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The Role of TNF-Alpha in ALS: New Hypotheses for Future Therapeutic Approaches

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1. Introduction

The pathophysiological origins of neurodegenerative disorders are a complex combination of both environmental and genetic factors. However, in many of these disorders, processes such as inflammation and oxidative stress activate common and final pathways leading to toxicity and cellular death. High levels of oxidative damage within the brain and the activation of neuroinflammation factors are a prominent feature in patients with Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), Amyotrophic Lateral Sclerosis (ALS) and inherited ataxias (Halliwell, 2006; Lin & Beal, 2006). Regarding the immunological point of view, the brain was considered an immune privileged organ because it was isolated from the systemic circulation by protective blood-brain barrier that controls the infiltration of pathogens, the transition of pro or anti inflammatory factors and peripheral blood cells (Itzhaki et al., 2004). Despite that, in recent years, the relationship between neuroinflammation and neurodegeneration has been described with particular attention to the lymphocytes activation and cytokines production (Appel, 2009; Tansey et al., 2007). Moreover, it is well known the implication of glial cells in the progression of neurodegeneration: they are involved in many types of damage, they migrate to the damaged cells and also they have a role in clearing the debris of the dead cells. Through such processes, microglia releases reactive oxygen species, proinflammatory cytokines, complement factors, and neurotoxic molecules, leading to further neuronal dysfunction and death (Heneka et al., 2011; Lasiene et al., 2011). In addition, the implication of the peripheral system and its participation in the cellular mechanisms that direct to neurodegeneration, as white blood cells, is well documented (Calvo et al., 2010; Ghezzi et al., 1998; Gowing et al., 2006).

Many data from autoptic spinal cord and blood examinations of the ALS patients, animal and cellular models support an immune system involvement in ALS pathogenesis. Since

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1984 the presence of an autoimmunity component in ALS was proven when immunoglobulin depositories have been described in spinal cord (Donnenfeld et al., 1984). At present the implication of the neuronal and non-neuronal immunological cells and activation of the inflammatory processes have been extensively described in ALS (Engelhardt et al., 1995; Henkel et al., 2004; Troost et al., 1990).
Starting from literature data about implication of the innate and adaptive immunity in ALS, we would like to point out the role of the TNF alpha (TNF-α) system and its interactions in ALS pathway with particular attention to SOD1 protein, the most important player in the ALS pathogenesis. We will focus this book section on TNF-α cytokine because its involvement both in immunological pathways and in oxidative stress is known in ALS disease. Moreover we will try to define the immunological actors that exert a protective function and how they could be used in a possible therapy.

2. ALS and immunity

In the last decades, increasing numbers of experimental and clinical observations have reported inflammatory reactions in ALS tissues which indicate the involvement of both the innate and adaptive immune responses (Fig. 1) (McGeer & McGeer, 2006; Moisse & Strong, 2006; Sta et al., 2011; Weydt et al., 2002).
So far it is not clear how immune system is involved in ALS disease, whether the adaptive or innate immunity has a major role and whether immunity is part of damaging or neuroprotective response to the pathological process.

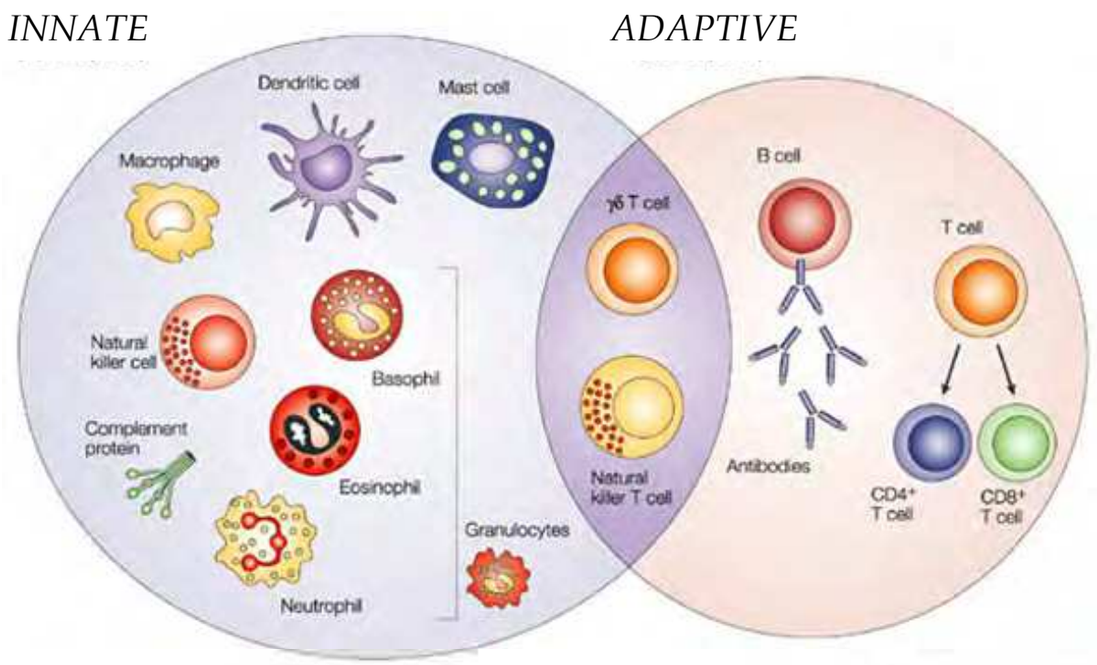

Fig. 1. Innate and adaptive immunity.

2.1 Innate immune system

Innate immunity is naturally present and is not stimulated by antigens or mediated by antibodies. It is therefore non-specific and is executed by a variety of cells: granulocytes, as eosinophils and basophils, white blood cells as natural killer and mast cells. Instead, microglia belongs to the central nervous system and is involved in the local innate immunity. Inflammation is one of the aspects of the innate immune response.

Interactions between innate immune system, brain and neurodegenerative diseases are known (Ghezzi et al., 1998; Gowing et al., 2006) and it has been reported that mast cells, macrophages, dendritic cells, microglia, complement and cytokines participate in limiting the damage (Calvo et al., 2010).

Innate system was found activated in central and in peripheral system of ALS patients (Chandels et al., 2001; Elliott et al., 2001; Sta et al., 2011).

Several studies regarding peripheral innate immune system changes in sporadic ALS reveal that there are increased levels of circulating monocytes and macrophages (Harman et al., 1991; Hemnani et al, 1998). The presence of T cells, IgG, activated microglia, macrophages, and reactive astrocytes, as well as other indications of inflammation are found in ALS spinal cord tissue (Henkel et al., 2004; Engelhardt et al., 1995; Troost et al., 1990).

