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TITOLO TESI DI DOTTORATO DI RICERCA: IMMUNE SYSTEM AND CENTRAL NERVOUS SYSTEM CROSSTALK: FOCUS ON IL-18 IN NEURODEGENERATIVE AND PSYCHIATRIC DISEASES

BIO/13

Tesi di dottorato di:

Dott. Ilaria Palladino

Coordinatore del corso

Prof. Giorgio Pantera

Firma

Tutore

Prof. Giuseppe Scapigliati

Firma

Co-tutore
Dott. Paola Bossù

Firma

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INTRODUCTION

1. INTRODUCTION

The immune system (IS) and the central nervous system (CNS) are the two main adaptive systems that provide for the functional integrity and regulate the homeostasis of the whole organism. Convincing experimental evidence, exponentially growing in the recent years, shows that immunity and brain interact to influence health. Indeed, the two systems share many analogies both for the function of their leading cells and for the way of action.

In the past, neuroimmunology was concerned mainly with the manner in which the IS interacts with the CNS in pathological conditions, such as autoimmune disorders. More recently, new levels of intersection and commonalities have emerged: for instance, it is now known that cells within the two systems communicate via morphologically similar physical connections and that they share molecular mediators of communication, such as transmitters and signalling molecules (Kerschensteiner et al., 2009). In addition, cells from both systems show targeted migratory behaviour (neurons mostly but not exclusively during development, immune cells throughout life). Finally, a very interesting functional characteristic that unites the two systems is their ability to connect and carry information from and to distant parts of the body: neurons using their long processes (dendrites and axons) and the immune cells as a result of their mobility. The immune system, through lymphocytes, secretes endocrine substances and neuropeptides so as the central nervous system. The communication of all these molecules produced occurs through receptors that are proteins located on the cell surface that transmit signals within the cell following the binding with the mediators for which have specific affinity. For instance, a list of common surface molecules that act as receptors includes the Toll-like receptors (TRLs), which were originally thought to be primarily functioning in immune cells, but that are now also found in neuronal cells, where they appear to perform a cell-autonomous function (Farina et al., 2007).

Similarities between the two systems are also found in the rules that govern the motility and the direction of movement of the neurons and immune cells. In fact, both types of cells are able to map a precise itinerary in response to attractive or repulsive signals produced by other cells present among their

migratory route or at the final destination. Thus, lymphocyte movement within lymphoid organs appears to be the result of recognition of molecules and structures produced and laid down by stroma cells in precise fashion (Bajenoff et al., 2006). Similarly, neurons migrate and extend their processes also through complex stromal structures after specialized signals that include samaphorins, signalling molecules that are key players in neuronal guidance, and that recently were found to be expressed and used also by lymphocytes (Mizui et al., 2009).

Until a few years ago it was believed that the brain was "an immunological privileged organ" inasmuch isolated from immune and inflammatory response by blood brain-barrier. This view has been drastically changed by a lot of studies that have shown as the brain can exhibit a wide range of immune and inflammatory response, beside being an important regulator of immune system.

A lot of experimental evidences show a crosstalk between the immune system and the nervous system by a bidirectional communication. The efficient coordination of the interaction between these systems guarantees the maintenance of the physiologic state, that if missing, may confer a risk for the onset of pathological processes.

1.1 THE IMMUNE SYSTEM: PERIPHERAL AND BRAIN RESPONSES

The immune system has the responsibility to defend the body against pathogenic insults, which may be represented not only by exogenous agents of infectious nature (as in the case of pathogenic microorganisms), but also from endogenous substances potentially harmful originated from injury, trauma or altered physiological processes.

Basically, two types of defensive processes are outlined in the immune system: the innate or natural immunity and the acquired or specific immunity. The natural immune response represents the first line of protection of the organism against pathogens and it is mainly based on non-specific defence mechanisms, existing since the birth of the individual. This

is the oldest system of defence against infection from the evolutionary point of view. The main components of innate immunity are the barriers of the organism with peculiar physical or chemical proprieties, such as the skin or the gastric acid contained in the gastric mucosa and different types of cells and soluble molecules. Among the latter, cells with phagocytic activity such as neutrophils, macrophages and dendritic cells and cells with cytotoxic activity or natural killer cells (NK), as well as the molecules belonging to the complement system and cytokines are all important players of the non specific response and immediate attack to pathogens. Following the natural immune response, a sequence of cellular and molecular events takes place which constitutes the acquired immunity, able to enhance the defence mechanisms triggered by the innate immunity. This process is able to activate immune responses highly specific for the different pathogenic macromolecules and to respond in an ever more powerful to repeated exposure to the same antigen, as it is characterized by the onset of a "immunological memory" which allows a persistent protection thanks to the presence of specialized immune cells functionally quiescent but capable of surviving for long periods. The main effector mechanisms of specific immunity are humoral immunity, mediated by antibody producing B lymphocytes and cellular immunity mediated by T lymphocytes. The latter, comprise two distinct subpopulations: cytotoxic and helper T cells, both incapable to recognize soluble antigens, but able to bind processed antigens presented by molecules of the major histocompatibility complex, through specific T cell antigen receptors (TcR). The immunocompetent cells that possess the specialized machinery required to efficiently process and express the antigens in this way are named antigen presenting cells (APC), and include macrophages, B lymphocytes and dendritic cells. Once T cells recognize and bind to the antigen-MHC complex, the APC send out additional co-stimulatory signal to activate T cells, so initiating a new adaptive immune response. While T cytotoxic lymphocytes exert their function directly killing the cells infected by the antigens, T helper cells secrete cytokines to activate the proliferation and the differentiation of other T cells, B lymphocytes and macrophages (Abbas et al., book).

By the anatomical point of view, the immune system consists of a complex network of organs and tissues, connected by blood and lymphatic vessels, that work together to prevent infection or injury-mediated damage throughout the body. The thymus, bone marrow, spleen, lymphoid system including lymph nodes and mucosal associated lymphoid tissue, as well as the circulatory system, are all components of the peripheral IS, as indicated on the basis of the collective definition for all immune responses that take place outside the brain and together are responsible for the creation, transport and function of immunity in mammals. However, also the CNS is under immunological surveillance, and even though the mechanisms are different from the periphery, immune responses occur in the brain (Hickey WF, 1999). Notably, an active interplay exists between brain and IS and increasing evidence points out that circulating immune signals exert an effect on the brain, as well as brain derived signals may influence immune reactions. The interaction element between the blood born cells and molecules of the immune system and CNS components is the blood brain barrier (BBB), which, differently from the past when it was mainly considered a static barrier preventing infiltration of immune elements in the brain, is today seen as an active regulatory interface that controls the exchange between the brain and the periphery and is modified and affected by circulating events and by events on the CNS side (McAllister AK, 2009). A series of different immune cells interact with the BBB to mediate immunity at this side. First of all, perivascular macrophages resides between the astrocytes and the vessel wall and their role is to phagocyte cellular debris and the depletion of these seems to increase meningitis infection (Williams et al., 2001; Polfliet et al., 2001). Moreover, mast cells have been identified to be associated with vessels in specific region of the CNS also if their role in CNS immunity and in BBB regulation has not been clarified (Wilhelm et al., 2005). It has been demonstrated that breakdown of the BBB is a critical component of several disease as multiple scleroses, edema and brain trauma. The breakdown of the BBB accompanies the primary insult in these disorders allows the entry of plasma component, immune molecules and cells, which leads to neural dysfunction and ultimately neural degeneration. New evidence also suggests that BBB dysfunction might be a

widespread component of many different neurodegenerative disorder as Alzhaimer's disease (Browman et al., 2007) and BBB dysfunction has also been proposed as a cause of this pathology (Pluta et al., 2006; Daneman, 2012).

The brain is protected by damage at several levels: by the interaction with the IS, by the BBB and by several resident cells. The most important resident cells that have the role to defend CNS by damage are some glial cells that constitute the microglia. These brain specific immune cells, are macrophage-like, originate from bone marrow and migrate to the CNS, where develop into glial cells. They are constitutively present in the brain were they actively survey the tissue by monitoring their extracellular space and cellular neighborhood, but are mobilized as a result of injury or infection and form the first line of cerebral defence. Once activated, they have an extension more robust and more branched than their resting counterpart. In CNS parenchyma, resident microglia are monocyte lineage cells, which enter in the CNS during development and are involved in neural development, innate immune response and promoting wound healing, can act as antigen-presenting cells to initiate an adaptative immune response (Ajami et al., 2007; Streit et al., 2005). Moreover, a subset of microglia cells interact with the CNS vessels and have been shown to regulate immune cells passage across the BBB (Persidsky et al., 1999; Hudson et al., 2005).

Recent study have shown that other cells over microglia are present in CNS. In fact, as I mentioned earlier, macrophages and mast cells, associated with meninges and perivascular spaces, may be maintained by replacement from blood monocytes (Ajami et al., 2011; Ajami et al., 2007; Ransohoff et al., 2010). Following CNS inflammation the number of myeloid cells increases as a result of infiltration from the blood and the proliferation of the resident microglia cells augments as well. Moreover, albeit the CNS was previously believed to be inaccessible to T cells as an immune privileged site, recent studies have instead proposed that memory T cells can enter the CNS from the blood into the cerebrospinal fluid (CSF) (Ransohoff et al., 2003) by a complex trafficking pattern mediated by chemokines and chemokines receptors.

1.2 BRAIN: ORGANIZATION AND FUNCTIONS

The nervous system is responsible for sending, receiving, and interpreting information from all parts of the body and is coordinated by a complex network of neurons. The brain is the center of the nervous system and it is a highly complex organ both from the anatomic point of view that interaction. During the cerebral development, few weeks after conception, at the level of the cephalic end of the neural tube, begin to be evident three bulges: the forebrain, the midbrain and the hindbrain. Seven weeks after the conception the forebrain and the hindbrain are clearly divided. The forebrain will be divided in telencephalon and in diencephalons: telencephalon will give rise to neocortex, basal ganglia and systemic limbic; diencephalon in thalamus and hypothalamus. The hindbrain will be divided in metencephalon that will give rise to pons and cerebellum and in mielincephalon will give rise to medulla oblongata. (Fig.1) Each part of the brain is connected with the others by various connections to exchange the information and each region underlying a specific behavior.

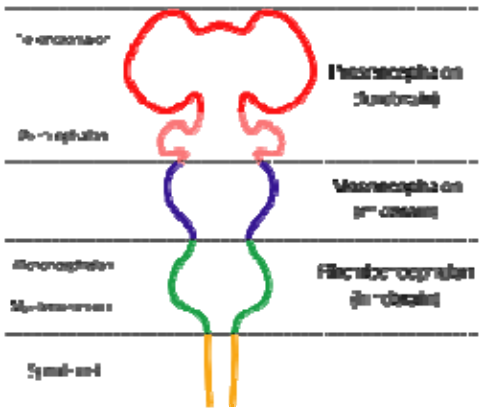


Fig.1 Subdivisions of the embryonic vertebrate brain. These regions will later differentiate into forebrain, midbrain and hindbrain structures

Begin to know this important system starting from its basic unit: the two principal classes of specific cells called glial cells (o glia) and neuronal cells (or neurons).

The glial cells are 10-50 times more numerous than neurons and are arranged to surround the cellular body, dendrites and axons. These cells perform different functions:

- Act as support to neurons giving shape and structure to the nervous system;
- Act as phagocytic cells by removing the fragments of cells due to injury (microglia);
- Improve the efficiency of nerve signals;
- Two type of glial cells (oligodendrocytes and Schwann cells) give rise to myelin;
- Other glial cells (radial glia) guide the migration of the neurons and their axons during neurogenesis;
- Other glia cells (astrocytes) make up the blood brain-barrier.

Some glia cells, in particular the microglia cells, have a direct modulation on the neurons that are the other cell population of the brain. Microglia cells in fact, as already mentioned before, are activated at cerebral level following a brain injury in an attempt to repair the damage. Microglia produce pro-inflammatory cytokines, which could contribute to augmented local inflammation, release of toxic molecules contributing on neuronal damage. In some situations, the role of microglia has been found to be beneficial, since activated microglia can reduce in Alzheimer's disease, Aβ accumulation by increasing its phagocytosis, clearance, and degradation (Frautschy et al., 1998; Qiu et al.,1998). Microglia can also secrete a number of soluble factors, such as the glia-derived neurotrophic factor (GDNF), which are potentially beneficial to the survival of neurons (Liu et al., 2003).

The neurons, that are the main brain cells, are principal players of the complex networks that underlie behaviour and cognitive functions. These cells are formed by three areas: the cellular body (where there is the nucleus), dendrites (extension of the cell body) and the axon (single extension of the cell). Dendrites originate from the cell body and branching form arborizations which represent the main equipment to receive messages coming from other nervous cells. Axons extend for long distance from the cellular body transmitting messages to other neurons. Axons can transmit electrical signals called action potential that are nervous impulses generated at the level of the origin of the axon (cone emergency). The potential of the signal does not change along the path and it has the specific characteristic to

be a signal of “all or nothing”. Moreover this signal can regenerate at regular intervals along the axon. The nervous system uses the action potentials to receive, analyzed and disseminate the information. These signals are stereotyped through the nervous system and to increase the conduction velocity, the axons are surrounded by a lipid envelope of myelin. The casing is interrupted at regular intervals by the nodes of Ranvier and in these sites the energy of the action potentials can regenerate. Neurons can also communicate with other neurons at the level of the synapse by the release of chemical molecules called neurotransmitters. Neurotrasmission (or synaptic transmission) is communication between neurons as accomplished by the movement of chemicals or electrical signals across a synapse. For any interneuron, its function is to receive input "information" from other neurons through synapses, to process that information, then to send "information" as output to other neurons through synapses. Consequently, an interneuron cannot fulfil its function if it is not connected to other neurons in a network. A network of neurons (or neural network) is merely a group of neurons through which information flows from one neuron to another. Every neural network headed by a specific neurotransmitter, underlie specific behaviour and cognitive functions. For example, the principal neurotransmitters which are associated specific cognitive functions are the following:

- Serotonin. Serotonin plays a very important role in a range of brain functions and it is synthesised from the aminoacid tryptophan. Within the brain, serotonin is localised mainly in nerve pathways emerging from the raphe nuclei (a group of nuclei at the centre of the reticular formation in the Midbrain) pons and medulla. These serotonergic pathways spread throughout the brainstem, the cerebral cortex and the spinal cord. Serotonin has been linked with a wide variety of functions, including the regulation of sleep, pain perception, body temperature, blood pressure and hormonal activity. This neurotransmitter, moreover seems to be involved in psychotic disease as schizophrenia inasmuch modulates the release of dopamine in several brain region causing the known symptoms of this illness.
- Dopamine. Dopamine is classed as a monoamine neurotransmitter and is concentrated in very specific groups of neurons collectively called the basal

ganglia. Dopaminergic neurons are widely distributed throughout the brain in four important dopamine pathways: mesolimbic, mesocortical, nigrostriatal and tuberoinfundibular. A decreased or an increase of brain dopamine concentration is a contributing factor in several diseases as Parkinson, Huntington and schizophrenia illness.

- Noradrenaline. Noradrenaline is classed as a monoamine neurotransmitter and noradrenergic neurons are found in the locus coeruleus, the pons and the reticular formation in the brain. These neurons provide projections to the cortex, hippocampus, thalamus and midbrain. The release of noradrenaline tends to increase the level of excitatory activity within the brain, and noradrenergic pathways are thought to be particularly involved in the control of functions such as attention and arousal.

