PhD PROGRAM IN TRANSLATIONAL AND MOULECULAR MEDICINE

DIMET

UNIVERSITY OF MILANO BICOCCA

SCHOOL OF MEDICINE AND SCHOOL OF SCIENCE

Activation state and functionality of dendritic cells from peripheral blood of amyotrophic lateral sclerosis patients

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Se vuoi andare veloce,vai da solo.
Se vuoi andare lontano, cammina in compagnia.

Alla mia famiglia, ai miei amici, a chi mi protegge da lontano
perché solo grazie a loro sono la persona che sono.

Summary

Abbreviation list

DCs: Dendritic cells

MHC: Major histocompatibility complex

APCs: Antigen presenting cells

PRR: Pattern recognition receptors

PAMPs: Pathogen-associated molecular patterns

TLRs: Toll-like receptors

LPS: Lipopolysaccharide

DAMPs: Damage-associated molecular patterns

HMGb1: Chromatin-associated protein high-mobility group box 1

HSP: Heat shock protein

LN: limph nodes

CDP: common derived progenitor

NK: natural killer cells

CMP: common myeloid progenitor cells

CLP: common lymphoid progenitor cells

LC: Langherans Cells

T_H1: T helper 1

T_H17: T helper 17

T_H2: T helper 2

pDC: plasmacytoid dendritic cells

TCRs: T cell receptors

IL: interleukine

TNFα: Tumor necrosis factor α

ALS: Amyotrophic lateral sclerosis

NIV: Non invasive mechanic ventilation

PEG: percutaneous endoscopic gastrostomy

RIG: radiologically inserted gastrostomy

CFS: cerebro spinal fluid

CNS: central nervous system

ALSFRS-r: amyotrophic lateral sclerosis functional rating scale revised

TGFβ: tumor growth factor beta

IFNγ: interferon gamma

CTR: healthy controls

EDTA: ethylenediaminetetraacetic acid

FACS: fluorescence-activated cell sorting

MFI: mean fluorescence intensity.

Chapter 1: Introduction

1.1 Innate Immunity

All living organisms have evolved strategies to protect themselves from infections. In higher organisms these varied and numerous strategies are performed by the immune system.

The mammalian immune system is composed by two different, but interrelated arms: the innate and the adaptive immune system.

The innate immune system is evolutionarily ancient and represent the first line of defense against infectious organisms as it rapidly controls the replication of the infecting pathogens and it is highly efficient to combat infections. It comprises myeloid cells, including granulocytes, mast cells, macrophage, dendritic cells (DC) and soluble factors (antimicrobial peptides and complement pathways). The innate immune system detects infection using a limited number of germ-line encoded receptors able to recognize invariant molecular structures typical of classes of microbes.

The adaptive immune system is based on antigen-specific responses of T and B lymphocytes. It is characterized by specificity and uses randomly generated, clonally expressed, receptors produced via somatic recombination. This allows to generate a highly specific and vast repertoire of lymphocytes

that leads to the generation of immunological memory. It appears only recently, at the time of the differentiation of vertebrates, it is highly specific, but temporally delayed. It is involved in the elimination of pathogens in the late phases of infection because it takes three to five days to produce sufficient numbers of clones and to differentiate into effector cells. Moreover primary immune responses of T and B cells can be induced only in organized secondary lymphoid organs. [1]

The combination of innate and adaptive immunity makes hosts able to recognize and eliminate invading pathogens with high efficacy and minimal damage to self and it is also able to provide protection from re-infection with the same pathogen.

The adaptive immune system cannot distinguish between self and non self because of the use of randomly generated receptors for antigen recognition. It must be instructed regarding the origin of an antigen by the innate immune system that can determine whether an antigen is derived from infectious non self, innocuous non self or self. Janeway postulated that the innate immunity would use germ line encoded receptors (PRRs) to sense the presence of an infection via recognition of conserved microbial pathogen-associated molecular patterns (PAMPs). This PAMPs have to be absent from eukaryotic cells and unique to microbes, they have to be common to a broad class of of microbes so that a limited number of germ-line encoded receptors could detect all infections and, in the end, they have to be essential for the life

of the microbe so that they could not be easily eliminated via mutation.

The adaptive immune system is able to integrate information from PPRs and other different signals from innate immune system to decide the correct response for each infection. [2]

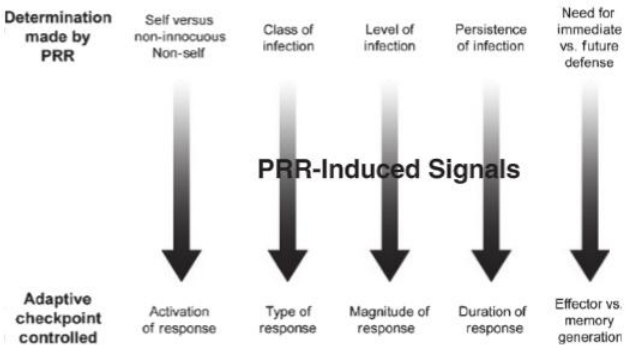


Fig.1 PRR-mediated control of checkpoints of adaptive immunity [2]

Pattern recognition receptors are able to detect the presence of an infection and PPRs –induced signal controls adaptive immunity. A failure in this system can lead to various immune pathologies including autoimmunity, allergy, immunopathology and failure to protect from infection.

Different PRRs react with specific PAMPs that can vary in their molecular structure and nature. They can be proteins, lipids, lipoprotein, components of the bacterial cell wall nucleic acids, and are able to activate specific signaling pathways leading to distinct antipathogen response.

When PAMPs are recognized and bound by PPRs they activate specific signal transduction pathways leading to the production of inflammatory cytokines and to the expression of many immune-related genes.

Among signaling receptors, Toll-like receptors (TLRs) are the best characterized family. They are the homologues of drosophila toll (a component of a signaling pathway that controls dorsoventral polarity in fly embryos). [4] Members of this family have a key role in the induction of immune and inflammatory responses in mammals. The first human toll to be characterized (TLR4) was shown to induce, like in drosophila, the activation of NF-κB signaling pathway leading to the expression of a large number of cytokines and costimulatory molecules fundamental for the adaptive immunity [5]

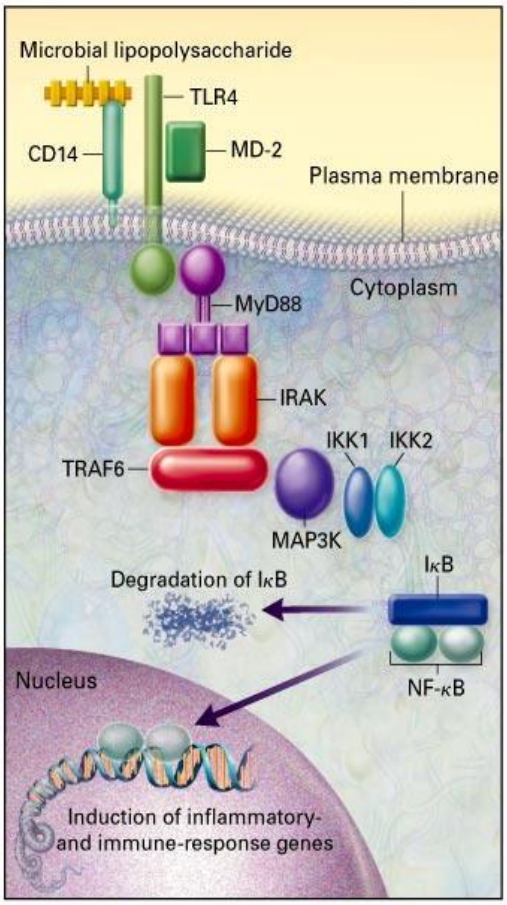


Fig.2 The signaling pathway of Toll Like Receptor 4 [5]

The recognition of lipopolysaccharide is mediated by three different gene products: CD14, toll-like receptor 4 (TLR4), and MD-2. The binding of lipopolysaccharide to CD14 leads to the association of CD14 with the TLR4–MD-2 complex and induce the dimerization of TLR4. The activation of TLR4 induce a signal transduction pathway that leads to the IκB degradation and to the relase of NF-κB, which moves into the nucleus and induces the transcriptional activation of a wide variety of inflammatory- and immune-response genes.

The adaptive immune system is able to recognize a pathogen only after it has been detected by the innate immune system. Antigen presenting cells expose peptides bound to MHC class II molecules on their surfaces and T cells use their antigen receptor to recognize those ligands. However these peptides could be either self or not self and T cells are not able to discriminate between the two categories. The recognition of the peptide-MHC- ligand is not sufficient to activate T-cells that requires at least to signals: one is the complex of a peptide and an MHC molecule and the other is a costimulatory signal mediated by, for example, CD80 and CD86 molecules on the surface of APCs. In the absence of this second signal T cells become permanently inactivated or apoptotic. The expression of CD80 and CD86 is regulated by the innate immune system. When TLRs and the other PPRs recognize a PAMP they induce the upregulation of these molecules on the APCs, so this occurs only when there is an infection. [5](Fig.3)

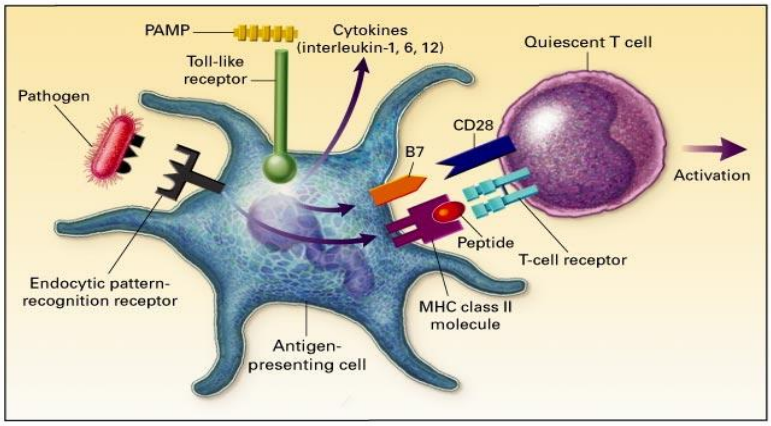


Fig.3 Interplay between Innate and Adaptive Immunity [5]

Recognition of PAMPs by PRRs generates signals that activate the adaptive immune system. Endocytic pattern-recognition receptors mediate the uptake and phagocytosis of pathogens by APCs (macrophages and DCs).Proteins derived from the microorganisms are processed and exposed in association with MHC class II molecules on the surface of the APCs and they are recognized by T-cell receptors. TLRs, instead, leads to the activation of signaling pathways that induce the expression of cytokines, chemokines, and costimulatory molecules.

PRRs are also able to recognize endogenous molecules released during cellular injury or stress conditions and non-infectious material that can produce tissue damage, causing the so called “sterile inflammation”. Some condition of sterile inflammation could be for example: ischemia riperfusion injury, tumor, autoimmunity, chronic inflammation and transplantation. Polly Matzinger proposed a new model called “danger model” in

which these molecules are defined damage-associated molecular patterns (DAMPs) (Fig. 4) [6]

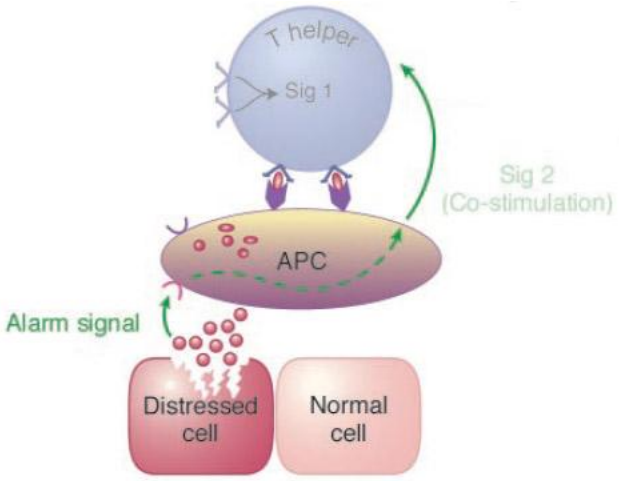


Fig.4 Danger Model [7]

APC are activated by endogenous cellular alarm signals from distressed or injured cells.

