a less stringent criterion including pre-operative cognitive dysfunction, depressive symptomatology, and the ability to perform daily routines, up to 19.1% of patient over the age of 64 undergoing orthopaedic surgery had POCD at 3 months (Ancelin et al. 2001). Neurological and cognitive complications were first described after cardiac surgery with cardiopulmonary

bypass (CPB), however POCD also affects non-cardiac surgeries (McKhann et al. 1997). The neurocognitive impairment clearly correlates with an increased vulnerability of these patients to further neurological degeneration / dementia, longer hospitalization time, greater co-morbidity and higher mortality risk compared to those that do not develop this postoperative complication. Although age and surgery are consistently reported as important risk factors in the development of cognitive dysfunction, the etiology of POCD remains elusive (Monk et al. 2008). Further studies have sought to identify pathogenic mechanisms due to perioperative factors [such as general *vs* regional anesthesia (Wu et al. 2006), hyperventilation, hypotension, hypoxia, psychoactive drugs and/or alcohol withdrawal], as well as patient-related factors including polymorphisms {apolipoprotein ε [APO ε] (Heyer et al. 2005)}, and co-morbidities such as underlying neurovascular / neurodegenerative disease and cancer (Newman et al. 2007). Although these studies have often been underpowered, lacking appropriate controls and not using similar batteries of tests for assessment, they also missed to prioritize the contribution of these factors in the development of this phenomenon (Newman et al. 2007).

1.16 Animal models of POCD

Due to the complexity of this condition and is classical definition as *multifactorial* pathology, animal models have been developed to identify pathogenic mechanisms and define putative targets for treatment. The first association between surgery and cognitive dysfunction in an animal model was developed in adult rats subjected to laparotomy and splenectomy. Herein, cognitive dysfunction persisted for up to three days postoperatively and was associated with an increase in transcription and expression of key pro-inflammatory cytokines together with glia activation in the hippocampus (Wan et al. 2007). In particular, increased IL-1β mRNA expression and glia activation in the hippocampus of splenectomized rats have bridged pro-inflammatory cytokines with memory impairment and POCD after a surgical procedure (Wan et al. 2007). Similarly, increased expression of IL-1β mRNA in the hippocampus of old mice has been reported following minor surgery, further elucidating that surgical trauma produces neuroinflammation, which results in cognitive impairment (Rosczyk et al. 2008).

The above-mentioned findings, alongside the clinical findings suggest that to trigger this process, a so-called danger signal is required to alert the immune system and elicit an adequate response to the original threat (Matzinger 2002; Harris and Raucci 2006). A surgical procedure is likely to trigger danger signals, hence producing an immune response. Surgical patients exhibit elevations of pro-inflammatory cytokines in both the central nervous system and in the systemic circulation and these may be associated with postoperative cognitive dysfunction (Buvanendran et al. 2006; Di Vita et al. 2006; Beloosesky et al. 2007). Major surgeries such as cardiac surgery or

orthopedic procedures in particular expose the subject to extensive trauma, blood loss, and tissue injury; all of these factors closely engage with the immune system to produce an inflammatory response (Buvanendran et al. 2006; Di Vita et al. 2006; Kobbe et al. 2008). Originally the concept of danger signal was only restricted to exogenous molecules, previously referred as PAMPS. Besides PAMPs, several endogenous molecules have now been found to interact and signal via the same receptors (Termeer et al. 2000; Johnson et al. 2004; Zedler and Faist 2006). The initiating mechanism whereby surgical trauma stimulates innate immunity is likely to involve DAMPs that are released from dead or dying (through non-apoptotic mechanisms) cells (Lotze et al. 2007); DAMPs can also be released during surgical trauma and include markers such as high-mobility group box 1 (HMGB1), which bind and signals through a family of relatively evolutionarily conserved PRRs (Levy et al. 2007).

TLR2, and TLR4, (and possibly TLR9) may be unique among TLR family members in their ability to recognize both exogenous and endogenous ligands, thus suggesting similarities between the mediators that drive the systemic inflammatory response to sepsis and aseptic tissue injury (Ohashi et al. 2000; Paterson et al. 2003; Akira and Takeda 2004; Kaczorowski et al. 2008). More recently, a correlation between endogenous proteins released after tissue injury and TLR4 has been suggested (Paterson et al. 2003; Mollen et al. 2006; Levy et al. 2007). A series of studies have been conducted using TLR4 deficient mice looking at the danger signal after acute tissue injury including hemorrhagic shock (Barsness et al. 2004), cardiac (Oyama et al. 2004), and hepatic (Tsung et al. 2005) ischemia/reperfusion models, showing

reduced inflammation and injury compared to wild types. Endogenous inducers not only engage with TLRs, but other PRRs are also involved in perpetuating and amplifying the inflammatory response (Medzhitov 2008). Advance glycation end-products, HMGB-1 and some S100 proteins activate the receptor for advance glycation end-products (RAGE), leading to downstream activation MAPK and NF-κB (Erlandsson Harris and Andersson 2004; Bianchi 2007). HMGB1 is a ubiquitous non histone DNA-binding protein which stabilizes the nucleosomal structure and facilitates gene transcription (Agresti and Bianchi 2003). Active and passive release of HMGB1 is seen in many inflammatory conditions ranging from sepsis to arthritis, acute lung injury, and stroke (Wang et al. 1999; Abraham et al. 2000; Kokkola et al. 2002; Lotze and Tracey 2005; Qiu et al. 2008). Recently HMGB-1 has been reported to bind to RAGE and TLR4 to mediate cytokine release, coagulation activation and neutrophil recruitment (Yu et al. 2006; van Zoelen et al. 2008). TLRs and the IL-1R also affect NFκB gene expression, and their signaling largely depends on the adaptor protein MyD88 (Kimbrell and Beutler 2001; Akira and Takeda 2004; Li and Qin 2005; Lotze and Tracey 2005).

1.17 Summary

In summary, major surgeries expose patients to extensive trauma, blood loss, and tissue injury; all of these factors effectively modulate the immune system to ultimately trigger an inflammatory response. Few studies have yet started to unmask the inflammatory component in POCD, in particular how neuroinflammation and microglia activation in the hippocampus following surgery associate with cognitive decline. The involvement of inflammation and cytokines has now been examined in this thesis, unveiling novel signaling pathways and mediators of the inflammatory cascade, representing feasible therapies to be translated into clinical trials.

1.18 Hypothesis

The hypothesis of this project is that inflammation plays a key role in the etiology of POCD.

1.19 Aim

The aim of this thesis is to investigate the inflammatory signaling pathways specifically involved with POCD, with a particular interest between systemic inflammation and local inflammation in the brain following surgery.

2 Chapter 2: Material and methods

2.1 Animal care

This research protocol complies with the regulation regarding the care, welfare and use of experimental animals published by the United Kingdom Animals (Scientific Procedures) Act of 1986 and under the Home Office (UK) license. Every effort was made to limit the number of animals and to ensure that any of the experimental groups experienced unnecessary suffering. Male C56BL/6 mice (Harlan, UK) 12-14 weeks old, weighting 25 - 30 g were used in this experiment and housed in groups of five in a 12-h-12-h light dark cycle, with controlled temperature (22-23°C) and humidity and free access to standard laboratory food and water. IL-1R$^{-/-}$ (kindly provided by Nancy Rothwell, Manchester (Labow et al. 1997)) were bred in house on a C57BL/6 background and age-matched to wild type counterparts. MyD88$^{-/-}$ (Adachi et al. 1998), TLR2$^{-/-}$ and TLR4$^{-/-}$ were obtained from the Kennedy Institute of Rheumatology and bred in house on a C57BL/6 background. Seven days of acclimatization were allowed before starting any experiment. All the animals were checked on a daily basis and evidence of poor grooming, huddling, piloerection, weight loss, back arching and abnormal activity, were excluded in the experiments.

2.2 Surgery

Animals were anesthetised with isoflurane (Total minimum alveolar concentration [MAC] of 1.5 ± 2 corresponding to air concentration of 2.1% - 30% FiO_2) (Abbot Laboratories Ltd., Queensborough, Kent, UK) (Engelhardt et al. 2006). Delivery of volatile agent was achieved with a semi-closed circuit. Concentration of volatile agent and oxygen were monitored before skin incision and at the end of each procedure (Datex-Engstrom AS/3; Instrumentarium Corp, Helsinki, Finland). Analgesia (buprenorphine - Buprenex®, 0.1 mg/kg) was given subcutaneously to reduce pain and pain-related systemic response and was administered immediately after anaesthetic induction and before producing the incision. Temperature was monitored throughout the surgical procedure with a subcutaneous thermometer (CSi8D Series, Omega, USA) and maintained within normal parameters between 36 and 37°C. Warming pads (Harvard apparatus) and a temperature-controlled light were used to achieve optimal equilibrium. Aseptic conditions were maintained throughout the procedure. The surgical area localized on the dorsal aspect of the left hind paw was meticulously shaved and disinfected with chlorhexidine gluconate 0.5% (BDH Laboratory Supplier, England). Sterile drapes were applied on the surgical field and onto the animal to limit risks of contaminations; autoclaved instruments were used throughout. The open diaphyseal procedure on the tibia was adopted from (Hiltunen et al. 1993) with some modifications. A longitudinal incision was made from the knee to the midshaft of the tibia under a dissecting microscope (Leica). The subcutaneous tissue was dissected and muscles isolated until revealing the patellar tendon and the tibial periosteum. A 0.5mm hole in the

proximal tibia was drilled with a 25-gauge needle just beneath and medial to the patellar tendon to enter the intramedullary canal. A 0.38 mm stainless steel rod was then inserted in the canal for 15mm circa until resistance was felt (figure 2.1). Subsequently, the fibula and the muscles surrounding the tibia were isolated, the periosteum stripped over a distance of 10mm circumferentially and an osteotomy was performed with scissors at the junction of the middle and distal third of the tibia.

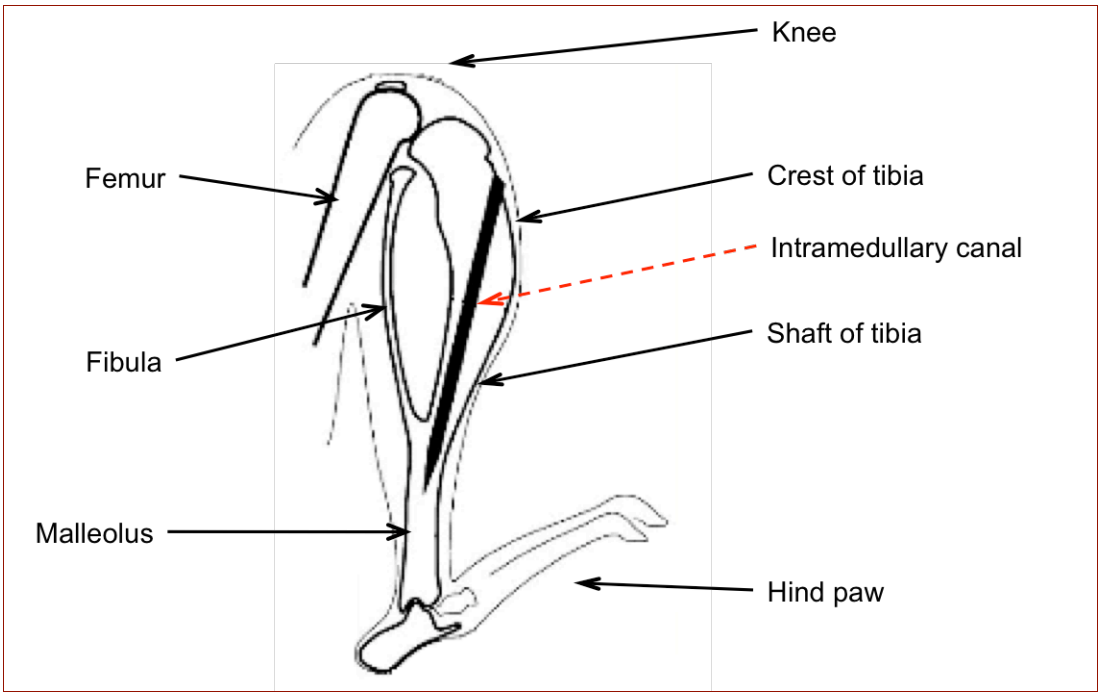


Figure 2-1: **Stabilized tibia fracture in a mouse.**
Adapted from (Gardner et al. 2006)

After producing the fracture the wound was irrigated and the skin sutured with 8/0 Prolene; intra-opaerative fluid loss was replaced with 0.5 ml of subcutaneously injected normal saline. The animal was allowed to recover in a warm box, once fully awake placed a clean standard cage. The entire procedure from induction of anesthesia to recovery lasted 15 ± 5 min.

2.3 Treatments

Minocycline (40 mg/kg in 0.1 ml/volume, Sigma, UK) was given intraperitoneally 2 hours prior surgery and daily thereafter until termination. Dosage was comparable to previous studies to achieve neuroprotective effects (Yrjanheikki et al. 1999; Bye et al. 2007). Minocycline was made fresh accordingly to the experimental schedule by dissolving it in phosphate buffer solution (PBS) (Sigma, UK) and minimizing precipitates formation. IL-1 receptor antagonist (IL-1 Ra) (Amgen, Kineret® 100 mg/kg, Nederlands) was given subcutaneously immediately before intervention. TNF neutralizing antibody (clone TN3, Sigma, UK, 100 µg in 0.1 ml/volume (Sheehan et al. 1989)) was dissolved in normal saline and injected either 18 hours before surgery or immediately after. Enrofloxacin (10 mg/kg, Baytril®) was given 2 hours prior to surgery and twice daily thereafter until assessment of outcome. Lipopolysaccharide (LPS) derived from Escherichia Coli endotoxin (0111:B4, InvivoGen, USA, 1 mg/kg) was dissolved in normal saline and injected intraperitoneally. Mice in the anesthesia without surgery treatment group received isoflurane (1.5 MAC for 20 minutes) and analgesia (buprenorphine

0.1 mg/kg) with no surgical intervention or other treatment. Control animals were injected with equivalent volumes (0.1 ml) of saline.

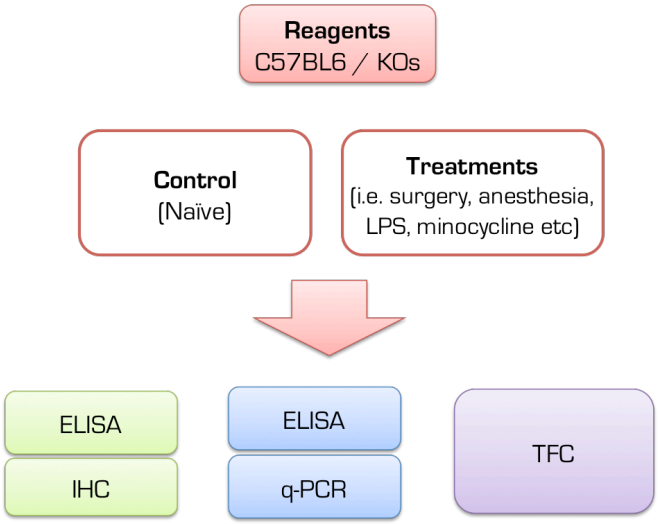


Figure 2-2: **Experimental groups flow chart.**

Mice were randomly assigned and treated according to each experimental setting. Animals were independently assessed for inflammatory markers and behavior using TFC. Blood was obtained from every animal for cytokine analyses (ELISA). Due to the differences in processing brain tissue for IHC or q-PCR, separate cohorts of animals have been used in each investigation. Assessment of hippocampal-dependent memory using TFC was also performed in different cohorts in order to obviate possible confounding effects of behavioral testing on inflammatory markers; no other analyses were performed on these animals.

2.4 ELISA

2.4.1 Blood collection

Blood was sampled transcardially after thoracotomy under terminal anesthesia 30 minutes, 2, 6, 12 hours and 1, 3, 7 days after experiments in the different cohorts and centrifuged at 3,600 rpm for 7 minutes at 4°C. Blood samples taken from animals without any interventions severed as controls. Plasma samples were stored at -20°C for further analysis. Plasma cytokine and HMGB-1 were measured using commercially available ELISA kits from Biosource, CA and Shino-test Corporation, Japan, respectively. The sensitivities of the assays were <3 pg/ml for TNF, <7 pg/ml for IL-1β, <3 pg/ml for IL-6 and 1 ng/ml for HMGB1.

2.4.2 Cytokine analyses

Cytokine levels were assessed with sandwich enzyme-linked immunosorbent assay (ELISA). Pre-coated 96-wells ELISA plates commercially available were used with few modifications. Briefly, standards and samples (50 μL) were incubated for 90 min at 37°C with 50 μL of biotin conjugate (protocol for IL-1β). All samples were run in duplicates. After washing all wells four times with washing buffer, samples were incubated with 50 μL of Streptavidin-HRP Working Solution for 30 min at room temperature (RT) on a shaker, and then washed again four to five times. 50 μL of Stabilized Chromogen was added to each well; after 30 min of incubation in the dark, the reaction was stopped

with 50 μL of Stop Solution. A colour reaction would occur in each well changing the solution from blue to yellow and the absorbance is read on a micro-platelet reader at 450 nm. Cytokine levels were calculated by a standard curve of the assessed cytokine. Manufacturer's data reported assay sensitivities and tested cross-reactivity for other cytokines. Mice from each treatment group were randomly assigned for assessment of either cytokine response or cognitive behavior, in order to obviate possible confounding effects of behavioral testing on inflammatory markers (Nguyen et al. 1998).

2.4.3 Brain ELISA

Each mouse was euthanized and quickly perfused with PBS. The hippocampus was dissected under microscopy on a frosted glass plate placed on top of crushed ice, then snap frozen and stored at -80˚C until processing. Each hippocampus was added to Iscove's culture medium containing 5% fetal calf serum (FCS) and a cocktail of enzyme inhibitors (in mM: 100 amino-n-caproic acid, 10 EDTA, 5 benzamidine-HCl, and 0.2 phenylmethylsulfonyl fluoride). The proteins were mechanically dissociated from tissue by means of sonication in a container plunged in ice. This consisted of 3 cycles of cell disruption each lasting 3 seconds. Sonicated samples were centrifuged at 10000 rpm for 10 minutes at 4˚C. Supernatants were collected and stored at -80°C until the ELISA was carried out. IL-1β was measured in the supernatant from hippocampal extracts, which were appropriately diluted prior to measurement to fall on the linear portion of the sigmoid curve, using a

commercially available ELISA kit (Bender Medsystem, CA). The sensitivity of the assay was 1.2 pg/ml. The ELISA kit was validated for use with brain tissues. Samples collected from mice treated with LPS (0111:B4, Invivogen, CA) 3 mg/kg i.p. were used to confirm reliability of dilution linearity and spike recovery.

2.4.4 Protein quantification

Protein quantification using the Bradford assay was carried out. Bradford dye concentrate (Bio-Rad) was diluted with double distilled water (ddH2O) in a 1:4 ratio respectively, following manufacturer's instructions. To make protein standard samples, a known concentration of bovine serum albumin (Bio-rad) was serially diluted with ddH2O to range in concentration from 1000, 500, 250, 125, 12.5 and 1.25 µg/mL. On ice, 10 µL of protein samples and 10 µL of protein standard samples were loaded in triplicate into a 96-microwell flat-bottomed clear plate. Bradford dye (200 µL) was pipetted into each well and left to incubate for 5 minutes. The plate was then placed in a spectrophotometer to measure the optical density of each sample at 595 nm. The standard curve created from the protein standard samples allowed for determination of the protein sample concentration and their subsequent normalisation to the ELISA readouts.

2.5 q-PCR

2.5.1 RNA Extraction

The hippocampus was rapidly extracted under a dissecting microscope, placed in RNAlater solution (Applied Biosystems, Ambion, Austin, TX) and stored at 4°C. Total RNA was isolated using RNeasy Kit (Qiagen, Valencia, CA) following manufacturer's instructions. Extracted RNA was treated with recombinant DNase I (rDNasa I) using a DNA-free™ kit (Applied Biosystems, Ambion, Austin, TX) and quantified using spectrophotometry (Nanodrop Spectrophotometer, Labtech, East Sussex, UK) to determine the volume of RNA equivalent to 1 µg to be used in the following PCR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) reverse transcription polymerase chain reaction (RT-PCR) was carried out to very the absence of any DNA contamination (figure 2.2).

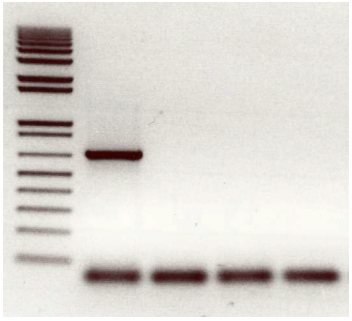


Figure 2-3: **RT-PCR product to verify absence of DNA contaminations.**

From left to right: 1kD protein ladder, cDNA (positive control), x3 samples assayed.

2.5.2 Production of cDNA

For the reverse transcription reaction, Moloney Murine Leukaemia Virus (M-MLV) reverse transcriptase (Promega, Madison, WI) was used following manufacturer's instructions. PCR amplification was carried out using oligo(dT) (MWG Biotech, London, UK) and recombinant *Taq* DNA polymerase (Invitrogen, CA) for GAPDH and cytokines of interest, following manufacturer's protocol (total reaction volume: 20 µL, including 1 µL cDNA). A T3 Thermocycler (Biometra, Goettingen, Germany) was used with the following settings: initialization step at 94ºC for 4 minutes, denaturation step at 94ºC for 30 s, extension step at 60ºC for 40 s for 30 thermal cycles, final elongation at 72ºC for 45 s and final hold at 4ºC. Oligonucleotide primers were purchased by MWG Biotech (London, UK).

PCR products were analyzed using 1.5% agarose gels. Briefly, 1.5 g of ultrapure agarose (Invitrogen, CA) was dissolved in 100 mL 1x tris-acetate EDTA (TAE) buffer under microwave heating. After cooling, 10 µL (1:10000 dilution) SYBR safe DNA stain (Invitrogen, CA) was added to the mixture to enable DNA visualization under ultraviolet light (UVP BioDoc-It System, Upland, CA). 10 µL of each PCR product was mixed with 2 µL of loading buffer (40% glycerol and bromophenol blue) and loaded onto the gel. Electric field was kept at 110 V for 30 minutes (figure 2.3).

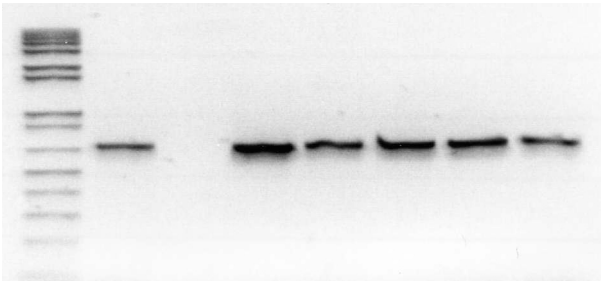


Figure 2-4: **RT-PCR product to verify presence of DNA for q-PCR**.

From left to right: 1kD protein ladder, cDNA (positive control), water (negative control), x5 samples assayed.

2.5.3 Quantitative real time polymerase chain reaction (q-PCR)

q-PCR was carried out to determine the level of mRNA expression of each cytokine in relation to endogenous control ACTB (β-actin). The endogenous control had a constant expression within cells that remained unchanged after treatment, thus allowing normalization to this value. Quantification was based on the fluorescent reporter probe method. A probe is a short DNA sequence complementary to part of the gene of interest, onto which a quencher dye (5' end) and a fluorescent reporter dye (3' end) are attached. When the two dyes are in close proximity, no fluorescence can be detected. However, during polymerisation, DNA polymerase cleaves the probe separating it from the quencher, hence the fluorescence increases.

The one-step q-PCR was performed on a Rotor-Gene 6000 (Corbett Life Science, Australia), using Assay-On-Demand premixed Taqman probe master mixes (Applied Biosystems, Ambion, Austin, TX). A ready-made reaction mix

was used containing *Taq* DNA polymerase, $MgCl_2$ and deoxyribomucleotides (Platinum® Quantitative PCR SuperMix-UDG, Invitrogen, CA) with gene-specific primers and fulorogenic probes. Total reaction volume was 20 µL of which cDNA was 5 µL (1:5 dilution of reverse transcription product). Taqman® gene expression assays for the gene of interests and controls were used for optimizing and validating the PCR. Precise primer sequences remain under proprietary copyright. Each sample was run in triplicate following the programme: 2 min incubation at 50ºC, 3 min pre-incubation at 95ºC, followed by 45 thermal cycles of 15 s at 95ºC and 40 s at 60ºC. Fluorescence was measured at the end of each cycle.

Relative gene expression was calculated using the comparative threshold cycle $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001; Wong and Medrano, 2005) and normalized to ACTB. This consists of setting a threshold cycle (Ct) value that represents a fixed value of fluorescence. Relative fluorescence of the gene of interest to the relative endogenous control ($\Delta C_{t,\ sample}$) is calculated by ($\Delta C_{t,\ cytokine} - \Delta C_{t,\ ACTB}$). Expression levels are then related to an external control, hippocampus from untreated animals, to give the Ct value by ($\Delta C_{t,\ sample} - \Delta C_{t,\ untreated}$). Relative expression can be obtained by $2^{-\Delta\Delta Ct}$. Results are expressed as fold-increases relative to controls.

2.6 Perfusion and cryostat sectioning:

Table 2-1: **Solutions**

0.2 M Phosphate buffer (PB) (2 L)
46 g Na_2HPO_4
9.1 g $Na_2H_2PO_4$
2 L dH_2O

0.1 M Phosphate-buffered saline (PBS) (2 L)
1 L 0.2 M PB
1 L dH_2O
18 g NaCl

0.1 M Phosphate-buffered saline with 0.3 % triton (PBS-T) (2 L)
2 L PBS
6 mL Triton X-100 (Promega)

0.1M Phosphate-buffered saline with 0.3 % H_2O_2 / 70 % methanol (30 mL)
9 mL 0.1 M PBS
21 mL Methanol
300 µL 30% H_2O_2

Heparinised phosphate-buffered saline (PBS-Hep) (2 L)
2 L PBS
2 mL (1000 U / mL) heparin sodium solution (Sigma)

10 % paraformaldehyde (2 L)
Prepare under fume hood and store at 4ºC
1.6 mL dH2O (heat to ~70ºC)
200 g paraformadehyde (dissolve in water)
1 mL NaOH (to aid dissolution)
2 L dH_2O
Cool solution to room temperature
Titrate to pH to 7.4 with HCl and/or NaOH
Filter and store at 4ºC

4 % Paraformaldehyde (2 L)
Prepare under hood, make immediately before use and store at 4ºC
1 L 0.2 M PB
0.8 L 10 % paraformaldehyde
Top up to 2 L with dH_2O

30 % Sucrose solution (2 L)
600g sucrose
2 L 0.1 M PBS

1 M Sodium hydroxide (NaOH) (2 L)
50 g sodium hydroxide pellets
Dissolve to 2 L with dH_2O

Mice were euthanized and perfused transcardially with ice-cold heparinized 0.1M PBS followed by 4% paraformaldehyde in 0.1M PBS at pH 7.4 (VWR International, UK). The brains were harvested and post-fixed in 4% paraformaldehyde in 0.1M PBS at 4°C and cryoprotected in 0.1M PBS solutions containing 15% sucrose for 24 hours (VWR International, UK) and then 30% sucrose for a further 48 hours. Brain tissue was freeze-mounted in OCT embedding medium (VWR International, UK). A metallic base was allowed to cool down to -20°C. OCT embedding medium was applied to the metallic base covering an area and the specimen attached vertically onto the prepared base. Additional embedding medium was placed around the specimen to completely cover it; rapid freezing aerosol spray was used to ensure that specimen maintained its vertical position. The specimen was left in the cryostat for at least 30 minutes until it was completely frozen. Once the specimen was ready, the metallic base was attached firmly onto the cryostat (Bright Instruments Huntingdon, UK). 25μm thick coronal sections of the hippocampus were cut sequentially in groups of 6 and mounted on Superfrost® plus slides (Menzel-Glaser, Germany). Slides were air dried overnight and stored at -20°C until further use.

2.7 Immunohistochemistry (IHC)

Sections from all of the treatment groups were run together in order to reduce variability. To begin the staining procedure, the sections were left at room temperature for 20 minutes before being transferred to 0.1M PBS. To reduce background staining caused by endogenous peroxidase the sections were quenched with a solution of 0.3% H_2O_2, 70% methanol and 0.1M PBS for 30 minutes. Sections were rinsed 3 times with 0.1 M PBS and incubated in 3% normal goat serum (NGS) blocking solution for 30 minutes. The rat monoclonal antibody, OX-42 (low endotoxin, clone M1/70.15) 1:200, directed against mouse CD11b (Serotec, UK) was used to label microglia. Visualization of immunoreactivity for OX-42 was achieved using the avidin-biotin technique (Vector Labs, UK) and a goat anti-rat secondary antibody (Chemicon International, USA) at a concentration of 1:200. After rinsing in phosphate buffer saline with triton X (PBST, 0.3% triton X, pH 7.5) all sections were incubated with ABC Vectastain Elite solution, but using a dilution of 1 in 200 for reagent A and B, instead of that suggested in manufacturers the user guidelines. ImmPACT DAB was used for the staining and colour was allowed to develop for 1 minute and 15 seconds. A negative control omitting the primary antibody was performed in all experiments (figure 2.4).

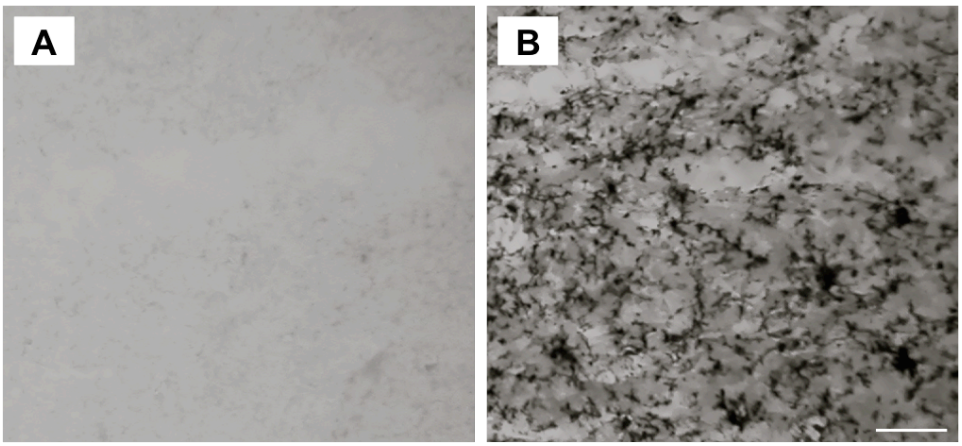


Figure 2-5. **Validation of microglia staining using CD11b**.

Panel A represents a slide in which the primary antibody has been omitted, thus reflecting the background staining. Panel B shows the complete technique with the staining (scale bar 50 μm, 20X).

The slides were then dehydrated in a stepwise manner through 50, 75, 90 and 100% ethanol for 10 minutes each. They were submerged in xylene for further 10 minutes and mounted under a cover slip using depex polystyrene (DPX) mounting medium (Raymond A Lamb, East Sussex, UK). Slides were air dried overnight under a fume hood and later kept in plastic boxes for storage. Immunohistochemical photomicrographs were obtained with an Olympus BX-60 microscope and captured with a Zeiss KS-300 colour 3CCD camera. The assessment of staining, by an observer blinded to the interventional group, was based upon a 4-point categorical scale (Colburn et al. 1997).

2.8 Behavioral assessment

2.8.1 Fear conditioning chamber

The memory assessment was performed in a dedicated conditioning chamber manufactured by Med Associates Inc. The back and the sidewalls of the chamber were made of aluminium, whereas the front door and the ceiling were of transparent Plexiglas. The floor of the chamber consisted of 36 stainless steel rods (1 mm diameter) spaced 0.5 cm apart (center-to-center). The rods were wired to a shock generator and scrambler for the delivery of foot shock. Prior to testing, the chambers were cleaned with a 5% sodium hydroxide solution. Background noise (60dB) was provided by means of a fan positioned on one of the sidewalls. An infrared video camera, mounted in front of the chamber captured behavior. The data acquired by the camera was automatically scored through a computer programme (Freezeframe, Med Associates Inc.).

2.8.2 Trace fear conditioning

On each day of testing, mice were transported to the behavioral room and left undisturbed for at least 20 minutes before placing them into the conditioning chamber. Freezing was recognized by the software as a total lack of movement, including absence of fur, vibrissae and skeletal movement (except for breathing). The percentage of time spent freezing over the total time spent in the chamber to accomplish the test was used to score memory and learning

abilities. A decrease in the percentage of time spent freezing indicated impairment of these abilities. Mice were trained and tested on two separate days. Training consisted of placing a mouse in a chamber, illuminating stimulus and house lights, and allowing exploration for 100 seconds. Thereafter, an auditory cue [2 kHz clicking via the solenoid; conditional stimulus (CS)] was presented for 20 s. The 2 seconds foot shock [0.95 mAmp; unconditional stimulus (US)] was administered 18 seconds after the CS (figure 2.5). This procedure was repeated twice, and mice were removed from the chamber 30 s later. With the termination of the trial, every mouse was taken individually within 30 minutes to the surgery room.

Seventy-two hour after training, mice were returned to the same chambers in which training occurred (context), and freezing behavior was recorded by the experimenter using time sampling (10 s intervals). At the end of the 5 min context test, mice were returned to their home cage. The time spent freezing as a percentage of the total time spent in the chamber during the context phase was used to score for hippocampal-dependent memory resulting from prior learning. A smaller percentage of freezing behaviour is indicative of greater degree of cognitive impairment.