In ALS there is morphological and neurochemical evidence for the proliferation and activation of microglia in areas of significant motor neuron loss, as spinal cord (Henkel et al., 2004; Kawamata et al., 1992; Moisse et al., 2006). This activation may be a consequence of stressed neurons that induced proliferation and activation of microglial cells activating complement system and pro-cytokine response involved in neuronal death (Fig. 2). Motor neuron loss and immune system activation may increase neuron stress leading to increase of neuroinflammation.

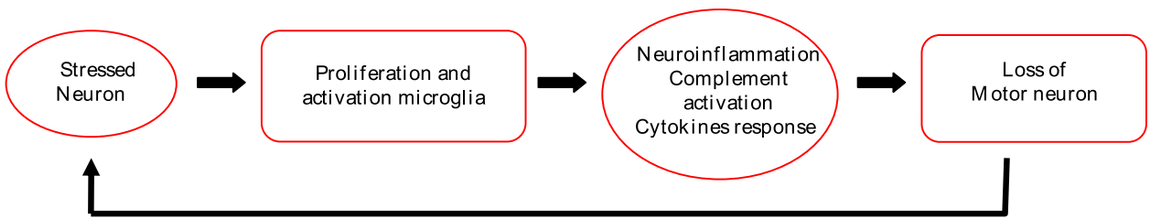

Fig. 2. Hypothesis of activation of innate immunity in ALS.

2.2 Adaptive immune system

As innate immunity, adaptive immunity has a role in ALS (Sta et al., 2011). Unlike innate immune responses, the adaptive responses are highly specific and they consist of antibodies, lymphocytes activation and cell mediated response. The cells of the adaptive immune system are B and T lymphocytes: B cells, which are derived from the bone marrow, become the cells that produce antibodies. T cells can cross-talk with neurons and microglia, and either damage or protect neurons from stressful stimuli (Alexaniu et al., 2001), also in spinal cord and brain (Chiu et al., 2008).

T-helper cells have been observed in proximity of degenerating corticospinal tracts; T-helper and T-suppressor cells, with a variable number of macrophages, have been found in ventral horns of the spinal cord (Troost et al., 1990).

Infiltration of T cells compatible with adaptive response have been found in the areas of motor neuron destruction in the CNS but no correlation was found between clinical

parameters and infiltrating T cells (Holmoy et al., 2008). The majority of T cells characterized in the infiltration were CD8+ cytotoxic T cells, but a substantial number of T CD4+ cells were also present (Beers et al., 2008).
Alterations of total lymphocyte count (Provinciali et al., 1988; Tavolato et al., 1975) and T subset distribution in peripheral system of ALS patients (Westall et al., 1983) have been reported. Low T cells numbers and decreased proliferative capacity in T cells are found in the blood of ALS patients (Holmoy et al., 2008).
As concerned CD8+ and natural killer T cells, they were found increased in ALS patients compared to control cohort (Rentzos et al., 2011).
Interestingly, ALS patients showed a reduction of CD4+/CD25+ regulatory T cells that are known to interact with the local microglia, reinforcing the hypothesis of the involvement of the adaptive immune system associated with neuroinflammatory process in ALS (Mantovani et al., 2009). Beers and colleagues (Beers et al., 2011) observed that regulatory T cells as CD4+/CD25+/FoxP3 correlated with disease progression; in fact, the number of T cells were found inversely correlated with disease progression rate.
Animal studies showed that in ALS model T cells deficiency decreases microglia reactivity and accelerates ALS disease progression; specific and progressive accumulation of monocytes/macrophages was observed along the length of degenerating nerve fibers and activated microglia was detected in spinal cord of ALS model mice (Chiu et al., 2009).
No infiltrating B-cells have been found even if a role of B lymphocytes in the pathogenesis of ALS has been hypothesized, as secreted autoantibodies by B cells identified in CSF and serum from ALS patients (Naor et al., 2009).
As concern antibodies, since the eighties the presence of IgG in serum or tissues of ALS patients has been documented (anti-ganglioside GM1, anti-sulfoglucuronyl paragloboside, anti-neurofilaments and anti-Fas) (Sengun & Appel, 2003; Yi et al., 2000). Indeed, IgG deposits have been demonstrated in motor cortex, spinal cord and in motor neurons from ALS patients (Donnenfeld et al., 1984; Engelhardt & Appel, 1990; Fishman & Drachman, 1995). Serum immunoglobulins from ALS patients showed enhanced binding to rat spinal cord cells *in vitro* (Digby et al., 1985), demonstrating cytotoxic effects when they were added to a motor neuron cell cultures (Alexianu et al., 1994; Demestre et al., 2005) and that the presence of an immune response to spinal cord cell membrane components in patients with motor neuron disease was a damaging event.
IgG from ALS patients reacts with the skeletal muscle DHP (bisognerebbe spiegare cosa è)-sensitive Ca^{2+} channels reducing the peak of the Ca^{2+} current and the charge movement in single cut fibres from the rat extensor muscle (Delbono et al., 1991). About 60% of ALS sera contained different monoclonal immunoglobulins: in particular IgG (72.7%) and IgM (27.3%) have been found (Duarte et al., 1991).

2.3 Cytokines

Different interactions have been found between innate and adaptive immunity in ALS, as concern cytokines involvement. Cytokines have an effect on the expression of other inflammatory factors and on each other, and these functional relationships are non-linear: the causal relationships of cytokines and disease are complex and difficult to prove (Marklund et al., 1992).
Several studies regarding immune system changes in sporadic ALS reveal that there are increased levels of circulating monocytes and macrophages, producing cytokines as IL-1

and IFN-γ, (Harman et al., 1991; Hemnani et al., 1998). Furthermore, high cytokine levels have been described in plasma, serum and cerebral fluid (CSF) from ALS patients and sometimes correlate with the clinical status (Kelly et al., 1994; Lee et al., 2005; Khule et al., 2009; Tateishi et al., 2010).

As concern inflammatory cytokines (IL-7, 9, 12, 17 and IL-1β) levels were found higher in CSF of sporadic ALS patients (Tateishi et al., 2010). IL-15 and IL-12 serum levels, they also have been found higher in patients with ALS (Rentzos et al., 2010). The same authors measured IL-17 and IL-23 levels in serum and CSF from ALS patients that were found increased compared to controls (Rentzos et al., 2010). TGF-β1 concentrations in the serum and CSF of ALS patients did not differ from controls, but TGF-β1 serum concentration was significantly higher in ALS patients at the terminal clinical status (Ilzecka et al., 2002). Higher amount of IL-6 has been found in sera and CSF from sporadic ALS patients and it has been related to hypoxemia severity rather than pathological condition (Moreau et al., 2005).