DAMPs are all endogenous factors. They are hidden from the immune system because they are usually sequestered intracellularly in physiological conditions. However these molecules can be released into the extracellular environment by dying cells and under stress condition. [8] Example of DAMPs are chromatin-associated protein high-mobility group box 1 (HMGb1), a nuclear protein that bind DNA in a non specific manner able to modulate transcription and chromatin remodeling [9], heat shock proteins (HSPs) [10] and purine metabolites such as ATP [11] and uric acid [12]

1.2 Dendritic Cells

DCs are a population of leukocytes with migratory property specialized for the uptake, transport, processing and presentation of antigens to T cells. [13] DCs comprise multiple subsets, all bone marrow derived, that differentiate from different precursors, have distinct functions and colonize different tissues (peripheral and lymphoid organs). [14]When they are in an “immature” stage their principal role is to patrol all peripheral tissue sensing the environment. Any encounter with products generated by tissue damage or microbes, initiate the migration of DCs to lymph nodes (LNs). DCs can sense the presence of those products thanks to microbial sensor and other receptors (PRRs) able to recognize PAMPs and DAMPs and they can start a signaling cascade that leads to the DC maturation. [15] Furthermore DCs are equipped with the biochemical machinery for processing and presenting antigens on MHC molecules. [16]APCs, and in this specific case DCs, usually present endogenous antigens (from self component or viral infection) on MHC class I, whereas they usually present exogenous antigens on MHC class II. The consequence of DCs-T interaction is not only T-cells proliferation, but DCs can also influence the subsequent development of these dividing T cells leading to immunity or tolerance and generation/activation of effector or regulatory T cells; moreover the interaction with DCs can instruct T cells to produce different pattern of

cytokines including those responsible for T helper 1 (T_H1) and T helper 2 (T_H2) polarization. (Fig 5) [17]

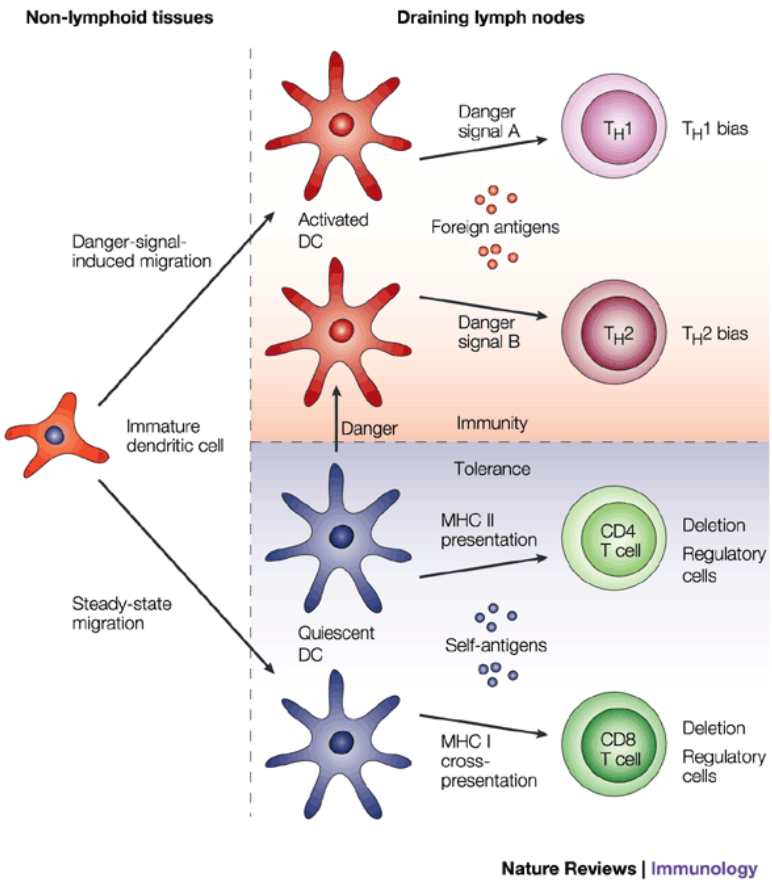


Fig 5: Dendritic – T cells interaction and its outcome. [18]

In the absence of microbial infections and related “danger” signals, there is a low level entry of DCs into lymphoid tissue, in which quiescent DCs help to maintain T-cell tolerance to self. DCs become activated and increase their rate of migration in the presence of microbial infection, inflammation and tissue damage. In lymphoid tissues activated DCs encounter antigen specific T cells and initiate the adaptive immune response.

1.2.1 DC heterogeneity and functions.

DCs are present in all the body, including lymphoid and non-lymphoid tissues, where they have different function such as T cell activation, interaction with others cell types (NK cells and B cells) and cytokine production. These different functions are performed by different subsets on the basis of their ontology:

- the conventional or classical DCs (cDCs),
- the Langherans cells (LCs),
- the plasmacytoid DCs (pDCs),
- the monocyte derived DCs (moDCs).

$CD34^+$ hematopoietic stem cells differentiate into common lymphoid progenitor cells (CLP) and common myeloid progenitor cells (CMP) that undergo another differentiation into $CD34^+CLA^+$ and $CD34^+CLA^-$ late progenitor cells. $CD34^+CLA^+$ cells then become $CD11c^+$ $CD1a^+$ Langherans precursor cells. These precursors in the blood migrate into skin epidermidis where they fully differentiate into Langherans cells. In the same way $CD34^+CLA^-$ cells differentiate into $CD11c^+$ $CD1a^-$ interstitial precursor in blood that migrate into the skin dermis and other tissues to become interstitial DCs. Without antigen/pathogen stimulation, both Langerhans cells and interstitial DCs may undergo a steady-state migration into the draining lymph nodes, where they may play a critical role in immune tolerance. Upon microbial invasion and inflammation, Langerhans cells and

interstitial DCs rapidly migrate into the draining lymph nodes. They undergo maturation and initiate primary immune responses. CMP and CLP also give rise to myeloid pre-DC1s and lymphoid pre-DC2s in bone marrow. They migrate into the blood and then to the lymphoid tissues. During bacterial infection, pre-DC1s ingest and kill bacteria and then differentiate into DCs and initiate adaptive antibacterial immune responses. During viral infection, pre-DC2s rapidly produce large amounts of type-1 IFN and then differentiate into DCs and initiate adaptive antiviral immune responses (Fig. 6).

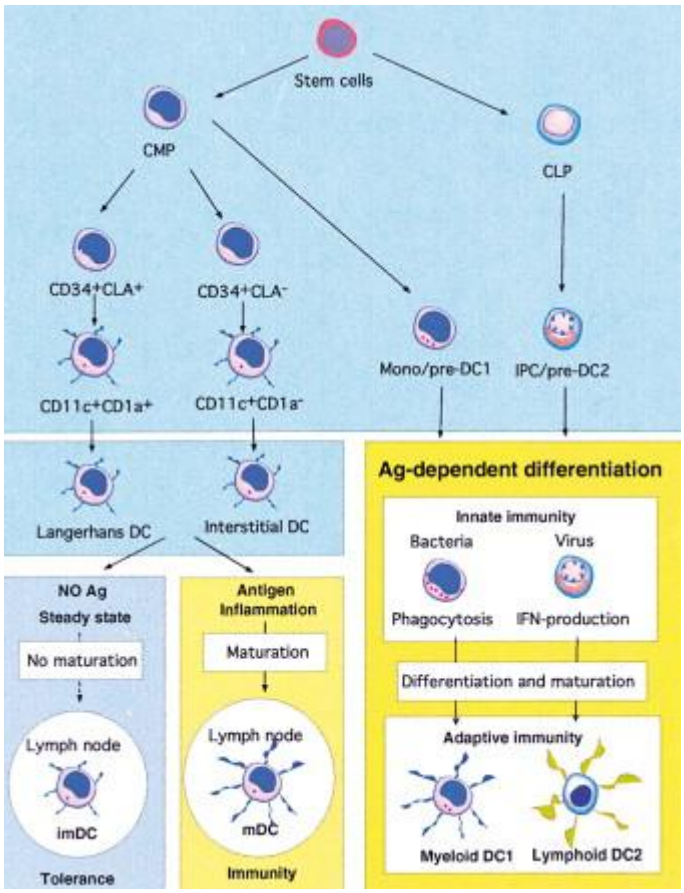


Fig 6: DC Development, Diversification, Maturation, and Function [15]

Schematic representation of Maturation process and function of different DCs subsets.

1.2.2 Human Dendritic Cells.

Human DCs arise from the bone marrow through a series of not well known precursor that may have both myeloid and lymphoid ancestor. [20] Thanks to recent studies were delineated a small number of DCs distributed in all mammals. (Fig 7)

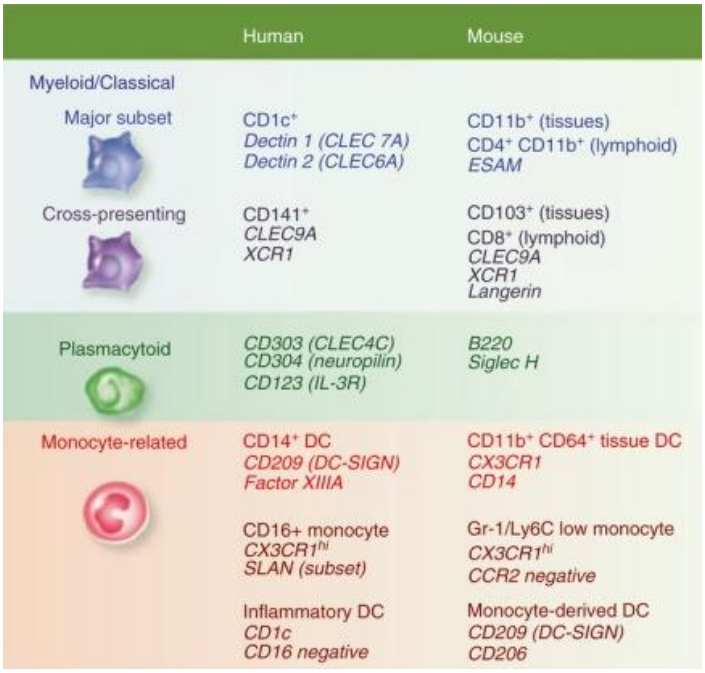


Fig 7: Surface markers of the major human DC population and their mouse homologues [21]

Myeloid DCs and mouse conventional DCs contain a major subset and a minor cross presenting subset. Plasmacytoid DCs are easily recognizable in a lot of species and monocyte related DCs include a subset of CD11b+ cells that may be homologues of CD14+. Inflammatory monocyte-derived DCs are heterogeneous.

All DCs in humans express high level of MHC class II (HLA-DR) and lack CD3, typical T-cells marker, CD19/20, typical B-cells marker and CD56, typical natural killer cells (NKs) marker.

CD1c $^{+}$ myeloid dendritic cells.

This subset of DCs are the major population of mDC in blood (approximately 1% of circulating mononuclear cells) and lymphoid organs. They were initially identified in blood as HLA-DR^{+}, $lineage^{-}$ with the expression of myeloid antigens such as CD11b, CD11c, CD13, CD33, CD172 and CD45RO [22]. In tissue human $CD1c^{+}$ express also CD1a, like Langherans Cells (LCs), but lack Langerin. They also appears more activated than their blood counterparts in terms of CD80, CD86, CD83 and CD40 expression [23] In LN $CD1c^{+}$ DCs are interdigitaling cells of T-cell areas. Tonsil and spleen also contain this DC subset.

$CD1c^{+}$ DCs are equipped with a wide range of TLRs that are fundamental for antigen uptake, transport and presentation. They respond well to LPS, flagellin, polyIC and R848 thanks to TLR1-8 and the expression of Dectin-1 and Dectin-2 suggest a role for these cells in anti-fungi immunity. Furthermore they express DEC205 and macrophage mannose receptor in a variable manner.

CD1c^{+} DCs are a good stimulator of naïve CD4^{+} T cells, but they are less efficient than CD141^{+} in antigen cross presentation to CD8+ T cells. [24]

They secrete many cytokines, TNFα, IL-8 and IL-10 when they are stimulated and also a small amount of IL-23 can be detected with a range of stimuli. This highlights the plasticity of DCs in different contexts and their ability to induce T_H1 or T_H17 response. [25]

CD141^{high} myeloid dendritic cells

CD141^{+} DCs are a subpopulation of myeloid dendritic cells represent the 0,1% of circulating mononuclear cells. Other CD141^{+} DCs were founded also in lymph node, tonsil, spleen, bone marrow and in non-lymphoid tissue such as skin, lung and liver. [26]

This human DCs subset, considered the homologue of CD8^{+}/CD103^{+} mouse DCs, have an augmented ability to take up dead or necrotic cells via CLEC9A, sense viral nucleic acids with TLR3 and to cross-present antigen to CD8^{+} T cells clones in vitro. They secrete TNFα, CXCL10 and interferon-λ. [26]

Plasmacytoid dendritic cells

pDCs are the most abundant DCs in blood. They lack CD11b, CD11c, CD13 and CD33: all myeloid markers, but express CD45RA, variable CD2 and CD7 and may harbor T-cell receptor and immunoglobulin rearrangement. pDCs are

distinguished from conventional DCs thanks to the expression of positive marker: CD123 (IL-3R), CD303 (CLEC4C, BDCA-2) and CD304 (neutropilin, BDCA-4). They are present in LN and they are rapidly recruited in condition of inflammation. They have enhanced secretory capacity and in particular they release type I interferons in response to viral infection maybe because they express high levels of TLR7 and TLR9. [27]

Langerhans cells

LC are able to form a network in the supra-basal epidermis and other squamous ephitelia where they reside. These cells express high levels of the C-type lectin, Langerin and CD1a, a non polymorphic class I MHC molecule. They also can be found in the LN para-cortex as langerin^{+} CD1a^{high} interdigitating cells. The function of LCs in immunity has been difficult to pin down. They can mature into potent cross-presenting DCs but also lack critical TLRs [30]and can induce regulatory T cells and IL22 production through CD1a-restricted antigen to autologous T-cells. [28]