2.8.3 Acoustic-cued behavioral chamber

Approximately 3 hours later the context test, freezing was recorded in a novel environment and in response to the cue. The shape of the chamber was changed from rectangular to triangular, ceiling colour was black instead of white, flooring rods were covered with a smooth surface, background noise was removed, and chamber was cleaned with 70% alcohol to provide a different smell. This made the environment substantially different, thereby limiting the influence of the hippocampus in the recognition of the chamber features. Mice were placed in the novel environment, and time sampling was used to score freezing for 3 min. The auditory cue (CS) was then presented for 3 min, and freezing was again scored (figure 2.5). Freezing scores for each subject were expressed as a percentage for each portion of the test. Memory for the context (contextual memory) for each subject was obtained by subtracting the percent freezing in the novel environment from that in the context.

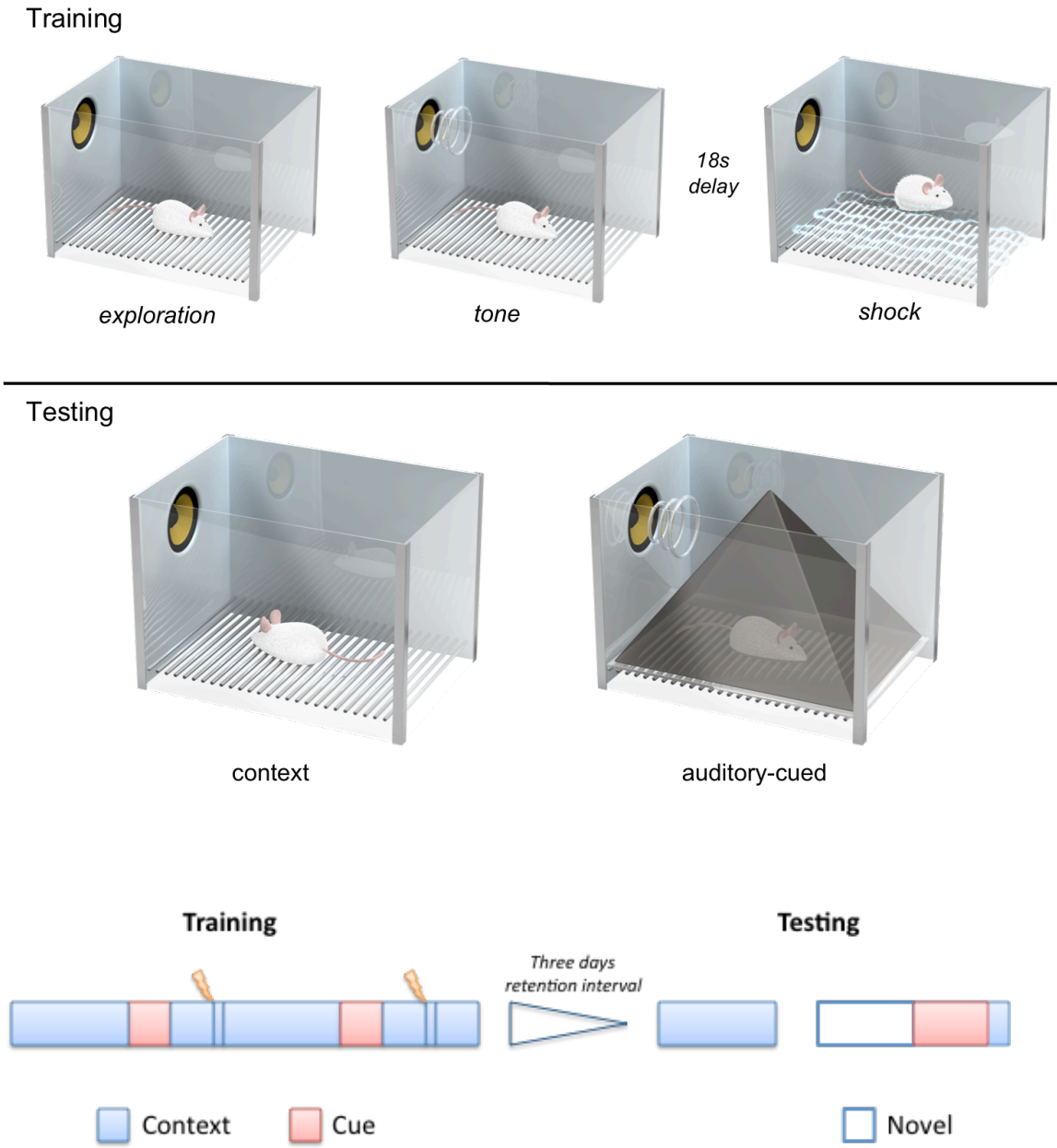


Figure 2-6: **Trace fear conditioning paradigm.**

Training consisted of exploration of the conditional box for 100 seconds (s) followed by an auditory cue (75-80 dB, 2 kHz), the CS, which was presented for 20 s. A trace interval of 18 seconds occurred before the 2 s foot-shock (0.75 mAmp), the US. This procedure was repeated twice, after an inter-trial interval (ITI) of 100s for a total duration of ~ 5 minutes. Mice were removed from the chamber 30 s later. After three days, rodents were returned to the same chamber for 270 s exposure to the context. Three hours thereafter they were placed in a different environment for the tone retrieval. After 135 seconds baseline, a tone was played, lasting 135 seconds. Overall percentage of time spent freezing was recorded at all stages, as a marker of memory performance.

2.9 Data analysis

Statistical analyses were performed using GraphPad Prism version 5.0a (GraphPad Software, San Diego, CA). The results are expressed as mean ± SEM. Data were analyzed with one-way analyses of variance followed by Newman-Keuls post hoc test wherever appropriate. For categorical data, non-parametric Kruskal-Wallis followed by Dunn's test was used. A $p<0.05$ was considered to be statistical significance.

3 Chapter 3: results

3.1 RATIONALE

In order to initiate an appropriate immune response, living organisms have developed a system to counteract potential life threatening events such as infection and tissue injury. The mechanisms underlying this process are known as the inflammatory response, which ultimately aims to restore the correct homeostasis in the host organism after a challenge. To trigger this process, a so-called *danger signal* is required to alert the immune system and elicit an adequate response to the original threat (Matzinger 2002; Harris and Raucci 2006). A surgical procedure is likely to trigger danger signals, hence producing an immune response. Major surgeries, including cardiac surgery and orthopaedic procedures expose the subject to extensive trauma, blood loss, and tissue injury; all of these factors will engage with the immune system to produce an inflammatory response (Buvanendran et al. 2006; Di Vita et al. 2006; Kobbe et al. 2008). Originally the concept of danger signal was only restricted to exogenous molecules, described as pathogen-associated molecular patterns (PAMPs). PAMPs, such as bacteria, lipopolysaccharide (LPS), and viruses interact with specific receptors known as pattern recognition receptors (PRRs) and activate the innate immunity by releasing pro-inflammatory molecules such as cytokines. Besides PAMPs, several endogenous molecules have now been found to interact and signal via the same receptors (Termeer et al. 2000; Johnson et al. 2004; Zedler and Faist 2006). These endogenous molecules are described as alarmins and they include high-mobility-group box (HMGB) proteins, interleukins, cytosolic

calcium-binding proteins of the S100 family, heat-shock proteins (HSPs), defensins, cathelicidin, eosinophil-derived neurotoxin (EDN) and nucleosomes (Oppenheim and Yang 2005; Harris and Raucci 2006). Alarmins are rapidly secreted after tissue injury or necrotic cell death as well as from immunocompetent cells that can directly release them without dying. Alarmins also function as chemoattractant molecules to both recruit and activate antigen-presenting cells (APCs) of the innate immunity as well as other PRRs. Furthermore, they can mediate adaptive responses and they are essential to restore homeostasis and to reconstruct tissues after an insult (Bianchi 2007; Lenz et al. 2007). Thus, not only do pathogens elicit an inflammatory response, but also mechanical damage to the cells (i.e. trauma) can activate similar downstream effects (Zedler and Faist 2006).

Systemic and central release of cytokines after a surgical procedure may be associated with cognitive dysfunction (Moller et al. 1998; Gao et al. 2005; Kalman et al. 2006; Beloosesky et al. 2007). POCD is the emblem of this phenomenon and is defined as a subtle dysfunction in one or more cognitive domains (Caza et al. 2008). Some risk factors associated with POCD may not be easily modifiable and include patient-related factors (advanced age, genetic polymorphisms and co-morbidities), perioperative factors (general anesthesia, hyperventilation, hypotension, hypoxia, benzodiazepine use and/or alcohol withdrawal), and surgery (type, duration, complications, multiple interventions). The complexity as well as the multifactorial etiology of POCD requires animal models to further dissect each of these components to attempt a better understanding of this pathology. Based upon the findings that adult rats showed signs of cognitive decline and inflammation within the

hippocampus following splenectomy (Wan et al. 2007), we sought to understand the inflammatory response following orthopedic surgery in a mouse model of POCD. The putative role of inflammation and its correlation with memory abnormalities was also suggested after minor surgery in aged mice (Rosczyk et al. 2008). Evidence both from animal models and clinical studies report increased pro-inflammatory molecules both systemically and centrally following surgery (Buvanendran et al. 2006; Kobbe et al. 2008).

Herein the cytokine time-course and the postoperative inflammatory changes are described, both systemically and centrally. This suggests a time-dependent association between increased production of cytokines and cognitive abnormality following surgery. Targeting of the inflammatory challenge with minocycline, a non-specific anti-inflammatory compound, prevents the development of postoperative cognitive dysfunction.

3.2 Establishment of a surgery-induced cognitive dysfunction

To elucidate the consequences of surgery on learning and memory abilities, different groups of mice received one of the following interventions: surgery of the tibia under inhaled isoflurane (surgery), isoflurane only (anesthesia), or no interventions (naïve).

Trace fear conditioning is characterized by a brief interval between the termination of the tone and the onset of the shock. Such interval allows accurate assessment of hippocampal function by analyzing the freezing response (Chowdhury et al. 2005). Animals were conditioned during the training phase irrespectively of the treatment they received. Each animal was exposed to the same acquisition environment during the training phase prior to the intervention. Establishment of learning and working memory was rapidly achieved with no significant differences between groups. During the initial seconds, when the animal is placed in the new environment for the fist time, the low baseline freezing is indicative of no distress / anxiety (figure 3.1A). This reflects curiosity and interest for the subjects to explore the novel environment. The increase in freezing following the CS – US pairing is indicative of functional working memory (figure 3.1B-C), thus the animals are able to learn and form a similar working memory or short-term memory (figure 3.1E).

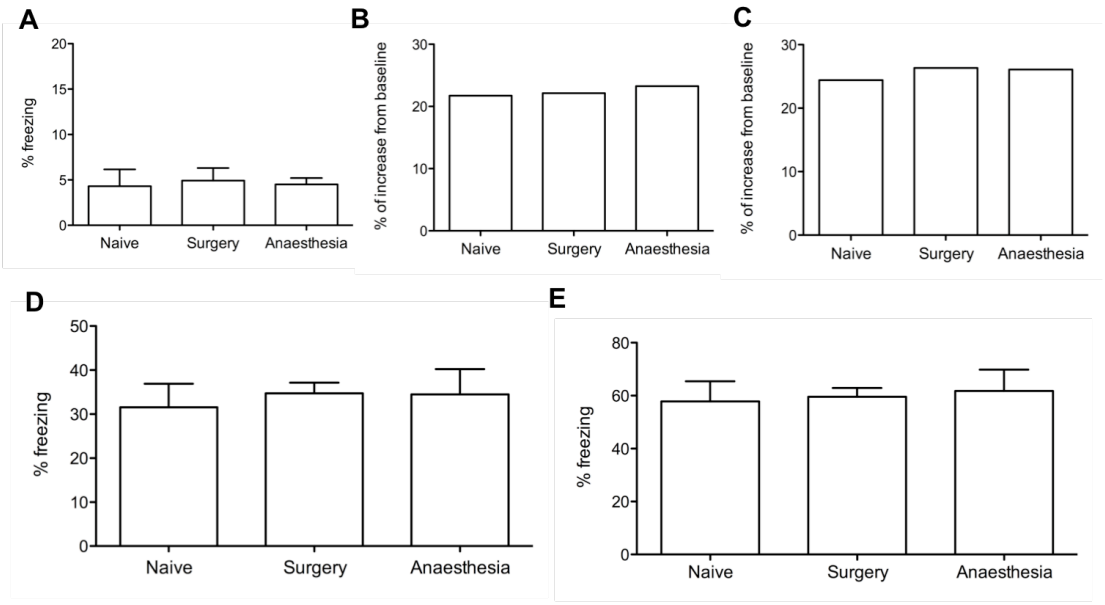


Figure 3-1: **Training phase during trace fear conditioning**.

Animals were collectively trained and then subdivided in three groups: untreated (naïve), tibia fracture under inhaled isoflurane (surgery), and isoflurane only with no intervention (anesthesia). Baseline freezing at the time of first exposure of these animals in the context is very low, indicating low levels of stress (A). Figure B indicates the increase of freezing response from Tone 1 to Tone 2, indicating working memory. Figure C indicates the increase of freezing response from Trace Interval 1 to 2, an indicator of working memory. Figure D indicates the working memory freezing response to context between tone – shock pairings. All animals demonstrate an equal working memory performance (E).

Immediately following training, animals were treated according to the group in which they were randomized. Mice in the surgical group or anesthesia underwent treatment within 30 minutes after training. Three days after treatment, rodents were taken back to the conditioning chamber and re-exposed to the same environment as during the acquisition phase. This time no cues (tone or shock) were used and the memory assessment relies only on the ability of each animal to retain memory for the environment (context) in which they were previously trained. When exposed to the context, the contextual retrieval task revealed a significant reduction of freezing behavior compared to the naïve littermates or animals undergoing anesthesia (figure 3.2A). Moreover, when the auditory-cued test was carried out two hours after the context retrieval task, a significant difference between surgical and naïve subjects was found in auditory cued-dependent freezing, with a significant reduction in surgical animals but no impairments in the overall freezing baseline (figure 3.2B).

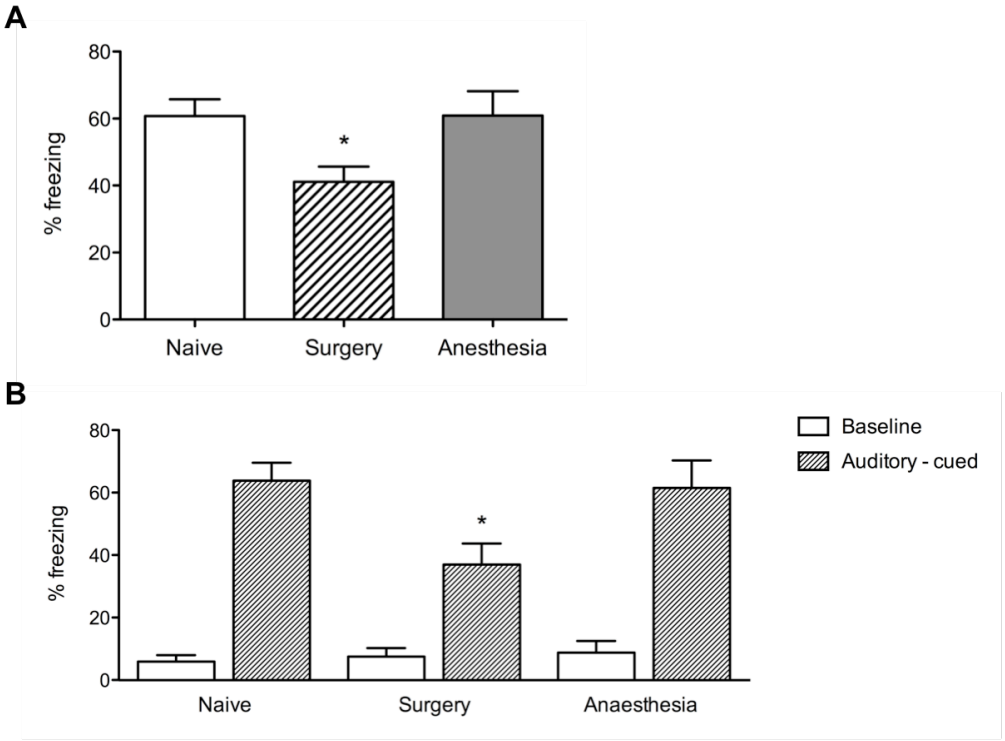


Figure 3-2: **Hippocampal-dependent memory recall is impaired following surgery.**

Contextual fear response, as measured by freezing behavior, is impaired in animals receiving surgery compared to naïve and anesthesia groups (**A**) (*p<0.05 vs naïve and anesthesia groups). Surgery-induced memory impairment is shown in the auditory-cued test in mice trained with trace fear conditioning (**B**). There is a significant difference between the groups in auditory-cued-related freezing behavior, suggesting disruption of auditory-cued, hippocampal-dependent, retrieval of memories after surgery. No difference was shown in the baseline freezing behavior showing no signs of anxiety (*p<0.05 vs naïve and anesthesia groups). Data are expressed as mean ± SEM (n = 10) and compared by one-way analysis of variance and Student-Newman-Keuls method.

3.3 Surgery elevates plasma concentration of inflammatory cytokines

In order to assess the temporal relationship between peripheral injury and systemic inflammation, plasma concentrations of IL-1β, IL-6 and TNF-α were measured postoperatively by ELISA. While the plasma concentrations of IL-1β and IL-6 were unchanged at 2 hours, these peaked at 6 hours increasing by 7-fold (IL-1β: 44.13 ± 7.35 pg/ml, $p<0.001$) and 20-fold (IL-6: 94.08 ± 15.68pg/ml, $p<0.001$) above baseline levels (IL-1β: 6.09 ± 1.31 pg/ml; IL-6: 4.08 ± 2.10 pg/ml), respectively. Levels of IL-1β and IL-6 remained unregulated 6-fold (IL-1β: 36.89 ± 6.54 pg/ml, $p<0.001$) and 5-fold (IL-6: 27.21 ± 4.53 pg/ml, $p<0.01$) compared to naïve animals 24 hours following surgery (figure 3.3A-B). Surgery did not significantly impact on systemic TNF-α release although a trend towards increase was noted during the early time - points (30-90 minutes). Intraperitoneal administration of LPS was used to establish the early changes that occur after PAMPs (figure 3.3C; 104.18 ± 17.36 pg/ml, $p<0.001$).

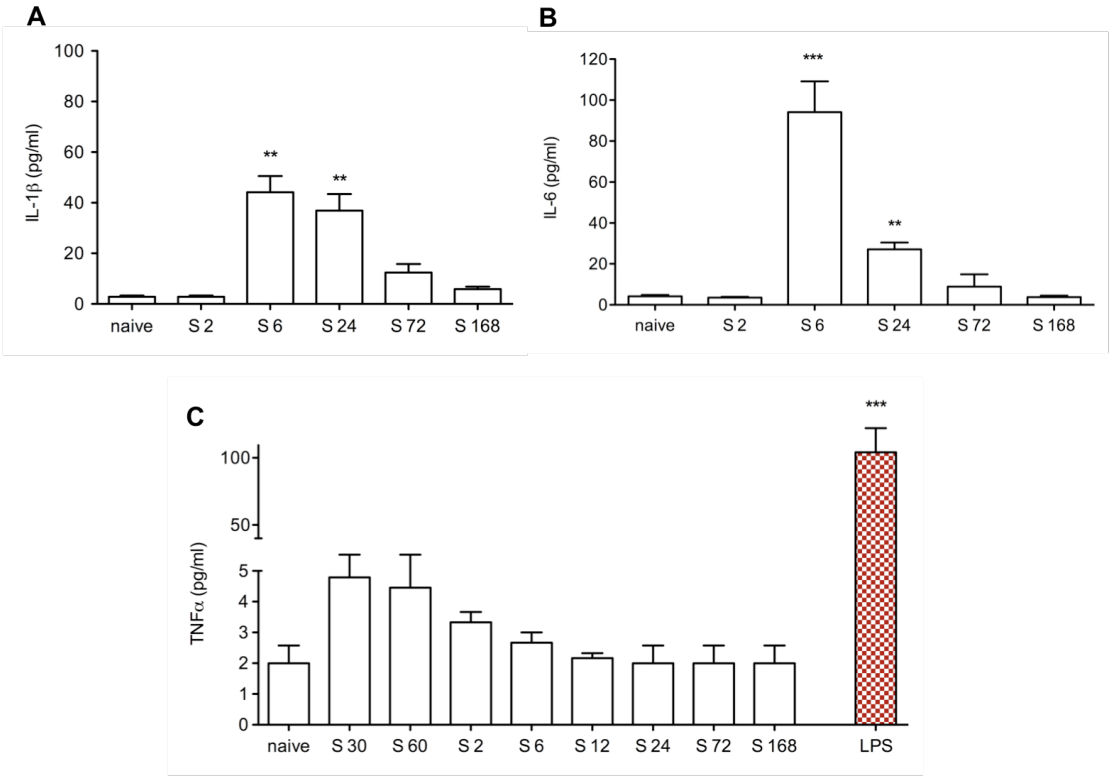


Figure 3-3: **Surgery-induced systemic inflammation.**

IL-1β and IL-6 levels in plasma were measured by ELISA at 2, 6, 24 or 72 hours post-intervention. Surgery resulted in increased plasma levels of IL-1β (**A**) and IL-6 (**B**) compared to naïve animals (**p<0.001 vs naïve). Levels of TNF remain undetected throughout the time points, with a positive trend at 30 and 60 minutes following surgery. Validation of these findings was achieved by administering LPS (1 mg/kg), which served as positive control (***p<0.001 vs naïve). Data are expressed as mean ± SEM (n = 6) and compared by one-way analysis of variance and Student-Newman-Keuls method.

In order to isolate the possible role of anesthesia in the inflammatory response following surgery, cytokines levels were measured following exposure to isoflurane. Administration of anesthetics alone produced no changes from baseline levels of any of these cytokines (figure 3.4). Furthermore, no changes in plasma cytokines were measure if the isoflurane exposure was increased from 20 minutes to 45 minutes. Also if the concentration of the volatile agent was increased from MAC 1.5 to 2 for 20 minutes, no effects on cytokines were detected.

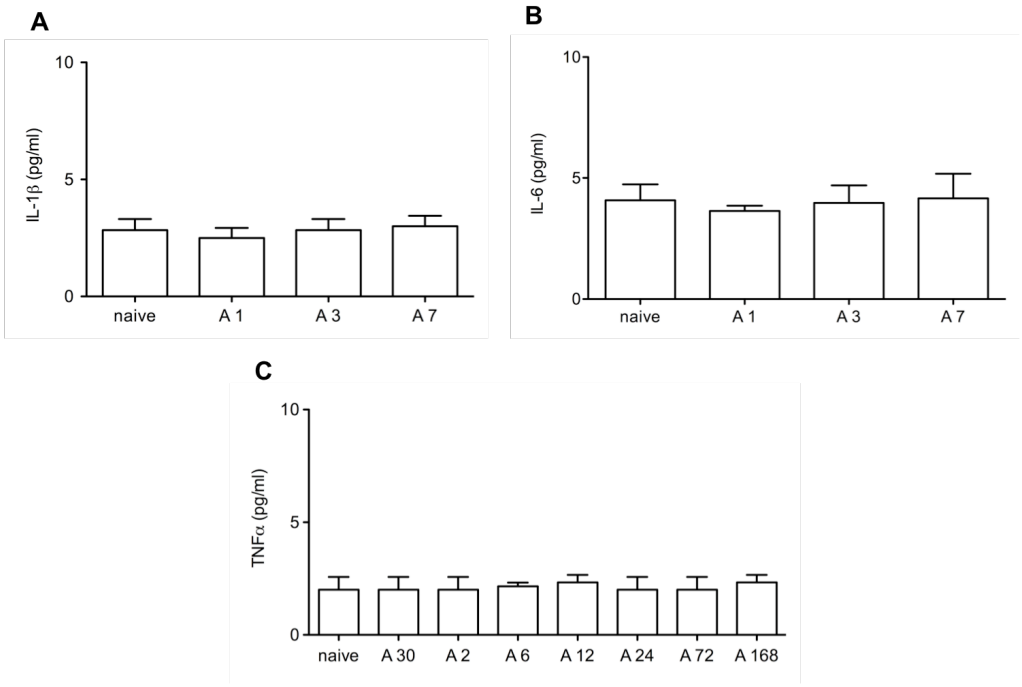


Figure 3-4: **No effects of anesthetics on pro-inflammatory cytokines in plasma**.

Mice were exposed to isoflurane MAC 1.5 (2.1% isoflurane, 30% FiO_2) for 20 minutes. None of the assayed cytokines, IL-1β, IL-6, and TNF were significantly increased from baseline levels (**A, B, C**). Data are expressed as mean ± SEM (n = 6) and compared by one-way analysis of variance and Student-Newman-Keuls method.

3.4 Surgery leads to central inflammation

Since IL-1β was increased in plasma after surgery and due to the association of this molecule with memory and cognitive abnormalities (Dinarello and Savage 1989; Lai et al. 2006), I investigated the putative central role for hippocampal IL-1β in this model of POCD. The first aim was to understand if the expression of IL-1β was influenced by surgery. Transcription of cytokines in the hippocampus was measured by qPCR at 4, 6 and 24 hours after surgery. Levels of IL-1β increased 2-fold in mice subjected to surgery at 6 hours following operation (figure 3.5A, $p<0.01$). In order to better define the central role of inflammation following surgery, I also measured levels of IL-6 as a downstream pro-inflammatory cytokine. As with IL-1, gene expression of IL-6 was also increased (approximately 4.5-fold) when compared to naïve animals at 6 hours (figure 3.5B, $p<0.001$).

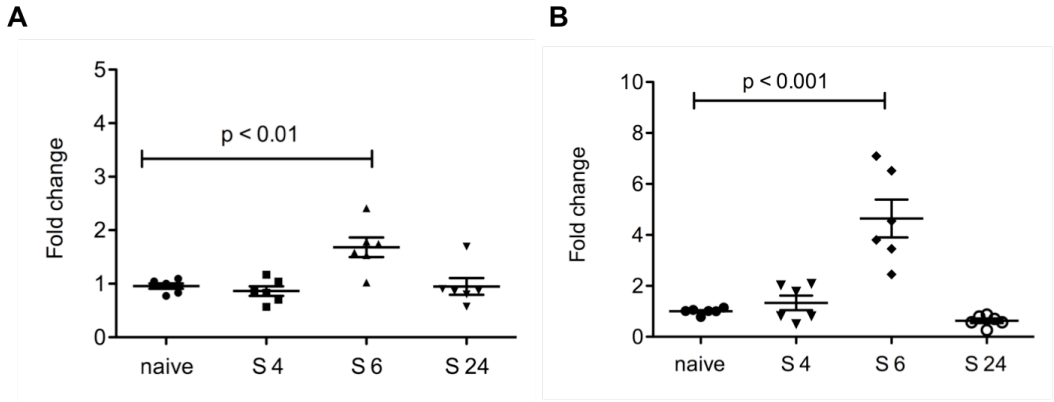


Figure 3-5: **IL-1β and IL-6 mRNA up regulation in the hippocampus**.

After extracting the mRNA, qPCR was used to measure the expression of IL-1β and IL-6 in the hippocampus. Levels of IL-1β peaked at 6 hours ($p<0.01$) and, to further corroborate these findings looking at a downstream effector, IL-6 was significantly up-regulated at 6 hours ($p<0.001$). Data are expressed as mean ± SEM (n = 6) and compared by one-way analysis of variance and Student-Newman-Keuls method with Bonferroni corrections.

These findings led to further investigate whether other inflammatory markers were induced in the hippocampus. Microglia cells are the resident macrophages of the CNS and is used to assess neuroinflammation in the brain. Post-surgical animals exhibited immunoreactive microglia in the hippocampus. Minimal immunoreactivity was reported in naïve animals in which cells maintained small cell bodies with thin and long ramified pseudopodia (figure 3.6A). Resting microglia shifted their state to a "reactive profile" after surgery, acquiring an amoeboid morphology with hypertrophy of the cell body and retraction of the pseudopodia. Reactive microglia displayed morphological changes including increased cell body dimensions, shortened and clumpy processes with higher levels of CD11b immunoreactivity compared to naïve animals. Microglial activation was reported at days 1 and 3 post surgery, returning to the baseline, resting state, by day 7 (figure 3.6 B-C-D; $p<0.01$, $p<0.05$ vs control). No changes in morphology were detected after exposure to isoflurane only.

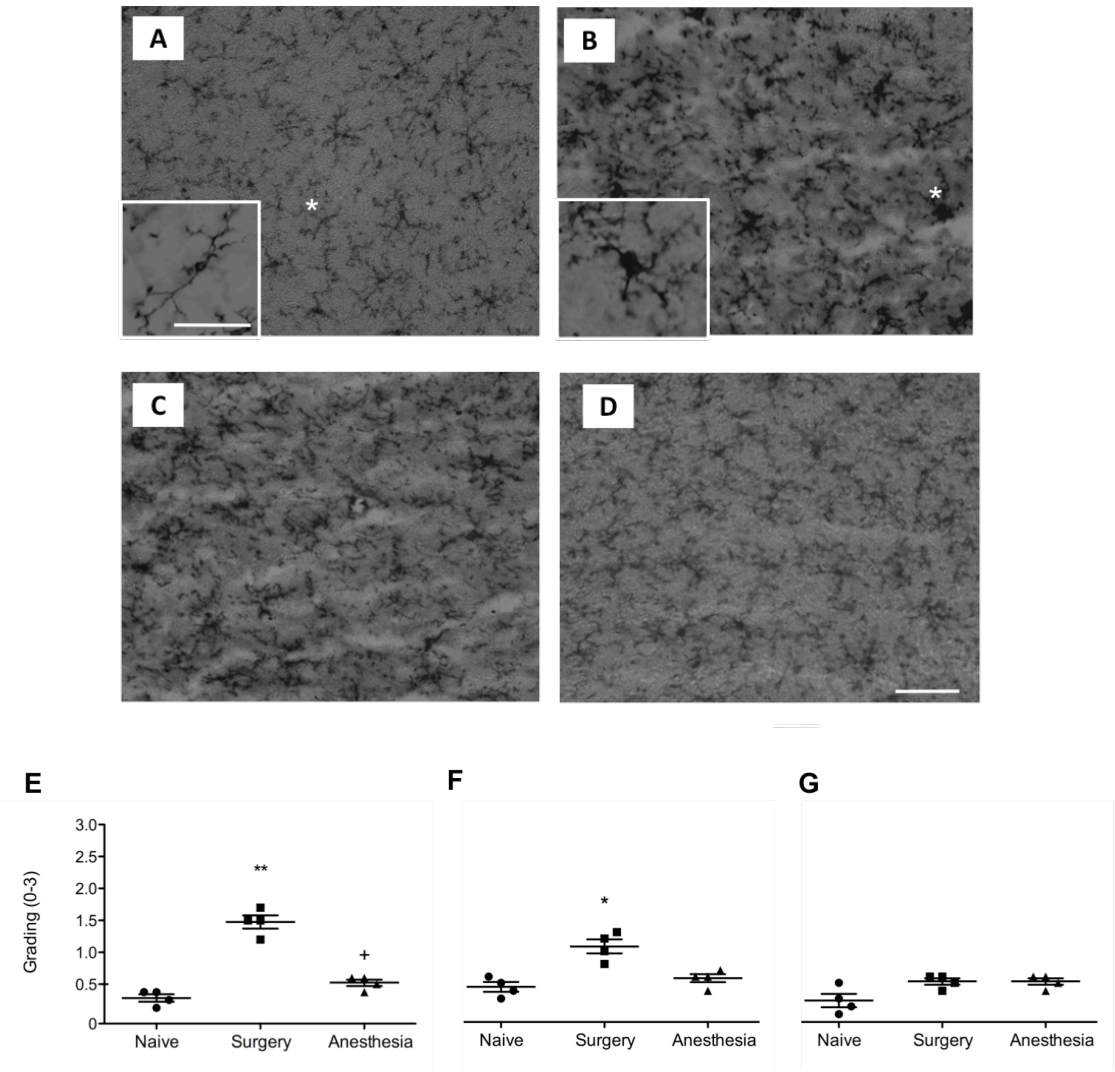


Figure 3-6: **Microglia activation following surgery**.

Hippocampi were harvested at days 1, 3, 7 after treatment and stained with anti-CD11b to indicate microglia activation following interventions. Photomicrographs were blindly scored and microglia activation was graded on a scale 0 (lowest) – 3 (highest). Reactive microglia were found at days 1 (**B**) and 3 (**C**) but not a day 7 (**D**) compared to untreated animals (**A**). Resting microglia (box A, 40X) shifted to a "reactive state" characterized by amoeboid hypertrophy of cell bodies, thickening of the proximal processes, decreased ramifications and clumped processes (box B, 40X). No changes were visible at day 7 (pictures show CA1, scale bar 50 μm, 20X). Immunohistochemical grading (0-3) (**E, F, G**) was presented for data illustration. At POD 1 clear microgliosis was present (**E**, *$p<0.05$ vs control, +$p<0.05$ vs anesthesia). Three days after treatment there were remaining signs of microglia activation in the surgery group (**F**, day 3, *$p<0.05$ vs control), but within 7 days levels returned normal (**G**) (n = 4). Non parametric data are presented with Kruskal-Wallis followed by Dunn's test.