Plasma concentration of TNF-α, TNF-R1 and TNF-R2 and their time course during disease progression were studied in ALS patients in order to assess the TNF-α system implication in ALS pathogenesis. In all plasma patients soluble forms of the TNF-α and its receptors are found increased already at disease onset and remain over the normal range during the disease progression time (Cereda et al., 2008). In addition TNF-α amounts have been found higher in sera from sporadic ALS patients but no correlation was found with the clinical criteria (Poloni et al., 2000; Cereda et al., 2008). TNF-α role in neurodegeneration will be further highlight in the next paragraph.

3. Tumor necrosis factor-alpha (TNF-α)

The Tumour Necrosis Factor Alpha (TNF-α) is a pro-inflammatory cytokine produced by monocytes/macrophages and activated by mast cells, endothelial cells, fibroblasts, neurons and glial cells during acute inflammation and it is responsible for a wide range of cell signals about cell viability, gene expression, homeostasis control and synaptic integrity.

TNF-α was described for the first time by Carswell et al. in 1975 as a protein component of serum of mice stimulated with bacterial antigens, and was brought to light the ability to induce death in cancer cell lines in vitro and in vivo to destroy transplanted sarcomas. Characteristically, this cytokine was able to cause tumor cells death without compromising the viability of healthy cells. The subsequent isolation and molecular characterization of the gene have provided information on the structure and functioning of this molecule.

3.1 TNF-α gene

The gene coding for TNF-α is located on chromosome 6 within the region encoding the Major Histocompatibility Complex (MCH), HLA in human, between the HLA-DR class II and HLA-B class I genes (Fig. 3). Its location and strict linkage disequilibrium present between some alleles of class I and class II genes has permitted to hypothesize associations between TNF-α alleles and some diseases. The gene for TNF-α include about 3 Kb and contains four exons (almost 80% of the protein is codified by the exon four) and three introns.

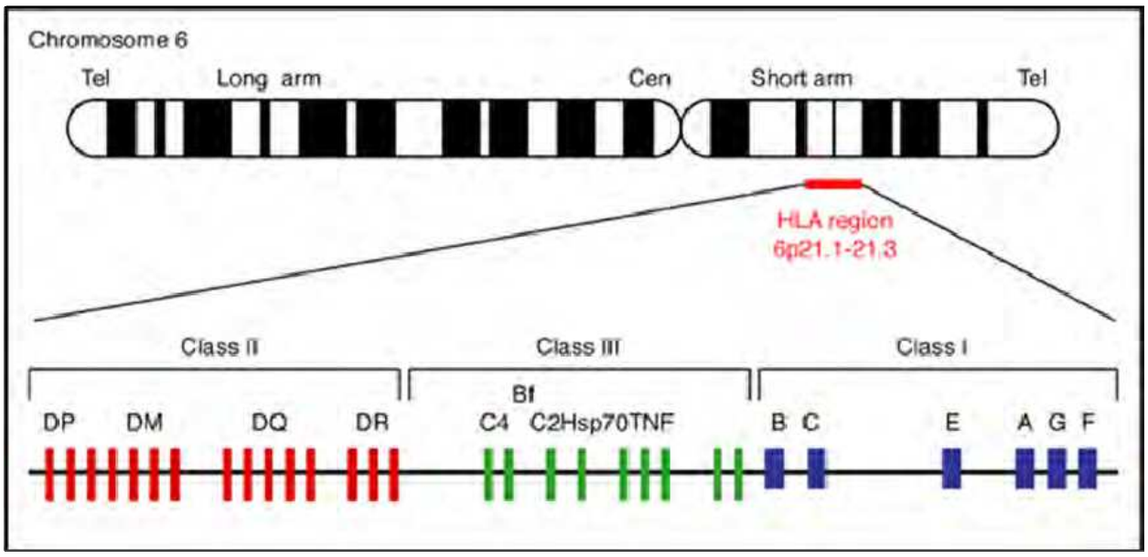

Fig. 3. Localization of TNF-α gene on chromosome 6 (6p21.3)

TNF-α gene codifies for a protein of 233 amino acids with a molecular weight of 25.6 kDa. TNF-α is, at the beginning, a transmembrane protein of 212 amino acids associated to homotrimers; the N-terminal portion loses 76 amino acid by cleavage of TNF-α converting enzyme (TACE or ADAM-17), producing a soluble monomeric TNF-α form (17 kD) and next, the trimetric form of 51 kD (Bazzoni & Beutler, 1996; Reddy et al., 2000).

The trimer is the biological active soluble form because of its ability to bind its receptors. However, the trimeric soluble form spontaneously tends to dissociate into a monomeric inactive form, that is a physiological process that allows to limit the deleterious effects of excessive concentration of TNF-α (Smith et al., 1987).

TNF-α response to a variety of extracellular signals is very rapid and transient and includes a transcriptional component as well as posttranscriptional events. Its transcriptional control occurs predominantly at the level of transcriptional initiation.

The approximately 1000 base pairs of the TNF-α gene's 5' flanking region contains a number of important regulatory elements that affect TNF-α transcription in response to various stimuli. The basic promoter region is defined by TATA box sequence, located about 20 bp upstream from the transcription site and about 200 bp form the translation start codon. Multiple potential regulatory sites, including consensus sequences for the AP-1 (Activator Protein-1) and AP-2 (Activator Protein-2) sites, the cAMP-responsive element, and sequences similar to the NF-kB, (Nuclear Factor kappa-light-chain-enhancer of activated B cells) sequences found in immunoglobulin and cytokine regulatory elements are present in 5' flanking region. This sequence has been demonstrated to be responsive to LPS (lipolysaccharide) and TNF-α stimulation. The 3' untranslated region contains a sequence element affecting posttranslational control of TNF-α through mRNA stability and translation efficiency.

Many polymorphisms have been described in TNF-α promoter region (-308, -857, -863, -238, -1031) defining its correlation with TNF-α mRNA amounts: wild-308G allele is responsible for a higher transcription gene (Helmig et al., 2011), as the A mutated allele at position -857 results in a high production of TNF-α (McCusker et al., 2001).

As concerned -238 polymorphism, has been described a direct effect on gene expression, although studies suggest that this region contains a strong repressor site (-280 to -172). -238 TNFG/A allele genotype may be in linkage disequilibrium with a functional polymorphism that impacts TNF production (Liu et al., 2008).
Several of these polymorphisms have been studied extensively in some diseases, mainly -308 and -857 SNPs.
The mutated allele -308A is a marker of susceptibility to several autoimmune and inflammatory diseases such as lupus erythematosus, celiac disease and Alzheimer's disease (Candor et al., 2002 and 2004), but so far it has been published in a very large number of works in order to reach the correct conclusions on the role of these polymorphisms.
Most SNPs (-863, -238, -1031) do not affect the levels of expression but their pathologic involvement is related to the variation of allelic or genotypic frequencies.
As concerned SNPs in coding region, some polymorphisms are described (http://www.ncbi.nlm.nih.gov/SNP) but they are not correlated with any diseases or functions.