1.2.3 Dendritic Cells control T-cell polarization

After their generation in the bone marrow immature DCs migrate to sites in which pathogens can potentially enter. From these tissues, like skin and mucosae, they can homeostatically reach lymph nodes in which they interact with T cells. DCs activation by DAMPs or PAMPs leads to an acceleration of this migration process and to a maturation of DCs that undergo a functional change losing their endocytic capacity. T cells and DCs need to interact creating an immunological synapse and the fate of T cells after that contact depends on three factors:

1) the ligation of T cell receptors (TCRs) by peptides derived from pathogens presented by MHC class II molecules on the DCs surface (signal 1),
2) the presence and level of costimulatory molecules given that in absence of this co stimulation T cells become anergic (signal 2),
3) the cytokines secreted by DCs that are able to polarize T cells (Fig. 8) (signal 3). [29]

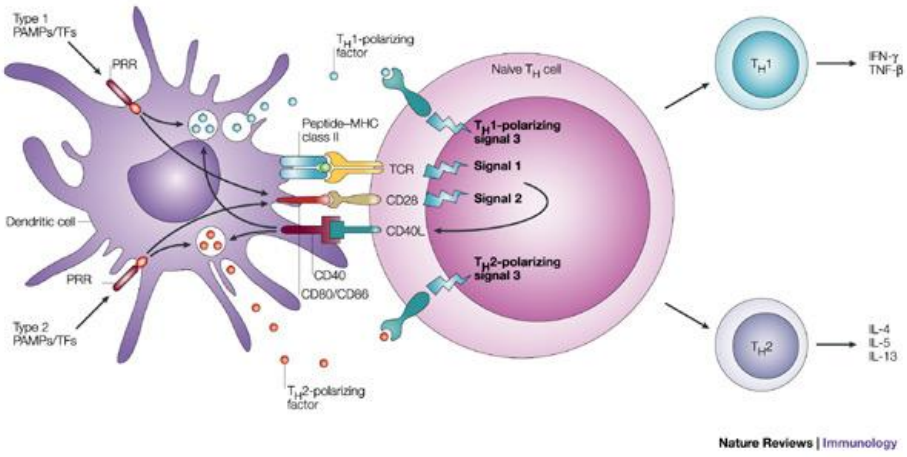


Fig 8: T cell activation and polarization need three signals derived from DCs [30]

The first signal required is the antigen-specific one. It comes from the engagement of TCR by MHC class II associated peptides. These peptides are the results of the processing of pathogen after their internalization. The second signal is represented by costimulation. It involves mainly the binding of CD28 on T cells with CD80 or CD86 that are expressed by DCs after ligation of PPRs by PAMPs or DAMPs. The third and last signal is the polarizing one. It is mediated by various membrane bound or soluble factors that promote the development of T_H1 or T_H2 cells. The nature of signal 3 depends on the activation of PPRs by PAMPs and DAMPs that could be responsible for the selective DCs priming for the production of high levels of T_H1-cell-polarizing or T_H2-cell polarizing factors.

The expression by DCs of T_H-cell polarizing factors depend on the way DCs are activated.

There are about three networks of inflammatory secondary messengers that are associated with the

development of effector T_H-cell subset. A first network is associated with the development of T_H1 cells and can be activated by viruses or intracellular bacteria. The response to these pathogens are dominated by TLR3, TLR4, TLR7 or TLR9 mediated production of type I interferon, IL18 and T_H1-associated chemokines, and to a lesser extent by IL12 and IFNα. This network promotes the development and function of T_H1 cells and cytotoxic T lymphocytes (CTLs). [31] A second network of factors is associated with infection with extracellular worms and the development of T_H2 cells. [32] The third network, instead, results in the downregulation of immunity and the induction of tolerance with the production of IL10 and TGFβ. [33] (Fig. 9)

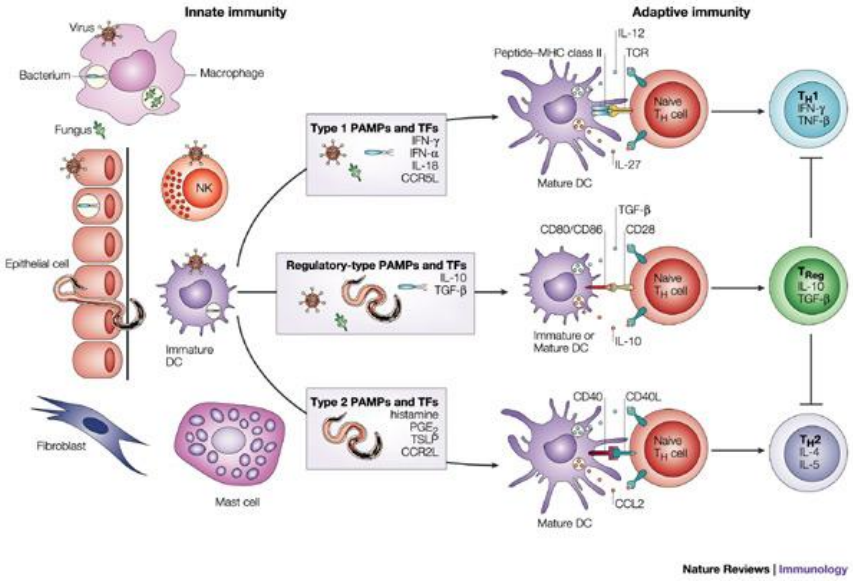


Fig 9: Different types of DAMPs and PAMPs influence DCs and T cells polarization. [30]

DCs can be polarized by type 1, type2 and regulatory type DAMPs and PAMPs and they can promote the development of naïve T cells into T_H1, T_H2 or T_{reg}

1.3 Cytokines: soluble mediator of immunity

Cytokines are small soluble protein that have a role in the interaction and communication between cells. The cytokine family includes:

- Lymphokine, made by lymphocytes
- Monokine, made by monocytes
- Chemokine, with chemotattic activity
- Interleukines (IL), made by leucocytes and able to have an action on other leucocytes.

These small proteins are able to exert their function in autocrine, paracrine or endocrine manner. They are produced by different cells type but the principal producer are T_H cells and macrophages. [34] Also dendritic cells are able to produce cytokines and they are susceptible to cytokine-mediated activation. [35]

Interleukin 1 and its family

This family of cytokines plays a fundamental role in up-regulation of inflammation and host defence. Until now up to eleven members of this family are identified but only five are completely characterized (IL-1α, IL-1β, IL-18, IL-1RA, IL-33) [36]

IL-1β is generated from its inactive precursors: pro-IL-1β. After the cleavage by caspase 1 the pro IL-1β becomes IL-1β that can bind to IL-1R1, which enables recruitment of the IL-

1RAcP co-receptor. A cascade of downstream events causes the activation of signalling proteins, such as mitogen-activated kinases (JNK, p38, ERK1/2), as well as transcription factors, including NFκB (p65 and p50 subunits) and c-Jun (a subunit of AP-1), which control the expression of a number of inflammatory and catabolic genes. [35](Fig. 10)

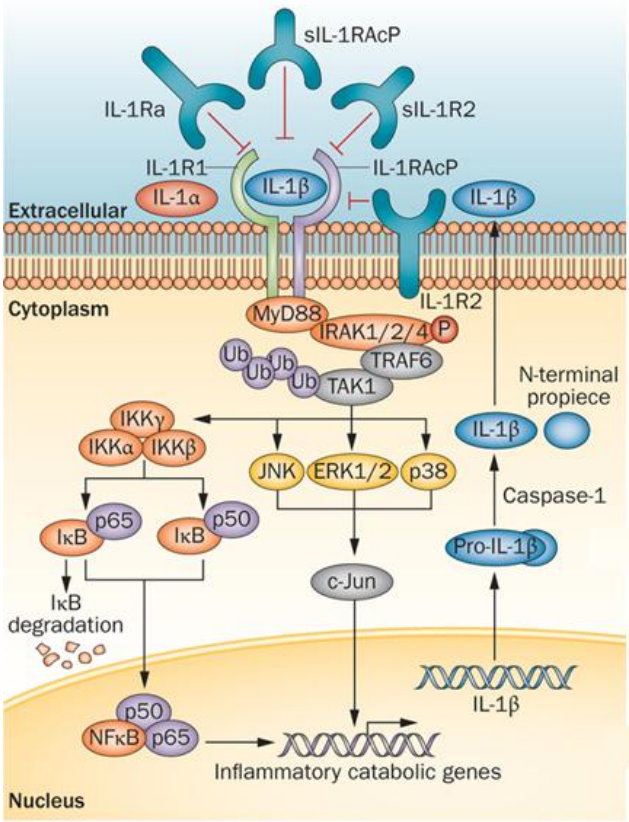


Fig 10: IL-1β synthesis and transduction pathway [37]

Schematic representation of synthesis and signaling transduction pathway of IL1β

Interleukin 6 and its family

IL-6 family is composed by seven members that have both pro-inflammatory and anti inflammatory properties and have an important role in hemopoiesis and innate and adaptive response. The members of this family are: IL-6, IL-11, leukaemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), oncostatin M (OSM), cardiotrophin-1 (CT-1) and cardiotrophin like-cytokine (CLC).

Activation of IL-6 signalling is mediated through the IL-6 or soluble IL-6 receptor/IL-6 complex in a process known as “trans-signaling”, unique example of a soluble cytokine receptor displaying agonistic effects. IL-6 is likely to be involved in the pathogenesis of inflammatory and autoimmune diseases through the dimerization of STAT3 and transcription of acute fase genes. (Fig.11)

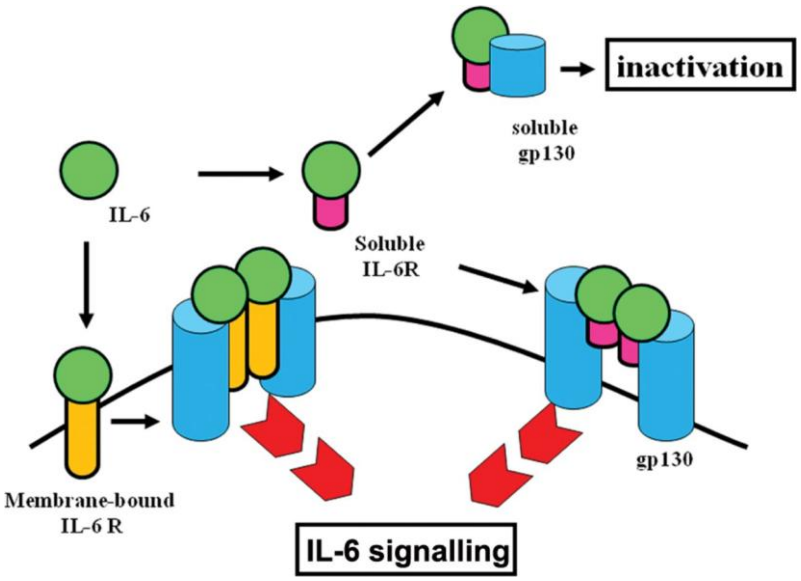


Fig 11: IL-6 signaling pathway. [38]

IL-6 can signal in two different ways. In the classical signaling pathway IL-6 binds to the IL-6R on target cells. Then the complex of IL-6 and IL-6R contacts the gp130. In the trans-signaling pathway a soluble type of the IL-6 receptor (sIL-6R) binds to IL-6 and interplays with gp130. Soluble form of gp130 can have inhibitory functions. Both pathway leads to STAT3 dimerization and nuclear translocation.