- Acetylcholine. Acetylcholine acts in cholinergic pathways that are concentrated mainly in specific regions of the brainstem and are thought to be involved in cognitive functions, especially memory. In fact, severe damage to these pathways is the probable cause of Alzheimer's disease.

Specific behaviour and cognitive functions can be associated non only with particular neurotransmitter but also with brain regions that underlie specific neural network (Fig.2).

Memory is the principal cognitive function and is particularly correlated with an area that is part of the forebrain and is one of the structure that composes the limbic system: the hippocampus. This area is involved in several function as emotional responces, spatial orientation and memory capacity. In paticular, the hippocampus is the area responsible to acquire new memories are gradually to transferre tham to neocortical stores where the memory are kapt by the process of memory consolidation. Moreover, the hippocamus is the structure througt which the memory can be evoked and than remember. For this, damage to this structures caused for exemple by neuronal death as in case of Alzheimer's disease, sekes above all severe loss of memory capacity. Another principal cognitive functions are the locomotion and the movement and several brain areas are responsible for this activity as cerebellum and struatum. In particular, the cerebellum is the portion of the brain responsible for the coordination of movement, balance,

equilibrium and muscle tone. It is located beneath the occipital lobe at the base of the skull. This area controls movement by processing and coordinating sensory input. The information is then sent to the motor nerves in order to produce fine motor movements. The cerebellum also calculates and corrects informational discrepancies in order to produce the desired movement. The striatum instead, is the largest nucleus of the basal ganglia. It receives direct input from most regions of the cerebral cortex and limbic and from sensorimotor and motivational regions of the brainstem arrives indirectly via relays in the thalamus. His role in motor skills is bound to his contact with the substantia nigra where by domanine via controls the movement activity. Damage in this neuronal network cause illness as Parkinson's disesae.

Basic functions necessary for life, such as fluid and electrolyte balance, feeding and energy metabolism, wake-sleep cycles, thermoregulation, stress responses, and sexual behavior and reproduction are regulate and coordinate by the hypothalamus that is a small area at the basis of the brain. This area receives direct sensory inputs from the smell, taste, visual, and somatosensory systems. It also contains within it sensors for such things as blood temperature, blood sugar and mineral levels, and a variety of hormones. Thus the hypothalamus receives sensory inputs necessary to detect challenges in both the internal and external environments. In addition, the hypothalamus receives inputs from forebrain areas including the hippocampus, amygdala, and cingulate cortex. These structures form the limbic lobe of the brain, receives highly processed sensory information from throughout the cerebral cortex. These inputs drive a wide range of emotional responses, and many of the phenomena we associate with emotional expression are mediated by the hypothalamus.

The cerebral cortex is the most highly developed and complex part of the human brain and is responsible for thinking, perceiving, producing and understanding language. The brain is dominates by two hemispheres: left and right hemispheres. Anatomists conventionally divide each hemisphere into four "lobes", the frontal lobe, parietal lobe, temporal lobe, and occipital lobe. The form of the hemispheres is the result of an elaborate folding of tissue where the reliefs are called laps and are separated from each other by

grooves to increase the cerebral surface. Every lobe has a specific function. For example, there are specific areas involved in vision, hearing, touch, movement, and smell. Other areas are critical for thinking and reasoning. Although many functions, such as touch, are found in both the right and left cerebral hemispheres, some functions are found in only one cerebral hemisphere. The parietal lobe is involved in the reception and processing of sensory information from the body; the frontal lobe is involved with decision-making, problem solving, planning, cognition and memory; the occipital lobe is involved with vision and the temporal lobe is involved with memory, emotion, hearing, and language. The informations are sent to the cortex, where are rielaborate, and than they are again again sent to other part of the brain.

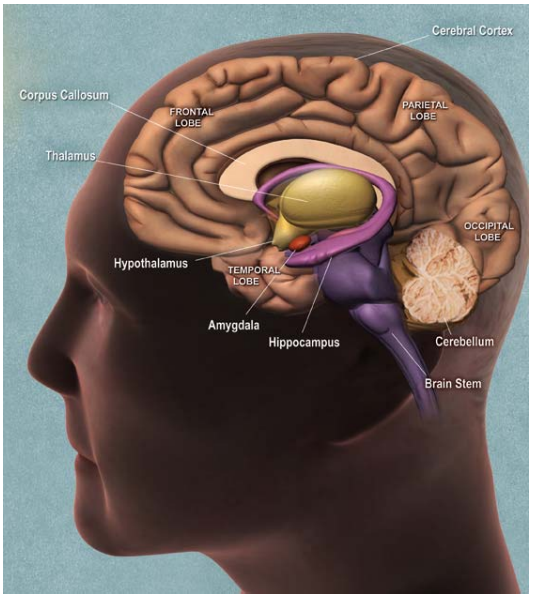


Fig. 2 Schematization of the main human brain areas.

A damage to the central nervous system in a brain area or in the production/recapture/action of one of the neurotransmitters, crusading by a pathogen agent or by an uncontrolled inflammatory event, induce several deficit in the all the brain. Injuries to the brain tend to affect large areas of the organ, sometimes causing major deficits in intelligence, memory, personality, and movement. These type of damage can be caused by trauma, edema or stroke events that induce neuronal death or by virus or bacteria leading for example to meningitis. Other damage of the brain can be

classified as neurodegenerative disease such as Alzheimer's disease characterized by a gradual death of individual neurons leading a progressive cognitive impairment. Mental disorder as psychosis such as schizophrenia may involve particular patterns of neuropsychological functioning related to various aspects of mental and somatic function. Yet is not know how these illness progress and what are the pathogenetic pathway that underlie them but they are shared by a neuro and peripheral inflammatory processes. In the human is difficult to study directly the neuroinflammatory process for practical reason and for this, result very interesting to investigate the peripheral inflammation that characterize the patients affected by psychosis or neurodegenerative disease.

1.3 NEUROINFLAMMATION AND CYTOKINES

Under optimal condition, integrated and concerted activation of the immune mechanisms leads to the elimination of the pathogen or insult and the repair of damage. In particular, the inflammation, mainly mediated by mechanisms of innate immunity and controlled from different processes of the immune response, has a defensive function. However, in case of chronic inflammation, when the regulation of immune processes is not adequate, the pathogen may persist and the mechanisms that normally protect the individual can cause tissue damage and trigger consequential pathologies which is reflected also as cause of damage in CNS. Since CNS has a low capacity for regeneration, it is extremely vulnerable to damage caused by inflammatory processes, which in district often lead to an alteration of the functionally and the irreversible loss of neurons. Neuroinflammation involves resident cells in the CNS (microglia, astrocytes, neurons) and, at peripheral level, different cells of the immune system (macrophages, monocytes, T and B lymphocytes, dendritic cells) and many soluble factors as cytokines. Then, pro-inflammatory cytokines, which have effects on both immune and nervous systems, are central players of inflammation and may contribute to brain changes during both physiological and pathological processes. As key mediators of a chronic neuroinflammation that drives

progressive tissue damage in the brain, cytokines could take part to the pathogenesis and progression of neurodegenerative diseases (Holmes et al., 2003; Mrak et al., 2009; Lampa et al., 2012).

The cytokines are a category of signalling protein molecules locally released by specific cells that carries out their function by binding specific receptors. When cytokines are produced by immune system cells are also called interleukin. These molecules can perform their action in an autocrine manner (acting on the same cell which has produced them) or in paracrine manner (by acting on adjacent cells) or endocrine when produced in large quantities. Cytokines have four main property:

- Pleiotropy: the ability of a cytokine to exert its effects on different cell types;
- Redundancy: more cytokines can mediate the same biological event;
- Synergism: the total effect of two or more cytokines is higher than their only addition;
- Antagonism: two or more cytokines are competitors.

With these capacities, they are involved virtually in all aspects of both innate and adaptive immune responses. Cytokines may play a pro-or anti-inflammatory activities depending on how they modulate the inflammatory response, that is by either amplifying or inhibiting it. Nowadays a lot of cytokines have been identified, whose role is under intense study by the researchers as some of them seem to be involved in various immune, neurodegenerative and psychiatric illness. For example, much interest is focusing on TNF- α and IL-1 β, two pro-inflammatory cytokines that are among the first players in the acute inflammatory response that characterizes many CNS diseases as stroke (Lambertsen et al., 2012), obsessive compulsive disorder (Gray et al., 2012), trauma and multiple sclerosis (Arvin et al., 1996).

Tumor necrosis factor (TNF-α) in fact is one of the main mediators of the acute inflammatory response whose produced by phagocytes, T lymphocytes, NK cells, mast cells and macrophages as a result by infectious agents and by gram-negative bacteria. TNF-α plays a central role in

initiating and regulating the cytokine cascade during an inflammatory response. It is produced as a membrane-bound precursor molecule of 26 kDa that is cleaved by the TNF-*α* converting enzyme to produce a 17 kDa active cytokine (Perry et al., 2001). The levels of TNF-*α* expression in the healthy brain are low, making it difficult to determine its precise role under physiological conditions. In inflammatory or disease states, TNF-*α* along with several other proinflammatory mediators and neurotoxic substances are predominantly produced by activated microglia. Neuronal production of TNF-*α* has been demonstrated (Breder et al., 1993), although brain-derived TNF-*α* is mostly synthesized by glial cells in response to pathological stimuli. Glial cells secrete both TNF-*α* and IL-1, which in turn, activate these cells in an autocrine manner to induce further cytokine production and astrogliosis. TNF-*α*, on the other hand, has been reported to have neuroprotective properties (Akiyama et al., 2000) in the AD brain.

Another pro-inflammatory cytokines closely related to TNF-α is the interleukin- 1 (IL-1), whose main role is to mediate the inflammatory response triggered by bacterial products such as lipopolysaccharide (LPS), a component of cell wall of Gram-Negative Bacteria. IL-1 is an important initiator of the immune response, playing a key role in the onset and development of a complex hormonal and cellular inflammatory cascade. This cytokine is mainly secreted by macrophages and other APC and its production can be further stimulated by the action of other cytokines such as TNF-α. Its mainly role is to promote both adaptive and innate immune response favouring the recall of both macrophages and T cells at sites of infection and stimulating the clonal expansion of B lymphocytes maturation. There are two forms of IL-1 calls IL-1α and IL-1β biochemically distinct but structurally related. IL-1 α / IL-1β exerts its function through the formation of a complex with type I receptor and accessory protein (IL-1 receptor accessory protein) (IL-1 RI and IL-1 RACP) starting the transduction cascade leading to activation of inflammatory genes. IL-1 α / IL-1β has a natural antagonist IL-1 RA functionally inactive, but able to inhibit its function. Both IL-1 α / β are produced as immature form with a signal sequence cleaved by interleukin-1 converting enzyme to produce the biologically active form of 17 kDa. These cytokines are involved in

inducing an acute inflammatory response, their action and production in normal decade then a few hours after stimulation by inflammatory event. In particular, elevated IL-1β has been detected in the CF and brain parenchyma within the early hours after brain injury in both humans and rodents (Winter et al., 2002; Woodroofe et al.,1991).Another pro-inflammatory cytokine, member of the IL-1 family (Ushio et al., 1996; Bazan et al., 1996), is IL-18, which is gaining more and more interest inasmuch it would play a prominent role in neurological and psychiatric disease and for this reason a lot of interest is taken from the entire system of IL-18 composed in the main way from the same IL-18 and from its natural inhibitor (IL-18 binding protein).

1.3.1 IL-18

IL-18 is a pleiotropic cytokine, involved in several pathological conditions and inflammatory diseases. This cytokine, working in collaboration with IL-12, is able to induce cell-mediated immunity response following an inflammatory stimulus. IL-18 induces natural killer (NK) cells and T-cell-helper type 1 to release another cytokine called interferon γ (INFγ) that is involved in the activation of macrophages and other immune cells. For this reason IL-18 was originally called INFγ-inducing factor (Okamura H et al., 1995). IL-18 presents some homologies with IL-1β which is another cytokine member of the IL-1 family. Indeed these molecules are structurally similar among themselves, in fact IL-18 and IL-1β are two of the few cytokines that are all-β-plated sheet. Moreover IL-18, as well as IL-1β, is synthesized in a 24 kD biologically inactive precursor form (proIL-18) which afterward, like proIL-1β, is cleaved into an active molecule (18 kD) by an intracellular cysteine protease called IL-1β-converting enzyme (ICE) or caspase-1 (Gu Y,1997). In turn, caspase-1 is produced as an inactive precursor, procaspase-1, that is activated by caspase-11 (Wang S, 1998). Caspase-11 is also induced as an inactive molecule called procaspase-11 which is activated by cathepsin B(Schotte P, 2000) via P38 MAPK

phosphorilation (Vanden Berghe W, 1998) which require NF-kB transactivation into the nucleus (Li X, 2002; Schauvliege R, 2002)

IL-18 is able to perform its biological function by binding to its receptor. IL-18 receptor (IL-18R) is an heterodimeric complex member of the IL-1/Toll like receptor superfamily that is composed by the IL-18 binding chain IL-18Rα, member of IL-1 receptor-related protein (IL-1RrP or IL-1R5), and by the nonbinding signalling chain IL-18Rβ or IL-18 accessory protein (IL-18RacP or IL-1R7); both chains are members of the IL-1 receptor super-family (Kato Z et al., 2003) and their peculiarity is to increase the affinity of the heterodimeric complex with the ligand so it is able to initiates signal transduction. Than, IL-18 binds to IL-18Rα, IL-18Rβ forms a high affinity between heterodimeric complex and the ligand allowing the recruitment of the IL-1R activating kinase (IRAK) via the adapter protein (MyD88). The autophosphorylation of IRAK induces the activation of TNFR-associated factor-6 (TRAF-6) resulting in phosphorylation of NF-kB-induced kinase (NIK) which in turn leads to activation of two IkB kinase (IKK-1 and IKK2) that lead to the phosphorilation of IkB. This latter step induces to the degradation and release of two components of NF-kB: p50 and p65. So, NFkB is free to migrate in the nucleus to induce the gene transcription.
IL-18 has the capacity to induce Th1 cells, NK cells, B cells and dendritic cells to produce INFγ. Moreover IL-18 promotes T cells, NK cells, mast cells and basophils to produce IL-4 and IL-13 (Hoshino T, 1999; Yoshimoto T, 1999), therefore it is able to stimulate both innate immunity and Th1, Th2 mediated responses.
Recent studies have show that IL-18 is present in the central nervous system in addition to periphery where its role in immune response is well known (Alboni et al, 2010). Indeed, it is constitutively present in the brain, it is produced by microglia, astrocytes, ependymal cells and by some habenular neurons (Sugama S, 2006) and its expression is increased in old processes (Dinarello et al., 2006) and in several pathologic condition, associated in psychiatric illness and neuropathological disease. IL-18 has been shown to inhibit the induction of LTP (Long Term Potentiation) in the dentate gyrus, without affecting the baseline neurotransmission (Curran et al., 2001;

Curran et al., 2003; Cumiskey et al., 2007). Recently, IL-18 has been found to play a diverse array of functions in non-immune tissues as the central nervous system (Alboni et al. 2010), and its abnormal expression has been observed both centrally and peripherally in neuropsychiatric disorders such as Alzheimer's disease and stroke (Bossù et al., 2008; Bossù et al., 2010). Indeed, IL-18 itself is able to stimulate microglia to increase the expression of ICE to produce other pro-inflammatory cytokines amplifying the inflammatory process. Recent studies have highlighted the overproduction of IL-18 in several inflammatory diseases indicating that IL-18 is a pro-inflammatory cytokine which may play an immunopathological role in many pathological conditions. A lot of studies performed both in *vivo* and in *vitro* have suggested an up-regulation of IL-18 gene expression in neuropathologic situations such as stroke, brain injury and Alzheimer's disease, candidating this cytokine as a crucial mediator of neuroinflammatory processes. Further data indicate that also stress contributes to increase IL-18 levels by activation of the hypothalamic-pituitary-adrenal (HPA) axis and suggesting that IL-18 may play also a role in the HPA axis regulation.