3.2 TNF-α protein function

The functions of TNF-α are biologically dependent on the amount of cytokine produced. If it is present in small amounts, TNF-α acts locally as autocrine and paracrine mediator of inflammation in leukocytes and endothelial cells, determines the expression of surface receptors for leukocyte migration, acting as an angiogenic factor, such as fibroblast growth factor and induces apoptosis in certain cell types. If it is present in high amounts, however, is distributed in the systemic circulation where it stimulates the production of IL-1 and IL-6 by leukocytes and the synthesis of acute phase proteins in the liver (such as fibrinogen) and activates the intravascular thrombus formation.
TNF-α exerts many of its effects by binding, as a trimer, the cell membrane receptor TNF-R1 of 55 kDa (p55) or TNF-R2 of 75 kDa (p75) belonging to the superfamily of TNF receptors, which also includes FAS, CD40, CD27 and RANK.
TNF-R1 is expressed in almost all tissues and may be activated by trimeric soluble form and also by membrane-associated form of TNF-α; TNF-R2 is expressed only by cells of the immune system and it is activated only by trimeric soluble form of the TNF-α. Differently from TNF-R1, TNF-R2 do not own a death domain (DD), and its activation may only induce the survival pathway (Fig. 4).
Instead, the binding of TNF-α to the TNF-R1 may cause both cell death or survival depending on which pathway is activated and it also depends on the second signal involved (Hsu et al., 1996; Darnay et al.,1997). Following the binding of TNF-α, TNF-R1 produces a conformational change that determines the separation of the intracellular death domain (DD). This dissociation allows the adapter protein TRADD to bind the domain of death (DD) to induce apoptosis or form a platform for the subsequent binding proteins to make cell survival. If TRADD directly binds FADD, which in turn recruits caspase-8, the apoptosis way is activated. High concentrations of caspase-8 induce its proteolytic activation and subsequent cleavage of downstream caspases, leading to cell apoptosis. Cell death induced by TNF-α plays a minor role compared to the role of this cytokine in inflammation. Its ability to induce apoptosis is in fact modest when compared to that of other members of the family as Fas and often masked by the anti-apoptotic effects.

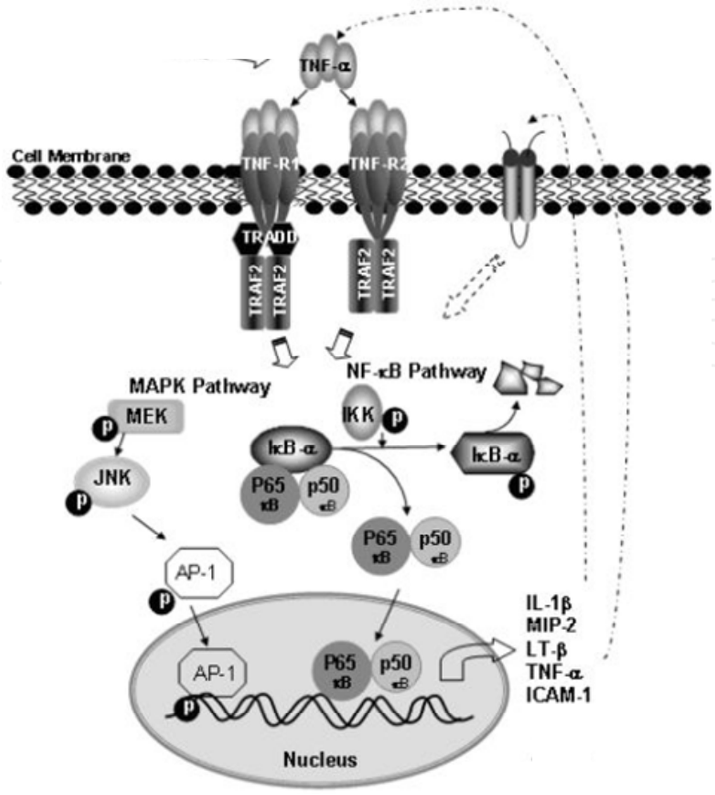

Fig. 4. TNF-α and TNFR pathway (Rosenquist M., 2003)

Both TNF-α receptors activate different survival intracellular signalling pathways, especially because of the IkB kinase (IKK) and the cascade of MAP kinases (MAPKs) that control the gene expression through NF-kB, and AP-1 respectively. In detail the term NF-kB refers to a family of five structurally-related transcription factors (p50, p52, RelA/ p65, c-Rel and RelB), all containing the Rel homology domain (RHD) within the N-terminus and acting as homo- and heterodimeric DNA binding complexes (O'Dea et al., 2010). Several studies showed that NF-kB activity is induced in most cell types in response to a broad variety of stimuli, ranging from cytokines, radiation and reactive oxygen species (ROS) (such as exposure to H_2O_2), with major roles in coordinating innate and adaptative immunity, cell activation and proliferation, survival, development and apoptosis (Ghosh et al., 2008; Vallabhapurapu et al., 2009).
TRADD recruits TRAF2 (TNF receptor-associated factor 2) and RIP (receptor interacting protein). TRAF2 in turn recruits the protein kinase IKK (inhibitor of nuclear factor kappa-B kinase), which is activated by RIP. The IKBα inhibitory protein that normally binds the NF-kB and inhibits its translocation, is phosphorylated by IKK and then is degraded, releasing NF-kB. The latter NF-kB is a transcription factor that translocates into the nucleus and mediates the transcription of a wide variety of genes and theirs products involved in survival and cell proliferation, in the inflammatory response and anti-apoptotic factors.

As concerns the activation of MAP kinase (MAPK), TNF-α induces a strong activation of JNK (c-Jun N-terminal Kinase), one of three major cascades of MAPK, evokes a moderate response of the p38-MAPK, and is responsible of a minimal activation of ERK. TRAF2 actives MEKK1 and ASK1 directly or indirectly, which phosphorylate MKK7, which in turn activates JNK. The latter AP-1 translocates into the nucleus and activates transcription factors such as c-Jun and AFT2. The way of JNK is involved in differentiation, cell proliferation and apoptosis.

3.3 TNF-α and nervous system

In the central nervous system TNF-α is produced by astrocytes, microglia and neurons in response to several stimuli both intra and extracellular, and seems to play a central role in the genesis and perpetuation of neuroinflammatory signal. An alteration in the regulatory mechanisms of TNF-α was found in a wide variety of disorders such as depression, carcinogenesis, and Alzheimer's disease. In the nervous system different TNF-α activities were defined, as inducer or inhibitor of neuronal apoptosis underlining how complex TNF-α pathway may be.