Interleukin 12 and its family

IL-12 is an heterodimeric cytokine composed by two subunits with different molecular weight named p35 and p40. It is produced by activated myeloid DCs and has a fundamental role in the differentiation and expansion of T_H1 cells. [39] IL-23, a cytokine involved in the development of many autoimmune diseases, such as inflammatory bowel disease [40], share with IL-

12 the p40 subunity. Other cytokines belonging to this family are IL-27 and IL-35. (Fig.12)

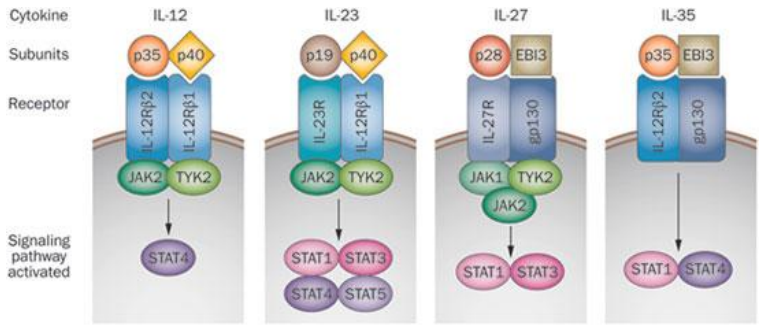


Fig12: IL-12 family. [38]

Cytokines belonging to IL-12 family share subunit and receptor components. In addition to their structural relationship, the IL-12 family members activate overlapping JAK–STAT-signaling pathways

Tumor Necrosis Factor α superfamily

The tumor necrosis factor α (TNFα) is a pro inflammatory cytokine whose disregulation was proposed to contribute to the pathogenesis of different disease such as autoimmune disease, insulin resistance and cancer. It was originally identified as products of lymphocytes and macrophages that cause the lysis of certain types of cells, especially tumor cells. [41] Its superfamily is composed of 19 ligands and 29 receptors that play highly diversified roles in the body. TNFα induces different types of signals including NF-κB activation, apoptosis pathways, extracellular signal-regulated kinase (ERK), p38 mitogen-

activated protein kinase (p38MAPK), and c-Jun N-terminal kinases. The pro-inflammatory effect of TNF is mediated through NF-κB–regulated proteins, such as IL-6, IL-8, IL-18, chemokines, inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and 5-lipoxygenase (5-LOX). Moreover, TNFα can induce the expression of TNFα itself through activation of NF-κB. [42]

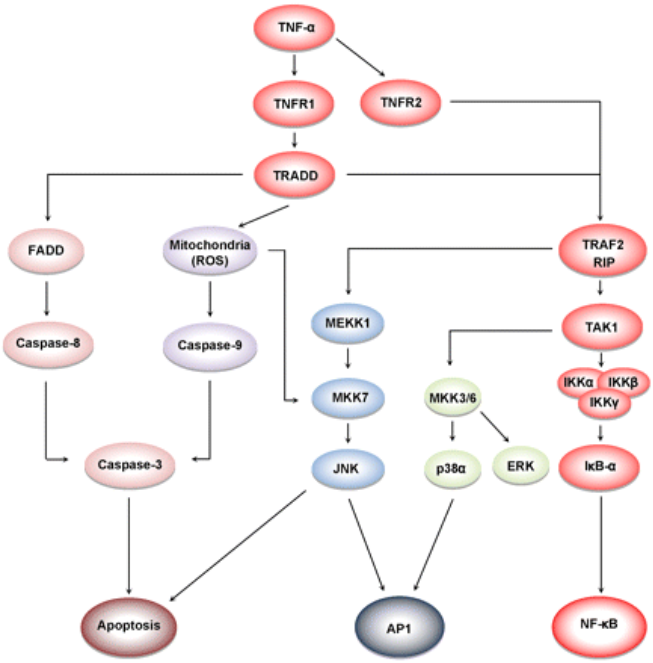


Fig 13: TNFα signaling pathway. [43]

TNFα binds two different receptor causing apoptosis, cellular proliferation through AP1 and NF-κB activation.

Interleukin 10

IL-10 is an anti-inflammatory cytokine produced by macrophage, dendritic cells, B cells and various subset of T cells. It exert its anti-inflammatory role through the inhibition of some pro-inflammatory cytokines (including IL-1α and β, IL-6, IL-12, IL-18, and TNF-α) and chemokine production (CCL2, MCP5, RANTES, IL-8, IP-10, and MIP-2).[44] Moreover IL-10 is able to inhibit costimulatory molecules expression on monocytes and macrophages and to prevent DC trafficking at lymph nodes with a consequent failure in recruitment and induction of TH1 cells from naive T cells, through an autocrine action that leads to inhibitions of chemokines production.[45]

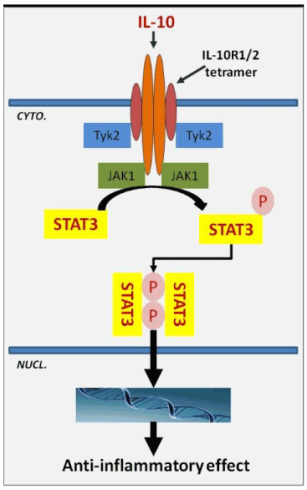


Fig 14: IL10 signaling pathway [46]

IL-10 binding starts an intracellular signaling pathway involving STAT3 as key translocation nuclear factor which induces the activation of specific gene encoding for anti-inflammatory factors.

Chemokines Superfamily: CCL2 and IL8

Chemokines are a group of cytokines able to induce chemotaxis as suggest their name (CHEMOtactic cytoKYNES) and they represent a family of low molecular weight secreted protein. Their secretion occurs in response to signals such as proinflammatory cytokines and they have a role is in the recruitment of monocytes, neutrophils and lymphocytes but, some of them, can exert other functions. Once induced, the directed migration of cells expressing the appropriate chemokine receptors (thanks to the binding to specific cell surface transmembrane receptors coupled with heterotrimeric G proteins, whose activation leads to the initiation of intracellular signaling cascades) occurs along a chemical ligand gradient, known as the “chemokine gradient”. This allows cells to move toward high local concentrations of chemokines. [47]

Chemokine are divided in different groups on the basis of the presence of differently conserved cysteine residues. In fact we have C-C chemokines including monocyte chemoattractant protein (also called CCL2), RANTES, MIP-1α and MIP-1β; C-X-C chemokines such as IL8; C chemokine like lymphoactin and CX3C chemokines represented by fraktaline. [34]

MCP-1, also known as CCL2, is a potent chemoattractant for monocytes. It is a member of C-C chemokine family and is produced by different cell types either constitutively or after induction by cytokines, oxidative stress or

growth factors. This protein is the most studied between human chemokines because has been reported that it can be useful in the treatment of some diseases such as multiple sclerosis [48], rheumatoid arthritis [49], atherosclerosis [50] and insulin-resistant diabetes [51]. CCR2 is the receptor for CCL2 and the receptor-ligand binding can result both in pro-inflammatory and anti-inflammatory action. The first one is dependent on CCR2 presence on APCs and T cells, whereas the second one is due to its expression on T_{reg}. In the CNS it is expressed mostly in astrocytes but also in neurons, microglia and macrophages after ischemia, hypoxia or exicitotoxicity and its excessive presence could exacerbate an injury. [81]

IL-8 also known as CXCL8 is the most powerful chemoattractant for neutrophils and was detected for the first time more than twenty years ago. The most interesting properties of IL-8 is its ability to change its expression levels. In healthy tissues, in fact, IL8 is express at very low levels, but it is rapidly induced by 10 to 100 fold in response to inflammatory stimuli such as TNFα, IL1, cellular stress or bacterial and viral products

1.4 Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease, also known as Lou Gehrig's disease, described for the first time by Jean Martin Charcot in 1800. It affects motor neurons in the cortex, brainstem and spinal cord (Fig. 15).

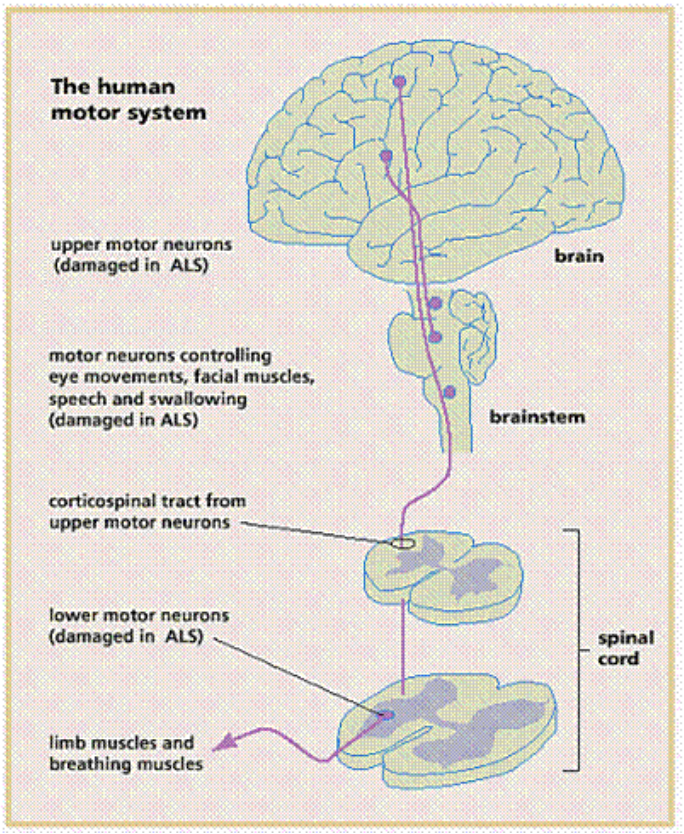


Fig 15: The human motor System [52]

Upper and lower motor neuron are damaged in ALS

The name of the pathology resumes all the major features of the disease. “Amyotrophic”, in fact, refers to muscular atrophy, and “lateral sclerosis” pertains to the scarring in the lateral aspect of the spinal cord. [53] ALS is a clinically heterogeneous disease characterized by muscles wasting, weakness, paralysis, swallowing impairment and respiratory failure that may occur in month or in years. These clinical features can also be accompanied or preceded by frontotemporal dementia. [54] The initial symptoms can vary in different people and also the site of onset can be different; ALS could affect muscles that control speech and swallowing, or hands, arms legs or feet. Other early symptoms include tripping, dropping things, abnormal fatigue of the arms and/or uncontrollable periods of laughing or crying. Since ALS attacks only motor neuron the sense of sight, touch, hearing, taste and smell are not affected, moreover in many people muscles of the eyes and bladder are not affected.

The clinical course of most ALS cases follows a progressive deterioration with a rapid and sometimes unpredictable progression. [55] Upper and Lower motor neuron are both involved, but the symptoms are different. Lower motor neuron engagement caused the majority of neurological manifestations during the late stage of the pathology and it is responsible of respiratory failure, the loss of the ability to swallow and the risk of aspiration. Upper motor neuron deterioration, instead, is responsible for weakness, muscle wasting and muscle spontaneous activity (fasciculation) and

cognitive impairment where frontotemporal dementia is present. [56]

ALS disease can be hereditary (FALS) or can be sporadic(SALS). Many genetic mutations has been described starting from the analysis of autosomal dominant familial case of ALS:

- the cytosolic anti-oxidant enzyme Cu^{2+}/Zn^{2+} -binding superoxide dismutase 1 (encoded by SOD1) [57] , transactivation response element DNA binding protein 43 (TDP-43 encoded by TARDBP) [58],
- fused in sarcoma RNA-binding protein (encoded by FUS) [59],
- angiogenin (encoded by ANG)
- an intronic hexanucleotide expansion in the gene encoding the chromosome 9 open reading frame 72 (C9orf72) [60].

Moreover it has been hypothesized that sALS is a multifactorial disease characterized by a complex interaction between potentially susceptible genes and environmental factor. The key component of ALS pathogenesis proposed until now are (Fig.16):

- Glutamate induced excitotoxicity [61]
- Cytoplasmic protein aggregates
- Oxidative injury [62]
- Alterated mitochondrial function [63]

- Cytoskeleton alterations [64]
- Axonal transport dysregulation [65]
- Immunomodulation [66]

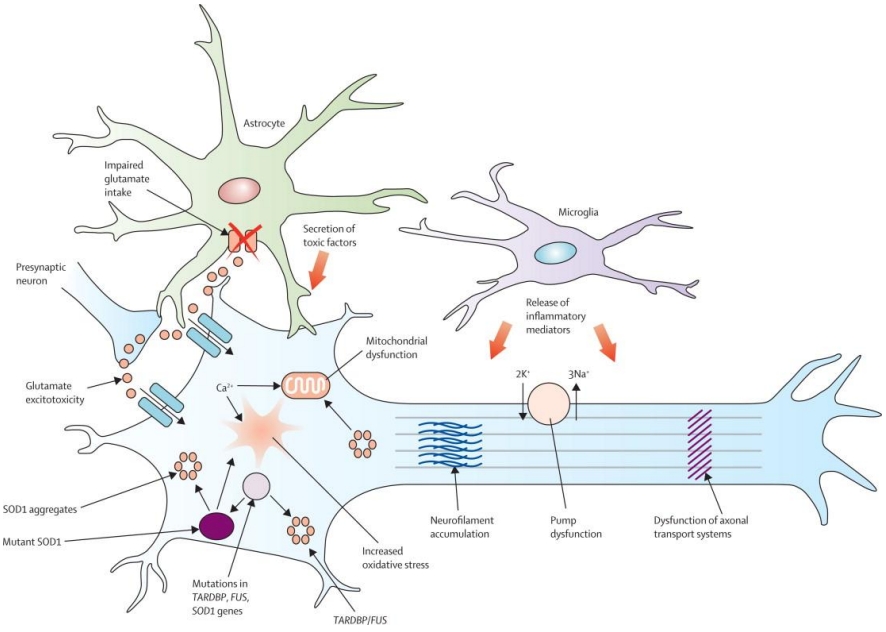


Fig 16: Cellular and molecular processes mediating neurodegeneration in ALS [67]

The mechanisms underlying neurodegeneration in ALS are multifactorial and operate through inter-related molecular and genetic pathways.