3.5 Targeting of the inflammatory response ameliorates POCD.

After having defined the inflammatory response in POCD, I aimed to understand whether targeting this component would ameliorate cognitive dysfunctions by reducing pro-inflammatory cytokines. The basic attempt looked at the effects of minocycline, a non-specific inhibitor of the inflammatory response (Amin et al. 1996). Minocycline, a second-generation tetracycline antibiotic with anti-inflammatory properties, has widespread effects on immunomodulators (MMPs, iNOS, COX-2, TNF, IL-1, macrophages, B cells, T lymphocytes, microglia) (Giuliani et al. 2005). This non-specific approach targets both the peripheral and central inflammatory response. Pre-operative administration of minocycline was able to effectively reduce the amount of systemic inflammation. Levels of IL-1β were significantly reduced by minocycline treatment both at 6 and 24 hours following surgery (figure 7.7A; 20.11 ± 3.35 pg/ml, 15.67 ± 2.62 pg/ml, $p<0.01$ vs surgery respectively). Minocycline had profound effects on IL-6, with a dramatic reduction at 6 hours and a significant decrease at 24 hours (figure 7.7B; 30.91 ± 5.15 pg/ml, 8.36 ± 1.39 pg/ml, $p<0.001$ vs surgery respectively). No significant changes were reported at any of the other time points assessed.

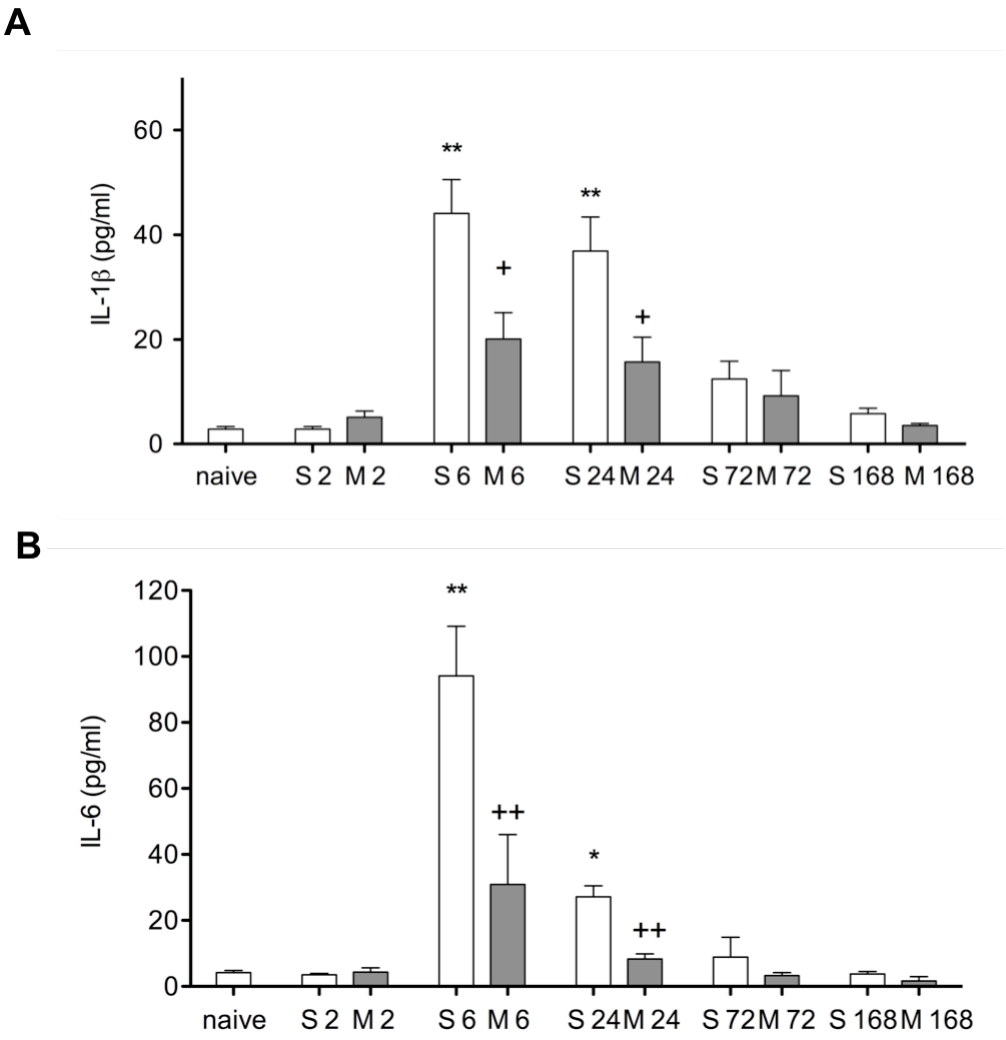


Figure 3-7: **Attenuation of the inflammatory response after minocycline**.

Mice were injected with minocycline (40 mg/kg) before surgery and daily thereafter until termination. Minocycline was able to significantly attenuate the amount of plasma IL-1β (**A**) and IL-6 (**B**) both at 6 and 24 hours post-intervention (+ $p<0.01$ vs surgery, ++$p<0.001$ vs surgery respectively). Data are expressed as mean ± SEM (n = 6) and compared by one-way analysis of variance and Student-Newman-Keuls method.

In order to correlate the role of minocycline to the surgical-induced behavioral abnormality, I first investigated the effects of this compound on microglia activation. There was a significant reduction in the amount of reactive microglia at day 1 post intervention (figure 3.8 B-C, $p<0.05$ vs surgery). Consistently, no signs of microgliosis were reported at POD 3 and 7.

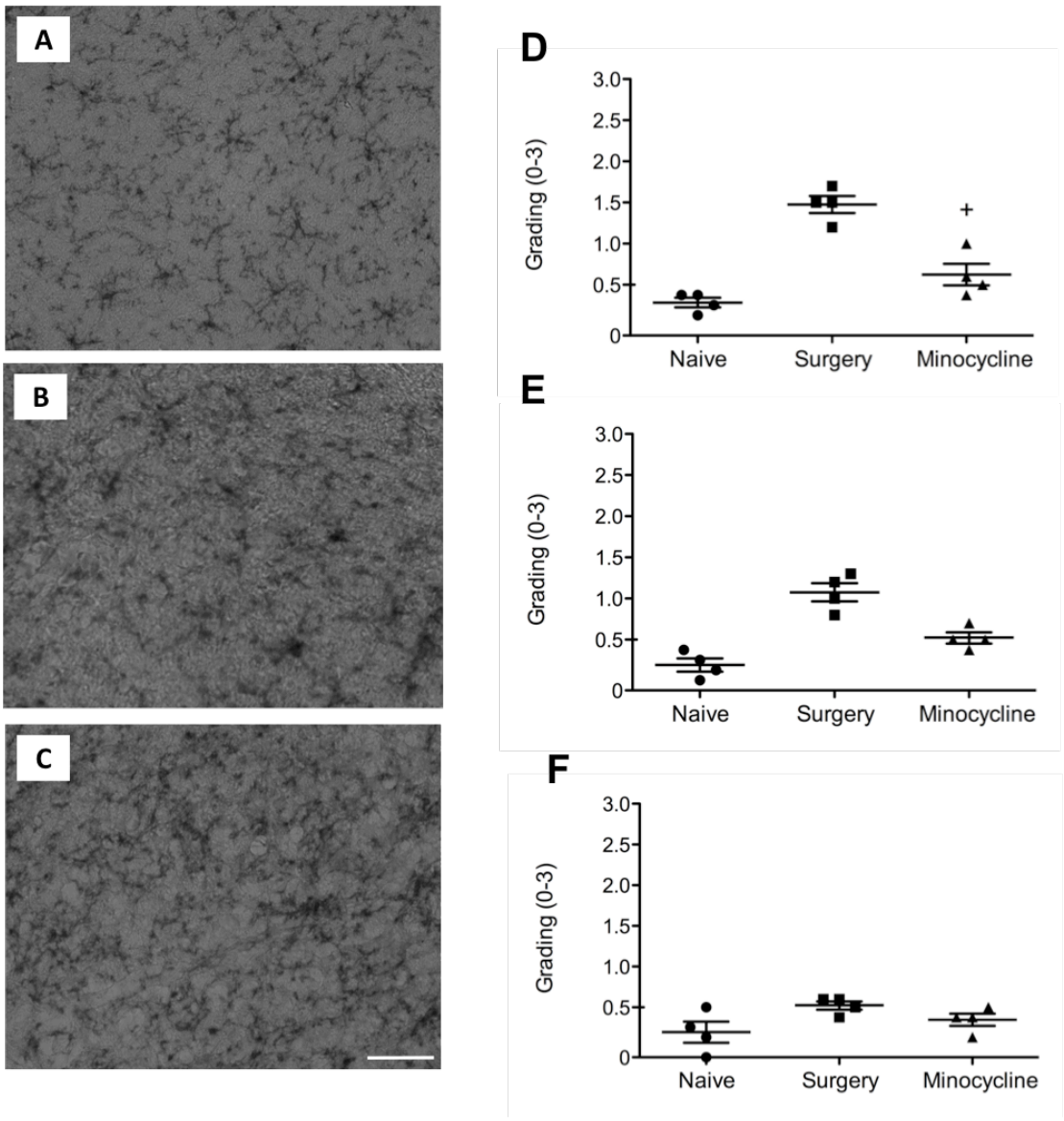


Figure 3-8: **Effects of minocycline on surgery-induced microgliosis**.

Hippocampi were harvested at days 1, 3, 7 after treatment and stained with anti-CD11b to indicate microglia activation following interventions. Photomicrographs were blindly scored and microglia activation was graded on a scale 0 (lowest) – 3 (highest). Administration of minocycline (40 mg/kg) pre-operatively and daily thereafter until termination was able to effectively reduced the level of reactive microglia at 1 day post-operatively (**B**). No significant changes were found at POD day 3 (**C**) (pictures show CA1, scale bar 50 µm, 20X). Figure A represent naïve animals. Immunohistochemical grading (0-3) (**D, E, F**) was presented for data illustration. At POD 1 the clear microgliosis present following surgery was effectively reduced by minocycline (**D**, +$p<0.05$ vs surgery). Both at POD 3 (**E**) and 7 (**F**) levels returned to normal (n = 4). Non parametric data are presented with Kruskal-Wallis followed by Dunn's test.

In order to establish a definite correlation between transcription of pro-inflammatory cytokines in the hippocampus and microglia activation, we also explored translation of IL-1β. Consistently with the 2-fold increase in IL-1β measured by qPCR, the expression of this cytokine was up-regulated 2-fold after 6 hours from surgery in the hippocampus (5.53 ± 0.89 pg/100μg of proteins, $p<0.05$) compared to naïve counterparts (figure 3.9A; 3.34 ± 0.39 pg/100μg of proteins). IL-1β expression in the hippocampus was not changed in animals treated with minocycline (figure 3.9A; 3.73 ± 0.21 pg/100μg of proteins).

In order to obviate the role of infection as a potential confounding factor in this model, an antimicrobial agent with no anti-inflammatory properties, enrofloxacin, was also given prior to surgery. Enrofloxacin failed to reduce levels of IL-1β centrally (5.19 ± 0.77 pg/mg of proteins, $p<0.05$ vs naïve) as well as in the systemic circulation. Furthermore enrofloxacin did not reduce the surgery-induced behavioral effect (figure 3.9B). These experiments were performed by Dr Mario Cibelli.

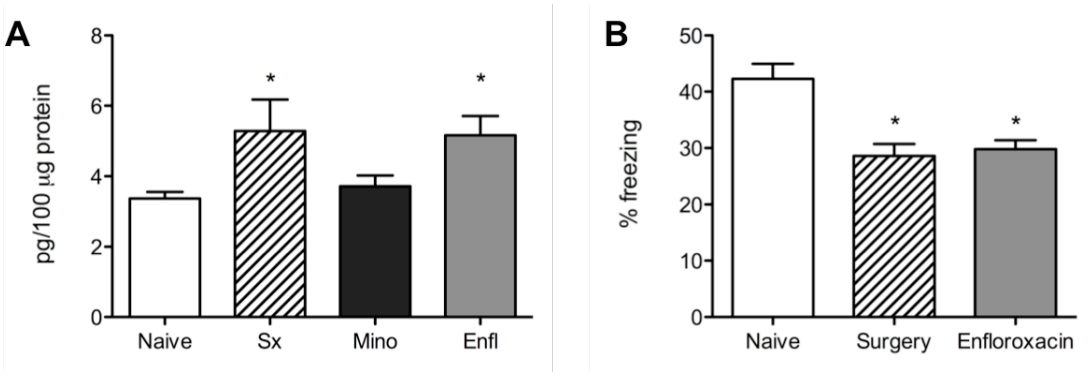


Figure 3-9: **Effects of enrofloxacin on POCD-associated behavior.**

Tissue collection was carried out 6 hours after treatment. Surgery (sx) resulted in increased expression of IL-1β in the hippocampus compared to naïve and anesthesia groups. Administration of minocycline (mino), but not enrofloxacin (enfl), mitigated surgery-induced hippocampal inflammation (*p<0.05 vs naïve and minocycline groups (**A**). Rodents underwent fear conditioning followed by tibia surgery under anesthesia (surgery), or the same surgical procedure with enrofloxacin (enrofloxacin) administration. Administration of enrofloxacin did not mitigate the surgery-induced, hippocampal-dependent, memory impairment (*p<0.05 vs naïve) (**B**). Data are expressed as mean ± SEM (n=7 for ELISA and n=34 for behavior) and compared by one-way analysis of variance and Student-Newman-Keuls method. Data presented with the permission of Mario Cibelli.

In order to assess the connection between systemic and central challenges in inflammation with POCD-associated behavior, animals were treated with minocycline and trace fear conditioning was carried out in order to assess memory retention. Conversely to what was reported following administration of the antibiotic enrofloxacin, which has no anti-inflammatory properties, contextual retrieval task revealed a significant improvement in memory retention following minocycline administration compared to untreated animals undergoing tibia surgery (figure 3.10A, p<0.05). In the auditory-cued test there was no difference in animals treated with minocycline (figure 3.10B). No changes were reported in the baseline freezing.

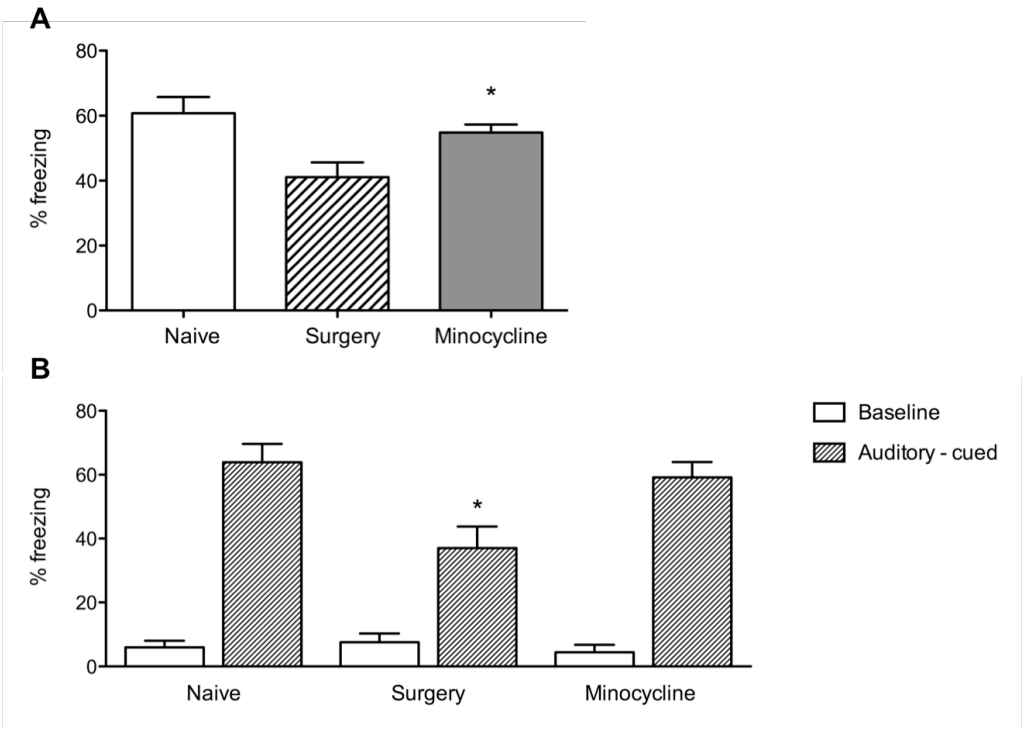


Figure 3-10: **Effects of minocycline on surgery-induced behavioral abnormality**.

Mice received either surgery under anaesthesia (surgery), Surgery under anaesthesia with minocycline (40 mg/kg, i.p.). Control comprised naive animals. Testing was conducted 3 days after intervention. Contextual fear response was significantly ameliorated after administration of minocycline (**A**) (+$p<0.05$ *vs* surgery). Hippocampal-dependent, surgery-induced memory impairment is shown in the auditory-cued test, in mice trained with trace fear conditioning (**B**). No difference was shown in the baseline freezing behavior or in the auditory – cued after treatment with minocycline. Data are expressed as mean ± SEM (n = 10) and compared by one-way analysis of variance and Student-Newman-Keuls method.

3.6 Discussion

The main objectives of this study were:

a) to characterize the inflammatory response associated with orthopedic surgery
b) to evaluate whether cytokines can mediate and ultimately orchestrate the behavioral abnormality associated with orthopedic surgery

To demonstrate the causal role of inflammation, minocycline has offered a protection to animals following surgery, suggesting that inflammation plays a pivotal role in the pathogenesis of POCD. Three main results have been accomplished: **(i)** anesthesia does not affect cytokine release; **(ii)** tibia fracture elevates systemic, namely IL-1β and IL-6; **(iii)** minocycline effectively reduces inflammation.

Anesthesia is known to affect the brain and nervous system at many molecular targets and these changes can alter brain functions for several days after exposure (Franks and Lieb 1994; Futterer et al. 2004). One of the most important features, and perhaps the reason why anesthesia accounts for a major breakthrough in modern medicine and surgery, lies on its ability of inducing temporarily loss of consciousness that is fully reversible. However, this concept of "entire reversibility" has been challenged from many authors and in different contexts. In particular, anesthetic agents have been correlated with memory impairment and as capable of interfering with different synaptic functions in the hippocampus (Culley et al. 2003; Wise-Faberowski et al. 2005). In this study the administration of a clinically used volatile

anesthetic agent, isoflurane, did not increase plasma concentrates of key pro-inflammatory cytokines including IL-1β, IL-6, and TNF-α. Despite a vivid debate on the role of anesthesia in POCD, these data provide no evidence of any causative relationship between anesthesia, cytokine production, and POCD (Simpson et al. 1961; Rasmussen et al. 2003; Wan et al. 2007).

It has been speculated on the possible role of cytokines in the development of POCD and it has now become a noteworthy concept in this area of research (Gao et al. 2005; Mathew et al. 2007). Few studies have described the effect of neuroinflammation in interfering with hippocampal-dependent memory processes (Wan et al. 2007; Rosczyk et al. 2008). Cytokine release is classically associated with "sickness behavior", which *per se* implies cognitive changes and even depression (Raison et al. 2006; Dantzer 2009; O'Connor et al. 2003). The peripheral cytokine response after surgery is a well-described phenomenon and the extent of the procedure / insult seems to directly correlate with the amount of inflammatory compounds released in the bloodstream. After skin incision, immunocompetent cells at the site of intervention rapidly release IL-6 and plasma concentrates peak at 8-24 hours (Minetto et al. 2006). Many factors have found to regulate IL-6 release (age, severity of trauma, length of operation) and have been proportionally correlated with the amount of cytokine released in the blood (Shenkin et al. 1989). Although there was an insignificant increase in TNF-α, this cytokine may still be responsible for the activation and release of IL-1β in the systemic circulation. This concept will be further analyzed in the following chapters. Plasma IL-1β was significantly high after 6 and 24 hours post-surgery. Both IL-1β and IL-6 are able to gain entry to the brain via relatively permeable

areas, especially in the CVOs and periventricular regions. Also, it has been shown that IL-1β can bind receptors and get through the intact BBB (Van Dam et al. 1993; Watkins et al. 1995). Neural afferents are known to be a fast and reliable pathway in the immune-to-brain communication, thus cytokines can gain free access to the CNS (Watkins et al. 1995). Elegant studies have reported that vagotomy attenuates classical symptoms of sickness behavior after systemic LPS or IL-1β administration (Bluthe et al. 1994; Dantzer 2004; Wieczorek et al. 2005). Since the orthopedic procedure performed in this study does not directly affect vagal afferent inputs, humoral, rather than, or in addition to, neural factors are likely to be involved. However, the rapid up regulation in mRNA expression in the hippocampus after 6 hours following surgery may suggest an involvement of neural afferents in this fast communication.

Within the CNS, cytokines closely converse with microglia cells, the immunocompetent cells of the brain. Pro-inflammatory cytokines such as IL-1β and IL-6 directly interact with the PRRs expressed on the surface of these cells. Upon activation, microglia exhibit discernible morphologic changes including enlargement of the cell body, thickening of the proximal processes and decreased ramification. In the active state, microglia are capable of mounting macrophage-type innate immunity and secrete pro-inflammatory cytokines, reactive oxygen species (ROS), excitotoxins (such as calcium and glutamate) and neurotoxins such as β-amyloid (van Rossum and Hanisch 2004). Activated microglia also inhibit neurogenesis in the hippocampus following endotoxemia, thereby exacerbating the extent of injury (Monje et al. 2003). Microglia are able to increase the synthesis and *de novo* production of

cytokines as well as ROS and MMPs within the brain, and this may account for the memory disruption found in POCD. The effects of the stress response, in particular the activation of purino receptors (P2X), may also intensify cellular degeneration and cytokine release by reducing intracellular K^+ levels and increasing caspase-1 activity (Di Virgilio 1995; Ferrari et al. 1997). In the CNS cytokines seem to have a biphasic effect; at low concentration and in the normal brain they are essential for a correct neuronal development and memory formation. However, at pathophysiological levels, they become dangerous and possibly responsible for the cognitive decline and neurodegeneration (Simi et al. 2007). This close interaction between cytokines and microglia may be responsible for the production of a highly hostile environment, and especially in a very vulnerable area such as the hippocampus, it may account for the inhibition of long-term potentiation (LTP), hence memory loss (Rothwell and Hopkins 1995; Griffin et al. 2006). An increase in the concentration of IL-1β, together with microglia activation, are causally related to the impairment of LTP as well as for a possible β-amyloid induced deficit in hippocampal function (Wang et al. 2004). Considering the resembling features of POCD with Alzheimer's disease (AD), also the action of isoflurane has been directly associated with increased Aβ oligomerization in H4 human neuroglioma cells stably transfected to express β-amyloid precursor protein – full length (APP – FL) (Xie et al. 2006; Xie et al. 2006). Thus cytokines and microglia are capable of producing a synergism whereby the signaling pathways involved in memory formation become damaged (Maher et al. 2006).

A significant increase in plasma concentrates of IL-1β was reported at 1 day

post-intervention. IL-1 activates distinct cellular signaling pathways to ultimately affect NFkB and p38 MAPK (Pinteaux et al. 2002; Hua et al. 2005). Post-receptor events greatly affect cytokine synthesis and *de novo* production. Besides acting on its cognate receptor, IL-1R, systemic IL-1β is likely to closely engage with TLRs, therefore enhancing the inflammatory effects seen in this model (Beutler 2004). Evidence for this mechanism will be later discussed in the context of LPS and endotoxemia.

To determine the contribution of peripheral cytokines with surgery-related cognitive dysfunction, minocycline, a tetracycline derivative with immunomodulatory properties was administered to surgical animals (Giuliani et al. 2005). Minocycline exerts its anti-inflammatory effects both systemically and centrally; it impairs the production of cytokines such as TNF-α and IL-1β but also up regulates anti-inflammatory cytokines such as IL-10 (Ritchlin et al. 2000; Bye et al. 2007). It also dampens systemic inflammation as a result of a direct inhibition of MMP-9 and T-lymphocyte transmigration and by blocking phospholipase A2 (Pruzanski et al. 1992; Brundula et al. 2002). Furthermore, neuroprotective effects including down regulation of proliferating microglia, inhibition of caspase-3 activity and apoptosis have all been reported after minocycline administration (Lee et al. 2003). It is likely that some of the effects we have reported after injecting minocycline, especially in the reduction of IL-1β, could be mediated by a direct inhibition of p38 MAPK, which would then turn off gene expression and release of pro-inflammatory cytokines (Hua et al. 2005; Nikodemova et al. 2006). Minocycline is known to inhibit microglia activation (Ekdahl et al. 2003) and, by also reducing cytokines, it was able to ameliorate the cognitive deficit following surgery.

These data suggest that cytokines production is a direct effect of surgery and no other confounding factors including infection and / or pain. In this attempt of reproducing a routine clinical setting, analgesia (buprenorphine) was administered and no clinical evidence of infection were seen in any of the animals. Previous studies using this murine tibial fracture model reported no evidence of clinical or histological sign of infection in any case (Harry et al. 2008). Administration of enrofloxacin, a wide spectrum antibiotic with no anti-inflammatory properties, did not improve any of the surgery-induced effects. Current work in the laboratory is looking at pain in greater details (hot plate for pain threshold and activity box for locomotor activity) without reporting any significant differences following surgery. Furthermore, using the trace fear conditioning, pain or movement impairment would reflect in increased immobility, thus higher freezing, whereas the opposite was observed in the surgical animals.

These data corroborate two other previous reports (Wan et al. 2007; Rosczyk et al. 2008) showing the association between surgery, inflammation and cognitive dysfunction. The mechanism whereby a peripheral surgical intervention initiates hippocampal neuroinflammation still remains unclear. Here we have reported a significant up regulation of key inflammatory cytokines in plasma after major orthopedic surgery. Despite these changes being transient and fully reversed by postoperative day 3, the memory impairment persists. This may hinge on the disruption of memory consolidation pathways, possibly by an effect of cytokines, during the processing of information from short-term (hippocampus) to long-term

memories in the neocortex (Kim and Fanselow 1992; Squire and Alvarez 1995; Anagnostaras et al. 1999; Frankland and Bontempi 2005).

We may be looking at a subtle form of POCD rather than a more chronic dysfunction. The incidence of both delirium and POCD is increased in surgical patients with advanced age, possibly due to the normal physiological changes in the immune activity / response. In the critically ill patients this translates in higher morbidity and mortality (Rockwood et al. 1999; Ely 2003). An increase in the concentration of IL-1β, together with activation of microglia, may contribute to the persistence of hippocampal-dependent cognitive dysfunction in the elderly (Miller and O'Callaghan 2005). Apart from age, there may be other factors capable of either increasing the duration and / or severity of the neuroinflammatory response to surgery. The supervention of a secondary infection may produce an anamnestic response. Furthermore, factors including co-morbidities such as underlying neurodegeneration (mild cognitive impairment [MCI]), cancer, metabolic syndrome, and gene polymorphism (APOe4) may produce a milieu in which the addition of surgery-induced neuroinflammation, creates a sustained cognitive dysfunction (Heyer et al. 2005; Caselli et al. 2007; Adamson et al. 2008). Because attenuation of the neuroinflammatory response with minocycline reduces circulating levels of pro-inflammatory cytokines and prevents the occurrence of post-surgical cognitive dysfunction, this represents a realistic target for therapeutic interventions in the clinical setting. Subsequent studies are aiming to elucidate the mechanisms whereby inflammation is produced and whether we can find novel and more specific targets for effective therapies.

4 Chapter 4: results

4.1 RATIONALE

Up regulation of peripheral pro-inflammatory cytokines, CNS inflammation, and the ensuing sickness behavior associate with innate immunity (Dantzer 2001). The mechanisms that underlie inflammation following surgery and general trauma share a number of similarities with infective pathways, but yet remain unclear. Following the effects ascribed to minocycline on the inflammatory response and POCD-associated behavior, I aimed to further investigate the putative pathways associated with the cognitive abnormality. The inhibitory effects of minocycline both on peripheral and central inflammation can be associated to p38 MAPK signaling and NFkB, the canonical pathways in IL-1 / TLRs signaling (Nikodemova et al. 2006; Cui et al. 2008).

TLRs and the IL-1 are pivotal structures whereby the innate immunity gets activated and communicates with the other components. The homologies between these PRRs are mostly found across the cytoplasmic region, whereas the extracellular domain largely differs between TLRs and IL-1Rs since the former has a leucine-rich repeat (LRR) motif and the latter contains three immunoglobulin-like domains (Akira and Takeda 2004; Takeda and Akira 2004). Despite the LRR motif being conserved across TLRs, several unique molecular patterns originated by both pathogens and endogenous ligands are recognized by each TLR in different cellular compartments (cell surface, endoplasmic reticulum, lysosomal compartments) (Zhang and Ghosh 2001; Yang et al. 2008). The involvement of PRRs in recognizing a wide

variety of microbial and infective agents is well recognized and described. PRRs are expressed on immune cells; macrophages and microglia in particular sense minimal changes in body's homeostasis thanks to the variety of PRRs expressed on their surfaces. Recognition of pathogens and potentially harmful substances strongly relies on TLRs, but many of the ligands that activate these receptors still remain to de discovered. Amongst all TLRs, TLR4 seems the most active interface to bridge microbial infection with sterile inflammation (Levy et al. 2006; Mollen et al. 2006; Kigerl et al. 2007). Its role in mediating inflammation after endotoxic shock and LPS in particular are now well established (Hoshino et al. 1999). More recently, a correlation between endogenous proteins released after tissue injury and TLR4 has been suggested (Paterson et al. 2003; Mollen et al. 2006). A series of studies have been conducted using TLR4 deficient mice looking at the danger signal after acute tissue injury including hemorrhagic shock (Barsness et al. 2004), cardiac (Oyama et al. 2004), and hepatic (Tsung et al. 2005) ischemia/reperfusion models, showing reduced inflammation and injury compared to wild types. Activation of these receptors by both exogenous and endogenous molecules leads to complex downstream signaling mostly relying on MAPK, JNK and NFkB transcription factors.

Despite the improved outcomes reported in TLR4 deficient mice following acute tissue injury and ischemia/reperfusion (Mollen et al. 2006), I aimed to first define the involvement of these receptors using animals devoiding the expression of MyD88 (figure 4.1).

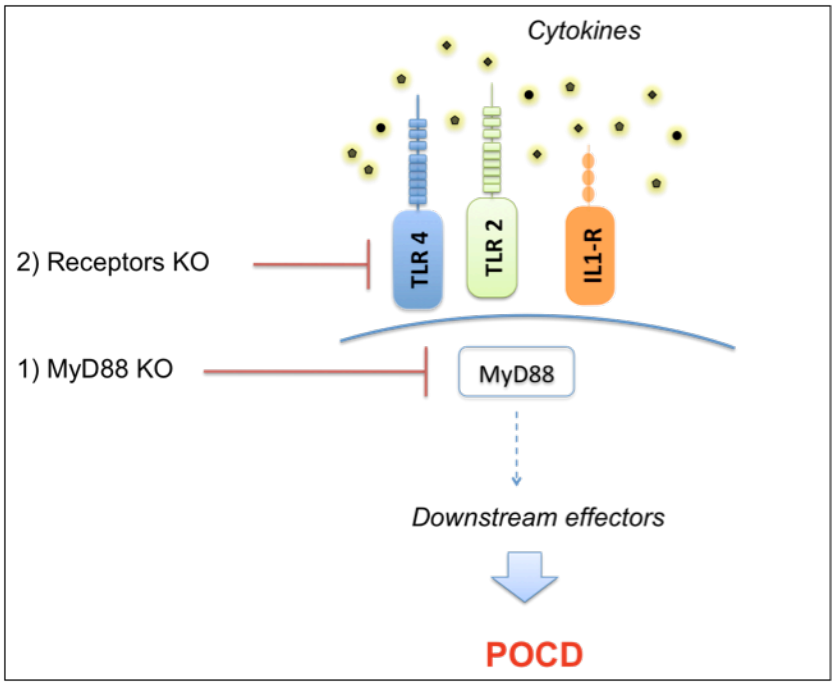


Figure 4-1: **Study plan**

Following the results with minocycline on inflammation and POCD-associated behavior, the aim was now to indentify putative signaling pathways involved with this dysfunction. The first set of experiments involved reagents devoiding in MyD88 expression (MyD88 KO). To follow-up from this initial investigation, I aimed to localize possible receptors involved in this inflammatory signaling. As MyD88 is shared in a number of pathways and receptors, I have focused on the role of IL-1R, TLR4 and TLR2 in POCD-associated inflammation.

Both TLRs and IL-1Rs affect NFkB gene expression, and their signaling largely depends on the downstream adaptor protein myeloid differentiation factor (MyD88), which constitutes the focal pathway to promote *de-novo* transcription of pro-inflammatory genes (Kimbrell and Beutler 2001; Akira and Takeda 2004; Li and Qin 2005; Lotze and Tracey 2005). Its involvement in mediating, sustaining, and facilitating the signaling following infection is pivotal. Upon understanding the role of MyD88 in the context of POCD, I sought to then target specific receptors as putative mediator of the inflammatory response and behavioral abnormality. Due to the involvement of TLR2 and TLR4 in sterile inflammation and trauma, alongside the importance of the IL-1 signaling in mediating sickness behavior and memory dysfunction, these mechanisms have been elucidated in POCD (Rachal Pugh et al. 2001; Paterson et al. 2003; Levy et al. 2006; Kaczorowski et al. 2008).

4.2 MyD88

4.2.1 Targeting MyD88 abrogates surgery-induced cognitive dysfunction

The effects of minocycline both on systemic and central inflammation highlighted the key role of the inflammatory response in the etiology of surgery-induced behavioral abnormality. Despite its putative action via p38 MAPK (Nikodemova et al. 2006), the non-specificity of this compound has only begun to answer the many questions associated with the signaling mechanisms in POCD. In order to address some of these questions, I opted to more selective approaches. Genetic reagents are able to offer this specificity, and, in particular, they have the potential to clarify the role of many biological functions in the absence of specific target molecules.