The IL-18 activity is modulated by IL-18 binding protein (IL-18BP). IL-18 BP is a 40 kDa protein that is constitutively expressed and appears to be the natural inhibitor of Il-18 activity binding selectively and with high affinity mature form of IL-18 preventing his interaction with IL-18Rα. Unlike soluble receptors for IL-18, IL-18BP does not have a trasmembrane domain; IL-18BP is a secreted protein with a high-affinity binding and ability to neutralize IL-18. Two human IL-18BP isoforms exhibited the greatest affinity for IL-18 with a rapid on-rate. Moreover, IL-18BP modulates the IL-18's activity in fact, in addiction to having neutralizing activity, it can induce a negative feedback regulation for IL-18 production to modulate in pathological circumstance the strongly increase of IL-18 (Novick, Schwartsburd, Pinkus et al., 2001). IL-18BP is constitutively present in the serum of healthy humans at molar excess compared with IL-18 (Novick et al., 2001) and it appears to be modulated during the life. In fact, since IL-18 participates in fundamental inflammatory processes that increase during the aging process (Dinarello, 2006) and IL-18BP serum levels tend to increase

in old age with evidence of strong increment in healthy centenarians (Gangemi et al., 2003), it has been suggested that IL-18BP retains a remarkable protective role by acting to limit the impact of age-related inflammation.

1.4 FROM NEUROINFLAMMATION TO BRAIN DISEASE: COGNITIVE AND BEHAVIORAL CORRELATES

Growing evidences have highlighted the association between specific mood disorders and altered immune function. More recently, a number of hypotheses have been forwarded to explain how components of the innate immune system can regulate brain function at the cellular and system levels and how these may underlie the pathology of disorders such as depression, Alzheimer's disease and several psychotic illness. The neuroinflammation seems to have effects on neurodegeneration and depression dysregulating the control and release of pro- and anti-inflammatory cytokines. In particular, pro-inflammatory cytokines, such as IL-1β, TNF-α and IL-18, which have a prevailing role in the immune response to pathogen in the periphery, have also a specific actions on neurons and circuits within the central nervous system. These cytokines, can act on the brain through different channels: or interacting from the periphery to the central, or acting directly in the brain after have been produced at cerebral site. There is in fact good evidence that they can originate both from the peripheral circulation following induction for example of LPS, as well as from local synthesis in neurons and glia (Dantzer et al., 2008; Raison et al., 2006). TNFα, together with IL-1β and IL-6 have well described actions on the hypothalamus including induction of fever, suppression of appetite and stimulation of the hypothalamic pituitary axis (HPA) to release corticotropin-releasing factor (Goshen et al., 2008; Yirminiya et al., 2011; Layé et al., 2000) property that has been viewed belong also to IL-18. Cytokines produced in periphery, particularly IL-1β, TNFα and IL-18, can enter the brain by an active transport system (Skinner et al., 1993; Gutierrez

et al., 2009), by the breakdown of the blood brain barrier and by the cerebrospinal fluid.

Once in the brain, these cytokines induce their own synthesis (Layé et al., 1994; Pitossi et al., 1997) and their expression are also regulated via the centrally generated response to stress by the activation of the HPA. It is the complex interplay between central and peripheral cytokines that ultimately influences the behavioural response to an environmental challenge. Than, during an inflammatory process, the principal events that characterize the damage at CNS levels are: massive breakdown of the blood brain barrier and typical changes in of the cerebrospinal fluid with a massive invasion of activated immune cells accompanied by the activation of resident microglia cells. These cells, when activated, produce several mediator to defeat the damage and in particular the mediators with pro- and anti-inflammatory proprieties that mostly collaborate to immunity response are the cytokines. For exemple, microglia cells are activated in the brain of patients with Alzheimer's disease and surround amyloid plaques in an attempt to eliminate them. Microglia produce pro-inflammatory cytokines such as IL-1β, IL-6, TNF-α, which could contribute to augmented local inflammation, release of toxic molecules contributing on neuronal damage (Akijama et al., 2000). Moreover, neuroinflammation and cytokines are associate with synaptic protein loss and these changes could contribute to cognitive impairments in neurodegenerative and neuropsychiatric illnesses (Rao et al., 2012).

The neuroinflammation has an effect on neurodegeneration and on specific mood disorders. In this context seems to be very interesting to focus the attention of the research on the crosstalk between inflammatory and central nervous systems and on the possible role as mediator of the cytokines, not only for understanding disease etiology, but also for selecting the best therapeutic approach.

1.5. NEURODEGENERATIVE DISEASE: ALZHEIMER'S DISESE

The Alzheimer's disease (AD) was so called after that the German neurologist Alois Alzheimer (1864-1915) described in 1907 the clinical signs of the neuropathology. The doctor in fact, led the autopsy of the brain of a woman who died as a result of an unusual mental illness and he showed the presence of agglomerate protein and neurofibrillary tangles that today we know to characterize the brain of AD patients.

The AD is responsible for two thirds or more of all diagnosed cases of dementia that affects approximately 3%, 19%, 47%, respectively, of the individuals of 65-74, 75-85 and 85 years. Disease are associated with several clinical signs. Among the first symptoms include the inability to create new memories and loss of short term memory. People with cognitive impairment, loss the ability to speak, to think, to remember past events and to develop a spatio-temporal orientation with the progression of the disease. A lot of patients also show delusion and change of personality. In the last stage of illness, patients are mute, immobile, incapable of understanding and the death occurs often due to respiratory failure. There are two types of AD identified by the age of onset of the disease: early AD, if it begins first of 65 years (early-onset) and late AD if it begins after of 65 years (late-onset). In late AD, disease lasts from 8 to 20 years, while in early AD the course of illness is generically more rapid and the death occurs within 5-10 years from the time of diagnosis.

Histological analysis by autopsies of the AD brain, shows the distinctive neuropathological characteristics. It can be observed devastating loss of synapses and neurons at the level of the hippocampus and the enthorinal cortex causing the first symptom of the disease: the mnestid deficit. Other brain areas are also affected by neurodegenerative process as septum, amydgala and several cortical areas with the progression of the disease, causing a huge deficit.

Cytological analysis shows dense spherical structures of 20-200 μm in diameter (senile plaques) outside the neurons and neurofiblillary tangles that accumulate within the cell bodies. It has been shown a strong correlation

between senile plaque in the enthorinal cortex and cognitive dysfunction (Cummings et al., 1996).

Autopsies performed on AD brains have shown that the neurofibrillary tangles appear initially in the limbic system and extend to the cortex with the progression of the disease, while the senile plaque appear first at the level of the frontal cortex and then to the rest of the brain, in parallel with increasing the severity of the pathology (Braak et al., 1998).

The senile plaque (or amyloid bodies) have a filamentary structure, constitute by protein and the main protein molecule present in the plaque is a peptide of 4 kDa (β-amyloid protein or Aβ), consisting of isoforms containing 39-43 aminoacids.

The genomic analysis has revealed that the Aβ isoforms of senile plaques derived by proteolytic cleavage from a protein called amyloid precursor protein or APP. This protein has a single trasmembrane site and it has three isoforms whit 695, 751 and 770 amino acids. Exactly the role of APP molecule is still not clear, but probably the extracellular portion acts for the cell-cell contact or cellular adhesion to the extraneuronal matrix, while the intracellular share seems to be associated whit the cytoskeletron. APP molecules are subjected to the action of different proteolytic enzymes. One of these enzymes is the α-secretase, which cuts the APP protein in site 687 generating two fragments not amyloidogenic. Although the β-secretase, acting at the level of the amino acid site 671, produces two fragments don't amyloidogenic. Than, a single proteolytic cleavage, by α-secretase or by β-secretase, generates non-pathologic peptides. However, a double proteolytic cleavage by β-secretase and γ-secretase (at the site 711), generates the isoforms Aβ1-40 and Aβ1-42 which, if present in high concentration, are not adequately degraded and they can become amyloidogenic and accumulate outside the neurons they form the senile plaques.

The neurofibrillary tangles inside the neurons of AD patients are constituted by filaments mainly composed by the tau protein. The function of the tau protein under physiological conditions is to assemble the tubulin for the formation of microtubules, a part of the cytoskeleton that allows the axonal transport. Studies in vitro have demonstrated that in pathological condition, such as AD, tau protein is in a state of hyperphosphorylation.

Hyperphosphorylated tau is not able to maintain keep together the fibrils of tubulin, so the microtubule formation is locked and the axonal transporters is consequentially compromised.

Many studies are focusing on the identification of the etiology of Alzheimer's disease and much interesting is to understand how the genetic can intervene on the onset of the illness. The AD, is characterized by a complex genetic, in fact only the 10% of cases have autosomal dominant inheritance. This 10% is made up of subjects who have in most cases, the presenile dementia (the disease occurs earlier than senile (before 60 years old). The remaining 90% are defined as sporadic cases with an onset of illness after 60 years old. Is interesting to note that 25-40% of late-onset cases have at least one or more kindred with the same disease. This therefore underlines that genetics may play an important role in the etiopathogenesis of AD. Screening tests have allowed us to study families characterized by early-onset AD to identify possible mutations responsible for the disease. Studies carried out both in vivo and in vitro have revealed a missense mutation that alters the amino acid in the side 717 of the APP. Studies carried on cultured cells and on the plasma of individuals which have this mutation in the APP gene, have higher levels of Aβ1-42 compared with health controls, as well as transgenic mice for APP gene produce increased amounts of Aβ1-42 compared to wilde tipe mice.

Studies of genetic association have identified two other mutations for early-onset AD: mutations of the gene for the presenilin 1 (PSEN1) and presenilin 2 (PSEN2). The presenelins are membrane proteins that appear to play a role in cell differentiation during embryogenesis and in the cellular transport of proteins. Even mutations of the genes of these two proteins are associated with an increased production of Aβ1-42. However, the mutations of APP genes, PSEN1 and PSEN2 are responsible for only 50% of cases of early-onset familiar AD and for this reason, remain undiscovered other genes that cause the disease. It is not clear how PSEN1 and PSEN2 mutation interact whit APP but it seems that what unites them in the developed of AD is the formation of Aβ1-42.

Other genetic association studies have shown a relationship between sporadic AD and the gene for apolipoprotein E (APOE). APOE is

synthesized primarily by astrocytes and its function is to sequester cholesterol and triglycerides from the debris of cells and to bring them in neurons where these molecules can be used for the formation of synaptic membranes. The APOE genes is polymorphic for three alleles: APOEε2, APOEε3 and APOEε4 and it would seem that the latter isoform (APOEε4) is associated with susceptibility to AD. This allele, however, represents only 50% of genetic component of sporadic late-onset AD and also this factor is neither sufficient nor necessary for the development of the disease. The APOEε4 is not a cause of AD but it is a risk factor for the disease, in fact, there are individuals that have both the alleles for APOEε4 but they haven't any symptoms of AD, while there are AD patients that do not show none of these alleles.

AD disease is therefore a very complex neurodegenerative disease characterized by numerous causes of both genetic and non genetic elements. Much researches are being carried out in order to understanding the factors inducing the formation of senile plaques, neurofibrillary tangles and causes of neuronal death. Recently, the more supported hypothesis is the β-amyloid cascade. In this case it is believed that Aβ is the primary cause of AD. Several observations have shown that mutations associated with AD increase the amount of Aβ42 (considereding this a pathogenic peptide), while others don't give much importance to the amount of Aβ42, but rather the relationship that increasing of Aβ42/Aβ40 could induce to AD. Finally, today the most widely accepted idea is that Aβ is present in soluble oligomeric forms that may represent the major pathogenic factors to disease. Oligomeric forms have been isolated from both brains of animal models of AD (Lesne et al., 2006) that from human brains with AD (Shankar et al., 2008) and parallel studies in vitro have demonstrated the strong cytotoxic power of these oligomers. It is not yet clear how the oligomeric forms are generated in vivo, but suggested two possible hypothesis. One possibility is that Aβ monomers initially form soluble aggregates that will give rise to future oligomers joining in insoluble protofibrils, fibrils and plaques. This hypothesis, therefore, considered pathogenic oligomers both soluble and insoluble (Shankar et al., 2008). An alternative hypothesis configures the existence of two distinct way: a pathogenic way that forms soluble

oligomers responsible for the disease and a not pathogenic way that forms insoluble aggregates and plaque (Catalano et al., 2006; Shanker et al., 2008).

According to the hypothesis of the cascade of β-amyloid, Aβ would seem to be the apex of the cascade triggering a process of neuroinflammation, brinding to the iperphosphorilation of tau protein through the activation of a cyclin-dependent kinases (CDK5) and to a reduction of synapses. This results in synaptic dysfunction, neuronal death and subsequent development of Alzheimer's disease (Pimplikar et al., 2009)

1.5.1. MILD COGNITIVE IMPAIRMENT (MCI)

Whereas the symptoms of AD performs when the pathology is already overt, and then is difficult to study the early stages of disease, the research is focusing on the study of subjects MCI (Mild Cognitive Impairment) as these individuals have a clinical picture that can be inserted between a normal condition and a very early form of dementia. The first major study focused on the characterization of clinical pathology of MCI was published in 1999 (Peterson et al., 2001). This study showed that MCI subjects are individuals who have a high risk of future cognitive impairment and progressive Alzheimer's dementia. The criterion adopted to define the state of MCI is based on five parameters (Peterson et al., 2008):

- Mnestic problems;
- Mnestic deficit in relation to the age of subjects;
- Cognition generally preserved;
- Daily activities unharmed;
- No form of dementia.

In Stockholm in 2003 there was an international conference on diagnostic criteria to include in the disease of MCI other forms of cognitive impairment in addiction to mnestic deficit (Winblad et al., 2004; Peterson et al., 2004). Two subtypes of MCI have emerged: amnesic MCI (characterized only by mnestic deficit) and non-amnestic MCI (characterized by deficit linked to not mnestic domains). It was proposed an

algorithm for diagnosing various type of MCI (Paterson et al., 2008). If the memory is impaired, clinicians considerer the patient as amnesic MCI, and if there is no obvious mnestic deficit but some cognitive impairment in other activities is shown (such language, executive functions and visuospatial tasks), it is believed that the patient is a non-amnesic MCI. Clinicians can also determinate which other cognitive domains may be compromised in MCI using neuropsychological tests. A diagnosis of MCI amnesic single-domain is assumed if the deficit involves only the mnestic activity (pure amnestic), while for amnestic multidomain MCI are considered patients that present in addition to memory impairment also other cognitive deficit. A diagnostic of MCI not amnestic single domain assumes that there is an impairment to only one other domain, while not amnesic multidomin MCI refers to a deficit in different domains that are not related to the function of memory. Following the clinical characterization of patients, the cause of cognitive impairment is determinated by neuroimaging studies and laboratory testes. Clinicians then determinate if the symptoms of MCI are due to a degenerative disease, vascular, psychiatric, or are a secondary consequence of another disease. In general, the type of amnesic MCI have an increased risk of elapse in Alzheimer's dementia whit a more rapid disease progression for multidomain than single domain, while non amnesic MCI seems to be a greater chance to convert into frontotemporal dementia with Lewy bodies. An extremely interesting observation indicates that the subgroup of amnesic single domain MCI (pure MCI) have a high selectivity, greater than all other subgroup, to develop in AD and not in other forms of dementia (Lopez et al., 2007).