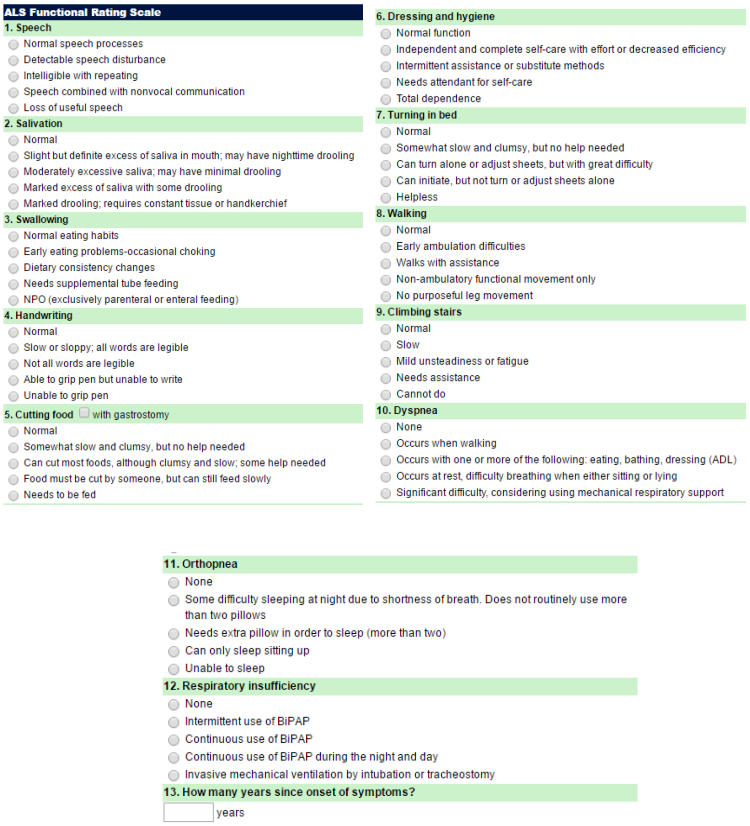
1.4.1 Diagnostic criteria for ALS

ALS is a disease really difficult to diagnose and this leads to an average delay of 15-18 month from onset of symptoms to diagnosis giving problems for the reduction of therapeutic window for potential neuroprotective therapies. There is no test or procedure to ultimately establish it, but is through a clinical examination and a series of diagnostic tests, often ruling out other diseases that mimic ALS. A comprehensive diagnostic workup includes most of the following procedures:

- Electrodiagnostic tests such as electromyography (EMG) and nerve conduction velocity (NCV)
- Blood and urine analisys
- Spinal tap
- X-rays including magnetic resonance imaging (MRI)
- Myelogram of cervical spine
- Muscle and nerve biopsy
- Neurological examination. [68]

After ALS diagnosis clinicians uses "The ALS Functional Rating Scale – revised" (ALSFRS-r) to evaluate the progression of pathology. The ALSFRS-r provides an extimation of the patient's degree of functional impairment and it is made on the basis of answer that patients give to the clinician . The

ALSFRS-r includes twelve questions about the patients level of functional impairment in performing one of some common tasks. Each task is rated on a five-point scale from 0 = can't do, to 4 = normal ability. (Fig. 17)



ALS Functional Rating Scale

1. Speech
- Normal speech processes
- Detectable speech disturbance
- Intelligible with repeating
- Speech combined with nonvocal communication
- Loss of useful speech

2. Salivation
- Normal
- Slight but definite excess of saliva in mouth; may have nighttime drooling
- Moderately excessive saliva; may have minimal drooling
- Marked excess of saliva with some drooling
- Marked drooling; requires constant tissue or handkerchief

3. Swallowing
- Normal eating habits
- Early eating problems-occasional choking
- Dietary consistency changes
- Needs supplemental tube feeding
- NPO (exclusively parenteral or enteral feeding)

4. Handwriting
- Normal
- Slow or sloppy; all words are legible
- Not all words are legible
- Able to grip pen but unable to write
- Unable to grip pen

5. Cutting food ☐ with gastrostomy
- Normal
- Somewhat slow and clumsy, but no help needed
- Can cut most foods, although clumsy and slow; some help needed
- Food must be cut by someone, but can still feed slowly
- Needs to be fed

6. Dressing and hygiene
- Normal function
- Independent and complete self-care with effort or decreased efficiency
- Intermittent assistance or substitute methods
- Needs attendant for self-care
- Total dependence

7. Turning in bed
- Normal
- Somewhat slow and clumsy, but no help needed
- Can turn alone or adjust sheets, but with great difficulty
- Can initiate, but not turn or adjust sheets alone
- Helpless

8. Walking
- Normal
- Early ambulation difficulties
- Walks with assistance
- Non-ambulatory functional movement only
- No purposeful leg movement

9. Climbing stairs
- Normal
- Slow
- Mild unsteadiness or fatigue
- Needs assistance
- Cannot do

10. Dyspnea
- None
- Occurs when walking
- Occurs with one or more of the following: eating, bathing, dressing (ADL)
- Occurs at rest, difficulty breathing when either sitting or lying
- Significant difficulty, considering using mechanical respiratory support

11. Orthopnea
- None
- Some difficulty sleeping at night due to shortness of breath. Does not routinely use more than two pillows
- Needs extra pillow in order to sleep (more than two)
- Can only sleep sitting up
- Unable to sleep

12. Respiratory insufficiency
- None
- Intermittent use of BiPAP
- Continuous use of BiPAP
- Continuous use of BiPAP during the night and day
- Invasive mechanical ventilation by intubation or tracheostomy

13. How many years since onset of symptoms?

_____ years

Fig 17: ALS functional rating scale - revised

(http://www.outcomes-umassmed.org)

Question comprised in ALSFRS-r test.

The thirteenth question isn't part of the ALSFRS-r, but it is useful to evaluated the disease progression calculating with the ratio between the ALSFRS-r score and the duration of the

pathology. This index give to the clinicians an indication of the velocity of the disease and makes them able to prognostic a fast progression ALS or a slow progression ALS.

1.4.2 Therapeutic option

To date there aren't treatments able to reverse the damage of ALS, they only can slow the progression of symptoms and prevent complications. The only medication approved by Food and Drug administration for the treatment of ALS is riluzole (used in clinic since 1995). [69] This drug, in partial percentage of patients, can slow down the progression of disease causing a modest extension of lifespan, but its mechanism of action remains still unclear. In addition of riluzole other drugs can be prescribed in order to mange ASL symptoms such as muscle cramps and spasms, spasticity, constipation, fatigue, excessive salivation, excessive phlegm, pain depression, sleep problems and uncontrolled outburst of laughing or crying. Other therapies could include breathing care (Non invasive mechanic ventilation, NIV) [70], physical therapy, speech therapy, nutritional support (percutaneous endoscopic gastrostomy, PEG or radiologically inserted gastrostomy RIG) [71] and psychological support.

1.4.3 Immune system involvement in ALS

External as well as intrinsic stressful events, pathological conditions or change in homeostasis can trigger an immune response. Accumulating evidence indicates that there is an involvement of the immune system in many neurodegenerative disorders, including ALS [72]. (Table 1)

Factors	**Cell/tissue expression**	**Prognostic value/rate of progression**	**Human/animal model**
NO↑, peroxynitrite↑, ROS↑ NOX2*↑	Neurons, astroglia, endothelial cells, macrophages, microglia, spinal cord	Fast	Human hmSOD1 mice
P2X7 receptor↑ CB2, Cox-2*↑	Astrocytes, macrophages, microglia, CNS	Fast	Human hmSOD1 mice
FasL/Fas-R↑, TNFL/TNFR↑ BCl-2 proteins↑	T cells ($CD8^+$), microglia	?	Human hmSOD1 mice
TLR2, TLR4↑ TRAIL↓	Microglia astrocytes	?	Human
C1q↑, C4↑, C3↑ C5a receptor CD88*↓	Microglia astrocytes, endothelial cells, CSF, spinal cord	Prognostic indicator slow	Human Nf-L knockout mice hmSOD1 mice
CRP↑	CSF, blood	Prognostic indicator	Human

Factors	Cell/tissue expression	Prognostic value/rate of progression	Human/animal model
S100 β*↑ CD14*↑	Astroglia microglia, CSF	Prognostic indicator slow	Human
RAGE ↑	Astroglia, microglia	?	Human
IgG↑, FcγR1↑ ICAM-1↑	Microglia T cells, CNS	?	hmSOD1 mice
CD11b↑, CD14↑, CD18↑, SR-A, CD68, MCP-1*↑ RANTES↑	Astrocytes, monocytes, macrophages, microglia spinal cord, CNS	Fast	Human hmSOD1 mice
DEC205*↑, CD86↑,CD83↑, CD54↑, CD40↑, CD1a↑	DCs, CNS	Fast	Human hmSOD1 mice
IFN-γ*↑, perforin ↑	T_h1, $CD8^+$ cells	Fast	Human
TNF-α*↑, IL-6*↑, IL-1β↑, IL-12, ↑ CCL2,3,5, CXCL1↑, CCR2↓	M1 microglia, T_h1 cells, monocytes, CNS, PBMC	Fast	Human hmSOD1 mice
IL-17A, IL-17, IL-18, IL-13, IL-23↑	CD8, mast cells, astrocytes, macrophages, monocytes, T_h cells, T_h17, PBMC, CNS	?	Human
T_h2: IL-4↑, IL-5↑, IL-10↑, TGF-β↑ CX3CR1 ↑	M2 microglia T_h2 and T_{reg} cells	Slow	Human hmSOD1 mice
ROS↓, iNOS↓ IGF-1↑	M2 microglia, T_h2, CNS, PNS	Slow	hmSOD1 mice

Factors	Cell/tissue expression	Prognostic value/rate of progression	Human/animal model
$CD4^+CD25^+/FoxP3^+$, IL-10, TGF-β↓	T_{reg} cells in blood and CNS	Fast	Human hmSOD1 mice

Table 1: Immune factor associated with ALS and animal models of ALS [85]

Summary of the immune-factors associated with ALS and their prognostic/rate of progression values

There is evidence of immune activation in biological fluids of ALS patients (blood and cerebro spinal fluid (CFS)), in the central nervous system (CNS) and in the ventral horns of spinal cord such as: presence of T-cell [82], IgG depositions[83], reactive astrocytes [84], macrophages[85], microglia [86] and others indication of inflammation. These observations are primarily based on post mortem studies that offer only a picture of the late stages of the disease. Genetic discoveries and the development of a murine model of ALS [73] helped to clarify some aspect of the pathology, even if there are still many unresolved questions.

Glial cells are non-neuronal cells present in the central nervous system (microglia and astrocytes) able to protect neuron homeostasis. Microglia are described as the brain-resident macrophage population deriving from monocyte precursors. These cells could have a role in ALS pathogenesis

since they become activated in response to a primary event, for example misfolded and aggregate proteins that act like a DAMPs triggering inflammation. [74] Other DAMPs such as ROS, HMGb1 and HSPs are reported to be overexpresed in the motor cortex and spinal cord of mice that have the mutant forms of human SOD1 gene (hmSOD1). Early activation of microglia has been hypothesized could have a protective effect on degenerating cells since T-cell deficiency and decreased microgliosis is associated with a worse outcome in ALS animal models [95]. However persistent activation of these cells during disease development could be a risky factor.

Also astrocytes could have neuroprotective or toxic function on motor neuron in ALS; in fact astrocytes are able to increase pro-inflammatory activity secreting complement protein, acute-phase reactants and proteinases, but they are also able to promote glutamate uptake reducing exicitotoxicity. [96]

In addition to microglia, while the disease progresses, other leukocytes with phagocitic abilities and APC undergo the same process of activation. [75]Also activated DCs were founded in post mortem tissue and animal models of ALS.

In ALS patients there is an immunological activation not only at the central level, but also muscle and nerves seems to be involved in this process, even if is not clear if CNS, spinal

cord and peripheral nerves are interested simultaneously or following a sequential process.

Most immunological mediators implicated in the disease are ubiquitously expressed. This evidence leads us to the speculation that they may be responsible for organs-organs crosstalk and disease propagation. [76] This spreading of immunological mediators become easier when occurs the breakdown of blood-brain barrier, conditions caused by the degeneration of endothelial cells that is reported in animal models of ALS [77]

There is also evidence of peripheral T cell population changes during the disease in line with that previously reported in human and mouse spinal cord specimens. [97] [98] The $CD4^+/CD8^+$ balance is altered and ALS patients displays a decrease of T_{reg} cells in blood compared with healthy controls [99]. In particular those patients in which the pathology has a faster development have lower Treg cells number in blood , suggesting a recruitment of these immunomodulatory cells from periphery to CNS. This reduction in blood may also alter immune tolerance with the risk of more widespread autoimmunity to protein such as neurofilament light chain in advanced ALS with an increase in blood levels of antibody against this protein. [100]

Many cytokines are up-regulated in affected tissues and peripheral blood of ALS patients including IL-6, TNFα and tumor

growth factor beta (TGFβ). Moreover microglia and T_H cells, present at CNS and spinal cord level, are able to release pro inflammatory cytokines such as IL-1β, TNFα, interferon gamma (IFNγ), IL-17 and anti-inflammatory cytokines like IL-4, IL-5, IL-10 And IL-13. In addition it is still not clear whether an individual cytokine can have an harmful effect or if it is necessary a synergism among different cytokines. Hensley et all have described an overexpression of TNFα, IL6 and IFNγ in the spinal cord of G93A SOD1 mutant mice, suggesting that different cytokines can synergize for the disease progression. [79]

The overexpression of some of these cytokines could be correlated with some disease parameters; for example high TGFβ (that is shown to exert neuroprotection during oxidative stress and excitotoxic events) levels have been positively correlated with longer disease duration and end stage disease. [80]

Not only cytokines, but also chemokines can be detrimental for the development of ALS because the change in inflammatory environment has also a chemo-attractant effect. In particular, CCL2 also known as MCP-1, a potent chemoattractant of CCR2-expressing myeloid DCs, microglia and macrophages is over expressed in CFS from ALS patients compared to the controls. There are also hypotheses, not yet confirmed, of a positive correlation between an increased MCP-1 and IL8 amount in biological fluids and a more rapid disease progression[87] [88] [89].