Adaptor molecules are crucial in signaling mechanisms; MyD88 has a pivotal role in activating and sustaining different inflammatory pathways in innate immunity. Animals lacking in the expression of MyD88 appear a suitable model to exploit the findings of POCD and investigate more specifically the role of putative pathways associated with surgery-induced cognitive dysfunctions. Indeed, one major limitation of genetic reagents concerns the ability of assuring a comparison with what is referred as “normal”, at least from a phenotypic standpoint. My first aim was then to establish a congruent learning curve in knockouts (KO), which would allow direct comparison with the wild type (WT) counterparts and the data obtained up to this time.

Trace fear conditioning was carried out as previously described. Acquisition and establishment of working memory during the training was also achieved with no significant differences between groups (figure 4.2).

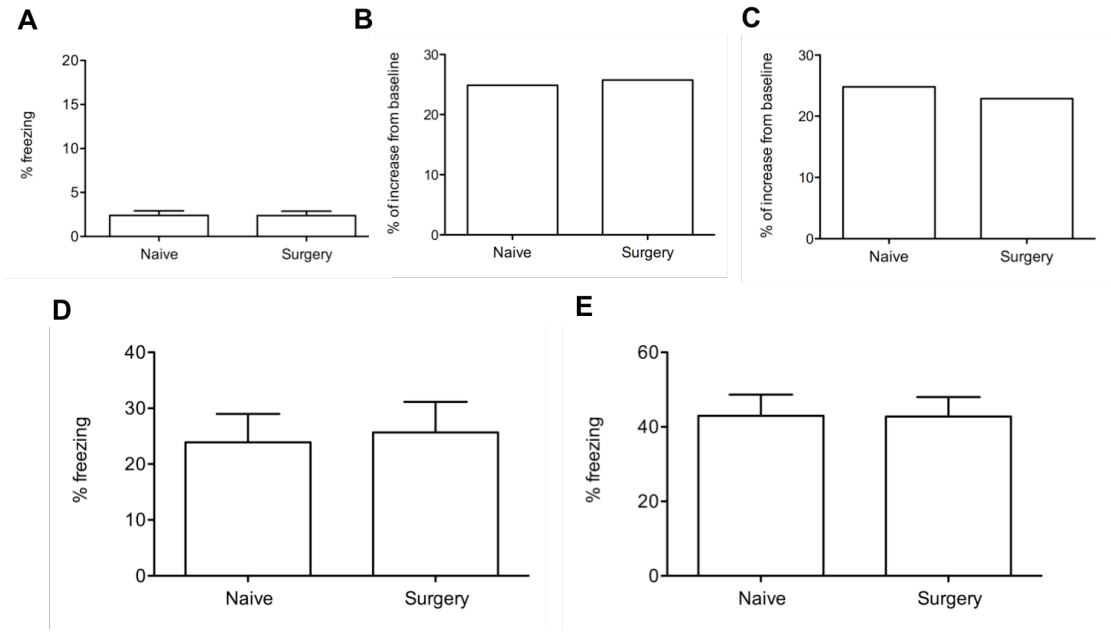


Figure 4-2: **Training phase during trace fear conditioning in MyD88$^{-/-}$**.

Animals were collegially trained and then subdivided in two groups: untreated (naïve) and tibia fracture under inhaled isoflurane (surgery). Baseline freezing at the time of first exposure of these animals in the context is very low, indicating low levels of stress (A). Figure B indicates the increase of freezing response from Tone 1 to Tone 2, indicating working memory. Figure C indicates the increase of freezing response from Trace Interval 1 to 2, an indicator of working memory. Figure D indicates the working memory freezing response to context between tone – shock pairings. All animals demonstrate an equal working memory performance (E).

Immediately following training, animals were treated accordingly. Due to the limitations in the availability of these reagents due to their low reproductive rate, I limited this investigation by comparing MyD88$^{-/-}$ naives with surgery only. Mice in the surgical group underwent treatment within 30 minutes after training. Three days after treatment, rodents were taken back to the conditioning chamber and re-exposed to the same environment as during training. Contextual retrieval task revealed no significant changes in freezing behavior when comparing naïve MyD88$^{-/-}$ to animals receiving tibia surgery (figure 4.3A). Also, during the auditory-cued test, no changes in freezing were reported. No changes in baseline freezing were reported as shown before (figure 4.3B). Data from WT counterparts are illustrated for comparison.

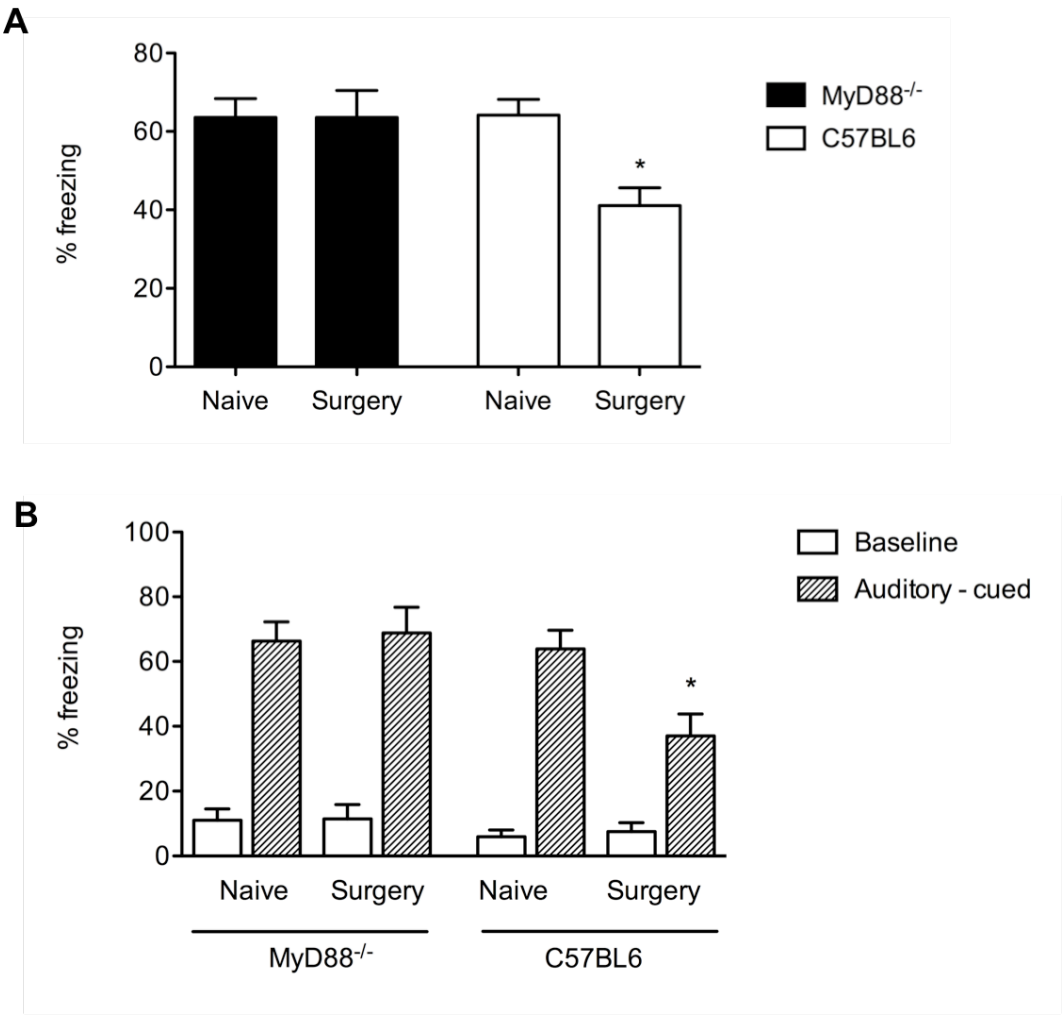


Figure 4-3: **Hippocampal-dependent memory following surgery in MyD88$^{-/-}$.**

Contextual fear response, as measured by freezing behavior, is the same in animals receiving surgery compared to naïve (**A**). Surgery-induced cognitive dysfunction in WT is illustrated for comparison. Surgery-induced memory impairment is shown in the auditory-cued test in mice trained with trace fear conditioning (**B**). There is no reduction in freezing following the auditory-cued retrieval test in MyD88$^{-/-}$. No difference was shown in the baseline freezing behavior showing no signs of anxiety. Data are expressed as mean ± SEM (n = 10) and compared by one-way analysis of variance and Student-Newman-Keuls method.

4.2.2 Surgery-induced elevation of systemic cytokines is significantly reduced

In order to further elucidate the putative relationship between systemic inflammation and behavioral abnormality, plasma levels of cytokines were measured in MyD88$^{-/-}$ animals. Based upon the findings on the WT, levels of IL-1β and IL-6 were assessed using ELISA at 6 and 24 hours. Following surgery, IL-1β was significantly reduced both at 6 hours (figure 4.4A; 14.27 ± 2.4 pg/ml, $p<0.001$) and 24 hours (10.04 ± 1.7 pg/ml, $p<0.01$) [WT values for comparison: 44.13 ± 7.35 pg/ml and 36.89 ± 6.54 pg/ml at 6 and 24 hours respectively]. IL-6 response was also attenuated in MyD88$^{-/-}$ with only a 1.5-fold increase at 6 and 24 hours (figure 4.4B; 19.79 ± 3.23 pg/ml; 6.89 ± 1.15 pg/ml, $p<0.001$ respectively) [WT values for comparison: 94.08 ± 15.68pg/ml and 27.21 ± 4.53 pg/ml at 6 and 24 hours respectively]. In order to understand the effects of anesthesia in these mice, MyD88$^{-/-}$ were exposed to isoflurane MAC 1.5 for 20 minutes and plasma cytokines were measured at 24 hours. Levels of both IL-1β and IL-6 remained at baseline (figure 4.4C, D). Corroboration and validation of the assays was also achieved by administration of LPS (1 mg/kg i.p.). LPS-injected animals served as negative controls. A drastic reduction in the amount of systemic inflammation was observed when compared to WT injected counterparts that displayed 8-fold increase in IL-1β (73.49 ± 12.24 pg/ml; at 24 hours, $p<0.001$ respectively) and 14-fold increase in IL-6 at 24 hours (figure 4.4C, D; 126.47 ± 21.07 pg/ml, $p<0.001$ respectively) (Adachi et al. 1998).

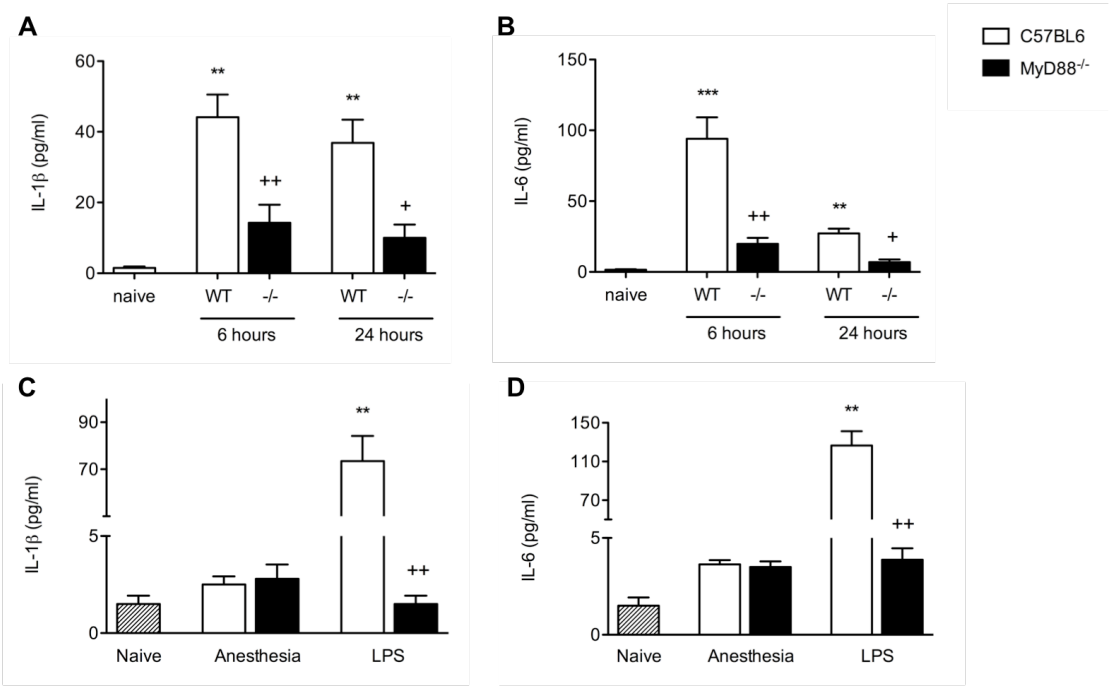


Figure 4-4: **Surgery-induced systemic inflammation in MyD88$^{-/-}$.**

IL-1β and IL-6 levels in plasma were measured by ELISA at 6 and 24 hours post-intervention. Surgery resulted in reduced plasma levels of IL-1β (**A**) and IL-6 (**B**) in MyD88$^{-/-}$ compared to WT (++p<0.001, +p<0.01 vs WT). LPS-induced systemic inflammation was not present in KO animals compared to exposed WT animals (**C**, **D** ++p<0.001 vs WT). No changes in systemic cytokines were reported following exposure to anesthesia only (**C**, **D**). Data are expressed as mean ± SEM (n = 6) and compared by two-way analysis of variance and Student-Newman-Keuls method.

4.2.3 Reduced neuroinflammation in MyD88$^{-/-}$

From the systemic effects of surgery in MyD88$^{-/-}$, I moved to the brain to investigate the central response following surgery. The strategy adopted here was to understand whether the reduced peripheral cytokines was accompanied by an attenuation of surgery-induced neuroinflammation. To address this question I focused on the hippocampus, looking at both the expression of IL-1β and the subsequent microglia activation. Translation of IL-1β in the hippocampus was significantly reduced at 6 hours following tibia surgery comparing to WT animals (figure 4.5; 3.47 ± 0.19 pg/100μg of proteins). Values from WT animals following surgery are illustrated for comparison (5.98 ± 1.23 pg/100μg of proteins, $p<0.01$ vs naive). No changes in IL-1β translation were found in naïve animals, either KO or WT.

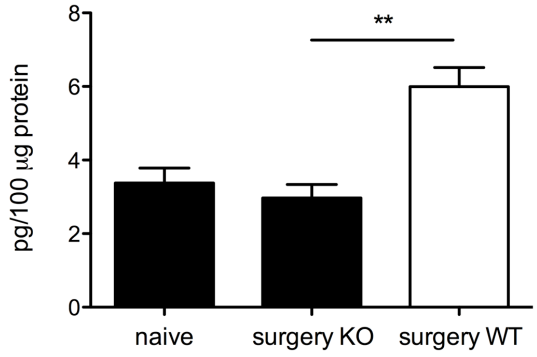


Figure 4-5: **Hippocampal IL-1β response following surgery in MyD88$^{-/-}$.**

Tissue collection was carried out 6 hours after treatment. Surgery in MyD88$^{-/-}$ resulted in no changes in the expression of IL-1β compared to naïve KO. There was a significant difference between KO and WT following the same surgical procedure (**p<0.001 comparison between surgery KO vs surgery WT). A positive control from LPS (1 mg/kg) treated animals was used to validate the readings. Data are expressed as mean ± SEM (n = 6) and compared by one-way analysis of variance and Student-Newman-Keuls method.

The findings on reduced hippocampal IL-1β prompted to me to investigate whether translation of IL-1 is casually related to microglia activation. Staining with CD11b in the hippocampus revealed no evident signs of reactive microgliosis in any of the treatments in MyD88$^{-/-}$ animals. In particular, no morphological changes in microglial conformation 24 hours post intervention were observed in MyD88$^{-/-}$ undergoing surgery. Resting microglia were found in animals undergoing anesthesia alone or LPS-exposure (figure 4.6).

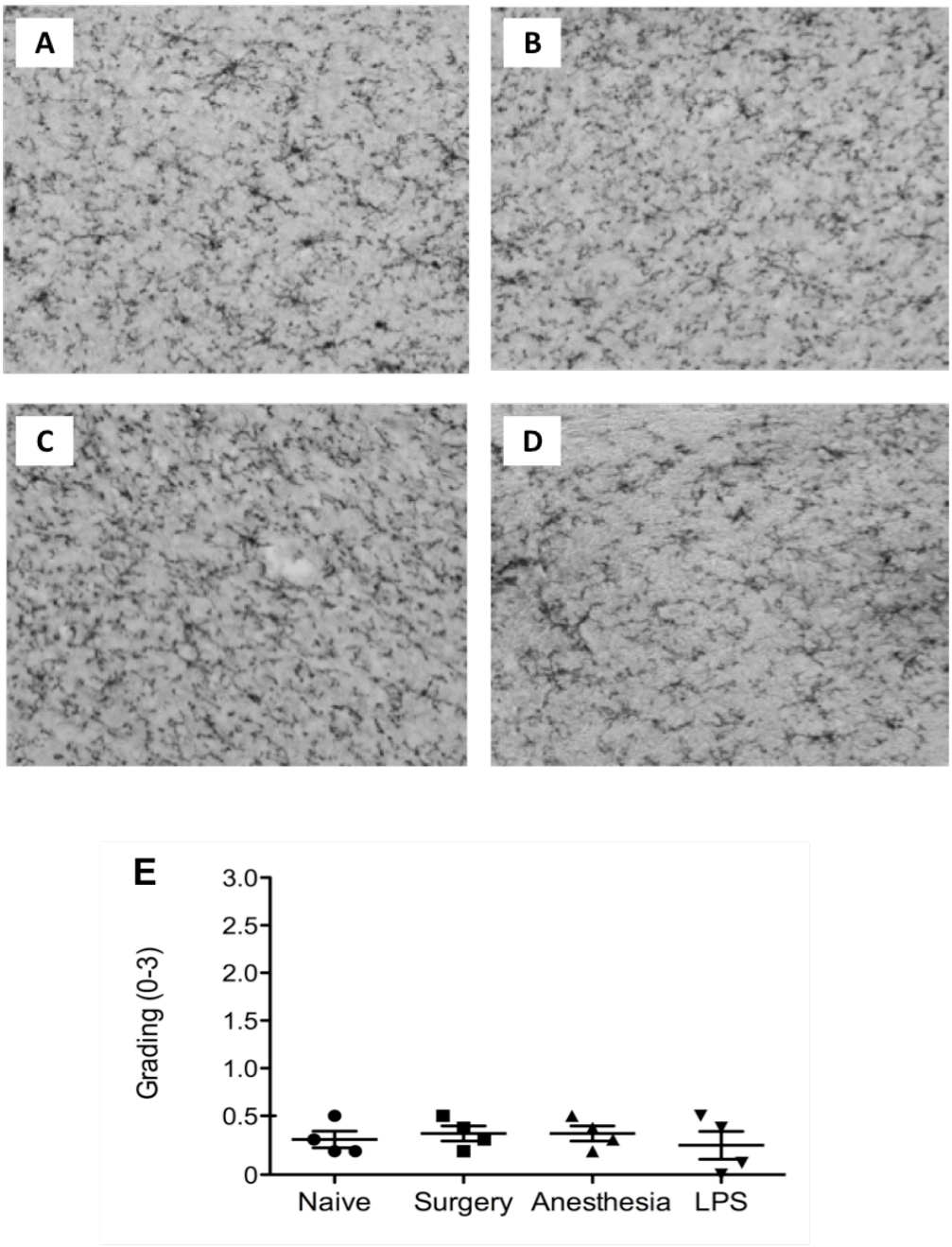


Figure 4-6: **Microglia activation following surgery**.

Hippocampi were harvested 24 hours post intervention and stained with anti-CD11b to indicate microglia activation following interventions. Animals were exposed to anesthesia only, underwent surgery of the tibia, or exposed to LPS. Untreated animals served as naïve. Photomicrographs were blindly scored and microglia activation was graded on a scale 0 (lowest) – 3 (highest). No signs of reactive microgliosis were observed in any of the treatments (**A** – naïve, **B** – anesthesia, **C** – surgery, **D** – LPS) (pictures show CA1, scale bar 50 μm, 20X). LPS slides from WT were used as positive control. Immunohistochemical grading (0-3) (**E**) was presented for data illustration. No significant changes in microgliosis were reported (n = 4). Non parametric data are presented with Kruskal-Wallis followed by Dunn's test.

4.3 IL-1

4.3.1 IL-1 receptor: a putative target dependent on MyD88 signaling

The findings on the MyD88$^{-/-}$ animals prompted interest for identifying putative targets (i.e. receptors) associated with POCD. A prime candidate with a downstream signaling pathway largely dependent on MyD88 is the IL-1 receptor (IL-1R). In order to understand the putative role of IL-1β and the IL-1R, I began my investigation looking at the inflammatory profile in animals lacking this receptor (IL-1R$^{-/-}$). Experiments focused on the crucial time points obtained from WT data, as previously highlighted. Levels of IL-1β and IL-6 were reduced in IL-1R$^{-/-}$ animals following surgery at 6 hours (IL-1β: 18.6 ± 3.1 pg/ml; IL-6: 29.78 ± 4.96 pg/ml, $p<0.001$ respectively) and 24 hours (figure 4.7A, B; IL-1β: 8.722 ± 1.45 pg/ml, $p<0.001$; IL-6: 14.79 ± 2.47 pg/ml, $p<0.01$). Consistently with the previous data, no changes were reported after anesthesia in either of these cytokines. The systemic response following administration of LPS was also significantly reduced in IL-1R$^{-/-}$ (figure 4.7C, D; $p<0.001$).

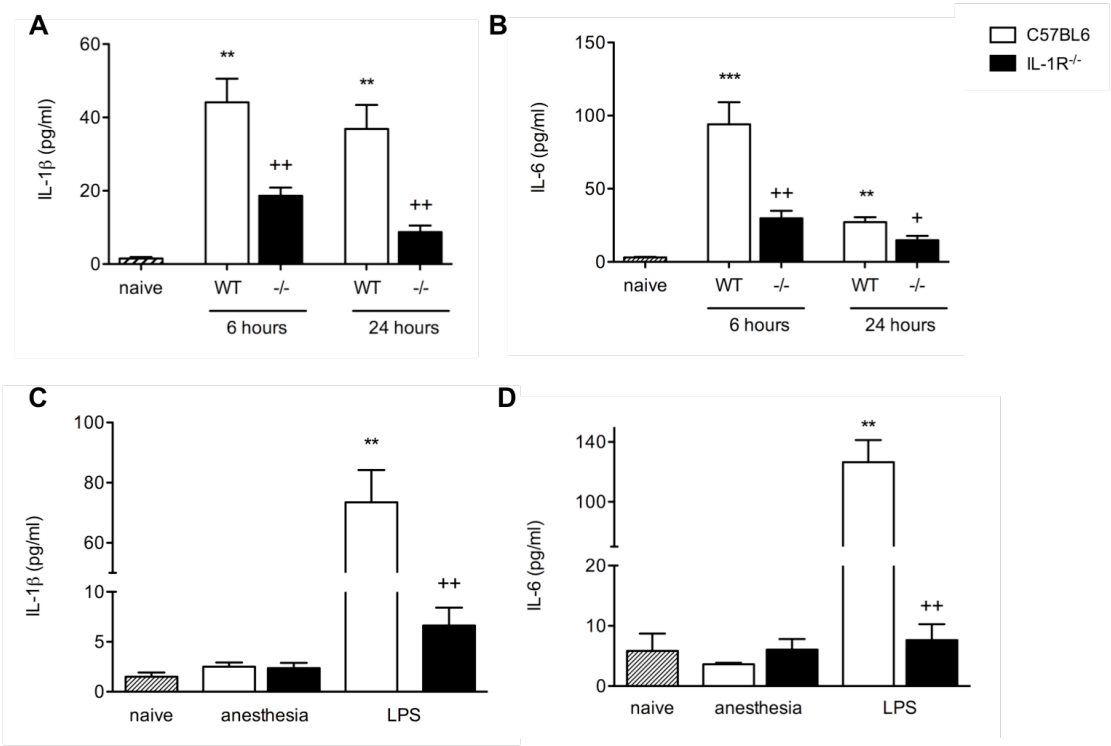


Figure 4-7: **Surgery-induced systemic effects in IL-1R$^{-/-}$.**

IL-1β and IL-6 levels in plasma were measured by ELISA at 6 and 24 hours post-intervention. Surgery resulted in reduced plasma levels of IL-1β (**A**) at 6 and 24 hours (++p<0.001 vs WT respectively). IL-6 (**B**) was also reduced in IL-1R$^{-/-}$ (++p<0.001 and +p<0.01 vs WT) at 6 and 24 hours post intervention. LPS-induced systemic inflammation was not present in KO animals compared to exposed WT animals (**C**, **D** ++p<0.001 vs WT). No changes in systemic cytokines were reported following exposure to anesthesia only (**C**, **D**). Data are expressed as mean ± SEM (n = 6) and compared by two-way analysis of variance and Student-Newman-Keuls method.

4.3.2 Neuroinflammation is significantly reduced in IL-1R$^{-/-}$

The systemic changes prompted me to look for neuroinflammatory effects. Levels of IL-1β in the hippocampus were significantly reduced at 6 hours in IL-1R$^{-/-}$, with no differences from naïve KOs (figure 4.8; 3.27 ± 0.15 pg/100μg of proteins). Injection of LPS in WT animals served to validate the readings. Levels of IL-1β following surgery in WT (5.98 ± 1.23 pg/100μg of proteins) are shown for comparison.

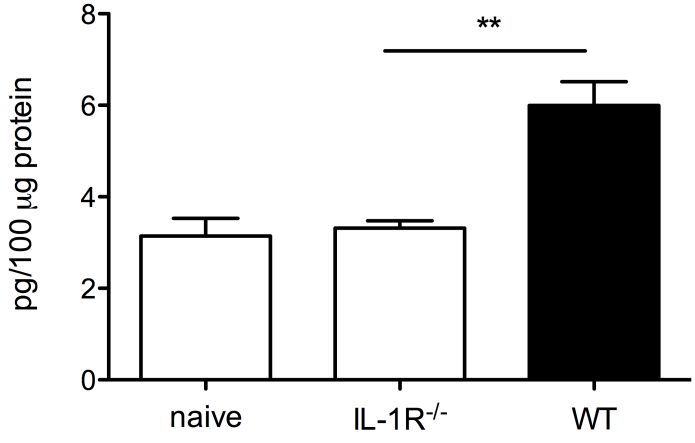


Figure 4-8: **No increase in hippocampal IL-1β following surgery in IL-1R$^{-/-}$.**

Tissue collection was carried out 6 hours after treatment. Surgery in IL-1R$^{-/-}$ resulted in no changes in the expression of IL-1β compared to naïve KO. There was a significant difference between KO and WT following the same surgical procedure (**p<0.001 comparison between surgery KO vs surgery WT). A positive control from LPS (1 mg/kg) treated animals was used to validate the readings. Data are expressed as mean ± SEM (n = 6) and compared by two-way analysis of variance and Student-Newman-Keuls method.

The reduction in the expression of IL-1β in the hippocampus led me to assess microglia activation 24 hours after surgery. Reactive microgliosis was significantly reduced following tibial fracture in IL-1R$^{-/-}$ (figure 4.9). Microglia maintained a resting state in all of the treatments, including anesthesia and LPS. Slides from LPS treated WT were used as positive control to assure validity of the immunohistochemical technique.

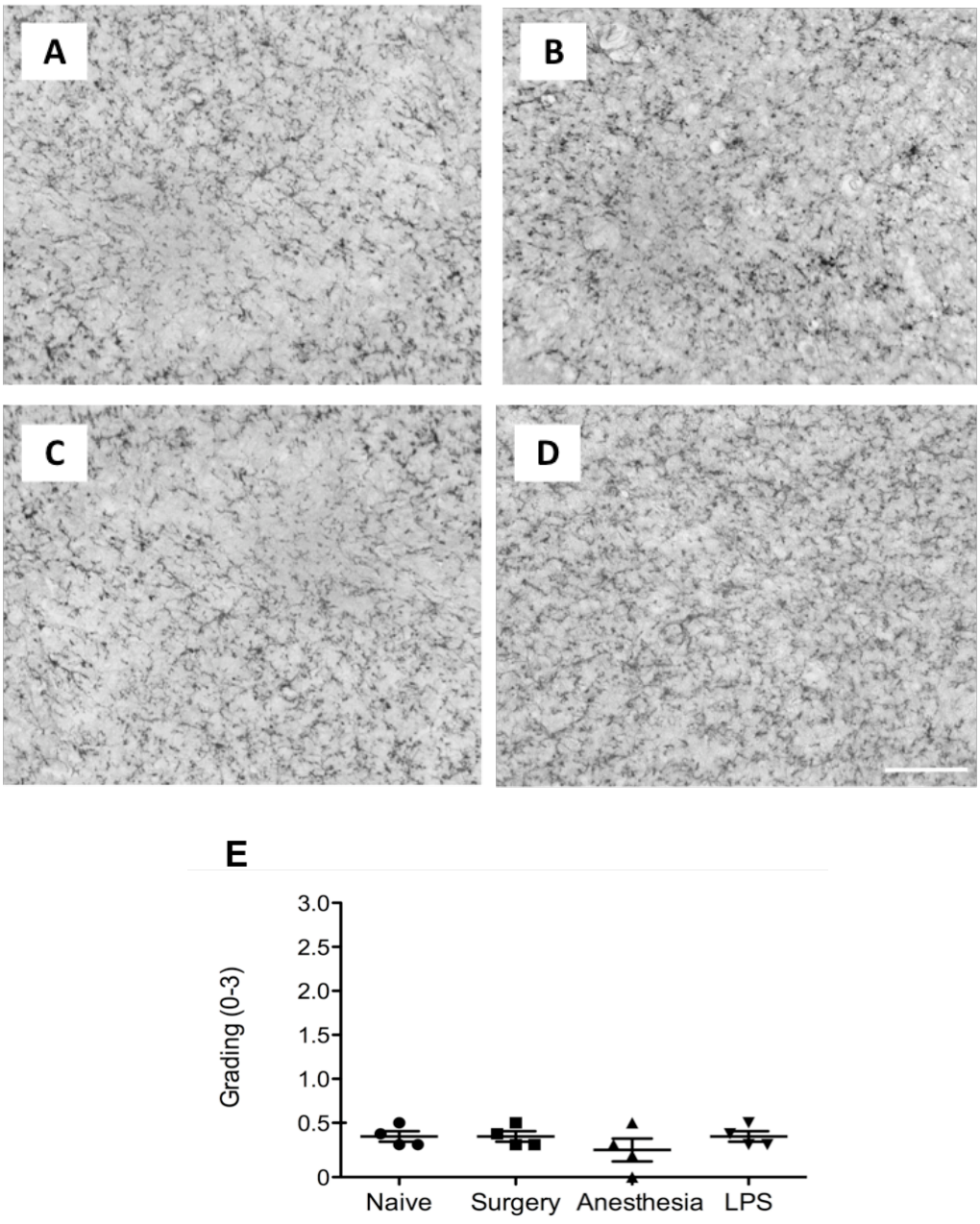


Figure 4-9: **Absence of microglia activation following surgery in IL-1R$^{-/-}$**.

Hippocampi were harvested 24 hours post intervention and stained with anti-CD11b to indicate microglia activation following interventions. Animals were exposed to anesthesia only, underwent surgery of the tibia, or exposed to LPS. Untreated animals served as naïve. Photomicrographs were blindly scored and microglia activation was graded on a scale 0 (lowest) – 3 (highest). No signs of reactive microgliosis was observed in any of the treatments (**A** – naïve, **B** – anesthesia, **C** – surgery, **D** – LPS) (pictures show CA1, scale bar 50 µm, 20X). LPS slides from WT were used as positive control. Immunohistochemical grading (0-3) (**E**) was presented for data illustration. No significant changes in microgliosis were reported (n = 4). Non parametric data are presented with Kruskal-Wallis followed by Dunn's test.

4.3.3 Blockade of IL-1 signaling ameliorates surgery-induced behavioral abnormality

In order to translate the information concerning the inflammatory profile in IL-1R$^{-/-}$ animals to cognitive behavior following surgery, trace fear conditioning was carried out to assess memory retention. Due to the limited availability of these reagents in the short-term, the involvement of IL-1 in POCD-associated behavioral abnormality was investigated using the IL-1 receptor antagonist (IL-1 Ra). Preemptive administration of IL-1 Ra (100mg/kg s.c.) to WT animals effectively ameliorated the surgery-induced cognitive dysfunction at three days post intervention as shown in the contextual retrieval task (figure 4.10). These experiments were performed by Mr Antonio Rei Fidalgo.

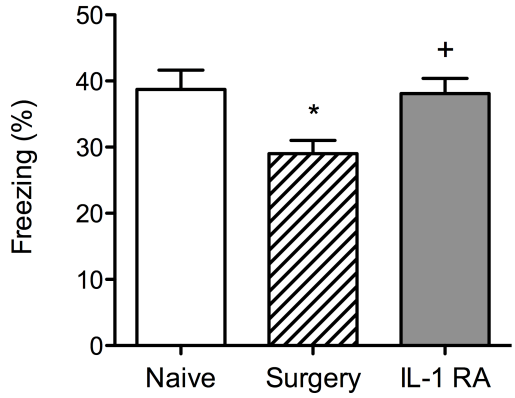


Figure 4-10: **Effects of IL-1RA on behavioural outcome following surgery.**

Contextual fear response following IL-1RA administration (100 mg/kg s.c.) mitigated the contextual fear memory impairment after surgery (*p<0.05 vs naïve, +p<0.05 vs surgery). Data are expressed as mean ± SEM (n = 8) and compared by one-way analysis of variance and Student-Newman-Keuls method. Data presented with the permission of Antonio Rei Fidalgo.