The study of MCI may therefore be used to understand the pathogenetic events that follows in the early stages of AD, not only to investigate the early stage of the disease, but if characterized by longitudinal clinical analysis, it would also help to identify the key processes that lead to the progression of AD in individuals. Identify subjects not yet sick, but with a high risk to develop AD, can be very interesting to understand the inflammatory parameters potentially involved in the disease and how the mediators of immunity may be over or under regulated at this preclinical stage of illness.

1.5.2. INFLAMMATION IN AD AND MCI

Alzheimer's disease is an example of a pathology characterized by chronic inflammation localized in the brain, where the molecules involved in the modulation of the inflammatory response are produced by cells of microglia and they play a role in the neuroinflammatory process. In particular, the key of the pathogenic events of AD would seem the protein β-amyloyd that seems to be at the basis of specific immune activator that occurs in the brain of AD. β-amyloyd, in fact, actives not only microglia cells (Akiyama et a., 2000) but also other immune cells recruited from the periphery to contribute to the neuroinflammatory process (Ciaramella et al., 2009; Ciaramella et al., 2009).

Numerous studies have attempt to investigate how inflammation can affect the pathogenesis and progression of the disease, through the analysis of the expression of various cytokines. Several studies have shown that IL-1 appears to be expressed in a manner more consistent in the cortical region most rich of plaque in AD patients. (Sheng et al., 1995; Sheng et al., 1998). TNF also appears to play a role in AD, in fact various studies have shown an increase of production of this cytokine in serum (Fillit et al., 1991), in the cerebrospinal fluid (CSF), in the cortex (Tarkowski et al., 1999) and glia cells of AD patients placed in culture after exposure to Aβ (Lue et al., 2001). IL-6 is another pro-inflammatory cytokine that appears to have a role in the pathology of AD (Braida et al., 2004). Maccioni et al., (Maccioni et al., 2009) have recently proposed a hypothesis according to which events of oxidative stress (Koseoglu et al., 2007; Zambian et al., 2004), repair mechanisms in brain damage and folic acid deficiency are sufficient signals to activate innate immunity by modifying the normal microglia activation. The microglia so activated produces an excessive production of IL-6, TNF-α and IL-1, that are cytokines whose levels seem actually elevated in Alzheimer's disease. The overproduction of these molecules may modulate the hyperphosphorilation of the tau protein and then create cell changes that can lead to neurodegeneration and neuronal progressive cognitive impairment. Moreover, another study seems to confirm the existence of a correlation between TNF-α and cognitive impairment, high serum level of

this cytokine may be associated whit cognitive decline that characterize AD patients (Holmes et al., 2009).

Among pro-inflammatory cytokines, IL-18 may contribute to the chronic neuroinflammation od AD. In fact, IL-18 participates in the initiation of the innate host response and can be normally detected in cortex, cerebellum, hippocampus, hypothalamus and striatum (Chulhaine et al., 1999; Bossù et al., 2012), mainly expressed in response to LPS by microglia and astrocytes (Conti et al., 1999). The importance of this cytokine in other neuroinflammatory and neurodegenerative pathological condition has been demonstrated both clinically and experimentally (Felderhoff-Mueser et al., 2005). Recently, IL-18 has been considered a possible phatogenetic mediator also in Alzheimer's disease. Several studies have shown a possible involvement of this cytokine in the pathology of AD, since it has been shown increased expression of caspase-1 (enzyme activator of IL-18) in AD patients (Pompl et al., 2003). Moreover it was found that the receptor of IL-18 and in particular the β chain indispensable for signal transduction, is major expressed at the level of lymphocytes of AD subjects. Furthermore, it was observed that polymorphisms of IL-18 gene promoter are associated with the risk of exhibiting Alzheimer's disease and with the course of the same illness (Bossù et al., 2007). Post-mortem studies carried out on the brains of AD patients, have allowed to quantify directly expression of IL-18 in situ in various brain areas (Ojala et al., 2009). In particular, this study shows a significant increase of IL-18 in the frontal cortex of AD patients compared to controls. Finally, it was shown that the blood cells of AD patients produce a greater amount of IL-18 compared with control subjects and that the high levels of this cytokines are correlated with high levels of cognitive impairment (Bossù et al., 2008).

An altered immunity profile was observed also in MCI. Galimberti et al. (Galimberti et al., 2005) have shown that a beta-chemokine with chemiotactic activity (MCP-1) is mainly produced both in MCI and in people that exhibit an early form of AD, while this molecules don't appears to be produced significantly during the most severe form of the disease. Other studies have shown that, as well as in patients converted in AD, MCI subjects also exhibit high levels of TNF-α (pro inflammatory cytokine)

compared to TGF-β (anti inflammatory cytokine) in the CSF, indicanding that patients with high risk to develop AD are characterized by a specific pro inflammatory profile (Tarkowski et al., 2003). Other studies have shown that PBMC (Peripheral Blood Mononuclear Cell) of MCI patients produce a greater quantity of pro inflammatory cytokines when stimulated with appropriate inflammatory stimuli such LPS, as IL-6 and IL-8 than anti inflammatory cytokine as IL-10 (Magaki et al., 2007). These results indicate that during the preclinical state of AD (during the stage of MCI), there is an alteration in the production of cytokines and this may suggest that inflammatory events precede the clinical development of the Alzheimer's disease. Based on all the research carried out it would appear that the inflammation has a significant role both in the pathogenesis and in progression of AD, also if all the processes which lead to the onset of the disease are not been clarified. Research is therefore strongly focused on clarification of molecules involved in the inflammatory process to realize how these act in the early stage of illness. To understand how IL-18 can be involved in AD, it can be helpful to discover new therapeutic procedures for this devastating disease.

1.6. A FORM OF PSYCHOSIS: SCHIZOPHRENIA

Psychosis is a syndrome, or rather a set of symptoms, which may be associated to many different psychiatric disorders. This can be considerate a set of symptoms in which the mental abilities of a person, his actual response and its ability to recognize reality, communicate and relate to others is impaired. Schizophrenia is the most common psychotic illness that affects 1% of the population. By definition, schizophrenia is a disorder that should last at six month or more, including at least one month of delirium, hallucinations, disorganized speech and behaviour. Delirium consist in an erroneous interpretation of reality and perceptions while hallucinations may occurs in any sensory modality. Many studies subdivided this pathology in five sizes: positive, negative, cognitive, aggressive/hostile, depressive/anxious symptoms.

- Positive symptoms: seem to reflect an excess of normal functions and include delirium, hallucinations, exaggeration of language, communication and behaviour.
- Negative symptoms: include five symptoms: affective flattening (limitation in the variety and intensity of emotional expression); alogia (limitation in the fluidity and productivity of speech and think); apathy (limitation in the capacity of action); anhedonia (inability to feel pleasure); deficit of attention.
- Cognitive symptoms: thinking disorder, inappropriate language, incoherence, difficulties of attention and learning often overlap with negative symptoms.
- Aggressive/hostile symptoms: hostility which manifests itself in verbal e physical violence, aggression, problems in impulse control and self-injurious behaviour often overlap with positive symptoms.
- Depressive/anxious symptoms: depressed mood, anxiety, guilt, tension, irritably and anxiety.

Biological bases of schizophrenia are not known. However, dopamine neurotransmitter plays a key role in hypothesis on five sizes symptoms just described. In particular, mesolimbic dopaminergic pathway, mesocortical dopaminergic pathway, nigrostriatal dopaminergic pathway and tuberoinfundibular dopaminergic pathway are the principal cerebral dopaminergic pathway but the first two are responsible of major symptoms that characterized schizophrenia.

- Mesolimbic dopaminergic pathway. This pathway projects from the cell bodies of dopaminergic neurons in the ventral tegmental area of the brain stem to the axonal terminals in limbic areas of the brain, as the nucleus accumbens. It is believed that a specific hyperactivity of this pathway mediate the positive symptoms of the psychosis.

- Mesocortical dopaminergic pathway. The cell bodies of this way are localized in the ventral tegmental area and project to the cerebral cortex. The role of this pathway is likely to mediate the negative and cognitive symptoms of schizophrenia. In fact, many researches believe that a hypoactivity of these neuronal systems is responsible for such behaviour. The dopamine deficiency of mesocortical dopaminergic neurons may be

causes either by a direct dopamine deficiency or by a secondary deficiency due to an excess of serotonin that mediates the activity of the same dopamine.

1.6.1 INFLAMMATION IN SCHIZOPHRENIA

There are many possible hypotheses about the origins of schizophrenia but there is not yet recognized a certain cause, but we can talk about risk factors, or rather conditions that predispose an individual to develop the disease than others. The genetic component is certain the most reliable risk factor in fact much research has been focused on this issue by the twin, family and adoption studies (Riley et al., 2005) and a myriad of geneses would seem to be involved in schizophrenia while the environment seems to play only a minor role (Schwarts and Susser, 2006). Inflammatory system seems to be involved in the schizophrenia pathology and this conception was already supported when researchers began to observe the psychotic disorders in 1845 (Rothermundt et a., 2001). Post-mortem studies have shown an up-regulation of several genes involved in inflammatory response in frontal cortex samples of schizophrenia subjects (Setre et al., 2007). Moreover, the probable involvement of inflammatory process in schizophrenia illness, is causing an increasing attention in pharmacotherapy to adopt anti-inflammatory drugs in schizophrenia treatment (Meyer et al., 2011).

Many evidence suggests that schizophrenia is mostly a neurodevelopmental disorder characterized in many cases by morphologic brain anomalies as ventricular enlargements (Vita et al., 2006) or decrease hippocampal volume (Nelson et al., 1998) and moreover children that develop schizophrenia during their adolescence show a tendency for impaired neurocognitive behaviour (Cannon et al., 2002) and neuromotor function (Murray et al., 2006). These damage could be caused by problems during pregnancy as prenatal exposure to infection (Remington and Klein, 2006). The possible interaction between infection and schizophrenia development is the stimulation of cytokines response (Patterson, 2009), perhaps an excess of

maternal inflammatory cytokines levels could be associated with neurodevelopment disorders (Dammann and Leviton, 1997) and with production of toxic agents by microglia and astroglia in the fetal brain that are harmful to neurons (Patterson, 2009). Recent studies have shown that cytokines play an important role in the development of nervous system (Deverman, 2009) and was seen by post-mortem study that an excess of cytokines levels may annoying the maturation of oligodendrocytes and of the white matter (Davis et al., 2003). Others study have hypothesized that cytokines may play a role non only during embryogenesis but also in the progression of schizophrenia and for this reason many researches are focusing on finding the circulating levels of pro and anti-inflammatory cytokines during schizophrenia illness (Meyer, 2011; Reale et al., 2011; Baker et al., 1996; Potvin et al., 2008). Possible link between infection and schizophrenia development is the stimulation of cytokine response and cytokine-centred hypotheses of schizophrenia pathogenesis have been recently proposed (Watanabe et al., 2010). In particular, IL-18 has been proposed, by recent data, to be connected to schizophrenia. In particular, five polymorphisms in genes related to the IL-18 pathway, including IL-18 receptor genes, have been associated with schizophrenia (Shirts et al., 2008) and some variants of the IL-18 gene have been recently related to the development of schizophrenia symptoms (Liu et al., 2011). Furthermore, the levels of IL-18 circulating protein have been found augmented in affected patients, as compared to controls (Tanaka et al., 2000; Reale et al., 2011).

AIM OF THE WORK

2. AIM OF THE WORK

It has been widely accepted that immune system and central nervous system are closely interlinked and that they actively communicate especially in case of damage. However, until now the processes that underlie this crosstalk are still not clear and, for this reason it was very interesting to focus my Ph.D study on this issue. In particularly, the attention is focused on the role of inflammatory cytokines, more specifically on IL-18, to investigate the possible role as mediator between peripheral inflammation and brain. This work was conducted along different steps to better focalize my topic of interest through different angles. A first line of research has been developed by an experimental study on rats to investigate how a peripheral inflammatory stimulus can induce several changes in cytokines production in different brain areas also occurring the possible repercussion of these changes in the behaviour. In fact, recently, a great interest has been focused on research addressed to better understand the role of brain cytokines in behavioral and cognitive performance following systemic inflammation, especially in the longer term after inflammatory insults, to realize what happens in the CNS after a peripheral inflammatory event. For this reason, a lot of studies have been conduced by peripheral injection of the bacterial endotoxin component lipopolysaccharide (LPS) of gram negative bacteria that models systemic infection, to induce neuroinflammation in order to analyze at brain level the synthesis of inflammatory cytokines, such as interleukin IL-1β, IL-6, and tumor necrosis factor (TNF)-α (Gatti et al., 1993; Pitossi et al., 1997). These inflammatory mediators, in turn, appear central in driving behavioral modifications, as in the case of the behavioral responses to LPS, known as sickness behavior, which is the acute consequence of cytokine elevation (Dantzer et al., 2001). Notably, endotoxin triggering can also induce protracted behavioral effects, as occurring in aging rats (Barrientos et al., 2006; Barrientos et al., 2009). Differently, investigations in healthy adults aimed at determining whether a single event of systemic inflammation can as such induce long-lasting modifications of behavior and brain cytokine synthesis are still limited. A single LPS administration results in delayed loss of neurons and cholinergic

innervations, leading to enduring behavioral alterations characterized by memory deficits and changes in exploratory patterns (Semmler et al., 2007) Moreover, TNF-α appears to convey inflammation from periphery to brain, where it is early and persistently released after LPS treatment and causes microglia activation and significant loss of neurons between 7 and 10 post-treatment months (Qin et al., 2007). Thus, it is predictable that additional cytokines could take part to neuroinflammatory phenomena leading to chronic progression of neurodegeneration. As I have already described, the pro-inflammatory cytokine IL-18 seems to be a key player in neuroinflammatory and neurodegenerative processes, with wide behavioral and cognitive effects (Felderhoff-Mueser et al., 2005; Alboni et al., 2010) but the time-dependent modifications of IL-18 expression within specific brain regions after a peripheral neuroinflammatory event have not yet been delineated. For this reason, to conduce an experimental study to characterize the long-term consequences of systemic inflammation on behavioral/cognitive performances and brain cytokine changes seems to be a very interesting research. Than, the aim of this first study of my Ph.D has been to analyze the motivational, spatial, mnesic, discriminative, and attentional functions, as well as to measure the levels of two cytokines TNF-α and IL-18 in several brain areas at 7 days and 10 months after a single intraperitoneal injection of high-dose LPS in rats (Bossù et al., 2012).