Moreover, electrophysiological experiments have shown a negative effect determined by TNF-α on neuronal function. Studies of hippocampal sections showed that the addition of pro-inflammatory cytokines decreased the long-term potentiation (Long-term potentiation, LTP), a correlate of learning and memory processes (Tancredi et al., 1992). The mechanism by which this occurs is still under investigation, but it has been suggested that activation of p38 plays a major role in reducing the early phase of LTP in response to TNF-α, while the protein expression changes would play a role in the late phase (Butler et al., 2004).

However, other studies have shown that TNF-α alone is not able to initiate apoptosis in the absence of a second signal and may actually prevent apoptosis in response to certain types of cell damage (Badiola et al., 2009). In fact, although apoptosis is primarily triggered by the TRADD, TNF-α may also be associated with cell survival signals, because it is also able to facilitate the binding of other molecules such as JNK and NF-kB, which were instead important in cells endurance, indicating how complex are the signalling pathways of TNF-α. In fact, TNF-α facilitates axonal regeneration, induces neuronal survival through the anti-apoptotic pathway mediated by NF-kB, limits the demyelination in experimental autoimmune encephalomyelitis, but these effects appear to be highly dose-dependent and related to the exposure time and interaction with other factors (Schwartz et al., 1991).

The protective effect of the cascade triggered by TNF-α has been documented experimentally: mice lacking the receptor for TNF-α were subjected to cerebral ischemia. This result was attributed to an increase of reactive oxygen species, suggesting that TNF-α could induce an antioxidant protection due to ischemic events (Bruce et al., 1996). Similarly, in a model of glutamatergic excitotoxicity, stimulation of TNF-R2 with TNF-α led to protection against this toxicity (Marchetti et al., 2004). In conclusion, these data suggest that TNF-α could affect neuronal viability in different ways depending on the receptor subtype involved and the presence/absence of secondary signals from endogenous or exogenous stimuli.

In neurodegenerative disease, as Parkinson's (PD) and Alzheimer's (AD), neuroinflammatory processes appear to play key roles in neuronal dysfunction and death (Hakansson et al., 2005). TNF-α was found increased in striatum and CSF from PD patients compared to controls (Nagatsu et al., 2000), and a large number of TNF-α immunoreactive glial cells were detected in CSN from PD patients (Imamura et al., 2003).

As concern AD, TNF-α was found upregulated in both CSF and serum, and its levels correlate with disease severity (Dickson, 1997; Fillit et al., 1991; Paganelli et al., 2002); examination of post-mortem AD brains reveals that TNF-α increased and co-localizes with Aβ plaques (Montgomery et al., 2011). AD patients showed elevated levels of TNF-α in the brain (Tarkowski et al., 2000) and in vitro studies have shown that TNF-α induces the production of Aβ peptides through the regulation of the gamma secretase complex (Blasko et al., 1999).

4. TNF-α system and ALS

Starting from TNF-α literature in ALS disease, the data often come from ALS animal model: TNF-α mRNA was found in the spinal cord of G93A mice in the early stages of the disease (4 months of age) in correlation with the astroglia activation (Hensley et al., 2003) . Moreover gene expression in mice increases with age up to a peak at the final stages of the disease (7-8 months). Although transcripts of both receptors, TNF-R1 and TNF-R2, have been identified in the spinal cord of the G93A rat (Elliot et al., 2001; Hensley et al., 2003). Yoshihara et al. (2002) have shown that the expression of TNF-α in the marrow of G93A mice overlaps with the activation of microglia already in a pre-symptomatic stage. Then, using immunohistochemistry approach authors found that TNF-α was located mainly at the level of motor neurons and microglia. Some genes involved in apoptosis showed the same pattern of TNF-α gene expression, suggesting a correlation between the inflammatory reaction and the apoptotic pathway (Yoshihara et al., 2002). To this regard, Hensley et al. (2003) have characterized the relationship between the inflammatory genes, oxidative stress and apoptotic events in the G93A mice. In the spinal cord of mice at the presymptomatic stadium expression of FADD and TNF-R1 and many members of the caspase apoptotic cascade were found increased; however, they were expressed at the highest level only in the early stage of the disease, during which an increased protein oxidation was also observed.
Kiaei et al. (2006) have seen an increase in immunoreactivity for TNF-α in sections of the spinal cord of G93A mice and familial or sporadic ALS patients; in G93A mice treatment with thalidomide and lenalidomide, drugs capable of inhibiting the expression of TNF-α, attenuates disease progression was. This result is a further confirmation of the hypothesis that TNF-α plays an important role in the pathogenesis of ALS, probably giving rise to an apoptotic pathway (Kiaei et al., 2006).
Veglianese et al. (2006), showed p38MAPK activation in the G93A mice in the presymptomatic stage at the level of motor neurons, and in later phase also in astrocytes and microglia. It has also been demonstrated to be involved in the activation of kinases upstream of MAPK pathway. An increased expression of both receptors of TNF-α is also observed in the presymptomatic stage, confirming the activation, mediated by TNF-α, of the signalling cascade that leads to MAPK. The MAPK seems to be implicated in the development and disease progression in G93A mice, as already said. Once activated it is able to phosphorylate neurofilaments, causing their accumulation within motor neurons, which is considered one of the pathogenetic features of ALS. In addition, p38MAPK is able to stimulate nitric oxide synthase in neurons and in glia, leading to the formation of peroxynitrite (Wengenack et al., 2004). The generation of SOD1 knock-out mice for TNF-α, however, has shown that the absence of TNF-α has no effect on axonal degeneration but influences onset, severity and progression of the disease in G93A mice; these data suggest that TNF-α, despite its high expression during disease, is not the only factor involved in the degeneration caused by mutations in SOD1 motor neurons in animal models (Gowing et al., 2006).

4.1 TNF-α level in peripheral blood of ALS patients

TNF-α and its soluble receptors, sTNFRs, were already found significantly higher in plasma of ALS patients than in those of healthy controls (Poloni et al., 2000). They found a significant correlation between levels of TNF-α and sTNF-R1 and sTNF-R2, confirming that a general activation of the TNF-α system occurred in ALS patients. Activation of the TNF-α system however did not correlate neither with the disease duration nor with the disease severity. Even after dividing the patients in two subgroups, with high and low TNF-α levels, they did not find any difference in terms of clinical parameters of the disease (Poloni et al., 2000).

Our research group analyzed the possible implication of TNF-α pathway in ALS pathogenesis (Cereda et al., 2008). We assayed both the levels of TNF-α and its soluble receptors in plasma from ALS patients overtime during disease progression. We assayed the concentrations of TNF-α and its soluble receptors in plasma of 88 patients with sporadic ALS and 40 healthy controls; blood sample from each patient was taken since two months after diagnosis up to death, or along 80 months. We found that circulating levels of TNF-α and its two soluble receptors were significantly increased in the plasma of patients with sporadic ALS. Our data show that TNF-α high plasma concentration is present already at disease onset in the majority of ALS patients and remains over the normal range during the whole disease progression time, even though it slightly decreases during disease progression.