4.4 TLR4

4.4.1 POCD is not reduced in TLR4$^{-/-}$ following surgery

MyD88 is shared in a number of innate immune signaling pathways. Aside the IL-1R, TLRs largely rely on MyD88 signaling. Recently, the association between TLRs and endogenous ligands during aseptic inflammation has been reported (Mollen et al. 2006; Zedler and Faist 2006). This would suggest a putative role for TLRs in the development and / or sustainment of the inflammatory challenge in POCD. A role for TLR4 has been described following bilateral tibia fracture (Levy et al. 2006), thus I sought to investigate the role of this receptor in POCD-associated behavior in our model. In order to validate and conduct a reliable memory assessment, learning and acquisition was measured in TLR4$^{-/-}$. Similarly to the previous findings both from WT and MyD88$^{-/-}$, also TLR4$^{-/-}$ showed consistent and uniform learning, with no differences in working memory between groups (figure 4.11).

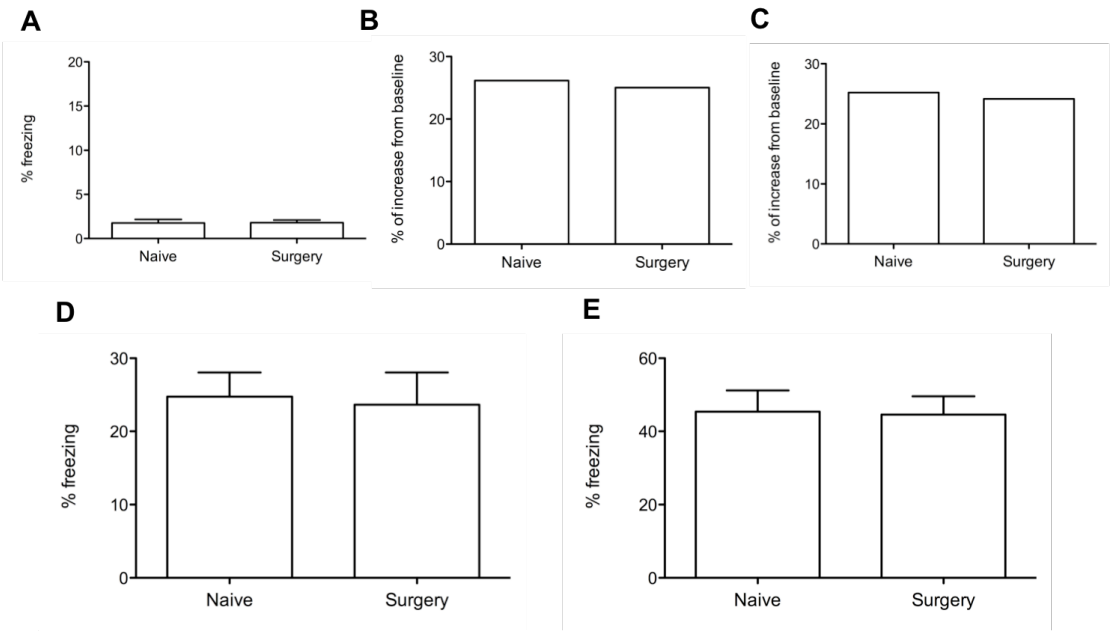


Figure 4-11: **Training phase during trace fear conditioning**.

Animals were collegially trained and then subdivided in two groups: untreated (naïve) and tibia fracture under inhaled isoflurane (surgery). Baseline freezing at the time of first exposure of these animals in the context is very low, indicating low levels of stress (A). Figure B indicates the increase of freezing response from Tone 1 to Tone 2, indicating working memory. Figure C indicates the increase of freezing response from Trace Interval 1 to 2, an indicator of working memory. Figure D indicates the working memory freezing response to context between tone – shock pairings. All animals demonstrate an equal working memory performance (E).

Immediately following training, animals were treated accordingly. Mice in the surgical group underwent treatment within 30 minutes after training. Three days after treatment, rodents were taken back to the conditioning chamber and re-exposed to the same environment as during training. When exposed to the context three days later, there was an evident reduction in the freezing response, resembling what shown in WT ($p<0.05$). The degree of behavioral abnormality present in $TLR4^{-/-}$ was very similar to what previously observed in WT. WT data are illustrated for comparison (figure 4.12A). Following the auditory-cued test, a significant reduction in the auditory-cue was also reported in $TLR4^{-/-}$ ($p<0.05$). No changes were reported comparing the auditory-cued response in KO and WT or in baseline freezing (figure 4.12B).

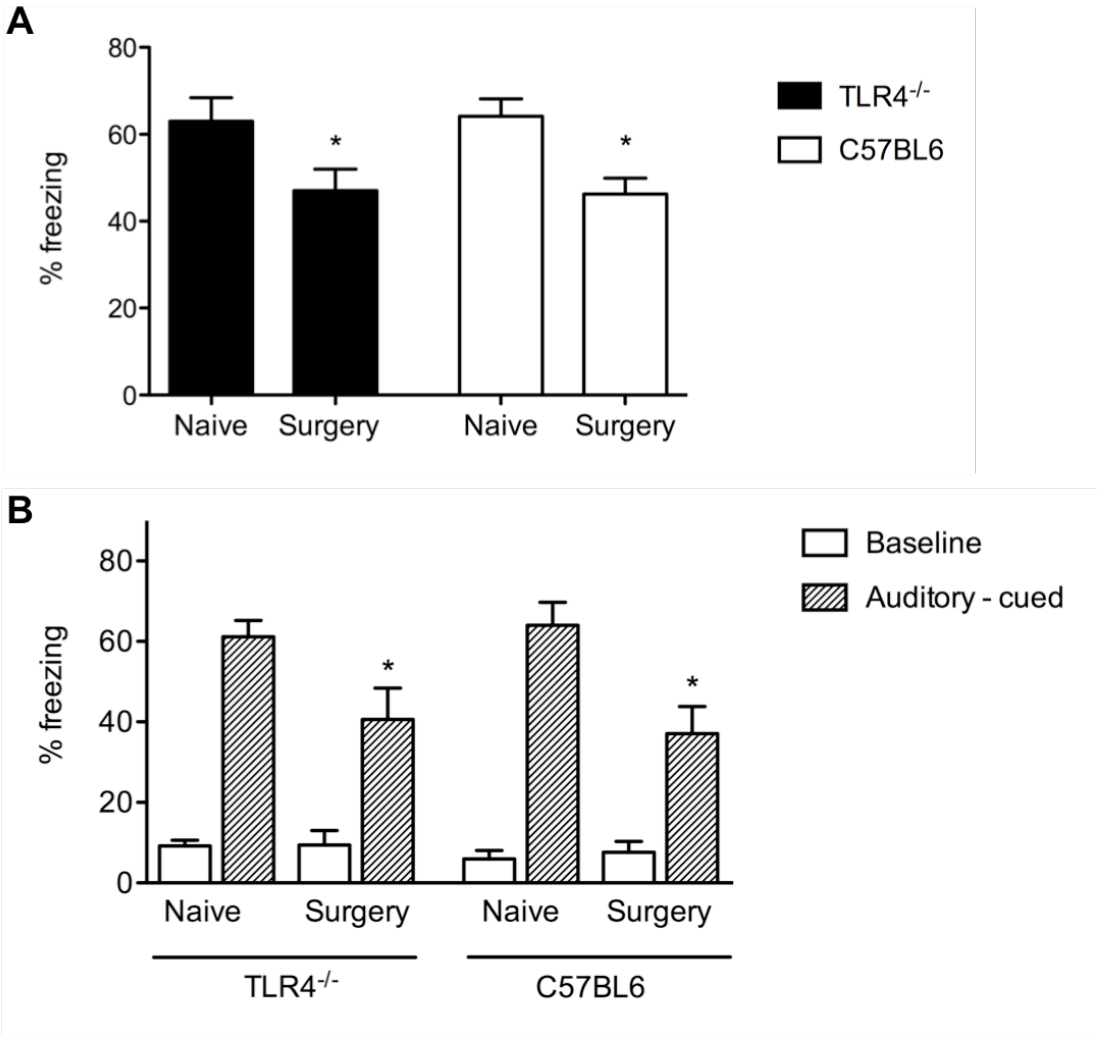


Figure 4-12: **Hippocampal-dependent memory impairment in TLR4$^{-/-}$ following surgery.**

Contextual fear response, as measured by freezing behavior, is impaired in TLR4$^{-/-}$ animals undergoing tibia surgery compared to naïve (**A**) (*p<0.05 vs naïve). Surgery-induced cognitive dysfunction in WT is illustrated for comparison. Memory abnormality is shown in the auditory-cued test in mice trained with trace fear conditioning (**B**). There is a significant difference between the groups in auditory-cued-related freezing behavior, suggesting disruption of auditory-cued, hippocampal-dependent, retrieval of memories after surgery. No difference was shown in the baseline freezing behavior showing no signs of anxiety (*p<0.05 vs naïve). Data are expressed as mean ± SEM (n = 10) and compared by one-way analysis of variance and Student-Newman-Keuls method.

4.4.2 Systemic inflammation is not modified in TLR4$^{-/-}$

TLR4 signaling is now established and well characterized, especially in relationship to infection and LPS. The possible involvement of TLR4 in other processes, such as aseptic inflammation or healing, still remains elusive and under investigation. Different endogenous ligands are likely to bind to TLR4 and activate downstream signaling, including HSPs, fibronectin extradomain A (tissue injury), and HMGB-1 (Miyake 2007). Following tibia surgery, a number of mediators including cytokines, tissue factors, and acute-phase proteins can bind to the receptor. Since no improvements following trace fear conditioning were reported after surgery, I sought to ascertain the relationship between inflammation and behavioral outcome following surgery. Levels of IL-1β and IL-6 were measured by ELISA at 6 and 24 hours (figure 4.13A, B). Despite an initial reduction in the levels of IL-1β at 6 hours ($p<0.001$ vs WT), no statistical differences between TLR4$^{-/-}$ and WT were reported at 24 hours post intervention (IL-1β: 23.57 ± 4.23 pg/ml and 30.88 ± 5.15 pg/ml; IL-6: 75.59 ± 14.6 pg/ml and 27.15 ± 4.52 pg/ml) [WT values for comparison: IL-1β: 44.13 ± 7.35 pg/ml and 36.89 ± 6.54 pg/ml; IL-6: 94.08 ± 15.68pg/ml and 27.21 ± 4.53 pg/ml at 6 and 24 hours respectively]. Consistently with the previous data, no changes were reported after anesthesia in neither of these cytokines. Administration of ultra-pure LPS to TLR4$^{-/-}$, which served as main ligand and activator of TLR4 signaling, resulted in no changes in pro-inflammatory cytokines as previously described (Poltorak et al. 1998; Qureshi et al. 1999; Medvedev et al. 2007) (figure 4.13C,D; $p<0.001$).

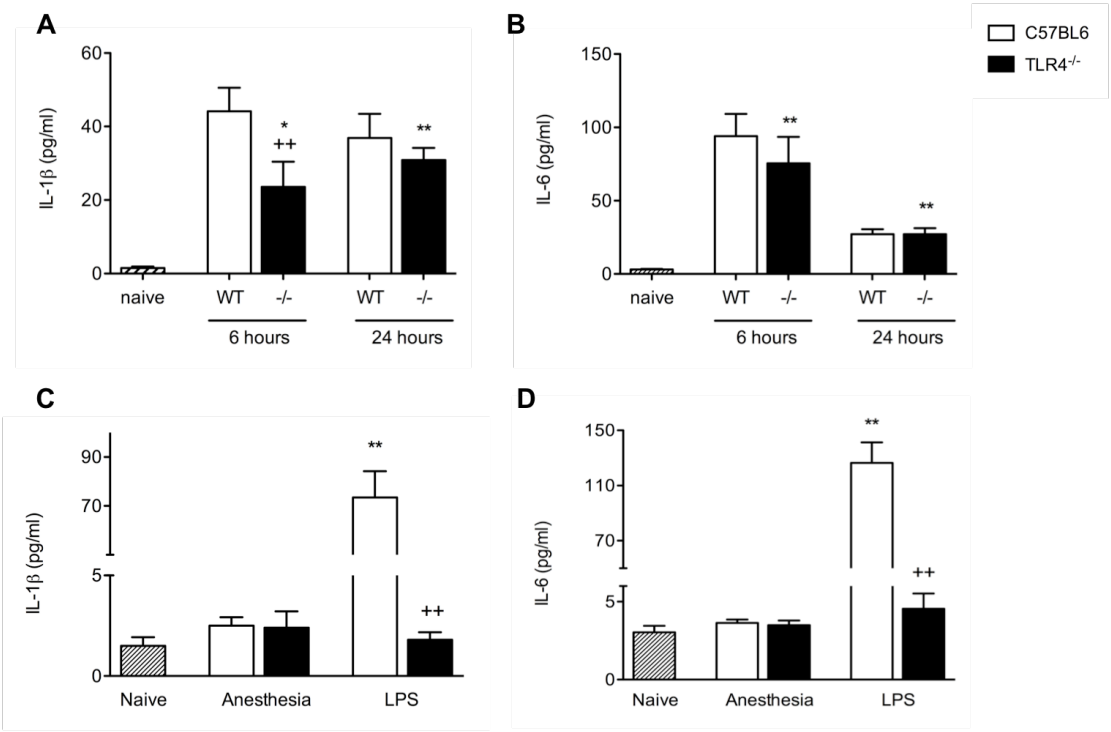


Figure 4-13: **Surgery-induced systemic inflammation in TLR4$^{-/-}$.**

IL-1β and IL-6 levels in plasma were measured by ELISA at 6 and 24 hours post-intervention. A reduction in IL-1β following surgery was observed at 6 hours, however no statistically significant changes could be found at 24 hours (**A**, ++p<0.01 vs WT, *p<0.01 vs naïve at 6 hours). No changes in IL-6 were measured when comparing TLR4$^{-/-}$ and WT both at 6 and 24 hours (**B**, **p<0.01 vs naïve). LPS-induced systemic inflammation was not present in TLR4$^{-/-}$ animals compared to LPS injected WT, both looking at IL-1β and IL-6 (**C**, **D** ++p<0.001 vs WT). No changes in systemic cytokines were reported following exposure to anesthesia only (**C**, **D**). Data are expressed as mean ± SEM (n = 6) and compared by two-way analysis of variance and Student-Newman-Keuls method.

4.4.3 Neuroinflammation and microgliosis is marginally decreased

In order to evaluate the effects of surgery on neuroinflammation, both IL-1β expression and microglia activation was measured in TLR4$^{-/-}$. At 6 hours following surgery, TLR4$^{-/-}$ displayed a reduction in hippocampal IL-1β, but not a complete obliteration as seen both in MyD88$^{-/-}$ and IL-1R$^{-/-}$ (figure 4.14; 5.14 ± 1.29 pg/100μg of proteins, $p<0.05$ vs surgery WT and naïve groups). IL-1β expression in the hippocampus was not changed in TLR4 naïve animals (3.34 ± 0.39 pg/100μg of proteins). Values from WT animals undergoing tibia surgery are illustrated for comparison (5.98 ± 1.23 pg/100μg of proteins).

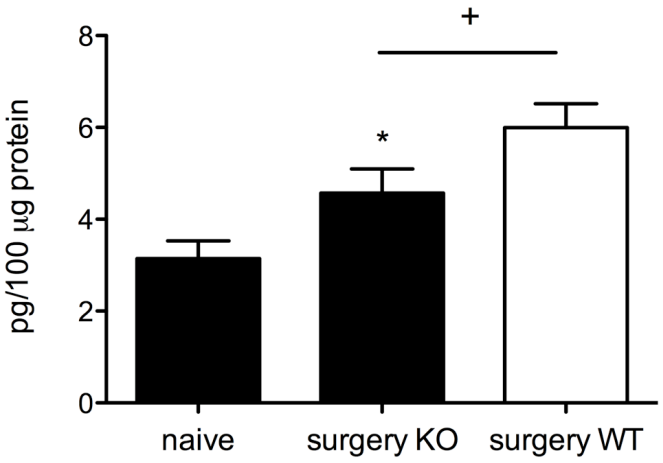
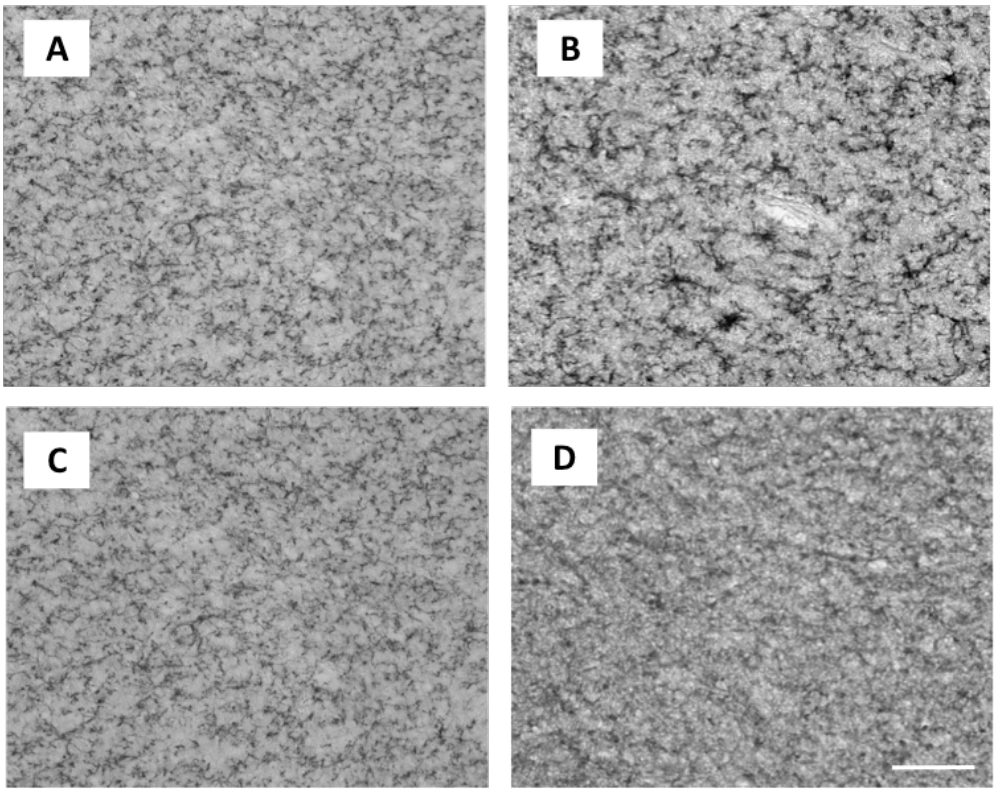


Figure 4-14: **Reduced, but not abolished, hippocampal IL-1β following surgery in TLR4$^{-/-}$.**

Tissue collection was carried out 6 hours after treatment. Surgery in TLR4$^{-/-}$ resulted in an increased expression of IL-1β (*$p<0.05$ vs naïve). There was a significant difference between KO and WT after surgery (+$p<0.05$ for comparison between surgery KO vs surgery WT). Data are expressed as mean ± SEM (n = 6) and compared by two-way analysis of variance and Student-Newman-Keuls method.

The partial amelioration observed at 6 hours both in plasma and hippocampal IL-1β prompted to understand the subsequent effects on microglia. At 24 hours following surgery there was a significant amount of reactive microglia compared to untreated animals ($p<0.05$). Neither anesthesia only nor LPS groups showed signs of microgliosis at this time point (figure 4.15).



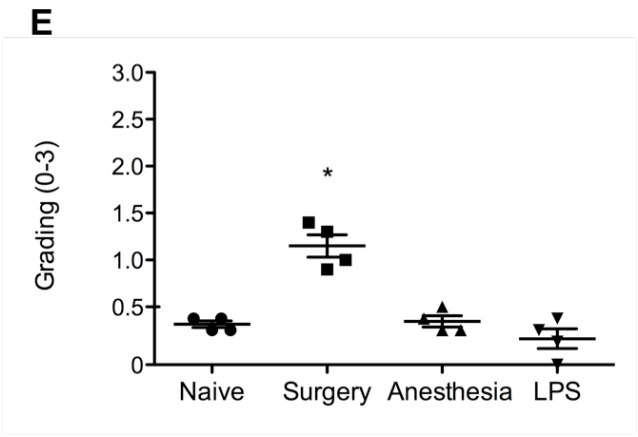


Figure 4-15: **Microglia activation in TLR4$^{-/-}$**.

Hippocampi were harvested 24 hours post intervention and stained with anti-CD11b to indicate microglia activation following interventions. Animals were exposed to anesthesia only, underwent surgery of the tibia, or exposed to LPS. Untreated animals served as naïve. Photomicrographs were blindly scored and microglia activation was graded on a scale 0 (lowest) – 3 (highest). Reactive microgliosis was observed following surgery, but not in any of the other treatments (**A** – naïve, **B** – surgery, **C** anesthesia –, **D** – LPS) (pictures show CA1, scale bar 50 μm, 20X). Immunohistochemical grading (0-3) (**E**) was presented for data illustration. Significant increase in reactive microgliosis was reported following surgery ($p<0.05$). No changes were observed in any of the other treatments ($n = 4$). Non parametric data are presented with Kruskal-Wallis followed by Dunn's test.

4.5 TLR2

4.5.1 TLR2$^{-/-}$ only show mild improvement on systemic inflammation

The second target amongst TLRs was TLR2. Similarly to TLR4, this receptor plays a dual role during infection and aseptic inflammation. TLR2 shares a conserved intracellular motif with TLR4 and are both associated with recognition of microbial ligands (Kurt-Jones et al. 2002; Yang et al. 2008). Even though the signaling pathway has been thoroughly investigated in the past years, endogenous triggers of TLR2 remain dubious. Recently, a role for TLR2 has been advanced in the recognition of DAMPs (hyaluraonic acid, HSPs, HMGB-1, uric acid), however its function and many endogenous triggers still remain undefined (Vabulas et al. 2002; Park et al. 2004; Scheibner et al. 2006). In order to further understand the role of this receptor and its possible association with POCD, systemic inflammation was assessed following treatment. Levels of IL-1β and IL-6 were measured by ELISA at 24 hours (figure 4.16A, B; IL-1β: 20.6 ± 3.43 pg/ml, $p<0.01$ vs naïve; IL-6: and 19.98 ± 3.3 pg/ml) [WT values for comparison: IL-1β: 36.89 ± 6.54 pg/ml; IL-6: 27.21 ± 4.53 pg/ml at 24 hours]. A reduction in the level of IL-1β was observed ($p<0.05$), however there was no significant difference when assessing IL-6. Consistently with the previous data, no changes were reported after anesthesia only in neither cytokines. LPS injection in TLR2$^{-/-}$ provided a strong positive control, cytokine levels reached similar values to treated WT (figure 4.16A, B; IL-1β: 65.77 ± 10.96 pg/ml; IL-6: and 118.9 ± 19.82 pg/ml, $p<0.001$ respectively) [WT values for comparison: IL-1β: 73.49 ± 12.24 pg/ml; IL-6: 126.47 ± 21.07 pg/ml at 24 hours, $p<0.001$ respectively].

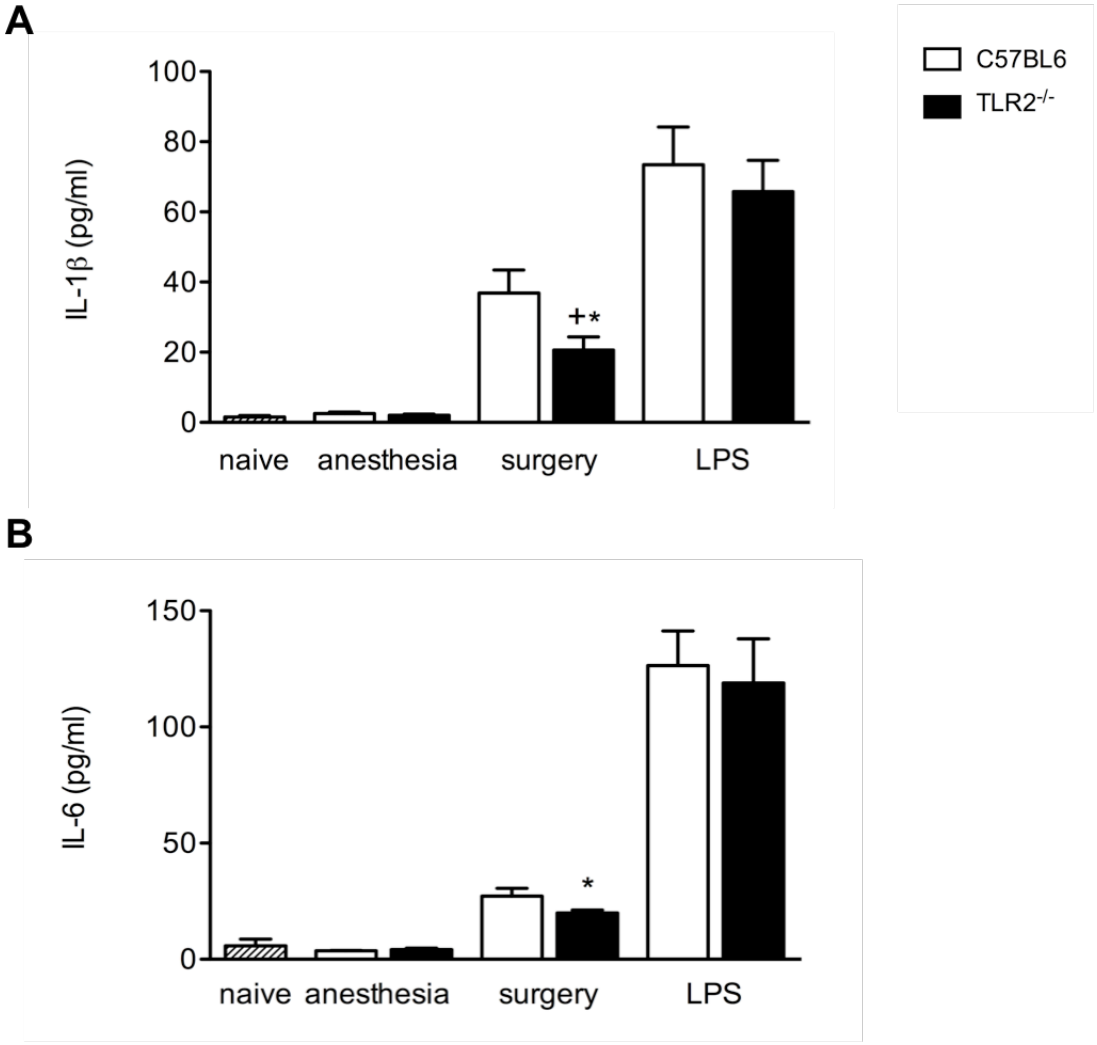
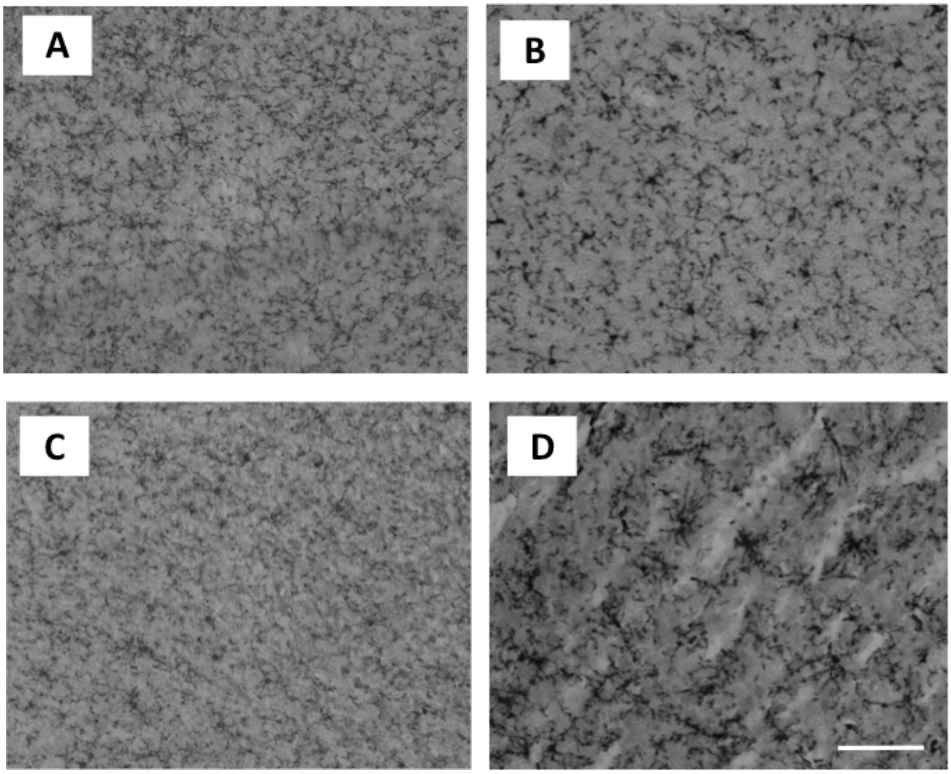


Figure 4-16: **Surgery-induced systemic inflammation in TLR2$^{-/-}$.**

IL-1β and IL-6 levels in plasma were measured by ELISA at 24 hours post-intervention. A reduction in IL-1β following surgery was observed at 24 hours (**A**, +/*$p<0.05$ vs naïve and WT groups). No changes in IL-6 were measured at 24 hours (**B**, *$p<0.01$ vs naïve). LPS-induced systemic inflammation was present in TLR2$^{-/-}$ animals compared to LPS injected WT, with no differences compared to WT in IL-1β and IL-6 ($p<0.001$ vs naive). No changes in systemic cytokines were reported following exposure to anesthesia only. Data are expressed as mean ± SEM (n = 6) and compared by two-way analysis of variance and Student-Newman-Keuls method.

4.5.2 Attenuated microgliosis in TLR2$^{-/-}$

To further elucidate the immune-to-brain signaling following surgery, microglia activation was measured in TLR2$^{-/-}$. Mild microgliosis was observed at 24 hours post intervention. Cells featured clumped processed and amoeboid cell bodies. However, following LPS administration in TLR2$^{-/-}$, significant microgliosis was observed ($p<0.01$), thus making the comparison with surgery less remarkable. No changes were observed following anesthesia exposure only (figure 4.17).



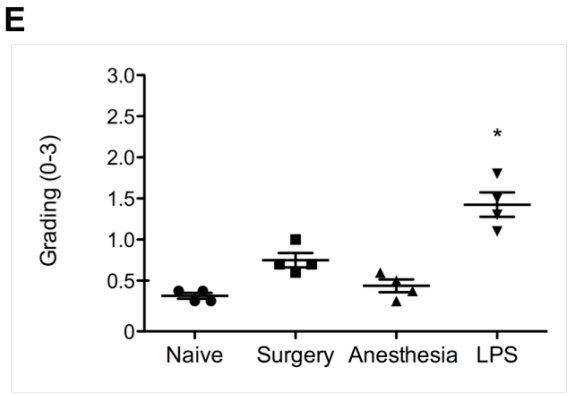


Figure 4-17: **Microglia activation in TRL2$^{-/-}$**

Hippocampi were harvested 24 hours post intervention and stained with anti-CD11b to indicate microglia activation following interventions. Animals were exposed to anesthesia only, underwent surgery of the tibia, or exposed to LPS. Untreated animals served as naïve. Photomicrographs were blindly scored and microglia activation was graded on a scale 0 (lowest) – 3 (highest). No signs of reactive microgliosis was observed following anesthesia exposure (**A** – naïve, **C** – anesthesia). Animals undergoing tibia surgery showed mild signs of reactive microgliosis (i.e. enlarged cell bodies, **B**). Animals treated with LPS expressed clear signs of reactive microglia (**D**) (pictures show CA1, scale bar 50 µm, 20X). Immunohistochemical grading (0-3) (**E**) was presented for data illustration. A significant increase in reactive microglia was observed in animals treated with LPS (**$p<0.01$ vs naïve and anesthesia groups). Mild microgliosis was reported following surgery ($n = 4$). Non parametric data are presented with Kruskal-Wallis followed by Dunn's test.

Table 4-1: **KOs summary**

		WT	MyD88	IL-1R	TLR4	TLR2
naïve	plasma	bl	bl	bl	bl	bl
	hippocampus	bl	bl	bl	bl	nd
	microglia	bl	bl	bl	bl	bl
surgery	plasma	+ +	- -	-	-/+	-/+
	hippocampus	+	- -	- -	-	nd
	microglia	+	- -	- -	+	-/+
anesthesia	plasma	- -	- -	- -	- -	- -
	microglia	- -	- -	- -	- -	- -
LPS	plasma	+ +	- -	-	- -	+ +
	microglia	+ +	- -	- -	- -	+ +

Legend: bl : baseline; - - : significant reduction; - :mild reduction; -/+ : no difference; + : increase; ++ : significant increase; nd: not determined

4.6 Discussion

Innate immunity is the most ancient and well conserved defence system shared throughout evolution from simple insects (*Drosophila*) to human beings (Kimbrell and Beutler 2001). Its key function is to discriminate infectious non-self pathogens and non-infectious self in order to activate a first line defence mechanism that alerts and protects the host organism against a possible threat (Medzhitov and Janeway 2000; Matzinger 2002). Recently, the role of PRRs has shifted from "microbial sensors" only to the so-called "danger signaling", expanding their action also into non-infective signaling mechanisms.