This first study has shown that IL-18 seems to be involved in the processes that lead to the progressive neurodegeneration and cognitive dysfunction that are characteristic of illness as Alzheimer's disease. Several studies have highlighted the possible role of this cytokine in AD and in particular, Ojala and collegues (Ojala et al., 2009), have done a post-mortem study carried out on the brains of AD patients that have shown a significant increase of IL-18 in the frontal cortex of AD patients compared to controls that is in perfect line with the result obtain in our work. It seemed therefore interesting to study the role of IL-18 in the cognitive impairment and, particularly, in the pathogenesis of AD. Many study were conduced to show the implication of IL-18 in the progression of AD disease and recently it was shown that the high levels of this cytokines are correlated with high

levels of cognitive impairment (Bossù et al., 2008). Since Alzheimer's disease displays its first symptoms only after the pathology is already established, or rather when the neuronal loss is already an advanced state, than it is impossible to study the inflammatory parameters in the early stages of pathology. For this reason the second part of my Ph.D study has focused on studying the possible implication of IL-18 in MCI subjects, who have a state of memory impairment which often convert in AD. This study was conducted in two phases: one at serum level and the other one at cellular level. Earlier we have quantized the levels of the IL-18 system in the serum of MCI in comparison to health controls to investigate the possible deregulation of these cytokine in MCI subjects and moreover to correlate these parameters with cognitive impairment of MCI to highlight the possible correlation between IL-18 systems and the cognitive impairment shown during the earliest phases of AD. At a later timer we have continued the work on MCI subjects to a cellular level. In particular, we have valued the levels of IL-18 and of other pro-inflammatory cytokines (TNF-α and IL-1β) in the cell supernatants obtained from PBMC in culture stimulated with LPS. This cellular cultures mime the brain cellular situation in course of damage. This study has been conducted on health controls in comparison with the amnesic MCI subpopulation (amnesic single domain MCI and amnesic multidomain MCI). This work can show if the cytokines examined can be predicted of disease. This work is still under study, but a preliminary result that has attracted a lot of interest in our focus, has been to note that IL-18 appears to have a role in AD and more in particularly, a role as predicted mediator of pathology qua only IL-18 is selectively more elevated in AD and in the subpopulation of MCI that appear to selectively convert in AD (amnesic single domain MCI) respect to controls. This results combined with the previous and with that conducted at serum level of MCI that have highlight the correlation of IL-18BP with cognitive impairment of MCI subjects, led us to the hypothesize that is very interesting to continue to investigate the role of IL-18 not only in neurodegenerative disease but also in psychotic syndromes.

Several studies have highlighted the hypothesis that cytokines may play a role in the progression of several psychosis syndromes and for this reason much attention of the current research has been devoted to find the circulating levels of pro and anti-inflammatory cytokines during schizophrenia illness that is the more commune form of psychosis (Meyer, 2011; Reale et al., 2011; Baker et al., 1996; Potvin et al., 2008; Watanabe et al., 2010). In agreement with the idea that imbalances of pro- and anti-inflammatory cytokines may contribute to the onset of psychotic symptoms and the progressive loss of brain tissue in schizophrenia, IL-18 has been proposed to be modulated during schizophrenia illness. The relationship between IL-18 and SCZ has been highlight both at gene level that at protein levels. However, the IL-18 biology in schizophrenia is still unclear and no data are available on the relationship between IL-18 and IL-18BP in affected individuals. Therefore, in my Ph.D we have felt interesting to evaluate also the conduct of IL-18 system in schizophrenia patients. Than, in the third part of my Ph.D study, we have measured the serum amounts of both IL-18 and IL-18BP in a group of schizophrenic subjects in comparison with healthy individuals (Palladino et al., 2012).

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3. 1. EXPERIMENTAL STUDY ON RATS

3.1.1. EXPERIMENTAL DESIGN

Fifty adult male Wistar rats (weighing 200 g at the beginning of the experiments; Harlan, Italy) were used.

The animals were pair-housed and kept under standard conditions with food and water ad libitum on a 12/12 h dark/light cycle (light on between 07:00 and 19:00 h). The animals belonging to the LPS-treated group (LPS, n = 26) were intraperitoneally (i.p.) injected with a single dose of LPS from E. coli, strain 055:B5 (5 mg/kg; Sigma, Italy) in 0.5 mL of volume, according to Qin and colleagues [16]. The animals belonging to the control group (CTR, n = 24) were i.p. injected with 0.5 mL of vehicle (sterile endotoxin-free PBS, Life Technologies, Italy). To avoid potential influence of behavioral testing on cytokine levels, cytokine assessment and behavioral testing were performed on different animals. Thus, 26 rats (LPS, n = 14; CTR, n = 12) were subjected to behavioral evaluations, while 24 animals (LPS, n = 12; CTR, n = 12) underwent brain cytokine analyses (Figure 1). Behavioral testing and cytokine analyses were performed after LPS challenge at two time-points, representative of long-term responses in its early (7 days) and late (10 months) phases. Following i.p. injections, animals were observed for the first 4 h and the presence of sickness behavior was evaluated in all animals by measuring changes in body weight and temperature. In addition, presence of piloerection and lethargy was observed for 1 min at five post-treatment time-points (0, 1, 2, 3, and 4 h). LPS injections induced significant body weight loss (one-way ANOVA: $F_{1,48} = 7.1$, $P < 0.01$) and body temperature increase ($F_{1,48} = 4.01$, $P < 0.05$). The number of animals showing lethargy and piloerection progressively increased as the LPS post injection hours went by, as indicated by Chi square test. Namely, in LPS animals the presence of piloerection became significant from the first hour onward and the presence of lethargy from the second hour onward ($\chi 2 = 6.0$;

d.f. = 1; P <0.01). According to other authors [16], an increased level of serum TNF-α was observed in treated animals at 4 h after LPS treatment (186.6 ± 37.5 pg/mL; mean ± SE), whereas in CTR rats this cytokine level was low and undetectable under our experimental conditions. At difference, serum IL-18 was undetectable in both LPS and CTR animals.

3.1.2. BEHAVIORAL ASSESSMENT

The behavioral assessment lasted two days and consisted of three tests: Elevated Plus Maze, Open Field with Objects, and Morris Water Maze.

3.1.2.1. ELEVATED PLUS MAZE (EPM)

The EPM raised 90 cm above the ground was formed by a wooden structure in the shape of a cross with four arms (50 cm × 10 cm). The north and south arms were opened, while the east and west arms were enclosed by 36-cm-high walls. The rat placed on the central platform was allowed to explore the maze for 5 min. The total time spent in the open and close arms was measured.

3.1.2.2. OPEN FIELD WITH OBJECTS (OF)

The apparatus consisted of a circular arena (diameter 140 cm) delimited by a 30-cm-high wall. During session 1 (S1), each rat was allowed to freely move in the empty open field and its baseline activity level was measured. During the habituation phase (S2-S4), four objects were placed in a square arrangement in the middle annulus of the arena and a fifth one was placed in the central area.

The five objects were: (1) a metal bar with a conical base; (2) a plunger; (3) a long steel rod; (4) a yellow rubber plug; and (5) a black cylinder with a plastic cup turned upside down on top of it. During the spatial change (S5 and S6), the spatial configuration was changed by moving objects 2
and 5 so that the initial square arrangement was modified to a polygon-shaped configuration, without any central object. During the novelty session (S7), the configuration was modified by substituting object 4 with a green plastic object shaped like a half moon. All sessions lasted 6 min; inter-session intervals lasted 3 min. Rats' behavior was recorded by a video

camera whose signal was relayed to a monitor and to an image analyzer (Ethovision, Noldus, Wageningen, The Netherlands). In S1 the following motor and emotional parameters were analyzed: total distance (in cm) traveled in the arena; percentage of distances traveled in a 20-cm peripheral annulus; number of central crossings; motionless time; number of defecation boluses. In S2-S4 total time spent in contacting object was analyzed. Contact was considered to have taken place when the rat's snout actually touched an object or when it sniffed the object for at least 1 s, but not when the rat leaned against, stood, or sat on the object. In S5-S7 the time spent contacting objects was expressed as discrimination index (d index), that is time exploring displaced (or novel) objects minus time exploring not displaced (or familiar) objects/total exploration time.

3.1.2.3. MORRIS WATER MAZE (MWM)

The rats were placed in a circular white pool (diameter 140 cm) filled with 24°C water made opaque by the addition of a-toxic acrylic color (Giotto, Italy). An escape platform (diameter 10 cm) was submerged 2 cm below the water level. Each rat was submitted to two consecutive phases (Place and Probe) consisting of eight trials (Place) and one trial (Probe). During Place, the rat released into the water from randomly varied starting points was allowed to find the hidden platform for a maximum of 120 s. When the rat reached the platform, it was allowed to remain there for 30 s. The inter-trial interval was 3 min. Then, the platform was removed and rats were allowed to swim for 60 s in searching for it (Probe phase). Rats' navigational trajectories were recorded by a video camera whose signal was relayed to a monitor and to the previously described image analyzer. In analyzing MWM performances the following behavioral parameters were considered: latencies to find the platform; total distances swum in the pool; mean swimming velocity; percentage of time spent in the rewarded quadrant (presence of platform) in the Probe phase; navigational strategies put into action in reaching the platform. The navigational strategies were classified in five main categories (Foti et al., 2011), regardless the platform was reached or not: Circling (C), that is, swimming in a 20 cm peripheral annulus, with inversion of swimming direction and counterclockwise and

clockwise turnings in the peripheral sector of the pool; Extended Searching (ES), that is, swimming around the pool in all quadrants, visiting the same areas more than once; Restricted Searching (RS), that is, swimming in some pool quadrants, not visiting some tank areas at all; Restricted Circling (RC), that is, reaching the platform by swimming only in the peripheral annulus; direct Finding (F), that is, swimming towards the platform without any foraging around the pool. Two researchers who were unaware of the individual specimen's group categorized the swimming trajectories drawn by the image analyzer. They attributed the dominant behavior in each trial to a specific category. Categorization was considered reliable only when their judgments were consistent.

3.1.3. ASSESSMENT OF PROTEIN LEVELS OF CYTOKINES IN SERUM AND BRAIN TISSUE

For serum cytokine measurement, blood samples were taken in terminally anaesthetized rats and collected in microfuge tubes. Samples were spun down and serum kept at −80°C until further use. For brain cytokine measurement, rats were sacrificed by decapitation and the brains were quickly removed and dissected on ice using a binocular dissection microscope. Frontal cortex, hippocampus, striatum, cerebellum, and hypothalamus were dissected on ice by a trained researcher
according to Glowinski and Iversen's method (Glowinski et al., 1996). All brain regions were extracted in 1 mL extraction buffer/100 mg tissue. Brain tissue samples were homogenized in an ice-cold lysis buffer containing 137 mM NaCl, 20 mM Tris–HCl (pH 8.0), 1% NP40, 10% glycerol, 1 ml PMSF 10μg/ml aprotinin, 1 μg/mL leupetin, and 0.5 ml sodium vanadate. The tissue homogenate solutions were centrifuged with 14000 × g for 25 min at 4°C. The supernatants were collected and stored at −80°C until analysis. Quantification of serum and intracerebral TNF-α and IL-18 protein levels were assessed by ELISA kit (Biosource, Invitrogen), according to manufacturer's instructions. The limit detection of assays was 4 pg/mL for TNF- α and 15 pg/mL for IL-18. Cytokine results, reported as picograms of the measured molecule per mL of serum (pg/mL) or for gram of tissue (pg/g) are expressed as mean values ± SEM. Where indicated, cytokine

amounts were also normalized to protein content. In this case, the concentration of total protein in the brain extracts was measured by Bradford assay (BioRad Laboratories).

3. 2. STUDY ON HUMANS

3.2.1. SUBJECTS

3.2.1.1. MCI STUDY

64 Mild Cognitive Impairment (MCI) subjects were recruited on the basis of the criteria of Petersen, were divided according to the characteristics of the cognitive impairment in amnesic pure (MCIa) and amnesic multidonimio (MCIm). 32 healthy control subjects (HC) were recruited and matched with MCI for age (Table 1.). The subjects underwent cognitive testing to determine the presence and degree of cognitive impairment assessed by MMSE score. MMSE (Mini Mental State Examination) is a test commonly used in neuro-cognitive test that measures orientation, language, verbal memory, attention, visual functions and mind control, by using the scores ranging from 30 (when the cognitive function is unharmed) to 0 that represents the maximum degree of cognitive impairment.

	HEALTH CONTROLS (HC) N=32	MILD COGNITIVE IMPAIRMENT (MCI) N= 64
AGE	69 ± 6	70 ± 6
MALES / FEMALES	13/19	35/29
MMSE (score)	/	27.5 ± 1

Table 1. Demographic characteristics of health controls (HC) and MCI subjects.

3.2.1.2. SCZ STUDY

Seventy-seven schizophrenic patients (SCZ) diagnosed according to the Diagnostic and Statistical Manual of Mental Disorders-IV edition (DSM-IV) (Spolitini et al., 2011) were recruited from outpatient clinics in Central Italy. Seventy-seven healthy control subjects (HC) were recruited in the

same geographic area and matched with the SCZ for age and gender. Exclusion criteria were: 1) treatment with anti-inflammatory or immunosuppressive medication; 2) overt infectious disease or auto-immune disease; 3) history of alcohol or drug dependence or traumatic head injury; 4) any past or present major medical or neurological illness. This research was carried out with the ethical approval of Santa Lucia Foundation institutional review board. Written informed consent was obtained from each subject after giving subjects a complete description of the study.

3.2.2. SERUM AND CYTOKINES MEASUREMENT

Serum samples were obtained from all subjects by centrifugation of clotted blood, and aliquots were stored at −80°C until cytokine assays were performed. Peripheral blood mononuclear cell (PBMC) were isolated by heparinized venous blood by centrifugation on density gradient (Lympholyte-H, Cederlaine, Hornby, Canada). 2 x 10^6 PBMC suspended in 0,5 ml of medium (RPMI 1640 medium, 2mM Glutamax, 25 mM Hepes buffer, 0,1 M sodium piruvate, 0,1 M of non essential aminoacids and 5 μg/ml of gentamicine (Invitrogen Life Technologies, Grand Island, NY) supplemented with 10% foetal bovine serum (Hyclone, Logan, UT), were placed in 48 wells culture plates and incubates ai 37°C for 18h in absense or presense of LPS (200 ng/ml; E.coli stain O55:B5, Sigma). After incubation, cell supernatants were collected and stored to -80°C until cytokine determination.

Total IL-18, corresponding to the total amount of free IL-18 and IL-18 bound to IL-18BP, was determined by enzyme-linked immunosorbent assay (ELISA), using antibodies unable to distinguish between the free and the bound form of IL-18. More in detail, coating antibody (clone 125–2 H), detecting antibody (clone 159-12B) and standard human recombinant IL-18, were used (MBL, Nagoya, Japan). IL-18BP was measured using commercial ELISA (R&D Systems Minneapolis, MN, USA) specific for IL-18BPa, the prevalent isoform in humans, in accordance with the manufacturer's instructions. The limit of detection for both assays was 12.5 pg/mL. Concentrations of free IL-18, namely unbound to IL-18BP, resulted from calculation based on the law of mass action, considering 1:1

stoichiometry in the complex of IL-18 and IL-18BP and a dissociation constant (K*d*) of 0.4 nM, as elsewhere reported in more detail (Novick et al., 2010; Migliorini et al., 2010). Standard human recombinants TNF-α and IL-1β were obtain from Peprotech (London, UK) while the pairs of antibodies (coating antobodies and detection antibodies) were obtain from Endogen (Woburn, MA).

3.2.3. STATISTICAL ANALYSIS

The data obtained by the experimental study on rats, were firstly tested for normality (Wilk-Shapiro's test) and homoscedasticity (Levene's test). Then, they were analyzed by one-way or two-way ANOVAs for independent (treatment, time) and repeated (trial, session, arm) measures followed by Tukey's HSD test. Non-parametric data related to piloerection and lethargy evaluation were analyzed by means of a Chi-squared metric.