Concerning DCs, it has been demonstrated their involvement in multiple sclerosis [90] experimental autoimmune encephalomyelitis [91], CNS infections [92] and tissue repair after an injury [93]. They can originate from infiltrating blood cells, choroid plexus, meninges and from microglia [94]. Henkel at al investigate their presence in the CFS and spinal cord of ALS patients looking at mRNA expression of marker for immature (CD1a, DEC-205) and mature (CD83, CD40) DCs and both subsets have been detected. The presence of immature and activated DCs, together with the presence in CFS of DC chemotatic proteins suggest that they could exacerbate the motor neuron injury expecially in those patients who had a faster decline and expressed higher levels of DCs marker mRNA. [52]

In conclusion, even if immune event seems not to be the triggering events of ASL, there is a significant contribution of inflammation and immune system involvement in the development of the pathology.

1.5 Aim of the thesis.

Several published data highlight the importance of chronic inflammation for neurodegeneration in ALS with an increased spinal cord recruitment of peripheral proinflammatory monocytes, DCs and T cells found in patients and animal models. Both $CD4^+$ and $CD8^+$ T lymphocytes infiltrates the brains of ALS patients and by interacting with glial cells could play a role in motor neuron degeneration. [101] Furthermore, in ALS patients, inflammation is not limited to the CNS but is present at the systemic level with higher levels of inflammatory serum cytokines, increased number of activated lymphocytes and higher expression of MHC class II on monocytes. Patients with a rapidly progression pathology also show low number of T_{reg} cells and increased levels of LPS in the plasma. High endotoxin levels may contribute to chronic low-grade inflammation as manifested in chronic disease such as Parkinson's disease and atherosclerosis. [104]

In order to become activated and infiltrate target organs, T cells need to interact with DCs, professional antigen presenting cells capable of priming T cells and skewing their responses toward an inflammatory or anti-inflammatory phenotype. [15]

Because of their unique capacity to shape immune responses, DCs play important roles in the pathogenesis of several inflammatory diseases including neuroinflammatory diseases. [102] Studies on human DCs demonstrate that in the

peripheral blood of patients with progressive neurodegeneration these cells are functionally altered and more prone to skew T cell responses towards a proinflammatory phenotype. [103]

To date no clear data are available regarding the functional state of DCs in peripheral blood of ALS patients. With the present project we propose to investigate the biology of peripheral blood DCs in ALS patients and their correlation with the clinical state and the disease.

In the present study we have examined circulating DC subsets in a large cohort of ALS patients taking into account their clinical phase. Ex vivo analyses of the frequency and expression of costimulatory, MHC and migratory molecules of purified DC subsets were performed and the capacity of purified DCs to spontaneously produce inflammatory cytokines and to respond to appropriate TLR agonists were investigated in vitro.

This study provides a detailed picture of the functional state of DCs in ALS patients at different clinical stages and lays the basis to understand how the cells that control inflammatory T cell activation contribute to disease progression

Chapter 2: Activation state and functionality of dendritic cells from peripheral blood

2.1 Cytofluorimetric analysis of CD1c^{+} DCs from peripheral blood of ALS patients and healthy controls

In this part of the study 10 healthy controls (CTR) (Table 2) and 20 ALS patients (Table 3), selected at NEMO (NEuroMuscular Omnicentre), were enrolled. Patients have been characterized taking into account their level of disability and the rate of disease progression. These two parameters were evaluated based on ALSFRS-R scale and Disease Progression Index [calculated as (48-ALSFRS-R score)/disease duration)] respectively.

Healthy control	Sex	Age
FCTR1	F	55
FCTR2	F	67
FCTR3	M	66
FCTR4	F	60
FCTR5	F	66
FCTR6	F	56
FCTR7	M	57
FCTR8	F	40
FCTR9	M	33
FCTR10	F	58

Table 2: Healthy Donors for cytofluorimetric analysis

Age and sex of Healthy Donors enrolled in our study.

FALS8	FALS7	FALS6	FALS5	FALS4	FALS3	FALS2	FALS1	**ALS**
M	F	F	M	M	M	M	M	**Sex**
69	65	53	44	72	64	69	66	**Age**
01/112013	01/122014	01/102008	01/092014	01/092010	01/012005	01/012014	01/10/2013	**Onset**
Bulbar	Spinal	Spinal	Spinal	Spinal	Spinal	Spinal	Spinal	**Type of Onset**
01/01/2014	01/05/2015	01/09/2009	01/12/2014	01/01/2012	01/10/2006	01/06/2014	30/01/2014	**Diagnosis**
877	392	2460	543	1598	3516	716	838	**Duration (day)**
26	46	45	29	12	18	20	36	**ALSFRS/48**
9	12	11	9	9	4	8	9	**ALSFRS Bulbar**
3	12	12	11	3	6	6	11	**ALSFRS Respiratory**
.	.	.	.	YES	YES	YES	.	**NIV**
YES	.	.	.	.	.	.	.	**TRACHEO**
.	.	.	.	.	YES	.	.	**PEG/RIG**

FALS17	FALS16	FALS15	FALS14	FALS13	FALS12	FALS11	FALS10	FALS9
M	F	F	M	M	F	F	M	M
63	71	57	67	63	75	68	52	62
01/01/2012	01/01/2009	01/01/2006	01/01/2012	01/10/2014	01/06/2013	01/12/2013	01/01/1993	01/10/2014
Spinal	Spinal	Spinal	Spinal	Bulbar	Bulbar	Spinal	Spinal	Spinal
01/10/2013	01/06/2011	01/01/2007	01/05/2014	01/10/2015	01/12/2013	01/07/2014	01/01/2009	01/02/2015
987	1840	3452	771	253	912	700	2707	485
12	16	8	29	43	9	26	4	25
4	6	5	11	10	1	11	0	9
6	10	3	12	12	6	6	4	12
.	.	.	.	.	YES	YES	YES	YES
.	.	.	.	.	.	.	.	.
YES	.	YES	.	.	YES	.	YES	YES

FALS18	M	50	01/09/2015	Spinal	01/12/2015	196	42	10	12	.	.	.
FALS19	M	72	01/06/2008	Spinal	01/10/2010	2097	17	9	3	.	.	.
FALS20	M	69	01/08/2015	Spinal	01/06/2016	27	32	12	12	.	.	.

Table 3: ALS patients for cytofluorimetric analysis

Age, sex and disease features of ALS patients enrolled in the study.

ALS patients and healthy donors were also described using the descriptive statistics depicted in Table 4.

	Case (n=20)	Control (n=10)
Age	63,55±8,32	55,80±11,19
Gender (M/F)	14/6	7/3
Alsfrs-r	25,5 [14 - 34]	.
Alsfrs-r Bulbar	9 [5,5 – 10,5]	.
Alsfrs-r Respiratory	8 [5 - 12]	.
Time from Onset to Diagnosis	10,67 [4,56 – 21,28]	.
Time from Onset to Evaluation	34,23 [20,86 – 91,39]	.
Time from Onset to NIV	48,38 [19,25 – 62,98]	.
Time from Onset to RIG/PEG	44,12 [21,77 – 138,33]	.

NIV (y/n)	12/8	.
RIG/PEG (y/n)	6/14	.
Tracheo (y/n)	1/19	.
Exitus (y/n)	1/19	.

Table 4: Descriptive Statistic of FALS and CTR population.

Median features of ALS and CTR populations.

Peripheral blood (10 mL) was collected from ALS patients and healthy donors and EDTA was used to block coagulation. After red blood cell lysis blood samples were stained with the selected antibody to evaluate the state of activation of DCs.

2.1.1 ALS patients have less circulating DCs than healthy controls

In the blood CD1c $^{+}$ DCs are described as CD11c^{high} CD123^{low} and represent the major subset of myeloid DCs. These cells show monocytoid morphology and express myeloid markers. A minor proportion of CD1c^{+} DCs express also CD14 and CD11b.

In the blood, apart from myeloid DCs, a subset of small resting B cells expresses CD1c; for this reason we selected our DCs population as CD1c^{+} and CD19^{-} (Fig: 18)

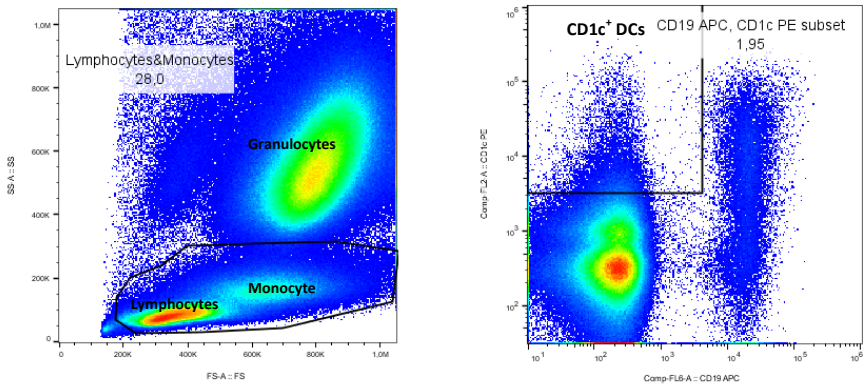


Fig 18: Pseudocolor plot of a blood sample

Left panel show all the cellular population in the blood of an healthy controls. Three differ populations can be identified: lymphocytes, monocytes and granulocytes. DCs are comprised in the monocyte population. In the right panel the CD1c^{+} CD19^{-} subpopulation is shown.

Taking advantage of Flow-Count™ Fluorospheres purchased from BD, we were able to quantify the absolute numbers of DCs in every sample analyzed.

The result of this analysis showed that ALS patients had significantly less circulating DCs than Healthy controls (Fig. 19).

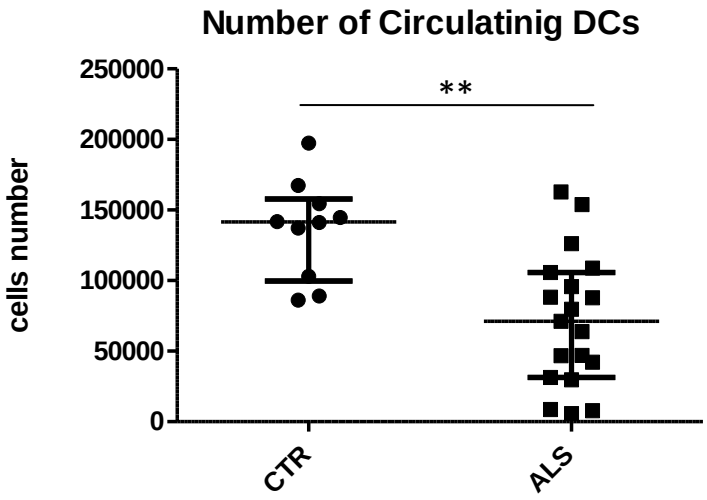


Fig 19: Absolute numbers of peripheral blood DCs in healthy controls and ALS patients

In peripheral blood of ALS patients there are statistically significative less (Wilcoxon Test p=0,0023) $CD1c^+$ DCs than in peripheral blood of CTR.

2.1.2 Peripheral blood DCs from ALS patients have significantly higher CD62L expression levels.

ALS patients showed lower numbers of circulating DCs compare to controls we decided to investigate the activation markers expressed by these cells. To this purpose we performed cytofluorimetric analysis taking into exam the mean fluorescence intensity (MFI) of different molecules of interest.

The first evidence we obtained from this data was that DCs of ALS patients expressed higher levels of CD62L than healthy controls. (Fig. 20)

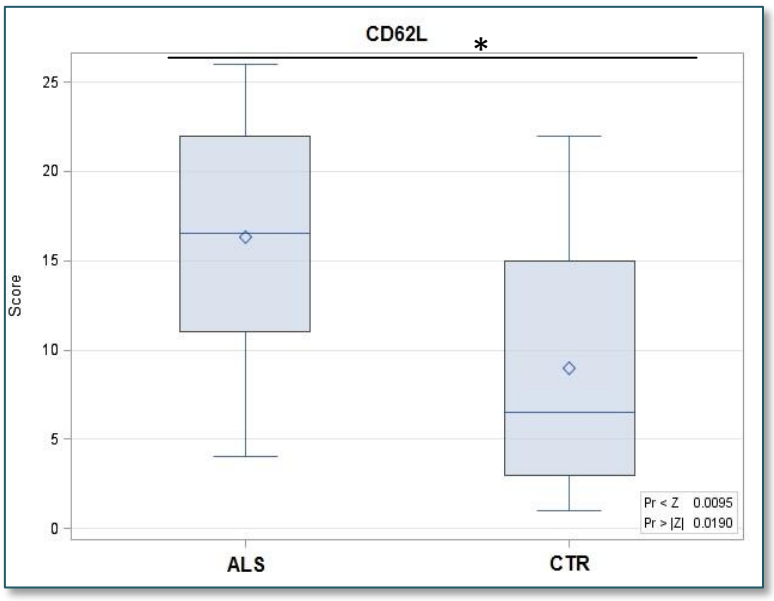


Fig 20: CD62L expression at the surface of blood DCs in ALS patients and healthy controls.