Sterile injury shares similarities and many symptoms of infection. The data here reported describe a pivotal role for MyD88 dependent signaling, in particular mediated by the IL-1R. The partial, but sufficient, reduction in pro-inflammatory cytokines reported in both MyD88 and IL-1 KOs suggest that these pathways are pivotal in POCD-associated behavior. However, since the inflammatory response was not completely abolished, this also prompts to understand whether non-MyD88 dependent signaling can mediate and perpetuate this inflammatory response. Some of these components will be further analyzed in the following chapter. Herein, I provide evidence for a minimal involvement of TLR4 in POCD-associated behavior and a marginal role for TLR2, which yet requires further understanding.

As reported in the previous chapter, cytokines play an important role in mediating the inflammatory response after surgery. The innate immunity is rapidly triggered and the immune-to-brain signaling ultimately leads to CNS

changes and behavioral abnormalities. Whether specific receptors / pathways can be defined in the context of POCD has yet not been investigated and the literature on aseptic inflammation and injury is still primordial. Both TLRs and the IL-1 largely mediate their action via MyD88, however a non-MyD88 signaling component has been recently suggested (Kenny and O'Neill 2008).

I first investigated the response to surgery in MyD88$^{-/-}$ and these animals appeared protected against the inflammatory-induced behavioral abnormality. Levels of both pro-inflammatory cytokines, including IL-1β and IL-6, and neuroinflammatory markers were significantly reduced in these animals. This suggests that this adaptor molecule exerts a pivotal role in surgery-induced behavioral abnormality. Humoral signaling and the possible recruitment of peripheral immune cells into the brain may account for the neuroinflammatory changes and POCD-associated behavior (Dantzer 2006; D'Mello et al. 2009). When MyD88 is absent, the danger signaling cannot perpetuate the inflammatory challenge, thus systemic cytokines are lower and reflect in an attenuated neuroinflammation and improved behavioral outcome.

Recently a dual role for MyD88 and TLRs in activating the innate and adaptive immunity via dendritic cells has been described (Hou et al. 2008). Using conditional knockouts, which allow selective targeting of a population of cells (in this case dendritic cells), more specific answers can be obtained. This would be advantageous in this model of POCD and is something that will be pursued in the future. Yet no evidence for a direct involvement of the adaptive immunity in POCD have been reported. Initially I had an interest in IFN-γ, in particular to correlate POCD with a chronic – autoimmune scenario.

Preliminary data looking at plasma levels of IFN-γ were assayed up to one month postoperatively but with no success (figure 4.18).

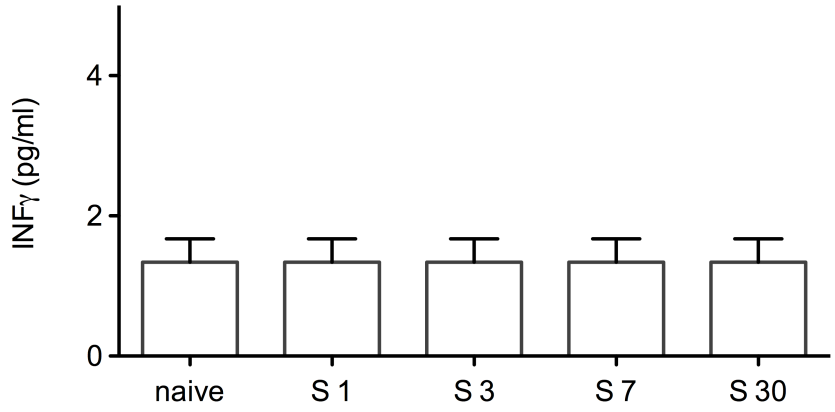


Figure 4-18: **IFN-γ after surgery.**

IFN-γ levels in plasma were measured by ELISA at 1, 3, 7, and 30 days post-intervention. No changes in the levels of IFN-γ were reported at any of the time-points. No changes were also reported following exposure to anesthesia only. A positive control was processed to confirm validity of the assay following manufacturer's instructions. Data are expressed as mean ± SEM (n = 6).

Even without characterizing specific cell types involved with POCD, these data demonstrate a pivotal role for MyD88 signaling. This led me to further investigate putative receptors associated with this molecule. TLR2, and TLR4, (and possibly TLR9) may be unique among TLR family members in their ability to recognize both exogenous and endogenous ligands (Ohashi et al. 2000; Paterson et al. 2003; Akira and Takeda 2004; Kaczorowski et al. 2008). TLR4 has partially shared mechanisms that involve MyD88 whereas TLR2 signaling relies entirely on this adaptor molecule (Barton and Medzhitov 2003; Akira et al. 2006). A series of studies have been conducted using TLR4

deficient mice looking at the danger signal after acute tissue injury including hemorrhagic shock (Barsness et al. 2004), cardiac (Oyama et al. 2004), and hepatic (Tsung et al. 2005) ischemia/reperfusion models, showing reduced inflammation and injury compared to wild types. More recently, a correlation between endogenous proteins released after tissue injury and TLR4 has been suggested (Paterson et al. 2003; Mollen et al. 2006; Levy et al. 2007). Endogenous inducers not only engage with TLRs, but other PRRs are also involved in perpetuating and amplifying the inflammatory response (Medzhitov 2008). In the absence of TLR4, animals showed clear signs of POCD, similarly to WT animals. TLR4 is classically involved with infection and sepsis (Poltorak et al. 1998; Qureshi et al. 1999), but has also been associated with aseptic inflammation (Kaczorowski et al., 2008; Levy et al., 2006; Paterson et al., 2003). Since the absence of TLR4 did not significantly impact on cytokine release and behavioral changes, this further corroborates the previous findings with the general antibiotic, enfloroxacin, suggesting that the effects reported in this model cannot be attributed to an infective origin. However, the importance of TLR4 has also been described in aseptic inflammation, danger signaling and tissue trauma (Ohashi et al. 2000; Smiley et al. 2001; Johnson et al. 2002). In particular, TLR4 has been pivotally described following bilateral femur fracture. In this study chimeric mice with a point mutation in the intracellular TLR4 signaling domain were used, showing that TLR4 mutant were protected following fracture (Mollen et al. 2008). In this model of POCD I did not use chimeric mice but I also reported a decrease in systemic cytokines and neuroinflammation, however this reduction did not appear sufficient to protect the animals from the behavioral abnormality. Also, the anesthesia

technique used in the bilateral femur model used sodium pentobarbital, which has known anti-inflammatory effects via NFkB and MAPK signaling pathways (Yang et al. 2007). Both TLR4 and TLR2 have also been studied in the context of acute brain injury, revealing a pivotal role for MyD88 but not for either of these receptors (Koedel et al. 2007).

In summary, MyD88 signaling crucially affects POCD. The IL-1R, but not TLR2 or TLR4, appear to be a sufficient target in ameliorating this condition. However, further studies including other TLRs, conditional KOs or double-KOs might address more specific questions. These data further prove the complexity and multifactoriality of this phenomenon, suggesting that a sufficient, even if not complete, reduction in cytokines has protective effects on POCD-associated behavioral abnormality. Because none of the analyzed receptors were able to fully abolish the inflammatory response, this yielded to further investigate whether putative MyD88-independent signaling affect POCD. Some of these mechanisms are explored in the following chapter.

5 Chapter 5: results

5.1 RATIONALE

Cytokines have broad physiological effects both on the periphery and in the CNS. As previously demonstrated, MyD88 and IL-1 have a pivotal role in mediating characteristic symptoms of sickness behavior as well as memory abnormalities and POCD-associated behavior. Inflammation relies on multiple factors and immune cells often recruit and produce cytokines in response to different stimuli, making the inflammatory response characteristic for a given insult. Aseptic inflammation, danger signaling and endogenous inducers not only engage with MyD88, but also other PRRs are involved in perpetuating and amplifying the inflammatory response (Medzhitov 2008). Both IL-1 and TNF have a broad range of effects with many synergistic interaction and both characterize many pathologies. Many primary functions of the immune system are regulated by these cytokines, but their mechanisms of action in particular at the post-receptor level yet remain unclear (Saklatvala et al. 1996).

TNF has a pivotal role in the initiation and amplification of the inflammatory cascade; it is involved in regulating chemokines and cytokines release, oxidative stress, recruitment of immune cells (leukocytes and neutrophils) and adhesion molecules, apoptosis, healing and tissue-specific repair mechanism (chondrocytes, osteoclasts, hepatocytes, neurons and adipocytes.) (McKellar et al. 2009). TNF also exerts neuromodulatory functions, in particular in regulating microglia and astrocytes activation in the brain. (Pan et al. 1997;

D'Mello et al. 2009)

After having defined a role for MyD88 signaling in POCD, I sought to investigate whether other molecules / receptors could account for POCD-associated dysfunctions. Following the findings that the innate immunity is closely involved with POCD, my aim was to understand if specific initiators could be targeted upstream of the receptors downstream cascade. TNF-α has a pivotal role in both directly and indirectly inducing other inflammatory markers, including cytokines, prostaglandins, nitric oxide, and adhesion molecules, making it a classic pleiotropic cytokine (Tchorzewski et al. 1993; McKellar et al. 2009). Despite the technical limitations in measuring this cytokine in plasma, I previously reported a positive trend after 30 minutes following tibia surgery. However, previous work for this laboratory has reported TNF-α in the hippocampus at 24 hours following splenectomy by RT-PCR, thus suggesting a putative role for this cytokine in the etiology of this phenomenon (Wan et al. 2007). Hence, I hypothesized that TNF-α may represent a putative initiator of the inflammatory response in POCD. Thus I opted to use selective antibodies to antagonize TNF-α pre-emptively.

5.2 Anti-TNF ameliorates surgery-induced cognitive abnormality

The first aim was to understand if the anti-TNF prophylaxis would have provided protection following surgery. To assess the effect on POCD-associated behavioral abnormality, trace fear conditioning was carried out as previously described. Since one anti-TNF group was injected 18 hours before surgery, this occurred before the acquisition phase. Thus, all other animals were injected with saline to account for any possible effect of the injection on learning capability. Acquisition and establishment of working memory was achieved with no significant differences between groups, similar to what was reported before (figure 5.1).

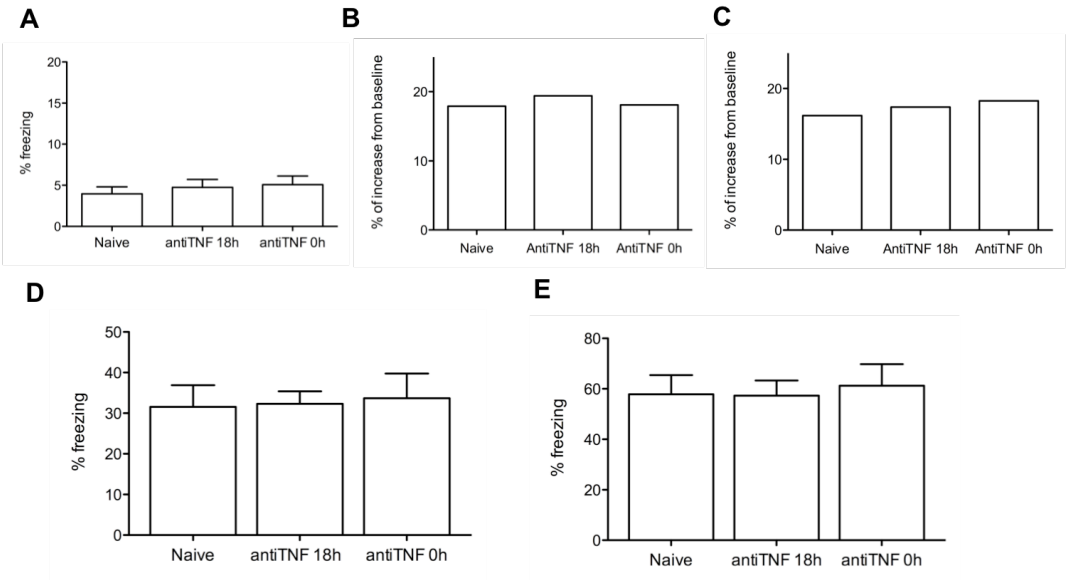


Figure 5-1: **Training phase during trace fear conditioning**.

Animals were collegially trained and then subdivided in three groups: untreated (naïve), tibia fracture under inhaled isoflurane with administration of antiTNF 18 hours before surgery (antiTNF 18h) and tibia fracture under inhaled isoflurane with administration of antiTNF immediately after operation (antiTNF 0h). Baseline freezing at the time of first exposure of these animals in the context is very low, indicating low levels of stress (A). Figure B indicates the increase of freezing response from Tone 1 to Tone 2, indicating working memory. Figure C indicates the increase of freezing response from Trace Interval 1 to 2, an indicator of working memory. Figure D indicates the working memory freezing response to context between tone – shock pairings. All animals demonstrate an equal working memory performance (E).

Immediately following training, animals were treated as follows. Mice in the surgical group received tibia surgery within 30 minutes after training. Prophylaxis with antiTNF was given 18 hours before surgery (antiTNF 18h) or the antibody was administered immediately after surgery still under anesthesia (antiTNF 0h). Three days after treatment, rodents were taken back to the conditioning chamber and re-exposed to the same environment as during the acquisition phase. The contextual retrieval task revealed no significant changes in freezing behavior compared to the naïve littermates in either treatment, perhaps with a more defined effect following the preemptive administration. No changes were reported in the auditory-cued test (figure 5.2B).

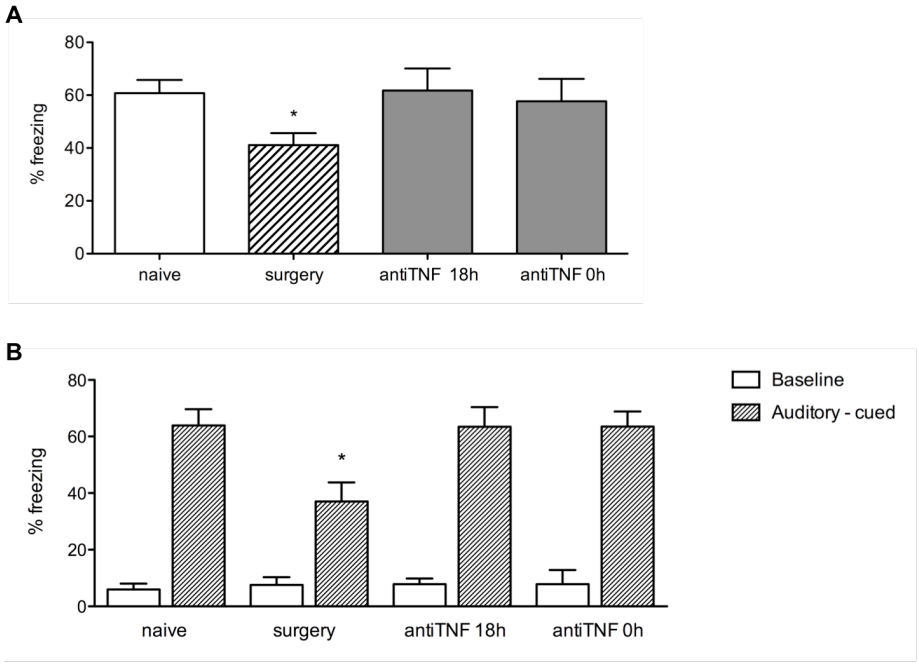


Figure 5-2: **Effects of antiTNF on behavioral outcome.**

Adult mice underwent surgery of the tibia under inhaled isoflurane (surgery), or the same surgical procedure with preemptive administration of antiTNF 18hours prior surgery (antiTNF surgery) or immediately after intervention (surgery antiTNF). Freezing to context after trace fear conditioning (**A**). Mice subjected to surgery exhibited reduced freezing to context when compared to naïve animals; in both cases, administration of antiTNF mitigated the contextual fear memory impairment ($p<0.05$). Hippocampal-dependent, surgery-induced memory impairment is shown in the auditory-cued test in mice trained with trace fear conditioning (**B**). There is a significant difference between the groups in auditory-cued-related freezing behavior, suggesting disruption of auditory-cued, hippocampal-dependent, retrieval of memories after surgery. No difference was shown in the baseline freezing behavior or after administration of antiTNF ($p<0.05$). Data are expressed as mean ± SEM ($n = 10$) and compared by one-way analysis of variance and Student-Newman-Keuls method.

5.3 Therapy with anti-TNF reduces systemic levels of IL-1 and IL-6

The promising effects of anti-TNF therapy on POCD-associated behavior led me to investigate the effects in relationship to other cytokines, namely IL-1β and IL-6. Due to the prolonged half-life of the antibody (>3 days) and the consistent results from the TFC assessment, I only focused on the prophylaxis 18 hours before surgery. The effects on systemic cytokines were remarkable. Anti-TNF successfully reduced the amount of systemic IL-1β by 6 hours after tibia fracture (fig A; 19.54 ± 3.26 pg/ml, $p<0.01$) and even further attenuated the response at 24 hours (10.22 ± 2.5 pg/ml, $p<0.001$). In order to corroborate the findings, in particular to relate this observation to the specificity of the therapy, I delayed the administration of the antibody up to 6 hours following surgery and re-measured the cytokine levels in plasma. As expected, the delay did not reduce the amount of plasma IL-1β at either of these time points (figure 5.3A; 50.09 ± 8.35 pg/ml and 35.6 ± 5.93 pg/ml) [WT values from untreated animals after surgery are reported for comparison: 44.13 ± 7.35 pg/ml and 36.89 ± 6.54 pg/ml]. To further corroborate the findings with IL-1β, I also assayed IL-6 as downstream effector from IL-1. Consistently, levels of IL-6 were similarly reduced both at 6 and 24 hours (figure 5.3B; 38.3 ± 6.38 pg/ml and 16 ± 2.6, $p<0.01$ and $p<0.05$ respectively) [WT values from untreated animals after surgery are reported for comparison: 94.08 ± 15.68pg/ml and 27.21 ± 4.53 pg/ml]. Also, the delayed treatment with the antibody did not provide any reduction in IL-6 (91.83 ± 15.31 pg/ml and 26.8 ± 4.47 pg/ml).

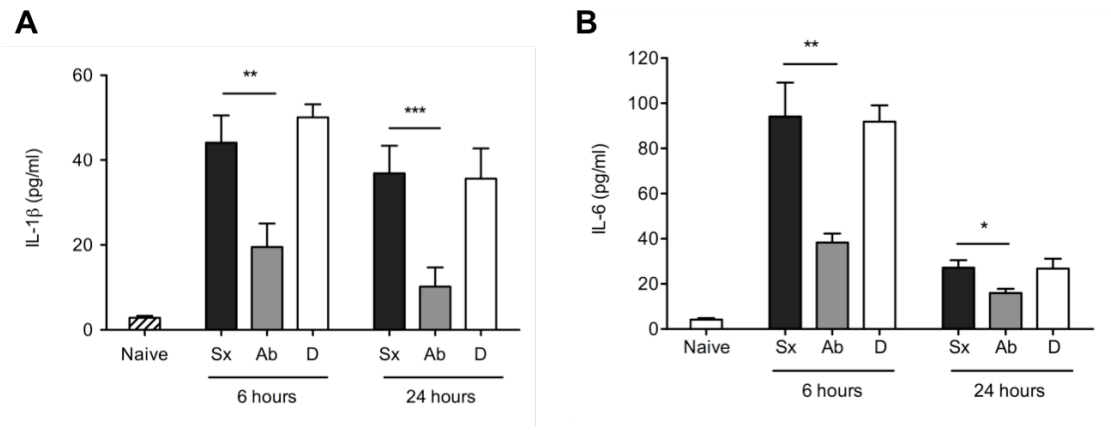


Figure 5-3**: Effects of antiTNF on systemic IL-1β and IL-6.**

Adult mice received surgery of the tibia under inhaled isoflurane. Preemptive administration of antiTNF 18 hours before surgery significantly reduced levels of IL-1β both at 6 (**p<0.01) and 24 hours (***p<0.001) (**A**). Delayed administration of the antibody did not provide any reduction in plasma IL-1β. Levels of IL-6 were similarly reduced after prophylaxis: both at 6 and 24 hours there was a significant decrease (**B**; **p<0.01, *p<0.05 respectively). Data are expressed as mean ± SEM (n = 6) and compared by one-way analysis of variance and Student-Newman-Keuls method. Sx=surgery, Ab=antibody, D=delayed administration of antibody

5.4 Reduced neuroinflammation follows antiTNF prophylaxis

From the action of the antibody on the systemic circulation, I then measured levels of IL-1β in the hippocampus. The novelty and interest of this experiment was to understand whether the action of the antibody on the periphery would also reflect in improved neuroinflammation. As both minocycline and the IL-1 Ra could freely diffuse in the brain and CNS, the antibody is limited in its ability of crossing the blood-brain barrier, thus allowing to speculate on the possible immune-to-brain signaling pathways (Gutierrez et al. 1994; Zhang et al. 2003; Skinner et al. 2009). Interestingly, when hippocampal levels of IL-1β were measured by ELISA, there was no

significant difference between animals treated with antiTNF and untreated counterparts (figure 5.4; 3.74 ± 0.23 pg/100μg of proteins). Values from WT animals following surgery are illustrated for comparison (5.98 ± 1.23 pg/100μg of proteins, $p<0.01$ vs naive).

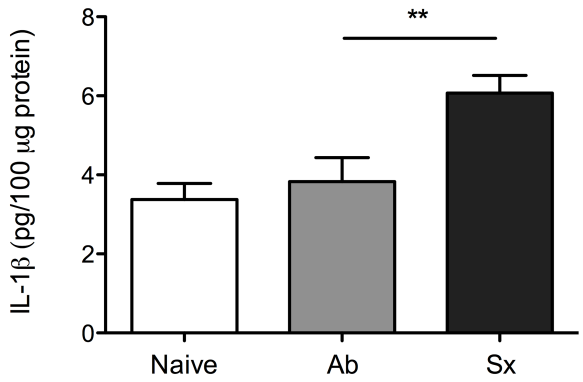


Figure 5-4: **Reduced hippocampal IL-1β following antiTNF.**

Tissue collection was carried out 6 hours after treatment. Surgery with preemptive administration of antiTNF resulted in no changes in the expression of IL-1β compared to naïve animals (**$p<0.01$). A positive control from LPS (1 mg/kg) treated animals was used to validate the readings. Data are expressed as mean ± SEM (n = 6) and compared by one-way analysis of variance and Student-Newman-Keuls method.

This led to further investigations of the relationship with microglial activation. The relationship between central IL-1β and further microgliosis was strengthened by the effects of antiTNF in reducing the amount of reactive microglia following surgery (figure 5.5; $p<0.05$ vs surgery only).

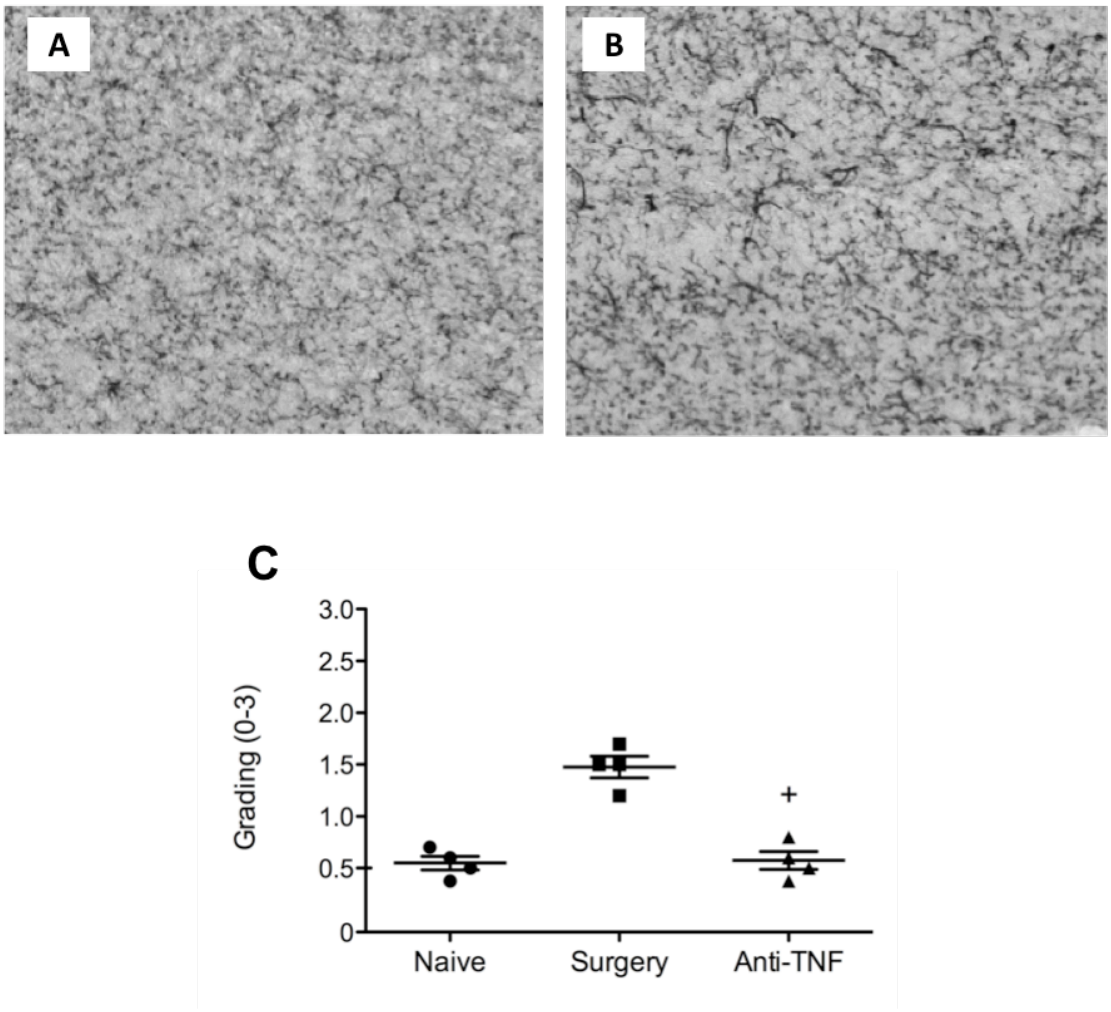


Figure 5-5**: Effects of antiTNF on reactive microgliosis in the hippocampus.**

Hippocampi were harvested at 24 hours after treatment and stained with anti-CD11b to indicate microglia activation following interventions. Photomicrographs were blindly scored and microglia activation was graded on a scale 0 (lowest) – 3 (highest). No signs of reactive microgliosis were observed following antiTNF treatment (**B**) compare to untreated animals (**A**) (pictures show CA1, scale bar 50 μm, 20X). LPS slides from WT were used as positive control (pictures show CA1, scale bar 50 μm, 20X). Immunohistochemical grading (0-3) (**C**) was presented for data illustration. Prophylaxis with antiTNF significantly reduced microglia activation at POD 1 (*$p<0.05$ vs surgery) ($n = 6$). Non parametric data are presented with Kruskal-Wallis followed by Dunn's test.

To recapitulate these findings, I have shown that administration of TNF-α antibody reduces IL-1β both systemically and centrally. This suggests that even though we cannot determine TNF-α in the circulation, this cytokine is an important player in mediating surgery-induced cognitive dysfunction. However, because either targeting of MyD88 or TNF-α produced only a reduction in inflammatory cytokines, but not a complete abolition, this intrigued me to identify whether the synergism of these cytokines, namely IL-1 and TNF-α, could account for the inflammatory response.

5.5 Role of TNF-α and IL-1.

In order to answer this question I administered anti-TNF to MyD88$^{-/-}$. As shown previously both MyD88$^{-/-}$ and IL-1R$^{-/-}$ displayed a detectable and similar amount of IL-1β, however when MyD88 received prophylaxis with the antibody and underwent surgery, levels of both IL-1β and IL-6 remained at baseline (figure 5.6A-B, $p<0.001$ and $p<0.01$ respectively versus MyD88$^{-/-}$ surgery only) [MyD88$^{-/-}$ levels following surgery at 6 and 24 hours are reported for comparison: IL-1β: 14.27 ± 2.4 pg/ml and 10.04 ± 1.7 pg/ml; IL-6: 19.79 ± 3.23 pg/ml and 6.89 ± 1.15 pg/ml].

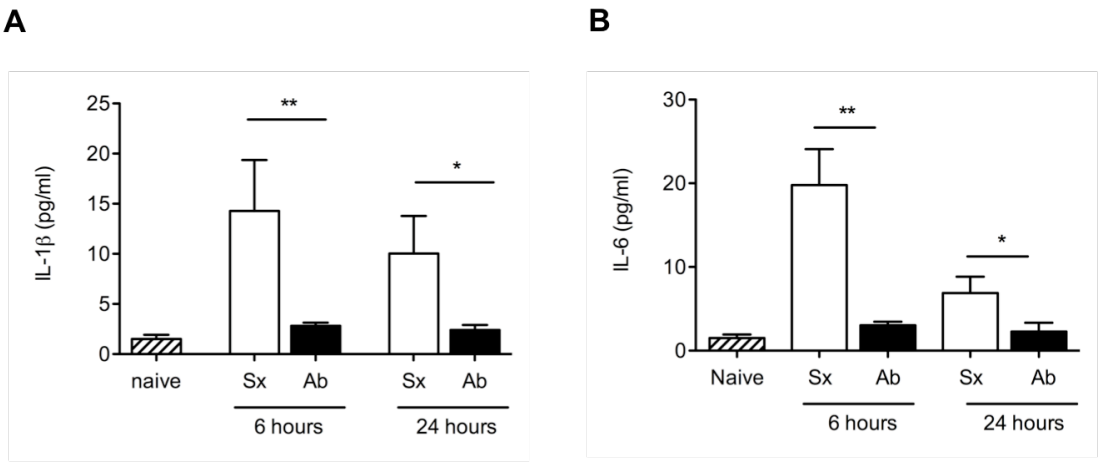


Figure 5-6: **Effect of prophylaxis in MyD88$^{-/-}$ on IL-1β and IL-6**.

Preemptive administration of anti-TNFα reduced the amount of systemic IL-1β as measure by ELISA to baseline both at 6 hours and 24 hours post intervention (A, **p<0.001, *p<0.01 compared to sx respectively). Levels of IL-6 were also measured; there was a similar reduction with baseline values at both time points (B, **p<0.001, *p<0.01 compared to sx respectively). Data are expressed as mean ± SEM n = 6 and compared by two-way analysis of variance and Student-Newman-Keuls method.

These findings from the MyD88$^{-/-}$ led to studies to reveal whether anti-TNF therapy could also protect TLR4$^{-/-}$, which displayed signs of inflammation correlated to POCD behaviour. Systemic inflammatory response following surgical intervention was abolished by pre-emptive administration of anti-TNF to TLR4$^{-/-}$.

Levels of both IL-1β and IL-6 were found at baseline (figure 5.7A, B) [TLR4$^{-/-}$ levels following surgery at 6 and 24 hours are reported for comparison:IL-1β: 23.57 ± 4.23 pg/ml and 30.88 ± 5.15 pg/ml; IL-6: 75.59 ± 14.6 pg/ml and 27.15 ± 4.52 pg/ml].

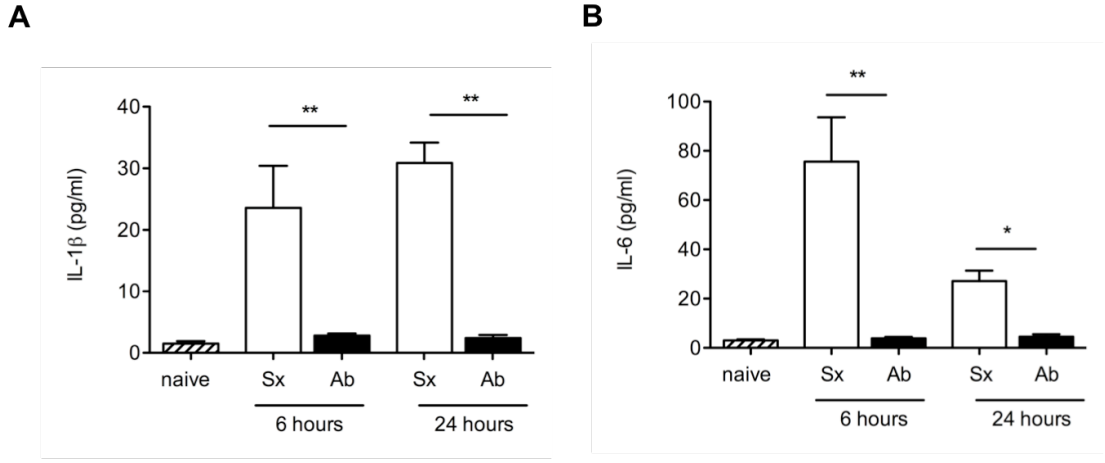


Figure 5-7: **Anti-TNFα prophylaxis in TLR4$^{-/-}$**.

Adult TLR4$^{-/-}$ mice underwent surgery of the tibia under general anesthesia (Sx), or the same surgical procedure with anti-TNFα prophylaxis 18 hours prior to surgery (Ab). The control group was composed of TLR4$^{-/-}$ naïve animals. Preemptive administration of anti-TNFα reduced the amount of systemic IL-1β as measure by ELISA to baseline both at 6 hours and 24 hours post intervention (**A**, **p<0.01 compared to sx respectively). There was a similar reduction in levels of IL-6, with values back to baseline at both time points (**B**, **p<0.001, *p<0.01 compared to sx respectively). Data are expressed as mean ± SEM n = 6 and compared by two-way analysis of variance and Student-Newman-Keuls method.