In order to detect statistically significant differences on the data obtained by human studies, comparisons between continuous variables were made using the Student's *t* test. Pearson's correlation coefficient was used to assess the relationships between continuous variables.

The level of statistical significance was defined as $P < 0.05$.

RESULTS

4. RESULTS

4.1.1. SEVEN DAYS FOLLOWING LPS TREATMENT

4.1.1.1. EPM

No differences in anxiety levels were evident between groups (Figure 2A). A two-way ANOVA (treatment x arm) revealed that all animals spent more time in the close vs. open arms (arm effect: $F_{1,10}$= 141.68, $P < 0.001$), without significant treatment effect ($F_{1,10} = 0.31$, P n.s.) and interaction ($F_{1,10} = 0.59$, P n.s.).

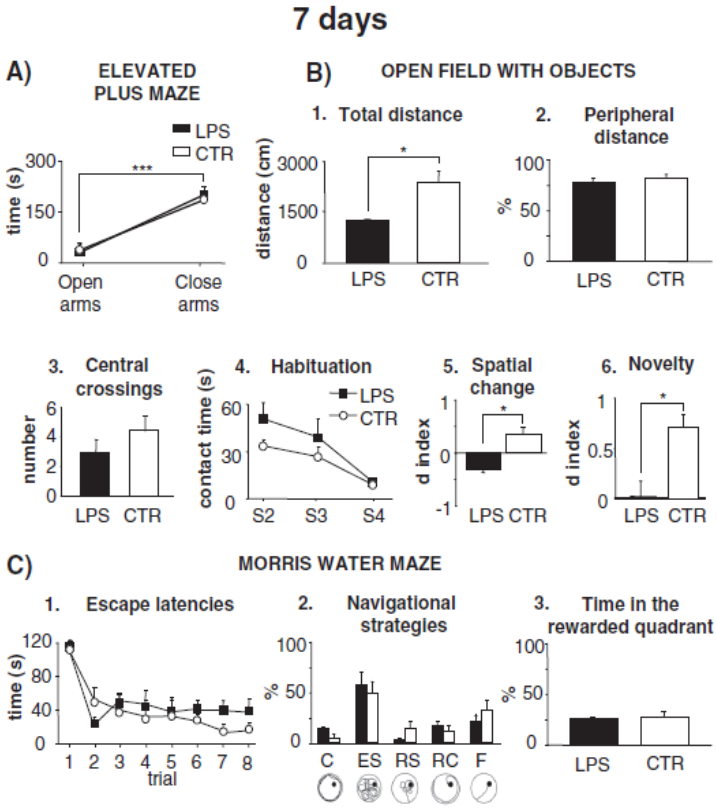
4.1.1.2. OF

In S1, LPS rats traveled around the arena significantly less in comparison to CTR (Figure 2, B1), while no significant treatment effect was evident on peripheral distances and on the number of central crossings (one-way ANOVAs: total distance: $F_{1,10} = 5.37$, $P < 0.05$; peripheral distance: $F_{1,10} = 0.86$, P n.s.; central crossings: $F_{1,10}=1.32$, P n.s.) (Figure 2, B2-3). All animals exhibited comparable levels of anxiety as indicated by the absence of significant differences between groups in the number of defecation boluses and motionless time (defecation boluses: $F_{1,10} = 0.01$, P n.s.; motionless time: $F_{1,10} = 1.52$, P n.s). In S2-S4, all animals showed habituation, progressively decreasing exploration time of objects (Figure 2, B4). A twoway ANOVA (treatment x session) revealed a significant session effect ($F_{2,20} = 9.93$, $P < 0.05$). Treatment and interaction effects were not significant (treatment: $F_{1,10} = 4.38$, P n.s.; treatment x session: $F_{2,20} = 0.60$, P n.s.). In S5, while CTR rats detected the new spatial arrangement and contacted displaced objects more than non-displaced objects, LPS rats failed to detect the new spatial arrangement (one-way ANOVA: $F_{1,10}=6.60$, $P < 0.05$) (Figure 2, B5). In S6, while CTR rats no more reacted to the spatial change, LPS rats persisted in not reacting to change ($F_{1,10}=0.91$, P n.s.). In S7, LPS rats failed to recognize the novel object. Significant treatment effect was found on d index ($F_{1,10} = 6.59$, $P < 0.05$) (Figure 2, B6).

4.1.1.3. MWM

Place. No significant differences in reaching the hidden platform were evident between experimental groups (Figure 2, C1-2). A two-way ANOVA (treatment x trial) confirmed that all rats displayed a progressive latency reduction as trials went by ($F_{7,70} = 15.47$, $P < 0.001$), without
significant treatment effect ($F_{1,10} = 1.30$, P n.s.), and interaction ($F_{7,70} = 2.15$, P n.s.). No differences were found in the swimming velocities (LPS rats _x : 21.70 ± 0.53 cm/s; CTR rats _x : 21.48 ± 2.44 cm/s; one-way ANOVA: $F_{1,10} = 0.79$, P n.s.) as well as in the total distances (two-way ANOVA: treatment effect: $F_{1,10}=0.81$, P n.s.; trial effect: $F_{7,70} = 30.09$, $P < 0.001$; interaction: $F_{7,70} = 2.01$, P n.s.). The analysis of the navigational strategies did not reveal any difference between groups.

Probe. No relevant differences were evident between experimental groups, as revealed by a one-way ANOVA on the percentage of time spent in the previously rewarded quadrant ($F_{1,10} = 0.07$, P n.s.) (Figure 2, C3).

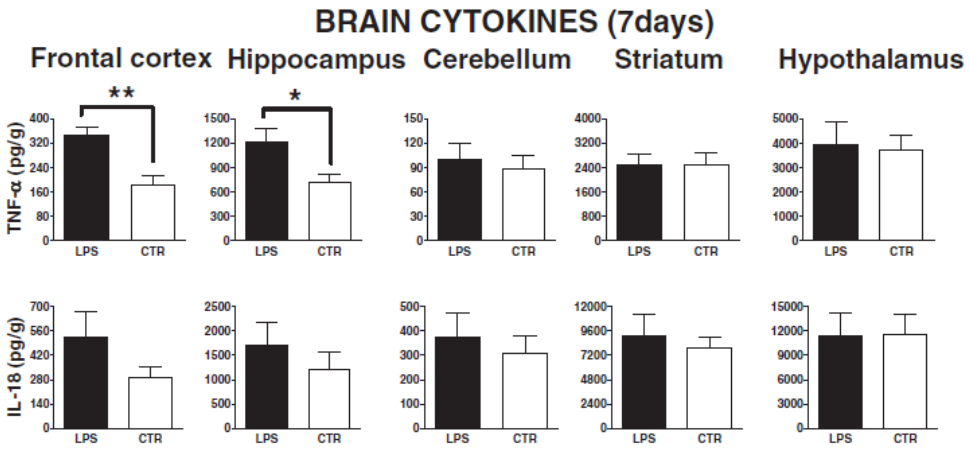


Effect of i.p. injection of LPS on behavioral performances at 7 days post-treatment. A. Mean time spent in the open and close arms by the animals in Elevated Plus Maze is depicted. B. Total distance (1), peripheral distance (2), central crossings (3), mean contact times with objects during habituation phase (S2-S4) (4), spatial change (5), and novelty (6) exhibited by the two experimental groups in Open Field with objects and depicted. C. Mean escape latencies to reach the platform (1), navigational strategies (2), and time spent in the rewarded quadrant (3) displayed by the two experimental groups in Morris Water Maze are depicted. In this figure, the circular figurines illustrate the typical explorative patterns of the five navigational strategies (C, Circling; ES, Extended Searching; RS, Restricted Searching; RC, Restricted Circling; F, direct Finding). Asterisks inside the graphs indicate the significance of comparisons between groups: * $P<0.05$, *** $P \leq 0.0001$.

4.1.1.4. CYTOKINE ANALYSIS

The levels of TNF-α and IL-18 in frontal cortex, hippocampus, cerebellum, striatum and hypothalamus measured 7 days after LPS or PBS injections (Figure 3). With regard to TNF-α (Figure 3, upper panels), its levels were significantly higher in LPS vs. CTR animals in hippocampus (one-way ANOVA: $F_{1,10} = 6.81$, $P < 0.05$) and in the frontal cortex ($F_{1,10} = 17.12$, $P < 0.01$). No significant differences in TNF-α levels were found between LPS and control animals in cerebellum, striatum, and hypothalamus. IL-18 levels (Figure 3, lower panels) were in general slightly more elevated in most brain regions of LPS, as compared to CTR animals, but at variance with TNF-α, the statistical significance was not reached in any of the analyzed cerebral regions. However, a trend towards IL-18 increase for LPS as compared to CTR rats was observed in frontal cortex (526.0 ± 141.6 vs. 290.3 ± 64.73; $P = 0.16$). In this experiment, cytokine data have been also calculated by normalizing to protein content instead than to tissue weight and very similar results

have been obtained with the two methods (not shown). No detectable TNF-α and IL-18 levels have been found in serum of both LPS and CTR animals.



Effects of i.p. injection of LPS on brain regional production of cytokines at 7 days post-treatment. The levels of TNF-α (upper row of panels) and IL-18 (lower row of panels) in LPS-treated and PBS injected control animals were asses 7 days post-treatment. Asterisks inside the graphs indicate the significance of comparisons between groups: * $P<0.05$, *** $P \leq 0.0001$.

4.1.2. TEN MONTHS FOLLOWING LPS TREATMENT

4.1.2.1. EPM

The two experimental groups did not differ on anxiety levels and all animals exhibited the standard open arms avoidance (Figure 4A). A two-way ANOVA (treatment x arm) indicated not significant treatment effect ($F_{1,12} = 0.59$, P n.s.) and interaction ($F_{1,12} = 0.10$, P n.s.), while arm effect was significant ($F_{1,12} = 6.91$, $P < 0.05$).

4.1.2.2. OF

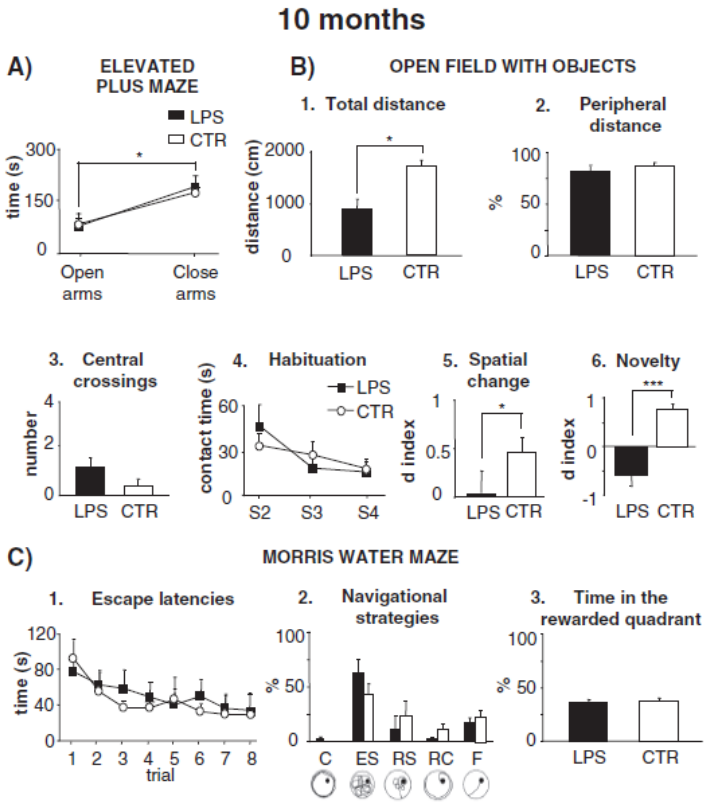
In S1, LPS rats traveled around the arena significantly less in comparison to CTR animals (Figure 4, B1), although significant differences between groups were not evident on peripheral distances and number of central crossings (one-way ANOVAs: total distance: $F_{1,12}=11.45$, $P < 0.05$; peripheral distance: $F_{1,12}=1.77$, P n.s.; central crossings: $F_{1,12}=2.78$, P n.s.) (Figure 4, B2-3). All animals exhibited comparable levels of anxiety as indicated by the absence of significant differences between groups in the number of defecation boluses and motionless time (defecation boluses: $F_{1,12}=0.58$, P n.s.; motionless time: $F_{1,12}=2.47$, P n.s). All animals habituated as the total amount of time spent exploring the five objects decreased from S2 to S4 (session effect: $F_{2,24} = 12.33$, $P<0.001$) (Figure 4, B4). Treatment effect ($F_{1,12} = 0.12$, P n.s.) and interaction ($F_{2,24} = 0.31$, P n.s.) were not significant. In S5, differently from CTR, LPS animals did not detect the spatial change (one-way ANOVA: $F_{1,12}=5.39$, $P < 0.05$) (Figure 4, B5). In S6, LPS rats did not still react to the spatial change, while CTR animals stopped to react to change ($F_{1,12}=0.71$, P n.s.). Once more, in S7, when the novel object was placed in the arena, LPS rats did not recognize it ($F_{1,12} = 21.65$, $P < 0.001$) (Figure 4, B6).

4.1.2.3. MWM

Place. No significant differences in reaching the hidden platform were evident between groups (Figure 4, C1-2). A two way ANOVA (treatment x trial) revealed that both groups displayed a progressive latency reduction as trials went by ($F_{7,84} = 7.02$, $P < 0.001$), without significant treatment effect

(F1,12 = 0.02, P n.s.) and interaction (F7,84=1.21, P n.s.). No differences were found in the swimming velocities (LPS _x : 19.73 ± 0.75 cm/s; CTR _x : 18.99 ± 1.46 cm/s; one-way ANOVA: F1,12=0.17, P n.s.) as well as in the total distances (two-way ANOVA: treatment effect: F1,12 = 0.99, P n.s.; trial effect: F7,84 = 4.84, P <0.001; interaction: F7,84=0.38, P n.s.). Furthermore, no significant differences between groups were observed on the navigational strategies.

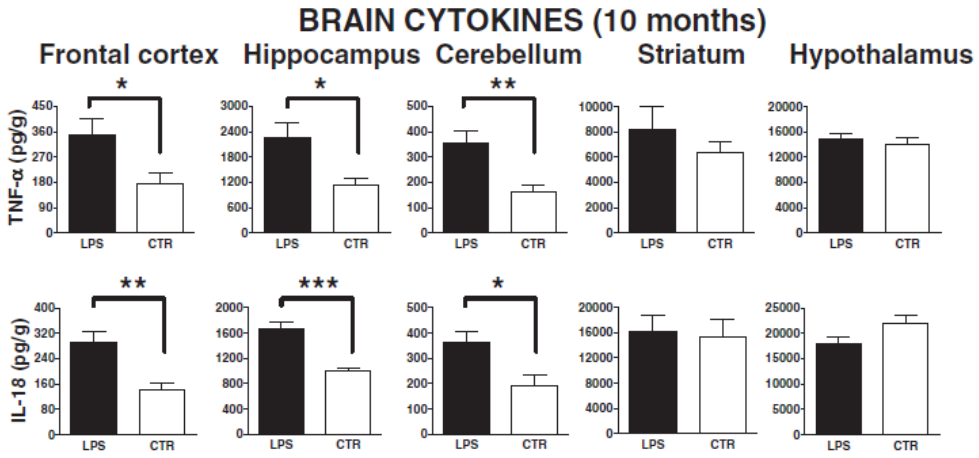
Probe. A one-way ANOVA on the percentage of time spent in the previously rewarded quadrant failed to reveal any significant treatment effect (F1,12=0.20, P n.s.) (Figure 4, C3).