$CD1c^+$ DCs of ALS patients (ALS) express significantly higher (Wilcoxon Test, p=0,019) levels of CD62L than $CD1c^+$ DCs of healthy controls (CTR).Medians are shown.

CD62L, also known as L- selectin, belong to the selectins family with P- and E- selectins. It is a calcium-dependent single chain transmembrane glycoprotein. L-selectin acts like a “homing receptor” because it is able to interact with ligands presents on endothelial cells and allow cell extravasation. [104]

Based on the lower number of DCs in the blood of ALS patients and the over expression of CD62-L by these cells we speculated that DCs are recruited in the CNS, where the disease cause an high level of inflammation, or in lymph nodes draining the inflammatory sites.

2.1.3 Peripheral blood DCs from ALS patients have higher CCR7 and CCR2 expression levels, but there is no difference in the expression of CCR5.

We have also evaluated the level of expression of inflammatory chemokines receptors for CCR7, CCR2 and CCR5.

These three molecules belong to the family of C-C chemokine receptors, G coupled hepta-helical receptors mainly expressed on hemopoietic cells. In humans chemokine receptors expression has been exstensively studied since it has been demonstrated their involvement in several disease such as: rheumatoid arthritis[104], multiple sclerosis [105], inflammatory kidney diseases[106] , and inflammatory bowel diseases[107]

CCR2 is the cognate CCL2 receptor and mediates the recruitment of circulating monocytes. [108] CCR2 is predominantly expressed on monocytes, but a functionally active CCR2 receptor has also been demonstrated in other cell types, such as vascular smooth muscle cells[109], endothelial cells[110], and fibroblasts. [111]

CCR5 share 75% homology with CCR2 but, despite their close structure, they bind different ligands and mediate a variety of effects. CCR5 is expressed on various cell populations including macrophages, dendritic cells and memory T cells in the immune system; epithelium, endothelium, vascular smooth

muscles, fibroblasts, microglia, astrocytes and neuron in CNS[112]. Natural ligands for these receptors are MIP1-α, MIP1-β, RANTES and MCP-2[113]. This receptor plays a role in the inflammatory response by directing the cells to the sites of inflammation and, in particular, ligands for CCR5 are able to augment the activation of T-cell responses and to enhance the production of antigen specific- T cells. [114]

CCR7, the receptor for CCL19 and CCL21, is expressed by semi-mature and mature DCs[115], thymocytes during defined stages of their development[116], naïve B and T cells[117], T_{reg} cells and a subpopulation of memory T-cells known as central memory T cells[118]. CCR7 is also expressed by different non immune cells and in particular in various malignancies. [119] This receptor mediate signals that control the migration of immune cells to secondary lymphoid organs and subsequently their positioning within defined functional compartments. Moreover, even if little is known about the mechanisms that regulate the trafficking of mature DCs to the lymph nodes via the afferent lymphatics, gene targeting has shown that CCR7 is essential for DC mobilization. [120]

Our data show that there is a trend concerning CCR2 (Fig. 21) and CCR7 (Fig.22) that are higher in ALS patients than in healthy controls, suggesting that chemokine signaling could be important in this disease. For CCR5, instead, we have not observed any differences between the two groups (Fig.23).

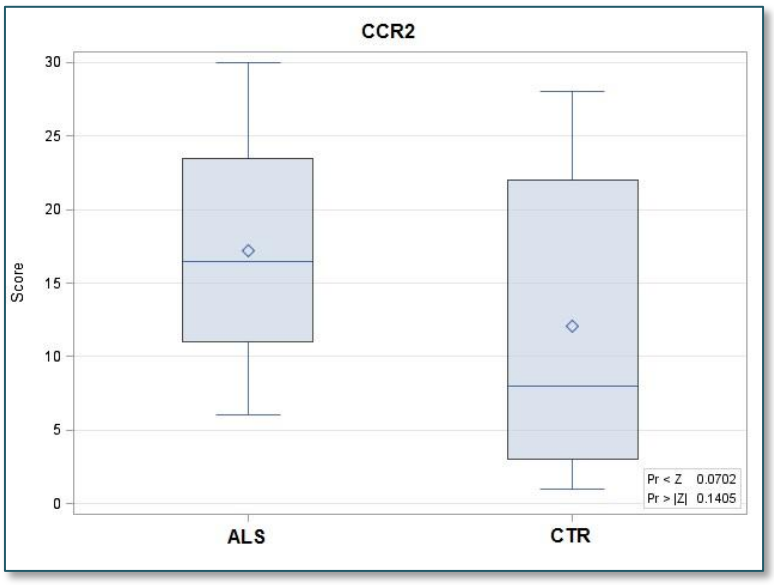


Fig 21: CCR2 expression at the surface of blood DCs in ALS patients and healthy controls.

$CD1c^+$ DCs of ALS patients have the tendency to express higher levels of CCR2 than $CD1c^+$ DCs of CTR, although the difference between the two groups is not statistically significant (Wilcoxon Test, p=0,1405). Data were shown in a box and whisker graph with median [Q1-Q3].

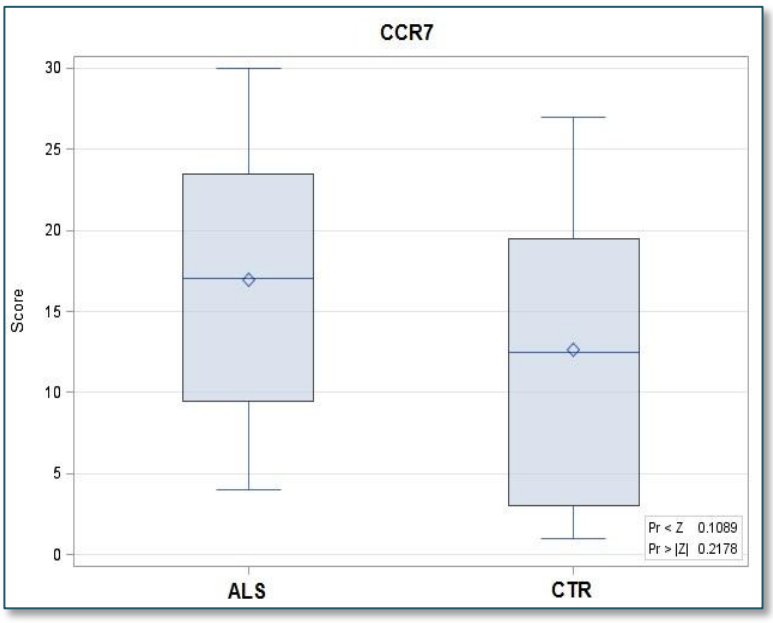


Fig 22: CCR7 expression at the surface of blood DCs in ALS patients and healthy controls.

CD1c+ DCs of ALS patients tend to express higher levels of CCR7 than CD1c+ DCs of healthy donors, although the difference between the two groups is not statistically significant (Wilcoxon Test, p=0,2178). Data were shown in a box and whisker graph with median [Q1-Q3].

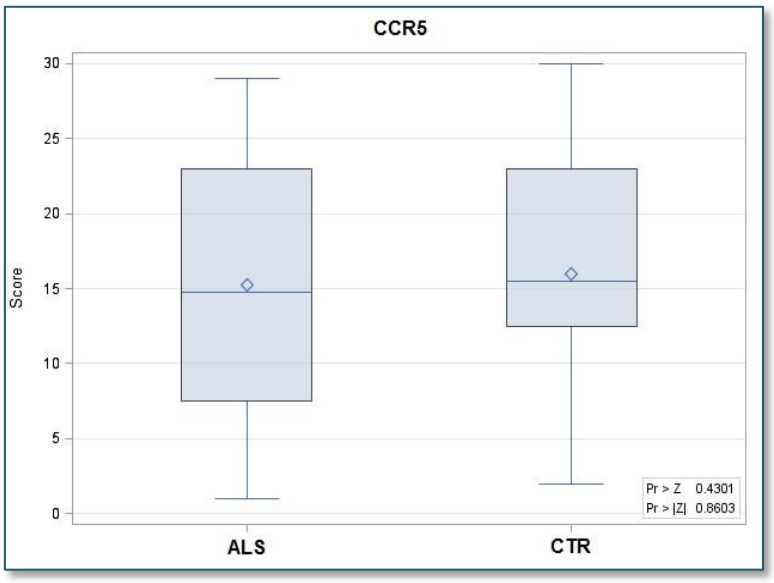


Fig 23: CCR5 expression at the surface of blood DCs in ALS patients and healthy controls.

No differences were observed between ALS patients and healthy donors (Wilcoxon Test, p=0,8603). Data were shown in a box and whisker graph with median [Q1-Q3].

2.1.4 Peripheral blood DCs from ALS patients and healthy donors express the same levels of MHC class II and costimulatory molecules.

In ALS patients, inflammation is not limited to the CNS but is present at the systemic level. This includes increased numbers of circulating activated lymphocytes, higher levels of MHC class II expression on monocytes and higher levels of inflammatory serum cytokines. MHC and costimulatory molecules (CD80, CD86, CD40) are able to provide signal 1 and signal 2 for the activation of T cells. For this reason we decided to evaluate also the levels of expression of these molecules. We observed that there were no differences between ALS patients and healthy donors. (Fig 24-28)

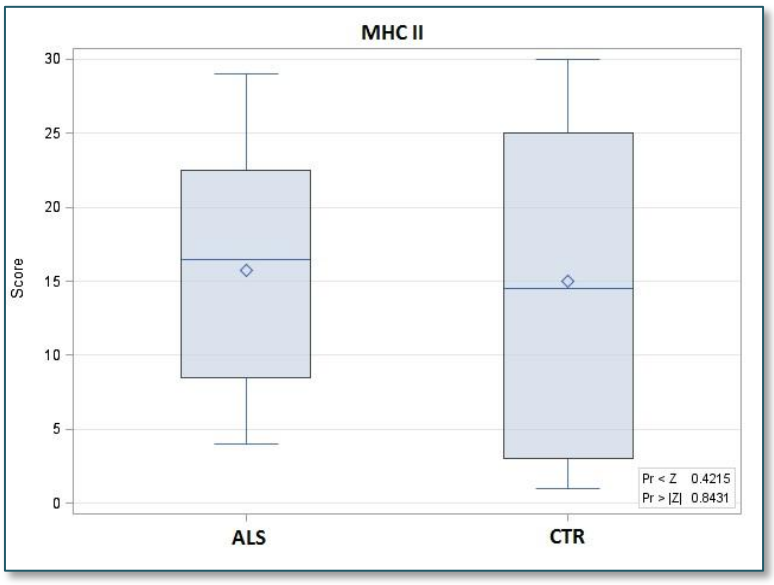


Fig 24: Expression of MHC class II molecule at the surface of blood DCs in ALS patients and healthy controls.

No differences are observable between the expression of MHC class II by CD1c+ DCs of ALS patients and CD1c+ DCs of CTR (Wilcoxon Test, p= 0,8431). Data were shown in a box and whisker graph with median [Q1-Q3].

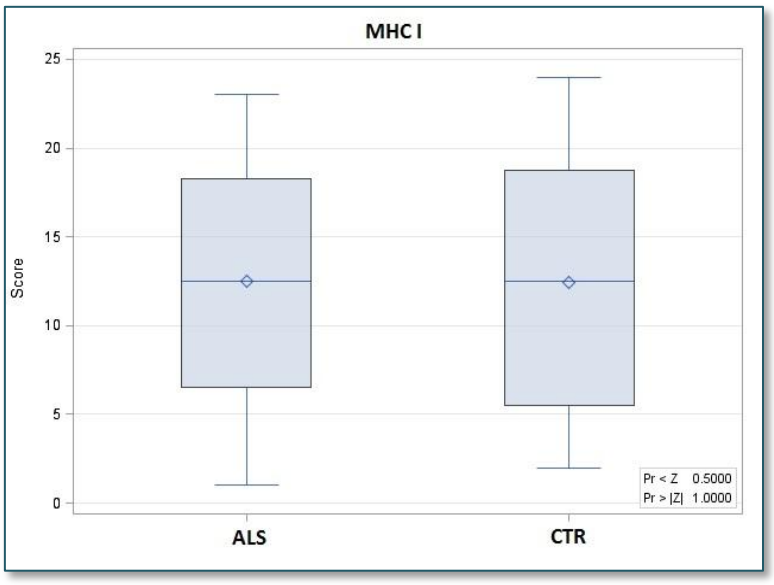


Fig 25: Expression of MHC class I molecules at the surface of DCs in ALS patients and healthy controls.

No differences are observable between the expression of MHC class I by CD1c+ DCs of ALS patients and CD1c+ DCs of CTR (Wilcoxon Test, p=1). Data were shown in a box and whisker graph with median [Q1-Q3].