Furthermore, as TLR4$^{-/-}$ displayed signs of neuroinflammation and microgliosis after surgery, this prompted to investigate the effects of the antiTNF prophylaxis on the CNS. Anti-TNF was able to reduce the amount of hippocampal IL-1β at 6 hours following intervention (figure 5.8; 3.74 ± 0.42 pg/100μg of proteins, p<0.01 vs surgery in TLR4$^{-/-}$).

Also, at 24 hours post intervention there were no signs of reactive microgliosis (figure 5.9).

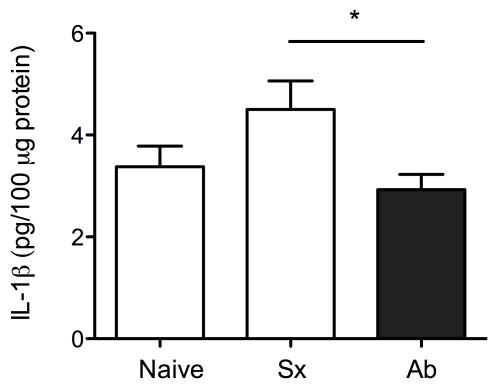


Figure 5-8: **Hippocampal IL-1 following prophylaxis in TLR4$^{-/-}$**.

Tissue collection was carried out 6 hours after treatment. TLR4$^{-/-}$ showed signs of neuroinflammation following tibia surgery but levels IL-1β were reduced following anti-TNFα prophylaxis (*$p<0.01$ vs sx). Data are expressed as mean ± SEM, n = 6 and compared by one-way analysis of variance and Student-Newman-Keuls method.

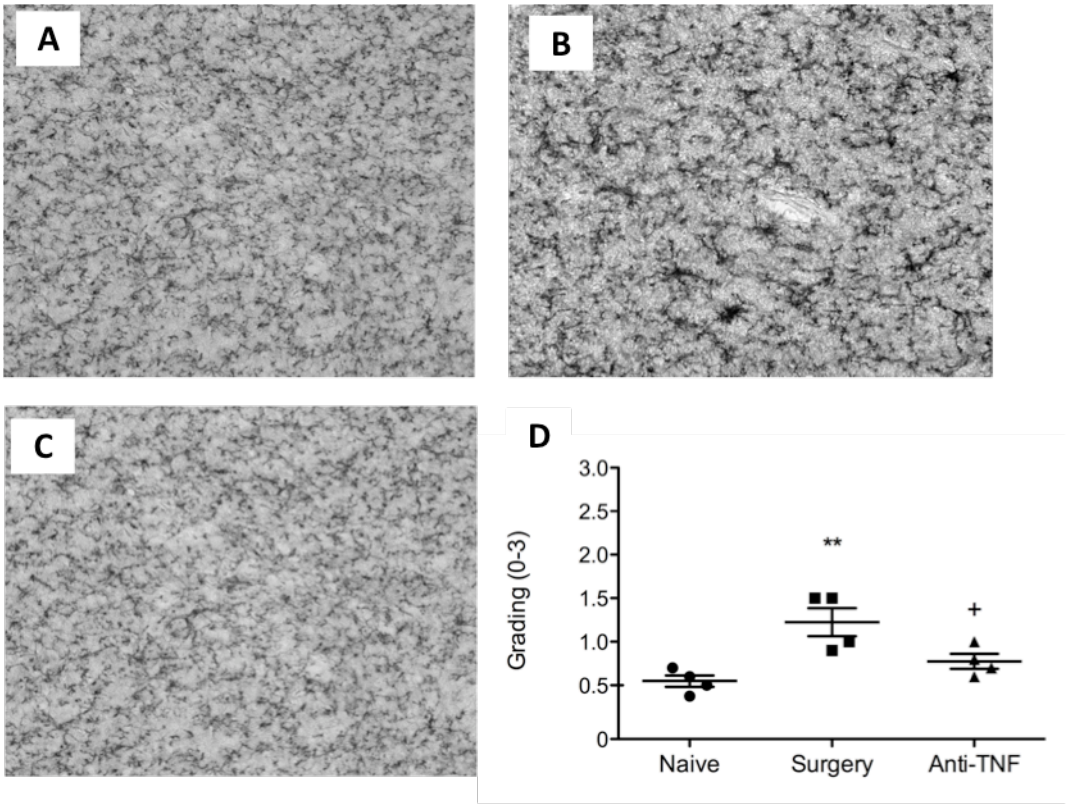


Figure 5-9**: Effects of antiTNF on reactive microgliosis in the hippocampus of TLR4$^{-/-}$.**

Hippocampi were harvested at 24 hours after treatment and stained with anti-CD11b to indicate microglia activation following interventions. Photomicrographs were blindly scored and microglia activation was graded on a scale 0 (lowest) – 3 (highest). Neither naïve (**A**) or mice treated with anti-TNFα (**C**) showed evidence of reactive microgliosis. Reactive microglia were found in TLR4$^{-/-}$ undergoing surgery as previously described (**B**, **p<0.01 vs naïve and ab groups). (pictures show CA1, scale bar 50 μm, 20X). Immunohistochemical grading (0-3) (**C**) was presented for data illustration. Prophylaxis with antiTNF significantly reduced microglia activation at POD 1 (+p<0.05 vs surgery) (n = 4). Non parametric data are presented with Kruskal-Wallis followed by Dunn's test.

5.6 Discussion

These data first show that anti-TNFα monoclonal antibody reduce the inflammatory burden following surgery, through the elaboration of IL-1β. These data suggest a role for TNF signaling on cognitive dysfunction and as a putative initiator of the inflammatory cascade that characterizes POCD. TNF targeting in WT was able to revert IL-1β to levels similarly to what observed in MyD88$^{-/-}$ after surgery. However, co-administration of anti-TNF to MyD88$^{-/-}$ or TLR4$^{-/-}$ completely abrogated the surgery-induced IL-1β response and downstream IL-6 production, suggesting a pivotal role for both these cytokines in the inflammatory response.

The importance of IL-1β in POCD and cognitive dysfunctions has been ascertained in a number of reports (Wan et al. 2007; Rosczyk et al. 2008). This is the first demonstration that preemptive targeting of TNFα as an early marker during post-surgical inflammation suppresses generation of IL-1, strengthening the role of cytokines in the etiology of POCD. In order to define the origin of the IL-1 response, I also looked at MyD88-dependent signaling and TLR4.

These data suggest a pivotal role of the initial peripheral response in the subsequent development of neuroinflammation and POCD-associated behavioral changes. In spite of the limitations of antibodies to permeate the brain, prophylaxis with anti-TNFα is primarily affecting the systemic inflammation, but it also provided a significant reduction in both hippocampal IL-1β and microglia activation. Although neural afferents may account for

signs of CNS inflammation (Dantzer 1994), these data may reveal the importance of humoral signaling in immune-to-brain signaling. Peripheral and central reduction of IL-1β through TNFα improved the behavioral performance, further elucidating and proving the inflammatory origin of surgery-induced cognitive abnormalities.

Due to the recent discoveries of independent MyD88 pathways in the IL-1 signaling (Kenny and O'Neill 2008), I investigated the response following surgery in animals lacking expression of this key adaptor molecule. MyD88$^{-/-}$ showed a reduction, even if not complete, in IL-1β following surgical stimulation. Dampening of the inflammatory challenge appears sufficient to provide a beneficial effect in POCD-associated behavior following surgery in MyD88. It is possible that here we report a *threshold effect* in which a certain level of IL-1 is sufficient to trigger enough neuroinflammation to impact on memory functioning, thus making behavioral changes. Combination of anti-TNFα prophylaxis to MyD88$^{-/-}$ was able to completely abrogate the response to IL-1β, suggesting a synergism with one another. The results from the TFC also suggest that the elimination of either cytokines will diminish cognitive dysfunction following surgical trauma (i.e. threshold effect).

The demonstration of the importance of MyD88 signaling in POCD led to further investigate whether targeting of specific receptors could ameliorate POCD symptoms. Due to the findings on the involvement of TLR4 during sterile inflammation and trauma (fracture) (Levy et al. 2006), I sought to also look at the inflammatory response in TLR4$^{-/-}$. Both IL-1β response and cognitive dysfunction after surgical stimulation were not eliminated. However, anti-TNFα prophylaxis was able to reduce the inflammatory challenge in

TLR4$^{-/-}$, further proving the key role of TNFα in the generation of IL-1β through a MyD88 independent pathway following surgery.

Herein I provide evidence for IL-1β working through MyD88 mechanism but, in addition, targeting of TNFα significantly ameliorates the cognitive dysfunction after surgery by reducing IL-1β. Therapy with TNF inhibitors already offers beneficial effects in other settings such as rheumatoid arthritis (Taylor and Feldmann 2009). Following a single preemptive administration we reported no evidence of infection or sickness behavior in this study, thus overcoming some of the limitations of these agents in facilitating postoperative complications, in particular infections. Whether the beneficial effects reported in this model of POCD following anti-TNF therapy will be retained in the clinic yet remains to be seen.

6 Chapter 6: results

6.1 RATIONALE

Systemic infection produces physiological and behavioral changes both in humans and animals. The ensuing sickness behavior is characterized by a decline in cognitive function, fever, decreased food intake, somnolence, hyperalgesia, and general fatigue (Dantzer 2004). Most of the symptomatic effects of infection can be correlated to neuroinflammation in different brain regions including the hippocampus, amygdala and limbic system (Annane 2009).

Cytokines have a pivotal role in orchestrating the inflammatory response after viral or bacteria infection and are essential in restoring homeostasis; these also affect behavior, especially memory and cognition (Pugh et al. 1998). LPS, comprising glycolipids from the outer membrane of Gram-negative bacteria, stimulates monocytes, macrophages, and neutrophils to produce cytokines and a plethora of other pro-inflammatory mediators. Since the first description of IL-1β as the endogenous pyrogen, numerous functions of this cytokine have been described in controlling endocrine, immune, and nervous functions. IL-1β is a classic mediator of the body's response to inflammation and has a pivotal role in immune-to-brain signaling, especially in modulating memory functions (Rachal Pugh et al. 2001). This molecule can be considered the prototypic multifunctional cytokine mediating widespread physiologic effects on CNS functions but also in effecting symptoms and signs in many disease states (Dinarello 1996; Murray and Lynch 1998).

Learning and memory processes largely rely on the hippocampus and this brain region expresses the highest density of IL-1 binding sites, making it particularly vulnerable to the adverse consequences of neuroinflammation (Parnet et al. 1994; Gemma et al. 2005). Although IL-1β is required for normal learning and memory processes, exogenous administration or excessive endogenous levels produce detrimental cognitive behavioral effects (Chen et al. 2008). Synergistic interaction between IL-1β and other cytokines, such as TNF-α and IL-6, enhances this cognitive dysfunction (Allan et al. 2005). Although microglia activation is observed in many different conditions ranging from neurodegenerative diseases, such as Alzheimer's disease (AD), to surgery-related complications (postoperative cognitive dysfunction), the putative association between cytokine release, microglial activation, and hippocampal-dependent memory dysfunction remains elusive (Wan et al. 2007).

Here the effects of LPS on cognitive dysfunctions are reported; I sought to further define the putative association between cytokine production and microglial activation, and their impact on cognitive function. Also, this work facilitates a comparison of two models of cognitive dysfunction, namely the surgery (aseptic inflammation) *versus* a similar behavioral abnormality, but produced by infection.

6.2 Postoperative complication (infection) exacerbates POCD

To elucidate the consequences of infection on learning and memory abilities, mice were exposed to lipopolysaccharide (LPS) one day following tibia surgery. I aimed to understand what would it be the effect of a superimposed complication, namely infective, in the setting of POCD and cognitive decline. Animals were conditioned during the training phase irrespectively of the treatment they received, as described previously. Establishment of learning and working memory was rapidly achieved with no significant differences between groups during the acquisition phase.

Immediately following training, animals underwent tibia surgery. One day following surgery, a group was exposed to an i.p. injection of LPS (1 mg/kg). Three days after surgery, rodents were returned to the conditioning chamber and re-exposed to the same environment, as the acquisition phase. When exposed to this context, the contextual retrieval task revealed a significant reduction in freezing behavior compared to the naïve littermates or animals undergoing surgery (figure 6.1A). Moreover, when the auditory-cued test was carried out two hours after the context retrieval task, a significant difference between surgical subjects and surgical with infection was found in auditory cued-dependent freezing, with no impairments in the overall baseline freezing (figure 6.1B).

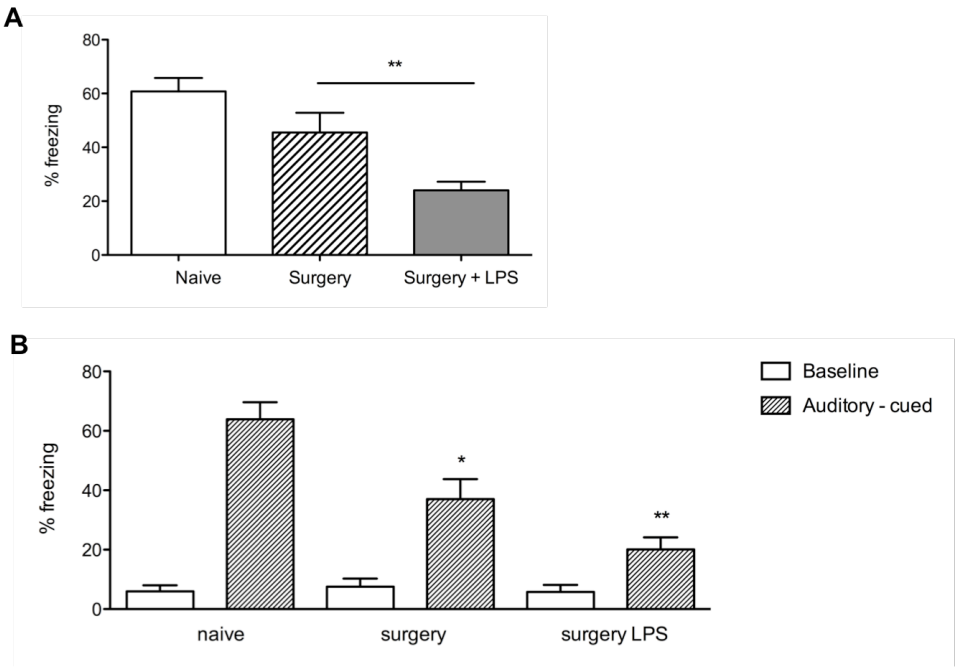


Figure 6-1: **Hippocampal-dependent memory recall in surgery after LPS infection.**

Contextual fear response, as measured by freezing behavior, is impaired in animals receiving surgery followed by LPS exposure (1 mg/kg) compared to naïve and surgery groups (**A**) (*p<0.05, ***p<0.01 vs naive group, ++p<0.05 vs surgery group). Surgery-induced memory impairment is shown in the auditory-cued test in mice trained with trace fear conditioning (**B**). There is a significant difference between the groups in auditory-cued-related freezing behavior, suggesting disruption of auditory-cued, hippocampal-dependent, retrieval of memories after surgery. No difference was shown in the baseline freezing behavior showing no signs of anxiety (*p<0.05 vs naïve and anesthesia groups). Data are expressed as mean ± SEM (n = 10) and compared by one-way analysis of variance and Student-Newman-Keuls method and student t-test for comparison between surgery and surgery with LPS.

These data aimed to reproduce a postoperative complication using LPS to induce a substantial and well characterized inflammatory response. Herein the data suggest an additive effect by exposing the animals to a complication following surgery, and current studies are investigating whether the association of these events is able to exacerbate, and sustain long-lasting, cognitive dysfunction.

The results with the addition of LPS led me to further investigate the inflammatory and behavioral responses in the absence of surgical intervention. While LPS has well-known effects on immunity and sickness behavior, the putative association between cytokine pathways, microglia and cognitive dysfunction still remains elusive. LPS is perhaps a more defined insult compared to the complex variables present in surgery, making it a suitable model to further elaborate.

6.2.1 Endotoxin-induced inflammation is modified by IL-1 Ra and in IL-1R$^{-/-}$

In order to define the inflammatory pathway following systemic LPS administration, I sought to assess changes of pro-inflammatory cytokines in plasma. TNF-α release occurred very rapidly and transiently; after 30 minutes it was significantly increased (104.18 ± 7.36 pg/ml, $p<0.01$ versus naive), peaking at 2 hours and returning to normal at 6 hours post-injection (figure 6.2A; $p<0.001$ versus naive). LPS evoked a robust systemic response that induced further cytokine release. Both IL-1β and IL-6 were significantly up regulated from 2 hours. IL-1β increased 4-fold and plasma levels continued to steadily increase until 24 hours (figure 6.2B; 73.49 ± 5.42 pg/ml, $p<0.001$ versus control). IL-6 expression was highly elevated at 2 hours, decreasing at 6 hours but still significantly detectable at 24 hours compared to naïve animals (figure 6.2C; 134.37 ± 8.43 pg/ml, $p<0.01$ versus control

respectively). During this time, animals showed classic symptoms of sickness behavior (reduced motility, poor grooming, huddling, piloerection, back arching). Levels of HMGB1 at 2, 6, and 12 hours post LPS were no different from baseline levels; a 1.5-fold increase in HMGB1 was observed from 24 hours after LPS and remained elevated up to day 3 (figure 6.2D; 25.77 ± 4.2 pg/ml, $p<0.01$, $p<0.001$ versus control). The systemic inflammatory response resolved after day 3 and all cytokine levels returned to baseline by day 7.

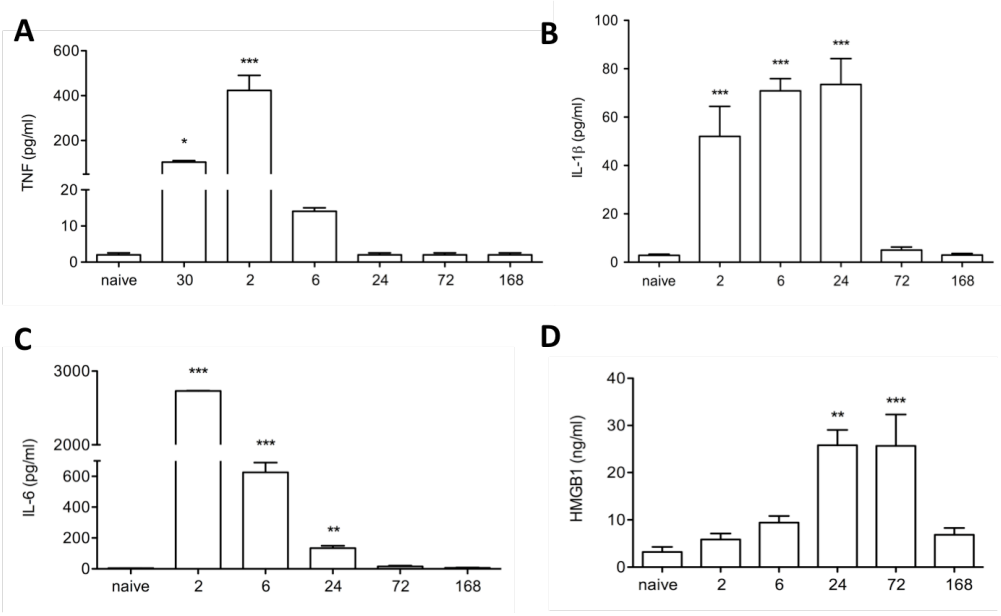


Figure 6-2: **Inflammatory response after LPS exposure**.

Mice were injected with LPS at time zero and plasma levels of TNFα, IL-1β, IL-6 and HMGB1 were measured by ELISA. TNFα was increased after 30 minutes and peaked at 2 hours, returning to baseline thereafter (**A**; *$p<0.01$; ***$p<0.001$ vs control). IL-1β was detected after 2 hours from LPS administration and levels continued to steadily increase until 24 hours (**B**; ***$p<0.001$ vs control). IL-6 expression was highly elevated at 2 hours, decreasing at 6 hours but still significantly detectable at 24 hours compared to naïve animals (**C**; ***$p<0.001$; **$p<0.01$ vs control respectively). Levels of HMGB1 started to increase at day 1 and until day 3 (**D**; **$p<0.01$; ***$p<0.001$ vs control). Data are expressed as mean ± SEM (n = 6) and compared by one-way analysis of variance and Student-Newman-Keuls method with Bonferroni corrections.

To assess the neuroinflammatory response to LPS we measured levels of IL-1β and IL-6 mRNA expression in the hippocampus. There was a 6.5-fold increase in IL-1β mRNA expression and a 15-fold increase in IL-6 in the hippocampus at 6 hours after LPS injection (figure 6.3; $p<0.001$ versus control respectively). In both cases the expression returned to normal values by 24 hours.

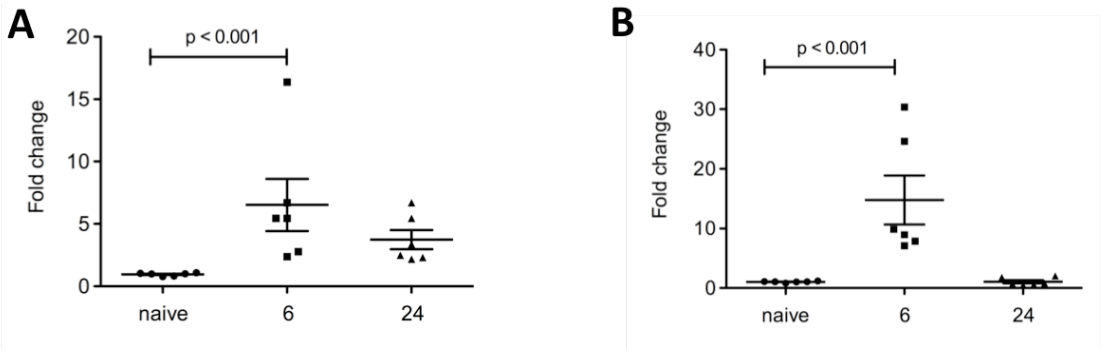


Figure 6-3: **IL-1β and IL-6 mRNA up regulation in the hippocampus**.

Increased mRNA expression of IL-1β (**A**) and IL-6 (**B**) was found at 6 hours after peripheral LPS injection in the hippocampus of mice using qPCR ($p<0.001$ vs control respectively); mRNA expression returned to normal by day 1. Data are expressed as mean ± SEM (n = 6) and compared by one-way analysis of variance and Student-Newman-Keuls method.

The increase in IL-1β both in plasma and in the hippocampus led to investigate whether blocking the IL-1 receptor could ameliorate the symptoms of LPS-associated cognitive dysfunction. A single preemptive dose of IL-1 Ra was able to significantly reduce plasma levels of IL-1β at 6 and 24 hours (figure 6.4A, 32.7 ± 5.45 pg/ml, 6.2 ± 1.03 pg/ml, $p<0.01$ and $p<0.001$ versus LPS respectively) [Untreated animals at 6 and 24 hours: 70.87 ± 5.09 pg/ml, 73.49 ± 5.42 pg/ml]. Similarly, levels of IL-6 were also reduced at the same time-points (figure 6.4B; 91.02 ± 15.17 pg/ml, 14.05 ± 2.34 pg/ml, $p<0.001$, $p<0.001$ versus LPS respectively). Corroboration of these data were achieved by injecting IL-1R$^{-/-}$ animals with the same dose of LPS and measuring cytokine expression in plasma. At 24 hours, time characterized by increased cytokines and clear sickness behavior, levels of IL-1β and IL-6 were comparable to the wild type mice that received IL-1 Ra treatment (figure 6.4A-B; $p<0.001$, $p<0.01$ versus LPS respectively). IL-1 Ra treatment however had no effects on HMGB1 levels, which maintained a similar pattern after LPS injection only and were unmodified in IL-1R$^{-/-}$ too (figure 6.4C).

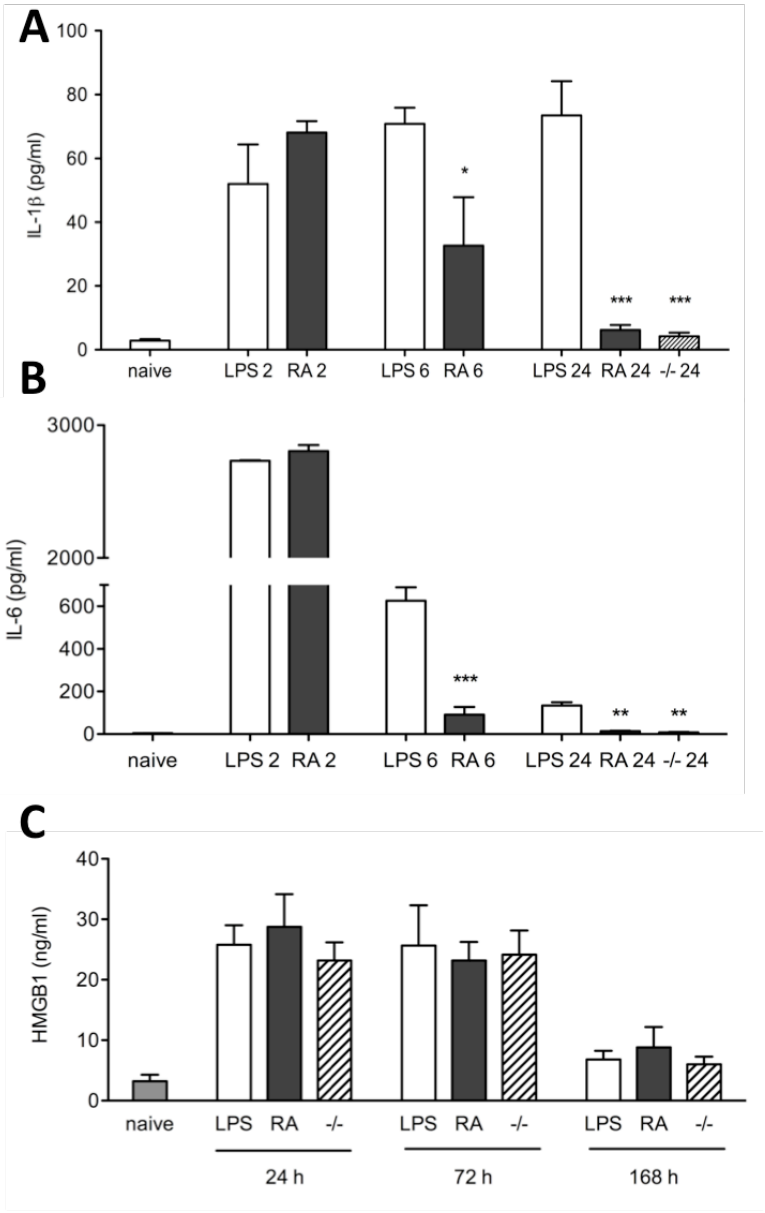


Figure 6-4**: Blocking IL-1 reduces systemic cytokine release**.

Animals received LPS (LPS) or treatment with IL-1 Ra immediately before LPS exposure (RA). Plasma levels of IL-1β and IL-6 were measured by ELISA at 2, 6, and 24 hours. Pre-emptive administration of IL-1Ra significantly reduced the amount of plasma IL-1β at 6 hours (**A**; **p*<0.01 vs LPS) and 24 hours (****p*<0.001 vs LPS). IL-6 followed a similar trend, with a strong decrease in plasma concentrates at 6 hours (**B**; ****p*<0.001 *vs* LPS) and at 24 hours (***p*<0.001 *vs* LPS). To corroborate the findings, levels of IL-1β and IL-6 were measured in IL-1R$^{-/-}$ (**A**-**B**, ****p*<0.001 and ***p*<0.01 *vs* LPS respectively). Blockade of IL-1 had no effects on HMGB1 release in plasma (**C**). Data are expressed as mean ± SEM, (n = 6) and compared by one-way and two-way (IL1R$^{-/-}$) analysis of variance and Student-Newman-Keuls method with Bonferroni corrections.

6.2.2 IL-1 blockade reduces LPS-induced microglial activation

The trascriptome findings prompted interest for other possible markers of neuroinflammation. Microglia, the resident immunocompetent cells of the CNS, were significantly up regulated in LPS-injected animals. Minimal immunoreactivity was reported in naïve animals in which cells maintained small cell bodies with thin and long ramified pseudopodia (figure 6.5A). Resting microglia shifted their state to a "reactive profile" after LPS exposure, acquiring an amoeboid morphology with hypertrophy of the cell body and retraction of the pseudopodia. Reactive microglia displayed morphological changes including increased cell body dimensions, shortened and clumpy processes with higher levels of CD11b immunoreactivity compared to naïve animals. Microglial activation was reported at days 1 and 3 post exposure (figure 6.5B,C; $p<0.01$, $p<0.05$ vs control), returning to the baseline resting state by day 7. Pre-treatment with IL-1Ra effectively reduced the number of reactive microglia at days 1 and 3 (figure 6.5E,F), with no changes at day 7. In order to corroborate these findings, we repeated the experiment using IL-$1R^{-/-}$ animals and exposing them to LPS. These knockout animals did not exhibit microglial activation following LPS treatment (figure 6.5G,H,I).

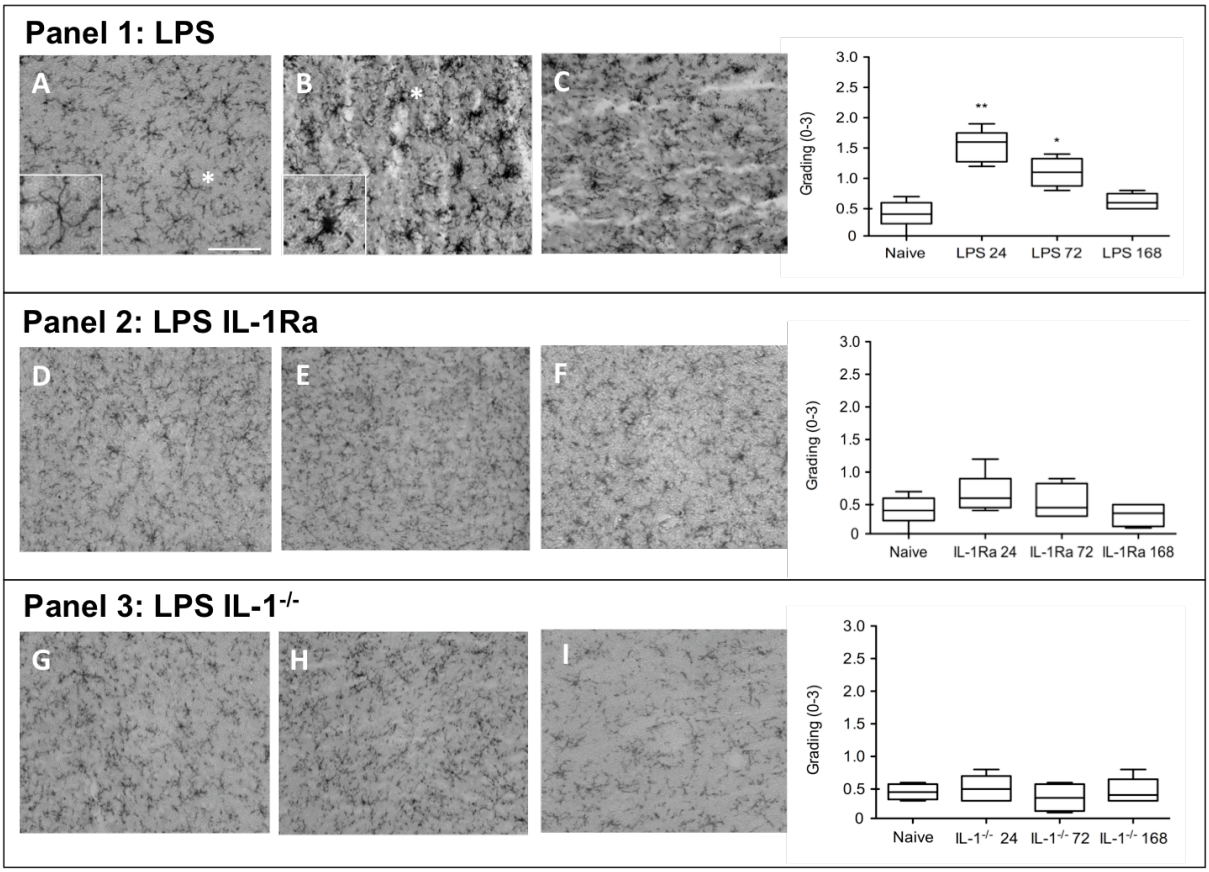


Figure 6-5**: Blocking IL-1 reduces microglia activation**.

Hippocampi were harvested at days 1, 3, 7 after LPS administration and stained with anti-CD11b. Pictures show CA1 (scale bar 50 μm, 20X) and photomicrographs were blindly scored and microglia activation was graded on a scale 0 (lowest) – 3 (highest). *PANEL 1: LPS*. Reactive microglia were found at days 1 and 3 after LPS injection (B-C) compared to naive (A). Resting microglia (box A, 40X) shifted to a "reactive state" (box B, 40X). *PANEL 2: IL-1Ra*. Reduction in the number of reactive microglia was observed after administering IL-1Ra both at days 1 and 3 (E-F), with no changes from controls (D). *PANEL 3: IL-1R$^{-/-}$*. Administration of LPS to IL-1R$^{-/-}$ did not induce microglia activation at any time point assessed (G-H-I). Immunohistochemical grading (0-3) illustrates panels 1, 2, and 3. One day after LPS administration we found clear microgliosis, which was attenuated by IL-1Ra treatment (day 1 ** $p < 0.001$ *vs* control, day 3 * $p < 0.05$ *vs* control). Significant reduction in microgliosis was found both after IL-1 Ra administration and in IL-1R$^{-/-}$ (n = 4). Non parametric data are presented with Kruskal-Wallis followed by Dunn's test.