Effects of i.p. injection of LPS on behavioral performances at 10 month post-treatment. A. Mean time spent in the open and close arms by the animals in Elevated Plus Maze is depicted. B. Total distance (1), peripheral distance (2), central crossings (3), mean contact times with objects during habituation phase (S2-S4) (4), spatial change (5), and novelty (6) exhibited by the two experimental groups in Open Field with objects are depicted. C. Mean escape latencies to reach the platform (1), navigational strategies (2), and time spent in the rewarded quadrant (3) displayed by the two experimental groups in Morris Water Maze are depicted. In this figure, the circular figurines illustrate the typical explorative patterns of the navigational strategies (C, Circling; ES, Extended Searching; RS, Restricted Searching; RC, Restricted Circling; F, direct Finding). Asterisks inside the graphs indicate the significance of comparisons between groups: * $P<0.05$, *** $P \leq 0.0001$.

4.1.2.4. CYTOKINE ANALYSIS

The increase of TNF-α already observed in the earlier post-treatment stage was maintained in the brain regions of LPS versus CTR animals (Figure 5, upper panels). In particular, the significant increase of TNF-α was maintained in the frontal cortex (one-way ANOVA: $F_{1,10} = 8.16$, $P < 0.05$) and hippocampus ($F_{1,10} = 9.91$, $P < 0.05$) and reached in cerebellum ($F_{1,10} = 11.86$, $P = 0.01$). Furthermore, not significant differences in TNF-α levels were observed in striatum and hypothalamus. Alike to TNF-α, IL-18 levels were significantly more elevated in LPS than CTR animals (Figure 5, lower panels) in frontal cortex ($F_{1,10} = 15.31$, $P < 0.01$), hippocampus ($F_{1,10} = 33.46$, $P < 0.001$), and cerebellum ($F_{1,10} = 8.49$, $P < 0.05$). Again, no significant changes in IL-18 levels occurred in striatum and hypothalamus. At this time point serum TNF-α and IL-18 levels have not been evaluated.



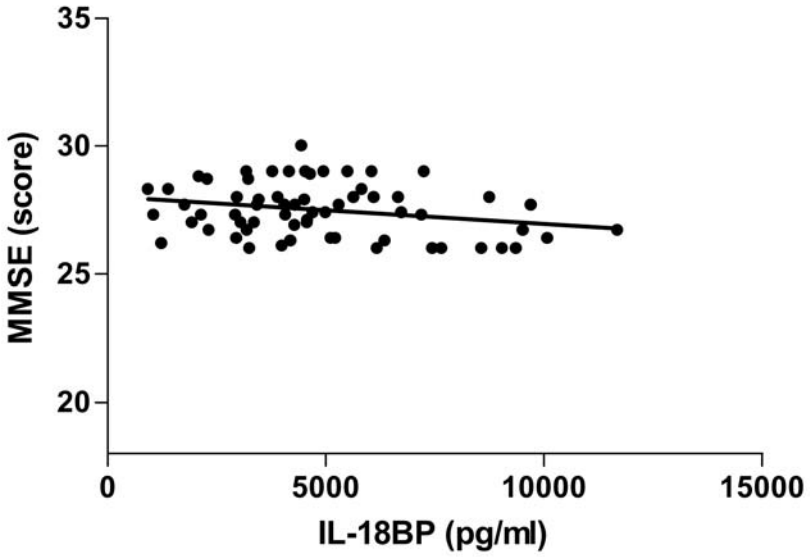
Effects of i.p. injection of LPS on brain regional production of cytokines at 10 month post-treatment. The levels of TNF-α (upper row of panels) and IL-18 (lower row of panels) in LPS-treated and PBS injected control animals were assed 10 month post-treatment. Asterisks inside the graphs indicate the significance of comparisons between groups: * $P<0.05$, *** $P \leq 0.0001$.

4.2. MCI STUDY:

The levels of IL-18 systems has been evaluated in serum of MCI and HC subjects but not significant differences have been found as reassumed in Table 2. However, correlating these levels with MMSE score of the subjects examined, we have found a significant correlation between IL-18BP serum levels and MMSE score of MCI patients (r= -0,2; p= 0,04).

	HC (N= 32)	MCI (N= 64)
IL-18 (pg/ml)	354 ± 21	438 ± 10
IL-18BP (pg/ml)	4562 ± 185	4899 ± 141
IL-18 free (pg/ml)	384 ± 36	252 ± 23

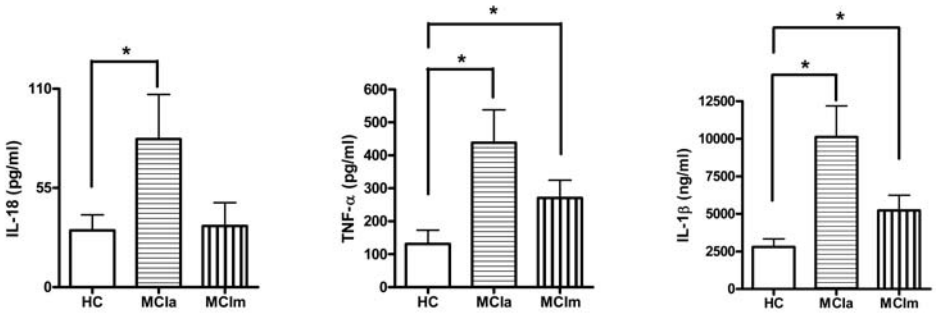
Table 2. IL-18, IL-18BP and IL-18 free concentrations (pg/ml) valued in serum of health controls (HC) and MCI subjects.



Correlation between the serum level of IL-18BP and the MMSE score of MCI subjects

The levels of IL-18, TNF-α and IL-1β have been evaluated in supernatants of PBMC of amnesic MCI subdivided in amnesic single domain MCI (MCIa) and amnesic multidomain MCI (MCIm) and of health controls. The PBMC were stimulated with 200ng/ml of LPS for 18h. The levels of TNF-α

are significant increased both in MCIa and in MCIm in comparison with HC (MCIa: 438 ± 99 pg/ml MCIm: 270 ± 54 pg/ml HC: 131 ± 41; MCIa *vs.* HC P= 0.002; MCIm *vs.* HC P= 0.04). Similar resuts was obtained for IL-1β, in fact, the levels of this cytokine is significant increased both in MCIa and in MCIm in comparison with HC (MCIa: 10118 ± 2079 pg/ml MCIm: 5230 ± 1017 pg/ml HC: 2811 ± 527; MCIa *vs.* HC P= 0.0001; MCIm *vs.* HC P= 0.03). Instead, the level of IL-18 are significant increase only in MCIa in comparison with HC (MCIa: 82 ± 24 pg/ml MCIm: 33 ± 12 pg/ml HC: 31 ± 8; MCIa *vs.* HC P= 0.02; MCIm *vs.* HC P ns)

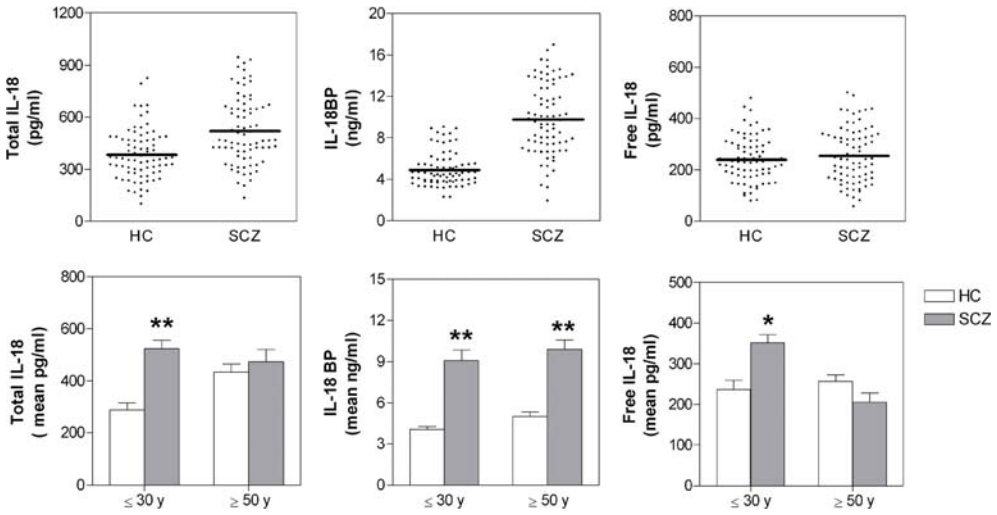


Concentration of IL-18, TNF-α and IL-1β in supernatant of PBMS stimulate with 200 ng/ml of LPS of HC (N= 16), MCIa (N=7) and MCIm (N= 13) subjects. Asterisks inside the graphs indicate the significance of comparisons between groups: * $P<0.05$.

4.3 SCZ STUDY:

4.3.1. SERUM LEVELS OF IL-18/IL-18BP

The concentration of both IL-18 and IL-18BP was evaluated for the serum of each subject. In order to define the levels of bioactive cytokine, free IL-18 was also calculated on the basis of IL-18 and IL-18BP results. Mean values of total or unbound IL-18 and its inhibitor concentration are reported in Table Table1,1, while individual values are reported in the upper panels of Figure Figure1.1. Total IL-18 levels were significantly higher in SCZ than HC ($P<0.0001$). Similarly, serum levels of IL-18BP were significantly higher in SCZ than HC ($P<0.0001$). Interestingly, serum levels of free, bioactive IL-18 were not significantly different between SCZ and HC. The levels of the three elements of IL-18 system were not significantly different between males and females and did not correlate significantly with illness duration and antipsychotic drug dosages.



Concentration of IL-18, IL-18BP and free IL-18 in serum of all HC (n = 77) and SCZ (n = 77) subjects (upper panels) and in two age subgroups of HC and SCZ subjects (lower panels), including subjects with age ≤ 30 years (HC: n = 15, mean age = 24.4 ± 0.7; SCZ: n = 18, mean age = 25.6 ± 0.7) and age ≥ 50 years (HC: n = 22, mean age = 56.5 ± 1; SCZ: n = 19, mean age = 56.1 ± 1.1. Asterisks inside the graphs indicate the significance of comparisons of SCZ vs. HC: * P< 0.001, ** P≤0.0001.

4.3.2. RELATIONSHIP BETWEEN IL-18, IL-18BP AND AGE

In order to evaluate a possible relationship between total IL-18 and IL-18BP levels, we first assessed the relationship existing between the two immune mediators both in SCZ and HC. A positive significant correlation was found in SCZ (r=0.245, P=0.03) but not in HC.

Afterwards, given that IL-18 might participate in age-dependent inflammation, we analyzed the direct relationship between the serum concentration of total IL-18, IL-18BP, free IL-18 and age in all subjects. Indeed, a significant positive correlation between serum total IL-18 levels and age was found in HC (r=0.268; P=0.018), but not in SCZ. No significant correlations were observed between the levels of IL-18BP and age in both HC and SCZ. Differently, an unexpected and highly significant inverse correlation was found between the serum amount of free IL-18 and age in SCZ (r=−0.432; P <0.0001) but not in HC.

In order to further investigate the age dependence of the IL-18 system, we compared the changes in the levels of IL-18-related molecules in HC and SCZ after dividing each main group into two subgroups formed by the youngest (≤ 30 years; HC, n=15 and SCZ, n=18) and the oldest (≥50 years; HC, n=22 and SCZ, n=19) subjects. As shown in the lower panels of Figure Figure1,1, SCZ showed an increase of total IL-18, as compared to HC, in the youngest (1.82-fold increase; P<0.0001) but not in the oldest individuals. The levels of IL-18BP were significantly higher in SCZ, as compared to HC, both in the youngest and the oldest subjects, although the increase was slightly higher in the youngest (2.2-fold increase; P<0.0001) than the oldest (1.96-fold increase; P<0.0001) subgroup. In contrast, the levels of free IL-18 were significantly higher in SCZ than HC as for the subgroup of the youngest subjects (1.48-fold increase; P=0.0006), while the biologically active cytokine has a tendency to decrease in SCZ *versus* HC in individuals with an age ≥50 years (1.24-fold decrease; P=0.068).

DISCUSSION

5. DISCUSSION

This detailed study confirmed the interesting role of IL-18 in several biological conditions. It is emerged that IL-18 is a mediator between periphery and brain and it may influences behavior and cognitive changes. Animal data confirm that IL-18 is a mediator between immune and central nervous systems, in fact brain levels of this cytokine are elevated even a long time following a strong peripheral inflammation. In addition, persistent behavioral and cognitive impairment can be observed in animals in relation with brain cytokine alteration.

In the first study reported, we showed that a single peripheral inflammatory insult induces persistent discriminative deficits accompanied by different production of TNF-α and IL-18 in selected brain areas. LPS treatment was able to elevate the expression of TNF-α in definite brain regions across the entire experimental period. In particular, since our first evaluation (7 days), TNF-α was significantly elevated in the frontal cortex and in the hippocampus and it remained significantly higher than in CTR animals also at 10 months. In addition, TNF-α levels were increased even in the cerebellum 10 months after LPS treatment. These results are in line with a previous study, where 2 months after peripheral LPS administration, a TNF-α mRNA increase was observed in the cortex and hippocampus, as well as cerebellar structures (Weberpals et al., 2009). Intriguingly, IL-18 levels significantly increased in frontal cortex, hippocampus, and cerebellum at the latest (10 months), but not at the earlier (7 days) stage of LPS-induced long-term neuroinflammation. According to their profile of expression, the two cytokines seem to differently participate in brain inflammatory response. Regarding TNF-α, our results support and further expand the view that this cytokine is an early and persistent mediator of the long-lasting inflammatory cascade (Qin et al., 2007). In fact, TNF-α might have a pivotal role in conveying inflammation from periphery to brain, as primary stimulus that promotes self-propelling mechanism of microglial activation, induces persistent brain production of pro-inflammatory cytokines including IL- 1β and, ultimately, leads to progressive neurodegeneration Differently from TNF-α, IL-18 might act as a delayed mediator of neuroinflammation, in

agreement with data obtained in experimental models of brain ischemia and trauma (Jander et al., 2002; Yatsiv et al., 2002). Regarding the possible molecular mechanisms, while quite a lot of evidence corroborates the hypothesis of an involvement of brain TNF-α in neurodegeneration and cognitive decline, even identifying some of its driving mechanisms (Qin et al., 2007; Janelsins et al., 2005; Medeiros et al., 2010), much less data are available about the molecular links between brain IL-18 expression and cognitive functions. In line with our data, IL-18 has been previously found constitutively expressed in the cortex, hippocampus, cerebellum, hypothalamus, and striatum (Culhane et al., 1998) and it is also detectable in astrocytes and microglia after LPS treatment (Conti et al., 1999; Wheeler et al., 2000). However, by means of immunocytochemistry analysis, other studies have shown, that IL-18 protein is mainly expressed in the neurons of the medial habenula and in the ependymal cells surrounding the lateral and the third ventricles, without significant staining in microglial cells or astrocytes (Sugama et al. 2002). Therefore, even though microglia might be a relevant cellular source of increased IL-18 in our experimental conditions, additional studies should be performed to identify the IL-18 producing cells within the brain in the presence of LPS mediated long-term neuroinflammation. Although with some fluctuations, we observed the expression of IL-18 protein (both constitutive and LPS-induced) in the brains of the rats analyzed both at 7 days and at 10 months. These data are in agreement with other results showing that IL-18 is present in immature and adult brain, as the cytokine is present in neonatal brain in normal conditions and during hypoxic-ischemic brain injury (Prinz et al., 1999; Hedtjärn et al., 2002) and it is involved in neuronal cell death during traumatic brain injury both in immature and adult brain (Yatsiv et al., 2002; Sifringer et al., 2007). In fact, although this cytokine has been generally associated with the amplification of age dependent inflammatory processes (Dinarello et al., 2006) and it may be relevant in age-related functional impairment (Frayling et al., 2007), its role in CNS might be important in both health and disease since the development, throughout the whole life. In neuroinflammatory conditions, given that brain cytokines function as an integrated network, the increased expression of IL-18, probably modulated