We hypothesised that in the majority of ALS patients TNF-α plasma concentration has already reached its peak at disease onset, remains high during all disease duration and starts to decrease at the end of the disease. This finding suggests that TNF-α pathway could be activated in the first stage of the disease and it decreases its effect with the progression of the disease (Cereda et al., 2008).

4.2 Polymorphisms and TNF-α transcription gene

Preliminary genetic analysis are documented in ALS only about (-308, -857) TNF-α promoter polymorphisms and they do not show statistically significant differences in allelic and genotypic frequencies (Cereda et al., 2008). In 2008 over 100 sporadic ALS patients' DNA samples collected at the Neurological Institute "C. Mondino" (Pavia, Italy) and DNA sample from 228 healthy controls were used to study polymorphisms of TNF-α, TNF-R1 and TNF-R2 genes by RFLP.

In our work we studied -308 G/A and -857 A/G. Moreover, we investigated MspaI polymorphism in exon 1 TNF-R1 gene, a SNP at +36 A/G positions, and Nla III polymorphism in exon 6 TNF-R2 that identified a SNP (T/G) at 196 codon, which leads to an amino acid substitution (Met/Arg). We found no statistically significant differences in allele and genotype frequencies between patients and controls for polymorphisms considered. In our recent work, we performed a molecular study of polymorphisms of many cytokines (IL-1α, IL-1β, IL-1R, IL-1R, IL-4Rα, IL-12, IFN-γ, TGF-β1, IL-2, IL-4, IL-6, IL-10) including -238 TNF-α on 70 ALS patients (unpublished data). Although no difference was found in allele frequencies of this polymorphism, we observed a statistical significance in AA genotype of the TNF-α -238 SNPs comparing ALS patients respect to healthy control. The most common G allele of -238 polymorphism of TNF-α gene is associated with high production of TNF-α (Huizinga et al., 1991; Wilson et al., 1997) but no data is available on the relationship between the minor allele and transcriptional level. Indeed the results obtained from studies on multiple sclerosis, a chronic inflammatory disease, suggest a possible protective effect of A allele in -238 position.

Our study shows that mRNAs of TNF-α were expressed at higher level in lymphocytes of sporadic ALS patients than in controls but there was not relationship with site of disease onset (spinal or bulbar), disease duration at the time of blood sample withdrawal or disease severity. We suppose that mRNA level increase may be due to an ALS disease's point common, as oxidative stress involvement, in all patients analyzed (Carrì et al., 1997; Ciriolo et al., 2002). Interaction between TNF-α, oxidative stress and SOD1 will be better described in the next paragraph.

4.3 TNF-α and SOD1 pathways

Several data support the hypothesis that TNF-α and SOD1 may not only take part to a common cellular pathway but they may also regulate directly and indirectly their self. In fact, an inverse correlation between the expression of SOD1 and TNF-α has been described: cytotoxic effects of TNF-α can be reduced by increasing levels of SOD1 as inhibition of SOD1 mRNA and protein may result in a decrease in the protective effect of SOD1 against inflammation (Meier et al.,1989; Wong & Goeddel ,1988).

In 2006 Afonso and collaborators demonstrated that, in U937 cells, TNF-α down-regulated SOD1 protein expression in a time-dependent manner (Alfonso et al., 2006). Afonso and colleagues performed different experiments treating U937 cells with TNF-α (10 ng/ml) for 1, 4 , 24 hours and their data showed a decline of SOD1 mRNA at 1 hour (22%), maximal suppressor at 4 h (54%) and lesser at 24h (38%).

Although SOD1 activity is modified in some specific situations, the direct effect of the proinflammatory cytokine TNF-α on SOD1 promoter has not been reported. Variable results were reported regarding SOD1 regulation by TNF-α (Chovolou et al.,2003), confirmed by gene expression studies that show the same tendency of SOD1 and TNF-α and suggest that these two genes may have a common system, or, at least, they may take part to the same one.

As concern SOD1 and TNF-α, it is well documented that both TNF-α and SOD1 pathways are regulated by reactive oxygen species (ROS) concentration and we suppose that oxidative stress is a common regulation point thought NF-kB activation.

The important role of ROS is reported in TNF-α signalling although it is unclear whether the TNF-α action may be producer or reducer of ROS concentration. In fact, TNF-α has been reported to increase ROS production from electron transport in mitochondria, plasma membrane NADPH oxidase and cytosolic phospholipase A2-linked cascade through signal transduction pathways triggered by TNFR-related proteins (Chandel et al., 2001; Micheau et al., 2003; Woo et al., 2000). Multimerization of TNFRs may lead to recruitment of TRAFs (TNFR-associated factors) by the receptors resulting in activation of kinases and transcription factors, such as c-Jun and NF-kB (Chandel et al., 2001).

About the reducer role, in a mice model it was demonstrated that TNF-α stimulation in mice deficient in TNF receptor-associated factor 2 (TRAF2) or p65 NF-kB subunit did not induce ROS accumulation, indicating that TRAF-mediated NF-kB activation normally suppresses the TNF-induced ROS accumulation (Sakon et al., 2003). ROS in lower concentrations may function as second messengers in mediating TNF-α activated signal transduction pathways that regulate the NF-kB system (Grisham et al.,1998; Janssen-Heininger et al.,2000).

As concern ROS role, Scott and collaborators demonstrated that ROS may up-regulate TACE activity and consequently, this increased activity may change TNF-α cleavage by TACE (Scott et al., 2011).

In fact, hydrogen peroxide serves as a messenger mediating directly or indirectly the activation of transcription factors such as NF-kB that mediates the induction of various proinflammatory genes (Schreck et al.,1991).
Regarding NF-kB, its pathway is also involved in SOD1, NF-κB was one of the first transcription factor shown to be redox-regulated. Rojo and colleagues (Rojo et al., 2004) showed that cell treatment with H_2O_2 initiates the PI3K/Akt cascades, which participates in NF-κB activation and in subsequent SOD1 transcriptional induction. Indeed, NF-κB binding site was identified in the human SOD1 promoter (GGTAAGTCCC) demonstrating that Akt-activated NF-κB presents increased binding to this sequence, mediating the up-regulation of SOD1 expression.
About ALS disease, we have already underlined the importance of NF-kB role in SOD1 activity, which altered expression and mutations are implicated in ALS disease. Our laboratory studied SOD1 mRNA expression (Gagliardi et al., 2010), we demonstrated that SOD1 mRNA level were altered in ALS patients. In fact we found that the SOD1 gene expression was increased in ALS patients than in controls population. Our unpubblished data show that TNF-α mRNA level is higher in patients' lymphocytes than controls as mRNA SOD1 gene. Unfortunately only few data are available about NF-kB and ALS, that may help to understand the relationship between SOD1 and ALS, so far NF-kB have been studied in ALS mouse model but the inhibition of NF-kB pathway has not effect on the progression of the disease (Crosio et al., 2011).