6.2.3 Hippocampal-dependent cognitive dysfunction following LPS

To relate the inflammatory response to hippocampal memory functioning, trace fear conditioning was used, in which mice are trained to associate a tone with a noxious stimulation (foot shock). The brief gap between the tone termination and the shock onset allows assessment of hippocampal integrity (Chowdhury et al. 2005; Comery et al. 2005). The high level of freezing seen in the naïve animals is indicative of good learning and memory retention. Contextual fear response shows a reduced immobility (freezing) at day 3, revealing hippocampal-dependent memory impairment (figure 6.6A; $p<0.005$ vs naïve trained). Pre-treatment with IL-1 Ra significantly ameliorated this cognitive dysfunction (figure 6.6B; $p<0.05$ vs LPS). No significant differences in the auditory – cued freezing were reported.

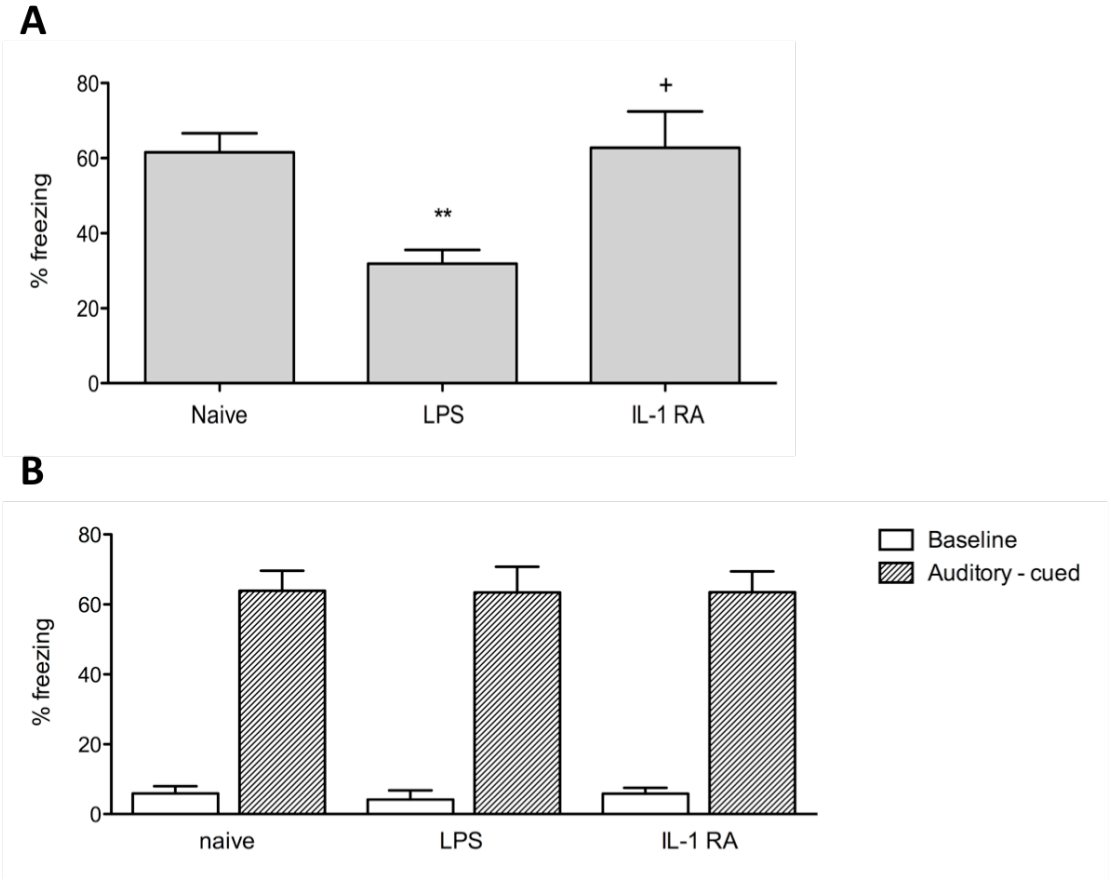


Figure 6-6: **Contextual fear response is ameliorated by pre-emptive IL-1 Ra.**

Within thirty minutes following training, mice were injected with LPS. Three days later, rodents were exposed to the same context in which fear conditioning was previously carried out. Contextual fear response reveals a clear hippocampal-dependent memory impairment (**A**, ** $p < 0.005$ *vs* naive). Pre-treatment with IL-1Ra abolished the main symptoms of sickness behavior and significantly ameliorates the memory retention at day 3 (+ $p < 0.05$ *vs* LPS). No changes in freezing were reported in the tone test (**B**). Data are expressed as mean ± SEM (n = 9 for acute behavior) and compared by one-way analysis of variance and Student-Newman-Keuls method.

6.3 Discussion

These data show that a sustained inflammatory challenge leads to neuroinflammation, microglial activation and hippocampal-mediated cognitive dysfunction. By blocking the IL-1 receptor, the feed-forward process that amplifies the inflammatory cascade is attenuated thereby reducing microglial activation and reversing the behavioral abnormality after endotoxemia. In the context of POCD, the additive effect provided by LPS exposure following surgery correlates with the findings of increased severity and prolonged cognitive dysfunction in humans (Moller et al. 1998; Abildstrom et al. 2000; Monk et al. 2008).

Cytokines play an important role in mediating the inflammatory response after infection or aseptic traumatic injury. The innate immunity is rapidly triggered after LPS, primarily via activation of toll-like receptor 4, TLR-4 (Hoshino et al. 1999). Activation of TLR-4 induces a multitude of pro-inflammatory cytokines via activation of transcription factors, NFκB (Andreakos et al. 2004). This prompt response provides a favorable environment for the synthesis and up regulation of both IL-1β and IL-6, which together contribute to the perpetuation of the inflammatory challenge. Also the rapid increase in TNF-α following LPS, which is reported as present already after 30 minutes, promotes synthesis of other cytokines and the initiation of the acute-phase response. It has been shown that IL-1β can bind receptors and translocate through the intact blood-brain barrier (BBB) (Van Dam et al. 1993). Neural afferents are known to be a fast and reliable pathway in the immune-to-brain signaling. Vagal-mediated signaling can rapidly induce brain cytokines and

manifest the classic symptoms of the acute phase response, including neuroinflammation (Dantzer 1994). As we have reported a significant increase in both IL-1β and IL-6 mRNA transcription at 6 hours in the hippocampus, the neuronal route may be the likely pathway to trigger the early activation of these genes and the initial changes in the CNS. Vagotomy was previously shown to partially attenuate sickness behavior both after LPS and IL-1β administration (Hansen et al. 1998), but not in the context of hippocampal-dependent cognitive dysfunction. However, recently has been also shown that peripheral monocytes can enter the brain causing sickness behavior. This process is strongly supported by TNF-α signaling, especially in activating microglia and recruiting active monocytes into the CNS (D'Mello et al. 2009).

Reactive microglia in the hippocampus interfere with memory processing. Peripheral cytokines affect brain homeostasis leading to microglia activation. Within the brain, cytokines interact with microglia cells. Pro-inflammatory cytokines can directly interact with many of the pattern recognition receptors (PRRs) expressed on the surface of these cells (Aloisi 2001). Upon activation, microglia exhibit discernible morphologic changes and secrete cytokines, reactive oxygen species (ROS), excitotoxins (such as calcium and glutamate) and neurotoxins such as amyloid – β (Hanisch and Kettenmann 2007). Activated microglia also inhibit neurogenesis in the hippocampus following endotoxemia, thereby exacerbating the extent of injury on memory processing (Monje et al. 2003).

To assess memory retention we used trace fear conditioning in which mice are trained to associate a foot shock with a given environment or tone

(Fanselow 1980). The extent to which an animal freezes to a context is largely dependent on the hippocampus (Maren et al. 1997). Hippocampal-dependent memory impairment was evident after 3 days post-LPS. Residual inflammation, primarily via reactive microglia, is possibly associated with this second phase behavioral abnormality. At these time points, levels of HMGB1 were also elevated and prompted us to further investigate the role of these factors in the development of cognitive dysfunction.

Targeting IL-1 ameliorates the cognitive abnormality by reducing microglia but does not affect HMGB1. IL-1β has a pivotal role in sustaining the neuroinflammatory response and closely interacts with memory processing and LTP (Vereker et al. 2000; Barrientos et al. 2002). Self-regulation and inhibition of IL-1β is normally achieved with the neutralizing action of endogenous IL-1Ra, which directly competes to the binding of the receptor (Arend 1991; Dinarello 2005). Transcription of endogenous IL-1Ra would normally occur temporally delayed from the synthesis of IL-1, thus following pharmacological intervention we aimed to block the receptor *a priori* impeding binding and limiting the damage mediated by the effector molecule. When the IL-1 receptor is disabled, either blocked pharmacologically (IL-1Ra) or by genetic intervention (IL-1R$^{-/-}$), the inflammatory response is not sustained as reflected by lower cytokine release and microglia activation, thus ameliorating the cognitive dysfunction as reported here. Treatment with IL-1Ra provides a significant improvement in cognitive dysfunction, confirming the crucial role of IL-1β in memory processes and behavior. However, since IL-1Ra is able to translocate directly into the brain (Skinner et al. 2009), we are unable to discriminate whether peripheral cytokines and/or *de-novo* production in the

CNS account for this cognitive dysfunction. Although there was a temporal correlation between microglia activation and late-release of HMGB1, neither IL-1Ra nor IL-1R$^{-/-}$ changes levels of in this cytokine. This evidence supports the notion that blocking IL-1 is sufficient to reduce the microglia activation and ameliorate the memory abnormality. However other receptors may be involved in sustaining this inflammatory challenge (figure 6.7); for example HMGB1 has been shown to activate TLRs and receptor for advanced glycation end-products (RAGE) and it has been reported as a key late pro-inflammatory mediator in sepsis, with considerable pathological potential (Wang et al. 1999; van Zoelen et al. 2009).

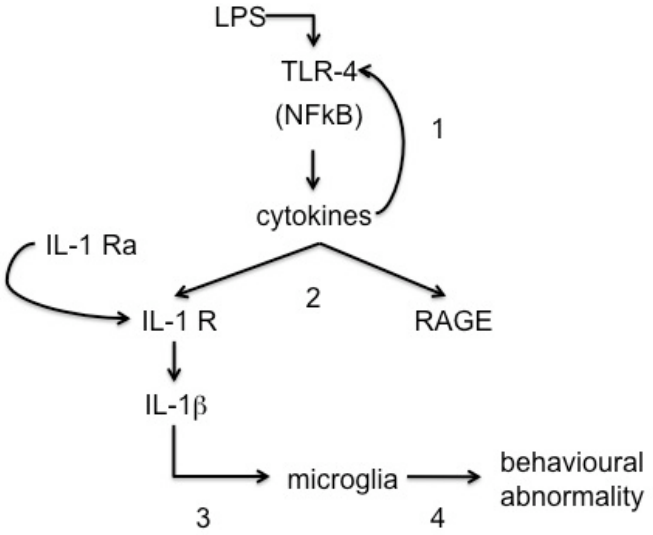


Figure 6-7**: Putative signaling mechanism.**

1) *TLR-4*. Activation of TLR-4 by LPS activates downstream signaling to induce gene transcription of pro-inflammatory cytokines (TNFα, IL-1, IL-6) and NF-κB activation.

2) *IL-1R*. The initial synthesis of IL-1β by TLRs signals to the IL-1R, engaging in a positive feedback loop to produces more IL-1. Blocking this receptor with preemptive IL-1Ra interferes with this amplification process, impeding binding and preventing cleavage and further processing by caspase-1. Other receptors are also involved in this inflammatory cascade, including RAGE. In this study the selective blockade of the IL-1R did not interfere with this alternative pathway.

3) Cytokines, in particular IL-1β, enter the CNS both via humoral and neuronal pathways. These changes are promptly sensed in the brain, leading to microglia activation.

4) Reactive microglia perpetuate the inflammatory response and further produce neurotoxic agents causing memory impairment and abnormal behavior. Chemotaxis and production of pro-inflammatory cytokines directly affect synaptic plasticity, inhibiting LTP and promoting long-term depression (LTD) in different hippocampal circuits. Targeting IL-1 and the resulting microglia activation appear to provide a beneficial effect in the context of cognitive dysfunctions and possible neurotoxicity.

Interestingly, if we compare the profile of HMGB-1 during infection or in aseptic inflammation, there are striking differences (figure 6.8). These discrepancies support the concept that this cytokine plays a different role accordingly. Surgery relies on the activation of the "*danger signaling*" and DAMPs, including HMGB-1, heath-shock proteins (HSPs), and tissue factors (Lotze and Tracey 2005; Zedler and Faist 2006). In this setting HMGB-1 is quickly released soon after the mechanical damage and tissue necrosis, and may be responsible for perpetuating the inflammatory response both acting on TLRs and MyD88-independent signaling (i.e. RAGE) (Erlandsson Harris and Andersson 2004; Park et al. 2004; Harris and Raucci 2006; Bianchi 2007; Muhammad et al. 2008). Following infection or sepsis, HMGB-1 has a different role and its activation is time-delayed and possibly sustained by already pre-elevated cytokines and circulating macrophages (Wang et al. 2001; Andersson and Tracey 2003; Czura and Tracey 2003). Advance glycation end-products (HMGB-1 and some S100 proteins) bind to RAGE leading to downstream activation of MAPK and nuclear translocation of NFkB, thus releasing further cytokine (Erlandsson Harris and Andersson 2004; Bianchi 2007). HMGB-1 can bind to both RAGE and TLR4 to mediate inflammation, coagulation activation and neutrophil recruitment (Yu et al. 2006; van Zoelen et al. 2008). These results first account for an involvement of RAGE signaling in POCD, further highlighting that infection is not a confounding factor in this model of POCD.

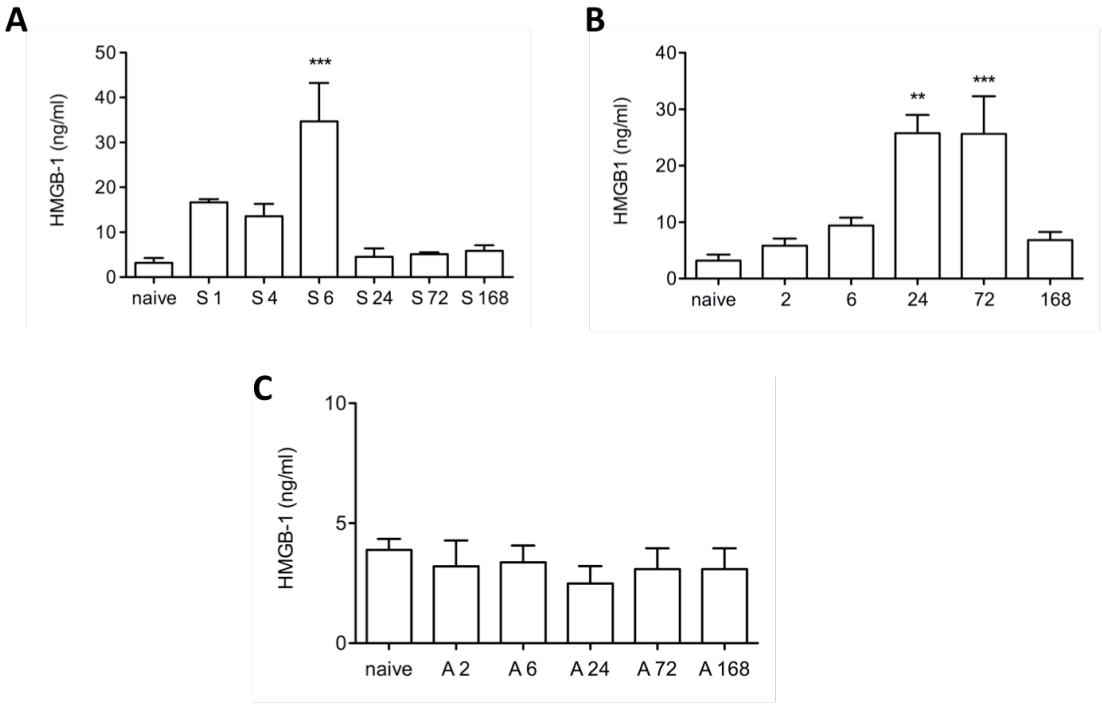


Figure 6-8: **HMGB-1 in surgery and infection**.

Mice either received tibia surgery under general anesthesia (**A**), i.p. LPS (1 mg/kg) (**B**), or isoflurane only (**C**). Levels of HMGB-1 were sampled at different time-points. Following surgery, a positive trend was already observed after 1 hours post incision and levels peaked at 6 hours (**A**; 34.69 ± 5.78 pg/ml, ***p<0.001 vs control). By 24 hours levels returned to baseline.

If animals were exposed to LPS, HMGB-1 was undetectable before 24 hours. Levels were increase after 1 day, and remained up regulated up to 3 days post exposure (**B**; 25.77 ± 4.2 pg/ml, **p<0.01, ***p<0.001 vs control respectively).

No changes were observed after exposure to anesthesia only (**C**) Data are expressed as mean ± SEM (n = 6) and compared by one-way analysis of variance and Student-Newman-Keuls method with Bonferroni corrections.

In summary, the beneficial effects on cognition reported in this study by targeting IL-1, preemptively, are encouraging. However, it is not possible to extrapolate these benefits to the setting of cognitive dysfunction that accompanies severe sepsis with multiple organ failure. In that clinical scenario there are complex inflammatory responses that are difficult to reverse (Riedemann et al. 2003). Clinical trials targeting IL-1 have been unconvincing in improving mortality rate, especially in sepsis (Marshall 2003). In this attempt to untangle the complexity of this condition, anti-cytokine therapy appears to be able to ameliorate the associated cognitive dysfunction. Inflammation clearly plays a pivotal role in mediating physiological as well as behavioral changes after LPS-exposure. Further studies are yet needed to ascertain whether selective targeting of other cytokine receptors can effectively prevent or ameliorate both the degree and length of cognitive decline.

7 Chapter 7: General discussion and conclusion

Postoperative complications are a major concern for the patient's quality of life. As the name suggests, complication from the Latin *complicare*, reflects in a number of tangled events and factors able to jeopardize an entire system, ultimately leading to death. The immune system is considered a fail-safe mechanism playing a pivotal role in maintaining and ensuring a correct homeostasis and protection to the organs. However, if dysregulated, cytokines may cause damage, and themselves, may be therapeutic targets in disease states.

In this modest attempt of clarifying the complexity of postoperative cognitive dysfunction (POCD), new pathways and molecules have been unveiled, alongside a potential for translating this research from the bench to the bedside. The multifactorial etiology of POCD has been described from many clinical trials, but studies have often been underpowered and anecdotal (Newman et al. 2007). Problems concerning cognitive domains have also been neglected and underestimated in the past; dementia and neurodegeneration are pivotal topics in medicine and neuroscience and require much more understanding.

Because both age and surgery are two main risk factors in POCD, pandemics are portended by the increasing aging population and the likelihood of those of advanced age requiring surgery and anesthesia. With cardiac and orthopedic procedures manifesting the highest incidence of cognitive abnormality, we opted to reproduce a clinical routine orthopaedic procedure using a mouse model to better dissect some of the underlying factors of

POCD. The pivotal interest of this thesis has evolved around the inflammatory response. Evidence for the involvement of inflammation in POCD are limited and often associated to the type of surgery. Cytokines and acute phase proteins are used as readouts to assess the extent of injury and the effects of surgical insults on the immune system. However, the putative association between these molecules and cognitive dysfunction has not been thoroughly studied. Work from this laboratory has first demonstrated the involvement of cytokines in surgery-induced cognitive abnormalities using a rat model of POCD. Herein, these data demonstrate that inflammation plays a pivotal role in mediating cognitive dysfunctions in mice and that by targeting specific pathways, or molecules, the inflammation can be attenuated, thus improving the cognitive disability. Starting from the initial anti-inflammatory effects provided by minocycline, I have been able to describe the involvement of specific MyD88 dependent signaling pathways as well as independent markers such as TNF-α and HMGB-1, which appear to have a role in POCD.

What has been reported here though raises further questions and novel issues to be addressed. In particular, assessing clinical patients after non-cardiac surgery the incidence of POCD is about 10% at 3 month post-discharge (Moller et al. 1998). Why “only” 10% of all patients have this phenomenon has been a daunting question where I have perceived limitations from the animal model. Cognitive domains and human cognition are extremely complex; the concept of *cognitive reserve* has been applied to POCD and this may explain why some people appear protected, perhaps by a more active neuronal network and synaptic plasticity. Indeed, these theories are possibly too challenging to be investigated in mice. However, this may also reflect the

inadequacy of tests being applied in the human system. Genetic polymorphism may be also a “discriminative” factor in identifying susceptible individuals. In this setting, animal models may offer added value by allowing targeting of specific genes either by knocking/out techniques. Prime candidates in this field include APOe4 and CRP (Abildstrom et al. 2004; Mathew et al. 2007). However, the new findings reported in this thesis may also prompt reasons for assessing polymorphisms in MyD88 and possibly TLRs. In a new era based on genetic testing and novel diagnostic tools, genotyping may add value in helping people with higher risks, upon identification of reliable biomarkers. Inflammation and cytokines are implicated in a number of disease states ranging from cardiovascular diseases (atherosclerosis, stroke, ischemia), rheumatoid arthritis, sepsis and cancer. Polymorphisms, deletions, and single nucleotide polymorphisms in inflammatory regulatory genes are thought to play a pivotal role in the pathophysiology of these conditions. Variations in the promoter region of TNF have been associated with higher susceptibility to infectious diseases, sepsis, and ischemic stroke (Hill 1999; Mira et al. 1999; Wang et al. 2009). Similarly, allele frequencies and genotype distribution of the IL-1 superfamily associate with susceptibility and differential outcome in severe sepsis (Fang et al. 1999). Studies on the role of genes in surgery and inflammation are yet very limited but the data reported in this thesis suggest a pivotal role for both TNF-α and IL-1 in surgery-induced cognitive dysfunction. Individuals with mutations in these genes may be at higher risk for cognitive disturbances following surgical stimulation. This knowledge should pave the way for the use immune-based therapies in personalized medicine.

The data reported here also suggest a pivotal role for MyD88 signaling in the etiology of cognitive abnormality. The role of this adaptor molecule in inflammation and cytokine production has been thoroughly detailed in the past, but novel pathways and alternative functions have been recently proposed. Studies that associate these classical inflammatory mediators with memory and behavioral abnormalities are very limited, especially in the context of surgery and aseptic inflammation. Herein a role for MyD88-dependent signaling in POCD has been described. IL-1 appears to have a pivotal role in the development of surgery-induced cognitive abnormality. I found surprising the limited effects of TLRs on POCD-associated inflammation and behavior. The complexity of POCD may still require TLRs activation, but the absence of both TLR2 and TLR4 appears insufficient to provide beneficial effects following surgical stimulation. This may be consistent with the *threshold theory* established following the experiments with anti-TNF prophylaxis. TNF-α clearly has a pivotal role in regulating the inflammatory response; early targeting of this molecule has provided beneficial effects both in terms of inflammation and behavioral outcome. Targeting TNF-α in this model has also strengthened the relationship between IL-1 and POCD, perhaps at an earliest time-point compared to administration of IL-1 Ra. Therapies with either anti-TNF-α or IL-1 Ra appear to protect against the cognitive dysfunction, however by targeting both molecules inflammation is fully abolished. This provides an insight on how MyD88-dependent and independent pathways synergise, further demonstrating the concept of *threshold theory* and how levels of pro-inflammatory cytokines closely regulate cognitive abnormality. If the inflammatory challenge following surgery

is sufficient to overcome a *threshold*, this leads to memory loss via sustained effects on the brain and hippocampus. Individual targeting of MyD88, IL-1 and TNF-α is able to prevent cytokines to reach this target, thus the effects on the CNS are minimal and no signs of behavioral abnormality are reported.

Anti-cytokine therapy here provided beneficial effects on POCD-associated behavior. Targeting of IL-1 either via MyD88-dependent pathways, genetic obliteration of the IL-1R, antagonism with the IL-1 Ra or antiTNF prophylaxis appear to reduce the overall inflammation and the associated cognitive abnormality. This is in line with the central role of IL-1 in sickness behavior and PAMPs-induced hippocampal dependent memory dysfunction, but also suggests a pivotal role for TNF-α in POCD. Prophylaxis, with IL-1 Ra or anti-TNF antibodies, is an amenable therapeutic option to be exploited in the clinical setting. Yet the use of anti-cytokines therapy is controversial, in particular with anti-TNF. Clinical trials in septic patients have not been as convincing as expected and there was only a minor improvement in mortality following therapy (Marshall 2003). It is possible that by administering these reagents, patients may be at higher risk for opportunistic infections and further complication after surgery. However most of the randomised clinical trials have not reported any differences in the incidence of infections in patients with anti-TNF treatment (Keane et al. 2001). Furthermore, anti-TNF has been the first cytokine to be validated as therapy for rheumatoid arthritis, where it continues to offer clear benefits to the patients (Brennan et al. 1989; Taylor and Feldmann 2009). In this thesis a single dose of anti-TNF was able to impart beneficial effects. Blocking cytokines may be a successful strategy able to dampen the inflammatory response, however cytokines are also

involved with the homeostatic and healing process following inflammation and trauma (Gerstenfeld et al. 2003). Following administration of IL-1 Ra, TNF-α antibody, or in MyD88$^{-/-}$ there was no evidence for impaired healing process following tibial fracture (personal conversation with Professor Jagdeep Nanchahal – unpublished data). Identification of vulnerable individuals who could benefit from these therapeutic options will be crucial in order to exploit these findings into the clinic.

As each individual may present with a different pattern of cytokine / inflammation, the concept of "cytokine disequilibrium" can be applied to POCD (figure 7.1). In the context of this thesis, I have demonstrated for instance how patterns of cytokines are defined by a given stimulus (infectious or aseptic injury). The magnitude of cytokines response, the time-course for the increase of each mediator and the specific molecules that are detected dramatically differ in each setting, making them distinct. In my opinion this is the knowledge that needs to be applied, and, although we tend to generalize providing "overall views" these may not reflect the individual. This knowledge should enable more therapeutic options as well as tailored-specific treatments according to each individual.

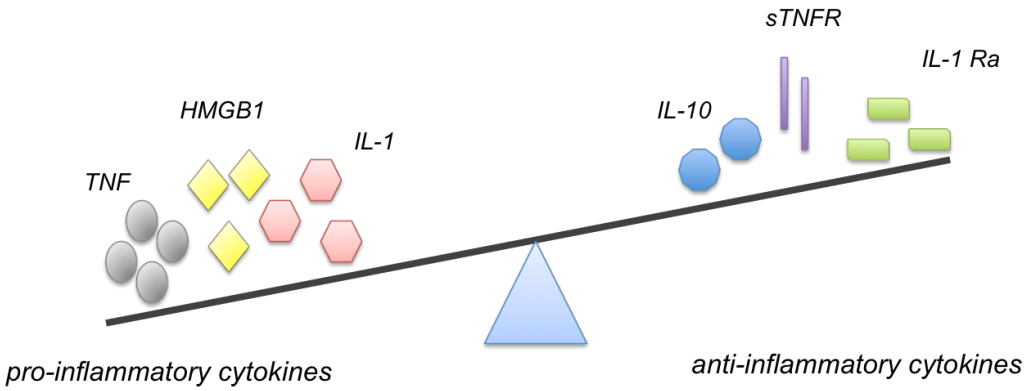


Figure 7-1: **Cytokine disequilibrium**
Modified from (Feldmann et al. 1996)

In my PhD studies I have proven the role of cytokines in inducing POCD. However, what induces cytokine production still needs to be investigated. Candidate pathways may include HMGB-1 and RAGE signaling. RAGE signaling has been associated with inflammation, oxidative stress, and metabolic dysfunctions (Lu et al. 2004; Torreggiani et al. 2009). Alternatively, surgical stress and reactive oxygen species are likely to up regulate cytokines after surgery, thus perpetuating the inflammatory challenge. The putative involvement of advanced glycation endproduct and their association also with diet and metabolism may prompt for other risk factors in POCD, which may include obesity and metabolic syndrome. Adipocytes are able to secrete factors and recruit chemokines, adhesion molecules, cytokines (TNF-α, IL-1β, IL-6 and monocyte chemoattractant protein 1 – MCP1), thus being active interfaces of the inflammatory response (Loffreda et al. 1998; Curat et al. 2004). As we have corroborated the crucial role of inflammation in POCD,

these factors should now be accounted for. Inhibitors of RAGE as putative activators of Aβ pathogenic pathways are also targets of ongoing clinical trials for dementia and Alzheimer's disease (AD), thus they may represent viable therapies also for cognitive dysfunctions (Mucke 2009).

The association between POCD and further degeneration (i.e AD) has been speculated but yet there is no evidence for a continuum between the two phenomena. This yields interest for the actual mechanisms underlying CNS inflammation and neurodegeneration; are microglia playing "*good cop, bad cop*" in these settings, what are the immune-to-brain signaling pathways responsible for this neuroinflammation, what are the significant risk factors to assess.

With the increased vulnerability of the elderly and a classic role for anesthesia as inducer of cognitive disturbances, there is a vivid debate on the effects of anesthetic agents on individuals. Neurodegeneration or AD-like symptoms caused by anesthesia have been reported, however the causative role of anesthetics in POCD has not been confirmed (Rasmussen et al. 2003; Culley et al. 2007). This work has reported no clear effects of isoflurane, a clinically used anesthetic agent, on the inflammatory cascade or POCD-associated behavior. However, it is possible that different anesthetic agents may provide different outcomes to susceptible patients. Intravenous anesthesia using propofol, a widely used anesthetic agent for induction and maintenance of anesthesia, showed improved surgical response and faster recovery time compared to isoflurane, thus reducing POCD in the elderly (Tan 2009). Anesthetic agents also closely interact with the immune system acting as

immunomodulators; propofol has renowned affects as an anti-inflammatory agent (Chen et al. 2005). This suggests that even if we have not found a direct effect of isoflurane on POCD-associated behavior, this does not preclude a role for anesthesia in cognitive dysfunctions.

Many questions still require answers; it has been a privilege to work on this project at this stage and I think this thesis has confirmed previous theories, revealed new findings and progressed towards a better management for this condition in the clinical setting. Whether the selective targeting of cytokines, or receptors, can effectively prevent or ameliorate cognitive dysfunction yet remains to be seen.

8 Future work

The results of this body of work have suggested a number of possible subsequent experiments to explore POCD's mechanisms. These experiments have the potential to further both preclinical knowledge as well as directly impacting on clinical practice, through the use of alternative therapeutic strategies.

Some questions that remain to be answered:

- to better define the immune-to-brain to communication in POCD, in particular how systemic cytokines can elicit neuroinflammatory processes. The role of microglia requires better understanding especially in the identification of their origin (infiltrating macrophages or microglia) and role

in the CNS (as harmful or protective cells).

- to explore the evidence for a direct link between surgery-induced neuroinflammation and the development and/or exacerbation of AD. Surgery can alter physiological processes in the host organism; in patients at risk from developing AD (i.e. with preexisting mild cognitive impairments), surgery may act as a "co-stimulus" able to prime the system and trigger both POCD and early-AD features in such susceptible individuals.
- test novel therapeutic interventions designed to mitigate cognitive dysfunction in clinical trials, based upon these preclinical data.
- further investigate the relationship between postoperative complications for example infection and co-morbidities such as rheumatoid arthritis and cancer, and their association with severity and duration of POCD in particular.

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10 Appendix

The work presented in this thesis has contributed to the following:

Patent: "Anti-cytokine therapies for POCD" Terrando, Monaco, Ma, Feldmann, Maze

Manuscript: "Systemic and hippocampal IL-1β-mediated inflammation underlie cognitive dysfunction following surgery" Cibelli, Rei Fidalgo, Terrando, Ma, Monaco, Feldmann, Takata, Nanchahal, Fanselow, Maze (submitted)

Manuscript: "How cytokines inform sepsis-induced cognitive dysfunction" Terrando, Rei Fidalgo, Vizcaychipi, Cibelli, Ma, Monaco, Feldmann, Maze (submitted)

Manuscript: "Anti-TNF prophylaxis prevents postoperative cognitive dysfunction" (in preparation)

Poster: "High mobility group box 1 and reactive microglia mediate late hippocampal dependent cognitive dysfunction after lipopolysaccharide endotoxemia" Terrando, Rei Fidalgo, Ma, Monaco, Maze, *Society for Neuroscience*, Chicago, October 2009

Poster: "Inflammation mediates postoperative cognitive dysfunction in mice after major surgery", Terrando, Cibelli, Rei Fidalgo, Foxwell, Maze, *European Society of Anesthetists*, Milan, June 2009

Poster: "Immune-to-brain signaling mediates cognitive dysfunction following surgery in mice" Terrando, Cibelli, Rei Fidalgo, Vizcaychipi, Ma, Feldmann, Maze, *Association of University Anesthesiologists*, Galveston, Texas, April 2009

Invited talk: "Inflammatory signaling in postoperative cognitive dysfunction", *Centre for Cerebrovascular Research*, University of California San Francisco, November 2009

Invited talk: "Inflammatory signaling in postoperative cognitive dysfunction", *Kennedy Institute of Rheumatology*, Imperial College London, September 2009

Invited talk: "Immune-to-brain signaling mediates cognitive dysfunction following surgery in mice", *Association of University Anesthesiologists*, Galveston, Texas, April 2009

Invited talk: "Inflammation mediates postoperative cognitive dysfunction (POCD) in mice after major surgery", *Institute of Child Health*, London – 1st Inaugural UCL and Imperial College London Academic training meeting in Anaesthesia, Pain and Intensive Care, January 2009