by TNF-α itself (Schmidt et al., 2004), could participate in perpetuating brain inflammation by stimulating the activation of the transcription factor NF-κB and the production of other pro-inflammatory mediators, such as IL-1β and TNF-α (Puren et al., 1998; Dinarello et al., 1999). As a consequence, the increase of IL-18 expression in the brain might influence progressive neurodegeneration and cognitive dysfunction. In fact, in both experimental and clinical settings, IL-18 appears to directly or indirectly interfere with the processes of memory consolidation, synaptic plasticity and/or neurogenesis (Bossù et al., 2010; Curran et al., 2001; Bossù et al., 2008). Noteworthy, the role of IL-18 in mediating the behavioral effects of LPS injection observed in our conditions is supported by a recent study showing that the expression of the IL-18 signaling receptor and of its short variant considered a potential negative regulator of IL-18, is tightly modulated by LPS and occurs in specific brain areas associated with the limbic system (Alboni et al., 2011). Overall, in spite of the intriguing association between behavioral/cognitive impairment and cytokine expression observed in the present research, we are aware that the present data do not allow defining the causal role of the increased TNF-α and IL-18 expression in behavioral and cognitive abnormalities. Similarly, they do not allow identifying at brain regional level the molecular mechanisms of chronic inflammatory network that could participate in long-term cognitive decline. Nonetheless, by taking together other authors' results and ours, it is possible to hypothesize that the peripheral administration of high-dose LPS could cause TNF-α dependent activation of microglia and subsequent increase of brain cytokines, like TNF-α itself, IL-1β, and IL-18 (Qin et al., 2007; the present study) which might concur in inducing persistent behavioral alterations (Semmler et al., 2007; the present study) probably caused by a long-term loss of neurons (Semmler et al., 2007; Qin et al., 2007).As early consequence of LPS treatment (7 days), we observed a motor hypoactivity and discriminative deficits (in OF) not associated to any spatial memory deficit (MWM Probe trial results) or anxiety expression (in EPM and OF). In particular, LPS rats exhibited marked discriminative deficits, when in OF spatial arrangement and saliency of the objects were changed. These results are in line with the impaired object recognition described in mice 4 days

after LPS treatment (Jacewicz M et al., 2009). These behavioral changes may be interpreted in terms of reduced motivation resulting in reduced responsivity toward the context. Nevertheless, it has to be underlined that the lack of spatial change detection in LPS rats might be ascribed also to an attentional deficit disrupting some, although not all, components of the spatial information processing. The late behavioral pattern (10 months) was similar to that displayed 7 days after LPS injection. While no modification of spatial memory or anxiety levels was observed, motor hypoactivity and discriminative deficits were once again present, indicating that a systemic inflammatory response evoked by a single LPS injection (5 mg/kg) is able to significantly affect cognitive functions even 10 months later. These results are in line with the report by Semmler and colleagues (Semmler et al., 2007) that described behavioral and cognitive impairment, neuronal loss in frontal cortex and hippocampus, as well as reduced cholinergic innervation in parietal cortex occurring up to 3 months after treatment in a rat model of sepsis evoked by high-dose LPS (10 mg/kg). Taking into account these observations, the long-term impairment of exploratory and discriminative functions observed in our LPS rats could be related to a reduced functionality of either cholinergic (Semmler et al., 2007) and dopaminergic systems (Qin et al., 2007). These two systems appear to be implicated the former one in the attentional tuning controlling discriminative behaviors (Sarter et al., 2001; Pepeu et al., 2004) and the latter one in the locomotory function and motivational drive to explore objects (Graybiel et al., 1997; Girault et al., 2004). Than, LPS induced cytokine alterations and it may influences behavior by affecting neurotransmitterial function, neuroendocrine activity, neuronal plasticity properties and brain circuitry (Capuron et al., 2011), mechanisms and functions whose evaluation is beyond the scope of the present study. Thus, whether and how a direct association between behavioral dysfunction and brain cytokine elevation indeed exists remains an open question.

These observations highlight that the inflammation induced cognitive/behavioral impairment, suggesting that both TNF-α and IL-18, although with a different pattern, might take part in the enduring deterioration of behavioral functions caused by systemic inflammation.

Furthermore, these findings support the recently hypothesized participation of IL-18 in the progressive neurodegeneration that occurs in some forms of dementia as Alzheimer's disease. In fact, a link between IL-18 and cognitive impairment seems confirmed in clinical studies of patients with progressive cognitive impairment. In particular, IL-18 and its natural inhibitor IL-18BP appear modulated in a different manner compared to control subjects, not only in individuals with AD, but also in subjects at risk of becoming AD. IL-18 and IL-18BP, seems to be altered in AD and subjects with an high risk to development AD. Moreover, other authors (Ojala et al., 2009) showed a significant increase of IL-18 in the frontal cortex of AD patients in comparison with healthy controls thus confirming that IL-18 is modulated in the periphery and in the brain during this illness.

For this reason, the second part of my Ph.D study has stressed this hypothesis or rather that IL-18 and moreover its molecular system can have a role in AD and more in particularly in the early stage of the disease. A lot of studies have investigate the implication of IL-18 as mediator in neuroinflammatory and neurodegenerative condition (Felderhoff-Mueser et al., 2005) and in particularly in AD pathology (Pompl et al., 2003; Bossù et al., 2010; Ojala et al., 2009). Interesting results were obtained by the research group I've worked with during my Ph.D about the implication of IL-18 in Alzheimer's disease. A study has shown non only that the blood cells of AD patients produce a greater amount of IL-18 compared with healthy subjects, but also that the levels of this cytokine are correlated with the cognitive impairment shown by patients (Bossù et al., 2008). This study has been the starting point for my work, in fact the aim of this part of the study was to measure the levels of IL-18 and its inhibitor in subjects with initial cognitive impairment when the dementia is not yet overt, namely the Mild Cognitive Impairment (MCI) condition. This study has shown that, also if there are not significant differences in the serum levels of the IL-18 system molecules between MCI and HC, a negative correlation between the level of IL-18BP and the MMSE score was found in MCI subjects. Since previous data had shown that in AD subjects the IL-18 levels (both in serum and blood cells) were associated with cognitive decline, this result led to hypothesize a different scenario between the early and late stages of

dementia, with increase of IL-18BP and increased IL-18 levels respectively in MCI subjects and AD patients who have higher decrease of cognitive abilities. These observations support the hypothesis that the IL-18 system may have a role both in the Alzheimer's disease and in the early stage of the illness but future studies will certain be necessary to better investigate the processes and the biological parameters that underlie the onset of Alzheimer's disease and its pathogenetic phase. Another interesting observation regarded amnesic MCI subjects, who have been subdivided in two subpopulations: amnesic single domain MCI (MCIa) and amnesic multidomain MCI (MCIm). After quantifying the levels of several pro-inflammatory cytokines in the supernatants of their in vitro stimulated blood cells, we have shown a significant increase of TNF-α and IL-1β in both MCIa and MCIm cell supernatants in comparison with HC, while a significant increase of IL-18, as compared to HC was found only in MCIa, the MCI subgroup with the highest risk to selectively convert in dementia of AD type. This result is in line with the previous observation that cell produced IL-18 is increased in AD patients (Bossù et al., 2008) suggesting that this cytokine may be considered as a specific and predictive parameter of AD onset.

In conclusion, based on the obtained data highlighting in both AD patients and MCI subjects an increase in cell production of IL-18 and significant correlations of IL-18 and IL-18BP levels with loss of cognitive abilities, we can claim that the inflammatory processes mediated by IL-18 may contribute to the onset of dementia, providing a further support to the hypothesis that inflammation may play an important role in AD.

This result suggest that this cytokine may have an early involvement in progression of cognitive impairment, possibly related to the neuro-inflammatory processes associated with AD.

Other studies have highlighted that this cytokine has also a role in some forms of psychosis as schizophrenia, where five polymorphisms in genes related to the IL-18 pathway have been associated with the risk of developing the disease (Shirts et al., 2008) and some variants of the IL-18 gene have been related to the progression of disease symptoms (Liu et al., 2011). Moreover also the levels of IL-18 circulating protein have been

found increased in affected patients, as compared to healthy controls (Tanaka et al., 2000; Reale et al., 2011). This last result has been also confirmed by our study (Palladino et al., 2012), in fact IL-18 system seems to be deregulated in schizophrenia. Moreover IL-18/IL-18BP alteration seems more pronounced in young schizophrenia patients, confirming the clinical data obtain in AD that suggested early involvement of the pathway of IL-18.

Than, the third study developed during my Ph.D, we expanded the analysis of the modulation of IL-18 system to another serious brain disorder characterized by deficits in thought processes, perceptions, and emotional responsiveness: the schizophrenia. We assessed the levels of both IL-18 and IL-18BP in SCZ and age- and gender-matched HC confirming that the total amount of IL-18 is higher in SCZ than in HC. Nevertheless, the main finding of this study is that the cytokine inhibitor IL-18BP is significantly increased in the serum of SCZ, as compared to HC. Interestingly, this elevation is about 40% higher than that of total IL-18. Thus, the evident increase of IL-18BP in SCZ creates a balance in the levels of active IL-18, which, in fact, appears comparable between patients and HC subjects. Moreover, we found a positive correlation between total IL-18 and its inhibitor only in SCZ and not in HC. This can be explained by the greater range of IL-18BP levels shown in SCZ in comparison whit HC. This result is in line with what occurs in autoimmune conditions, when IL-18BP levels are increased following the amplification of the IL-18 response (Novick et al., 2010) and these observations leads us to hypothesize that IL-18BP elevation in SCZ is linked to an early and disease-dependent increase of IL-18. Moreover, comparing the levels of IL-18 system molecules of SCZ and HC belonging to different age groups, we confirmed that in HC there is a trend of IL-18 to increase with age (Dinarello et al., 2006; Gangemi et al., 2003). A different condition, instead, appears in SCZ where this age-associated modulation of the IL-18 system seems altered, thus strengthening the concept of an IL-18 system perturbation associated with the disorder. We established that in SCZ subjects, as compared to age-matched HC, the increase of total and free IL-18 was observable only in the youngest individuals, where the elevation of IL-18BP did not seem to reach adequate

levels to reduce free IL-18 to HC levels, thus reflecting an imbalance which might favor a general inflammatory state in the early phase of the disorder. In agreement with the condition we have described for young SCZ patients, several studies have previously reported disease-related high levels of free IL-18, despite the concomitant over expression of IL-18BP (Novick et al., 2010; Mazodier et al., 2005; Ludwiczek et al., 2005), which is in accordance with the nature of the interaction between IL-18 and IL-18BP (Kim et al., 2000). A different situation can be seen in older individuals, where SCZ subjects with an age ≥50 years did not show levels of total IL-18 significantly different from HC, likely a result of the age-dependent increase of total IL-18 values occurring only in HC, which would progressively bring the cytokine levels of the latter to almost reach those of SCZ. Furthermore, in older SCZ the highly significant elevation of IL-18BP persisted and free IL-18 levels appeared slightly lower than those of HC. Then, we can hypothesize that in young SCZ, total and free IL-18 increase over normal levels, similar to what has been observed for other pro-inflammatory cytokines, results perhaps as a consequence of an inflammatory challenge at the maternal-fetal interface (Watanabe et al., 2010). Therefore, IL-18BP elevation might be interpreted as an effort of the organism to compensate the exaggerated inflammatory response linked to the disorder and this might become effective in balancing the IL-18-mediated pro-inflammatory reaction only in the later stages. Other studies in line with our obresvations, suggest that SCZ subjects are not characterized by a simple pro-inflammatory profile, but more likely the illness is accompanied by both pro- and anti-inflammatory activated forces, which may involve both monocyte and T-cell networks, probably as a product of an inflammatory system control (Drexhage et al., 2011; Miller et al., 2011). Yet, the sense of this perturbation in IL-18 system, characterized by elevated levels of IL-18BP here described for the first time in the context of schizophrenia, should be deciphered on a time-dependent basis, in the light of its ability to limit harmful inflammation and to contribute to increased susceptibility to infection and immune-related diseases (Meyer et al., 2011). The early increase in free IL-18, which fades away with age in SCZ, consistently points to the importance of considering age and duration of the illness when

discussing the immune-related pathophysiological mechanisms of schizophrenia.

CONCLUSIONS

6. CONCLUSIONS

During my Ph.D course, the complex study carried out has shown the interesting and multiple role of the pro-inflammatory cytokine IL-18. The results obtained, lead us to hypothesize that IL-18 may be a key mediator in the communication between the immune and the central nervous systems. This cytokine seems to play also a role in several pathogenetic processes that underlie cognitive and behavior functions characteristic of neurodegenerative disease and psychotic illness. IL-18 and its natural inhibitor seems to be involved in the pathogenetic early stage and in the clinical progression of Alzheimer disease well as in the illness progression of schizophrenia. These observations suggest that IL-18 has a central role in several pathological conditions. For this reason, the investigation of the action of this cytokine seems to be very interesting above all to discovery a possible disease marker. Then, further studies could investigate this possibility in more detail studying the modulation of molecular and cellular mechanisms of IL-18 in several pathology. In particular, it will be interesting to deepen this research to the biologic activity of IL-18 expanding the study to the entire IL-18 system. In fact, thought our works and others, seems to be clear by now that is not enough to considerer only the molecule IL-18, but it is essential to regard the IL-18/IL-18BP modulation, to value the actual amount of biological active IL-18. Seems to be very interesting to deepen the role of this cytokine in several pathological conditions as in Alzheimer's disease and in schizophrenia illness. In particular, there are two strands of thought for AD disease, in fact some researchers think that IL-18 should be protective for the Alzheimer's disease, while others consider it harmful. Then, to better investigate the real role of IL-18 in this pathology can be important to find future care for AD. This cytokine could be involved also in schizophrenia illness and in particular in its pathogenesis. Certainly, a lot of information still need to be discovered but it would seem clear that the inflammation is present, both in the brain and in peripheral levels, in the pathogenesis of AD as in the pathogenesis of SCZ. In fact AD is characterized by a chronic neuroinflammation, while an immunitary hypothesis is hypothesized for the

SCZ pathogenesis, in which it is believed that an inflammatory event to an embryonic state is the cause of disease.

The complex study carried out during my Ph.D course, has provided key elements for the evaluation of the role of IL-18 in the interaction between immunity and brain thought several different approaches. In fact, this study confirmed the potential diagnostic and therapeutic value of IL-18 in various disease brain on the basis of animal model and clinical studies, by in vivo and in vitro assays, animal behavior tests, cognitive and psychiatric analysis on patients.

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