5. Therapeutic strategies

5.1 Classic immunotherapy in ALS

Several trials both controlled and uncontrolled using immunomodulating agents have been conduced in patients with ALS. These have included plasma exchange, steroids, azathioprine, cyclophosphamide, recombinant human IFN, cyclosporine, immunoglobulin, glatiramer acetate, minocycline.
High-dose therapy with intravenous immunoglobulins was used in ALS, the rationale was strengthened by observations that Ig was effective in improving the muscle strength of patients with a paraproteinemic or conduction block polyneuropathy and also in other autoimmune neuromuscular disease. The authors (Meucci et al. 1996, Dalakas et al. 1994) concluded that IVIg had no apparent therapeutic role in improving the symptoms or arresting the progression in ALS patients (Meucci et al. 1996). Meucci et al included in the study seven patients with a diagnosis of definite or probable ALS according to El Escorial criteria. All patients were treated with intravenous infusions of IVIg 0,4 g/kg/die for 5 consecutive days, followed by monthly, two day infusions at the same daily dosage for 4 to 13 months. All patients were concomitantly treated with oral cyclophosphamide, 1-2 mg/kg/die, as this therapy is effective delaying the frequency of IVIg maintenance infusions in other diseases. The response to treatment was assessed by the Medical Research Council rating scale for muscle strength on ten muscles per limb, a clinical bulbar function scale, a modified Rankin disability scale. The effect of treatment on the progression of the disease was evaluated by comparing the monthly rate of progression of upper and lower limb muscle weakness before and during treatment. All patients continued to deteriorate during treatment, reflected by the worsening of scores after treatment compared with the scores before therapy. The monthly rate of progression of limb weakness during therapy

was not better and possibly worse than that estimated in the period before therapy. No major side effect was reported by the patients.

Dalakas and collaborators (Dalakas et al. 1994) used intravenous infusions of high-dose immunoglobulin administered once a month for 3 months (total dose 2g/kg, divided into two daily doses) in nine ALS patients (El Escorial criteria) with a rapidly progressive course of disease. The efficacy of treatment was assessed by objective measurement of maximum voluntary isometric contraction in all muscle groups of two limbs or with Medical Research Council sores, before and after therapy. All patients worsened during the study and, by the end of the third month, their mean total muscle scores had declined. The pace of progression did not change during the observation period. All patients tolerated the intravenous immunoglobulin infusions well and adverse effects were noted.

Another approach was designed by Drachman lab's group (Drachman et al., 1994), who assessed a more powerful and prolonged immunosuppression obtained by total lymphoid irradiation (TLI) in ALS patients. The discovery that TLI produces powerful immunosuppression in humans led to its use in the treatment of autoimmune diseases. The basic principles of TLI therapy involve the lymphoid organs while shielding non-lymphoid tissues and delivering the radiation in multiple small fractions. The study included thirty patients with ALS. The radiation field consisted of an extended mantle, a para-aortic field and an inverted-Y including the spleen. Patients received anterior and posterior irradiation 5 days/week at a rate of 1,8 Gy/day. Blood counts were obtained 1 to 3 times/week as needed to detect haematological toxicity. Four types of parameters of motor function were evaluated: quantitative dynamometry (4 pairs of muscles in the upper extremities and 5 pairs of muscles in the lower extremities), manual muscle testing, functional tests (swallowing, breathing), activity indexes. Tests of immune function were: leukocytes (absolute lymphocyte counts, decrease in CD4 cells, CD4/CD8 ratio), cell-mediated immunity (negative conversion of skin tests), humoral immunity (tetanus antibody response). To assess whether the effectiveness of immunosuppression had an influence on the course of ALS, they analysed the relationships between parameters of immunosuppression and the measures of progression of ALS. This analysis showed that evidence of more effective immunosuppression did not correlate with a more favourable disease course.

Another immunotherapy tried in ALS was liquorpheresis (Andrich et al. 1996; Finsterer et al., 1999) with no results both in sporadic and familial ALS.

IFNs alpha and beta cytokines can regulate the major histocompatibility complex and the presentation of antigens to T-cell receptors. They have been used in variable doses for up to 6 months in small trials in ALS patients with negative results, however the small sample size, the possibility inadequate dose and the short period of follow-up prevented definite conclusions about the efficacy of IFNs. For these reasons a study was undertaken in which recombinant IFNbeta-1a was used in a large patient population at a dose twice as large as that found to be effective in patient with MS. Beghi et al. (2000) recruited patients with 6 to 24 months history of confirmed ALS, that received 12 mIU of IFN subcutaneously three times a week for 6 months and were followed up for an additional 6 months. Medical Research Council scale, Norris scale, bulbar scores were used to assess disability; selected electrophysiologic measures were also used. There were no significant differences of disease progression and disability in patients treated with IFN. Common adverse events were flu-like syndrome, local erythema, gastrointestinal symptomps.

Glatiramer acetate (GA) is a synthetic copolymer composed of four amino acids, used in MS for reduction of the frequency of relapses. GA induces a wide variety of actions on T-cells and leads to generalized, antigen-non-specific alterations of various types of antigen presenting cells in such a way that they stimulate Th2-like responses. It blocks the release of TNF-α and interleukin in monocytes and dendritic cells. So GA has neuroprotective as well as immunomodulatory actions. Meininger and collaborators (Meininger et al., 2009) recruited patients with El Escorial definite, probable or laboratory probable ALS of less than three years duration. Patients were given 40 mg GA daily for a period of 52 weeks. The prospectively defined primary efficacy outcome was the slope of ALSFRS score, the secondary efficacy outcome was time to death, tracheostomy or positive pressure ventilation more than 23 h per day. Additional functional endpoints included mean change from baseline and across visits in ALSFRS score, manuasl muscle testing score and slow vital capacity. GA was shown to be safe and well tolerated, the most significant adverse event was the injection site reaction. This study suggested that glatiramer acetate didn't show any beneficial effect in ALS patients either for course or survival.

Minocycline has anti-apoptotic and anti-inflammatory effects in vitro in CNS, so several trials are planned or are in progress to assess whether minocycline slows human neurodegeneration. Gordon (Gordon et al., 2007) did a multicentre trial in which patients with diagnosis of ALS (according to El Escorial criteria) received minocycline escalating doses of up to 400 mg/day for 9 months (started at 100 mg twice per day and increased every week by 50 mg twice per day to the highest dose of 400 mg). The primary outcome measures was the changes in ALSFRS-r, the secondary outcome measures were forced vital capacity, manual muscle testing, quality of life, time of tracheostomy, chronic assisted ventilation, survival and safety. ALSFRS-r score deterioration was faster in the minocycline group of patients and greater mortality during the 9-months treatment phase was registered in the same group.

Adverse events were most commonly reported in the respiratory system, gastrointestinal system (nausea, diarrhoea, costipation), neurological system (dizziness, fatigue).

5.2 TNF-α and new approaches in immunotherapy

The discovery, in 1988, of a naturally occurring TNF-α inhibitor in human urine (Seckinger et al., 1990), which was identified as a soluble form of the TNF-receptor that acted by neutralizing the cytokine, opened the way to immunotherapy. Subsequently two TNF-binding proteins were purified that were capable of inhibiting the binding of TNF-α to cells (Engelmann et al., 1990). The identification of soluble TNF-α receptors paved the way for the development of soluble TNF-α receptors antibodies currently used for the treatment of several systemic inflammatory diseases, including rheumatoid arthritis, juvenile polyarticular rheumatoid arthritis, inflammatory bowel diseases, psoriatic arthritis and ankylosing spondylitis (Sfikakis et al., 2010).

There are three anti-TNF-α agents approved for clinical use: Etancercept, Infliximab, Adalimumab. The latter two are full-length bivalent IgG monoclonal antibodies specific for sTNF and tmTNF, whereas Etanercept is a genetically engineered Fc fusion protein generated from the extracellular domain of human TNF-R2 and functions as a decoy receptor to block sTNF, tmTNF and distinct ligands of lymphotoxin, a TNF-related protein (Tracey et al., 2005).

The important side effects that have been most extensively related to TNF-α inhibitors include: lymphoma (hepatosplenic T-cell lymphoma in young patients being treated for

Chron disease and ulcerative colitis), infections (fungal infections such as histoplasmosis, coccidioidomycosis, blastomycosis and tuberculosis), congestive heart failure, demyelinating disease, a lupus-like syndrome, induction of auto-antibodies, injection site reactions and systemic side effects (Scheinfeld et al., 2006).

Clinical trials examining the effects of TNF-α inhibition have been conducted on patients with Multiple Sclerosis (MS) and Alzheimer disease (AD).

Strategies to inhibit TNF-α in MS seemed promising in preclinical applications but have widely failed in human clinical trials due to the lack of therapeutic selectivity. During an open-label phase I trial, a monoclonal TNF-α antibody was infused into two human patients exhibiting rapidly progressing disease. Subsequently, in a double-blinded, placebo controlled, multicentered phase II study, 168 relapsing-remitting MS patients were administered Lenercept, a sTNF-R1 fusion protein that neutralizes TNF-α. Lenercept-treated individuals experienced higher occurrence of relapse and increased neurological deficits (Van oosten et al., 1996). The ineffectiveness of anti-TNF-α therapy in MS may be a consequence of divergent roles for the TNF receptors, considering that blocking TNF-R1 in mouse models dampens disease severity, while suppressing TNF-R2, the receptor that induces remyelination and harbors immunosuppressive properties, results in exacerbated disease (Arnett et al., 2001; Kassiotis et al., 2011). Recently, pharmacological agents selectively targeting TNF-R1 have been investigated. Using phage display technology, a TNF-R1 antagonist was developed and upon evaluation in mice it was found that administration of this selective antagonist improved clinical scores, reduced cerebral demyelination and suppressed the number of infiltrating inflammatory cells (Nomura et al., 2011).

TNF-α intervention in AD has been evaluated in open-labeled phase I clinical trials where perispinal extrathecal administration of Etanercept was administered weekly to a small number of patients ranging from mild to severe AD for a short duration of 6 months that claimed substantial cognitive and behavioural improvements, including verbal fluency and aphasia (Tobinick et al., 2006; Tobinick et al., 2008). Currently a phase II study is recruiting to evaluate the safety and tolerability of Etanercept in AD. These results seem promising but conclusions regarding the promise of such a therapeutic strategy should be reserved until after extensive chronic suppression of TNF-α activity is performed in preclinical models and double-blind human clinical trials have been conducted and results critically reviewed by the research community.

Trails on the immunological hypothesis in ALS are not yet established although TNF-α system implications have been described for a long time. This therapeutic approach was not considered using neither synthetic TNF-α-receptor inhibitors nor monoclonal anti-TNF-α antibody.

6. Conclusions

There is no doubt that TNF-α play key roles in degenerative conditions afflicting CNS, also in ALS. The precise role TNF-α plays remain highly controversial due to the complexity and pleiotropic nature of this cytokine and its activities during critical developmental and homeostatic cellular processes. Multiple factors determine whether TNF-α will exert deleterious or beneficial effects for neuronal survival and some of these differential actions relate to its duration of expression, concentration, receptor conformation. Despite the

elaborate and promising data collected thus far to assign function to TNF-α in neurodegeneration, surprisingly little is still known about the cellular and stage-specific roles of this cytokine.

The data reported in this chapter also underline the importance of TNF-α pathways in ALS pathology due to the interaction with SOD1 gene.

In fact, the data that demonstrated the down-regulation of SOD1 after treatment with TNF-α (Afonso et al., 2006), related to the up-regulation of SOD1 mRNA expression in ALS patients suggest to carry on the studies about TNF-α in ALS disease to better define the TNF-α function in neurodegeneration. A better understanding of SOD1 regulation related to TNF-α function may permit to develop novel immunotherapy application in ALS disease.

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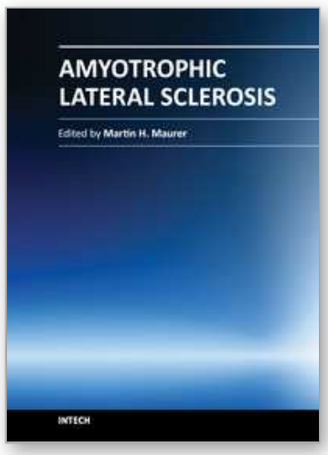

Amyotrophic Lateral Sclerosis

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Though considerable amount of research, both pre-clinical and clinical, has been conducted during recent years, Amyotrophic Lateral Sclerosis (ALS) remains one of the mysterious diseases of the 21st century. Great efforts have been made to develop pathophysiological models and to clarify the underlying pathology, and with novel instruments in genetics and transgenic techniques, the aim for finding a durable cure comes into scope. On the other hand, most pharmacological trials failed to show a benefit for ALS patients. In this book, the reader will find a compilation of state-of-the-art reviews about the etiology, epidemiology, and pathophysiology of ALS, the molecular basis of disease progression and clinical manifestations, the genetics familial ALS, as well as novel diagnostic criteria in the field of electrophysiology. An overview over all relevant pharmacological trials in ALS patients is also included, while the book concludes with a discussion on current advances and future trends in ALS research.

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