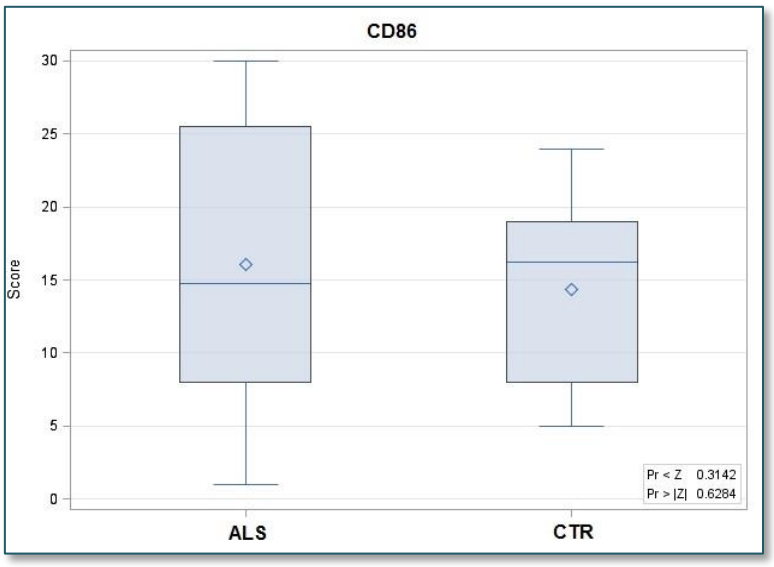


Fig 26: CD86 expression at the surface of blood DCs in ALS patients and healthy controls.

No differences were observed between the expression of CD86 by CD1c+ DCs of ALS patients and CD1c+ DCs of CTR (Wilcoxon Test, p=0,6284). Data were shown in a box and whisker graph with median [Q1-Q3].

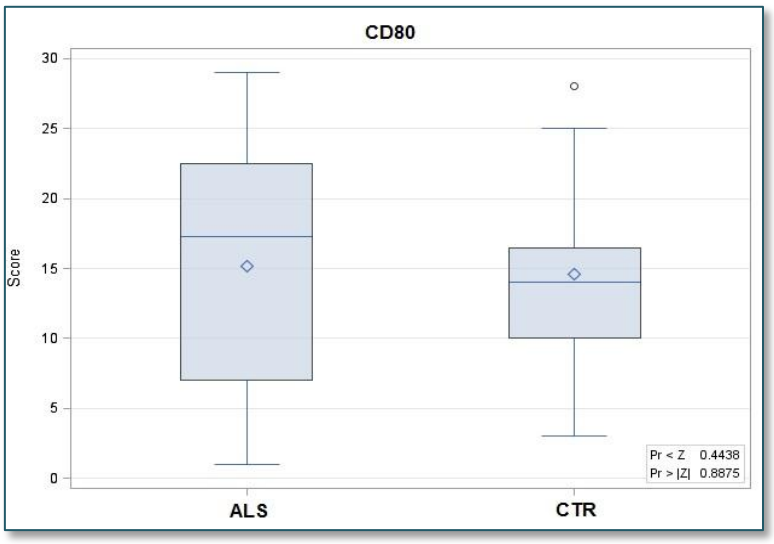


Fig 27: CD80 expression at the surface of blood DCs in ALS patients and healthy controls.

No differences were observed between the expression of CD80 by CD1c+ DCs of ALS patients and CD1c+ DCs of CTR (Wilcoxon Test, p=0,8975). Data were shown in a box and whisker graph with median [Q1-Q3].

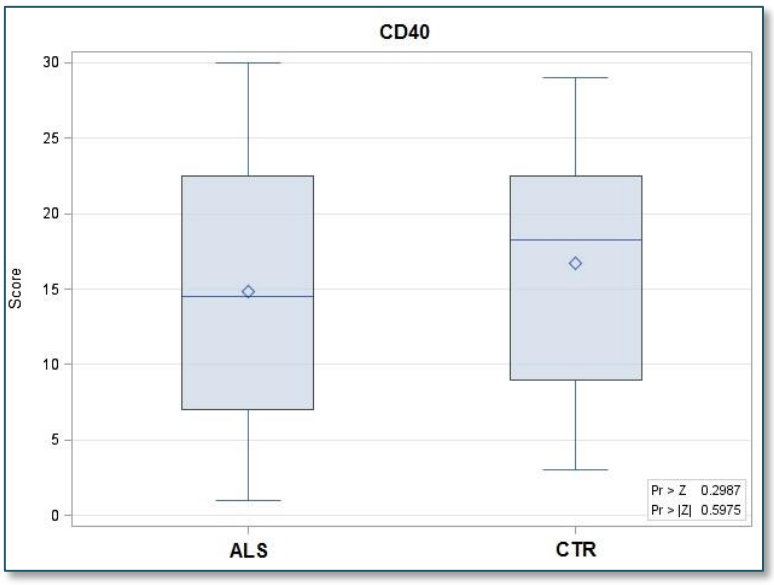


Fig 28: CD40 expression at the surface of blood DCs in ALS patients and healthy controls.

No differences were observed between the expression of CD40 by CD1c+ DCs of ALS patients and CD1c+ DCs of CTR (Wilcoxon Test, p=0,5975). Data were shown in a box and whisker graph with median [Q1-Q3]

2.2 Functional analysis of CD1c^{+} DCs from peripheral blood of ALS patients and healthy controls

Given that we found the number of DCs was lower in ALS patients than in healthy controls and expressed higher level of CD62L we decided to investigate the functional state of the CD1c+ cells. The purpose of this part of the study was to define whether DCs from ALS patients were in an activated state and prone to induce inflammation.

36 healthy controls (CTR) (Table 5), 25 neurological controls (CTRN) (Table 6) and 52 ALS patients (Table 7), all selected at NEMO (NEuroMuscular Omnicentre), were enrolled in this part of the study. Also in this case patients were characterized taking into account their level of disability and rate of disease progression evaluated using the ALSFRS-R scale and the Disease Progression Index [calculated as (48-ALSFRS-R score)/disease duration)] respectively.

CTR	Sex	Age
DCCTR1	F	71
DCCTR2	M	68
DCCTR3	F	63
DCCTR4	F	74
DCCTR5	F	58
DCCTR6	M	75
DCCTR7	F	58
DCCTR8	F	65

DCCTR9	M	65
DCCTR10	F	44
DCCTR11	F	57
DCCTR12	M	48
DCCTR13	F	58
DCCTR14	F	52
DCCTR15	M	50
DCCTR16	F	59
DCCTR17	F	50
DCCTR18	M	47
DCCTR19	F	62
DCCTR20	F	59
DCCTR21	M	57
DCCTR22	F	43
DCCTR23	F	59
DCCTR24	F	55
DCCTR25	F	72
DCCTR26	F	36
DCCTR27	F	33
DCCTR28	M	73
DCCTR29	F	65
DCCTR30	M	65
DCCTR31	M	54
DCCTR32	M	42
DCCTR33	F	62
DCCTR34	F	60
DCCTR35	F	34
DCCTR36	M	68

Table 5: Healthy controls for functional analysis

Age and sex of healthy controls enrolled in our study.

DCCTRN	Sex	Age	Disease
DCCTRN1	F	44	TARLOV CYST
DCCTRN2	F	56	TARLOV CYST
DCCTRN3	F	53	TARLOV CYST
DCCTRN4	M	49	STRUMPEL – LORRAIN DISEASE
DCCTRN5	F	78	PERIPHERAL POLYNEUROPHATY
DCCTRN6	M	46	DUCHENNE MUSCULAR DISTROPHY
DCCTRN7	F	67	MYOTOCHONDRIAL MYOPATHY
DCCTRN8	F	42	MYOTONIC DISTROPHY TYPE I
DCCTRN9	F	46	MYOTONIC DISTROPHY TYPE I
DCCTRN10	M	37	MYOTONIC DISTROPHY TYPE I
DCCTRN11	F	36	FSHD
DCCTRN12	M	48	MYOTONIC DISTROPHY TYPE II
DCCTRN13	M	42	MYOTONIC DISTROPHY TYPE I
DCCTRN14	F	53	MYOTONIC DISTROPHY TYPE II
DCCTRN15	M	49	MYOTONIC DISTROPHY TYPE I
DCCTRN16	F	59	TARLOV CYST
DCCTRN17	M	45	MYOTONIC DISTROPHY TYPE I
DCCTRN18	M	33	MYOTONIC DISTROPHY TYPE II
DCCTRN19	M	37	MYOTONIC DISTROPHY TYPE I
DCCTRN20	F	40	FSHD
DCCTRN21	F	50	MYOTONIC DISTROPHY TYPE I
DCCTRN22	M	44	MYOTONIC DISTROPHY TYPE I
DCCTRN23	M	65	TARLOV CYST
DCCTRN24	M	66	MYOTONIC DISTROPHY TYPE I
DCCTRN25	F	52	MYOTONIC DISTROPHY

Table 6: CTRN patients for functional analysis

Age, sex and disease of neurological controls enrolled in our study.

DCALS9	DCALS8	DCALS7	DCALS5	DCALS4	DCALS3	DCALS2	DCALS1	ALS
M	F	F	M	F	M	M	F	Sex
09/03/1939	19/02/1953	26/02/1944	18/10/1934	17/12/1956	18/02/1953	23/12/1938	28/10/1931	DOB
76	62	71	80	58	62	76	83	Age
01/03/2014	01/06/2014	01/06/2013	01/01/2014	01/04/2013	01/09/2014	01/09/2014	01/09/2014	Onset
Spinal	Bulbar	Bulbar	Bulbar	Bulbar	Spinal	Spinal	Bulbar	Type of onset
01/11/2014	01/02/2015	01/02/2014	01/10/2014	01/04/2014	01/03/2015	01/03/2015	01/03/2015	Diagnosis
36	43	9	16	30	35	31	26	ALSFRS/48
11	9	1	5	6	12	6	4	Bulbar
11	12	5	3	7	12	12	7	Respiratory
.	.	YES	YES	YES	.	YES	YES	PEG/RIG
.	.	.	.	.	.	.	.	TRACHEO
.	.	YES	YES	YES	.	YES	YES	NIV

DCALS18	DCALS17	DCALS16	DCALS15	DCALS14	DCALS13	DCALS12	DCALS11	DCALS10
F	M	F	M	M	F	F	F	F
30/08/1944	06/03/1952	15/01/1957	05/09/1972	25/09/1953	03/07/1954	16/03/1938	08/04/1941	23/03/1948
70	63	58	42	61	60	77	74	67
01/07/2013	01/09/2013	01/07/2014	01/09/2013	01/01/2015	01/12/2013	01/12/2013	01/06/2013	01/05/2013
Bulbar	Bulbar	Bulbar	Bulbar	Bulbar	Bulbar	Bulbar	Bulbar	Spinal
01/12/2014	01/09/2014	01/12/2014	01/06/2014	01/05/2015	01/10/2014	01/06/2014	01/01/2014	01/01/2014
43	32	23	40	42	24	20	22	12
12	8	4	10	10	5	5	3	5
12	6	10	12	11	5	6	6	7
.	YES	YES	.	.	.	YES	YES	YES
.	.	.	.	.	.	.	.	.
.	YES	.	.	.	YES	YES	YES	YES

DCALS27	DCALS26	DCALS25	DCALS24	DCALS23	DCALS22	DCALS21	DCALS20	DCALS19
F	M	M	F	M	M	M	M	M
01/07/1966	18/08/1953	02/10/1962	04/05/1939	24/01/1951	13/11/1941	04/05/1978	28/05/1955	06/07/1953
49	62	52	76	64	73	37	60	62
01/05/2014	01/10/2014	01/04/2014	01/01/2014	01/12/2014	01/07/2013	01/05/2014	01/11/2013	01/10/2014
Spinal	Spinal	Bulbar	Bulbar	Spinal	Respiratory	Bulbar	Bulbar	Bulbar
01/11/2014	01/02/2015	01/11/2014	01/01/2015	01/04/2015	01/10/2014	01/01/2015	01/04/2014	01/07/2015
37	33	11	23	18	31	30	40	37
9	11	4	5	7	6	9	12	12
12	12	6	6	6	7	11	12	12
.	.	YES	YES	.	YES	.	.	.
.	.	.	.	.	.	.	.	.
.	.	YES	YES	YES	YES	.	.	.

DCALS36	DCALS35	DCALS34	DCALS33	DCALS32	DCALS31	DCALS30	DCALS29	DCALS28
M	M	F	F	M	F	M	M	F
27/02/1944	07/11/1950	06/01/1947	30/04/1949	20/08/1967	10/01/1942	16/12/1940	08/07/1954	19/02/1962
71	64	68	66	48	73	74	61	53
01/06/2014	01/10/2013	01/06/2014	01/02/2015	01/12/2013	01/07/2014	01/06/2014	01/08/2014	01/03/2014
Spinal	Spinal	Spinal	Spinal	Spinal	Bulbar	Bulbar	Spinal	Spinal
01/11/2014	01/01/2014	01/07/2015	01/07/2015	01/01/2014	01/04/2015	01/02/2015	01/04/2015	26/10/2014
13	36	33	37	32	28	39	28	37
7	10	9	12	12	6	9	9	12
6	11	12	11	12	6	9	12	12
YES	.	.	.	.	.	.	.	.
.	.	.	.	.	.	.	.	.
YES	.	.	.	.	.	.	.	.

DCALS45	DCALS44	DCALS43	DCALS42	DCALS41	DCALS40	DCALS39	DCALS38	DCALS37
M	F	M	M	M	F	M	M	M
02/09/1948	16/06/1949	06/03/1970	26/03/1943	20/08/1967	03/07/1973	19/07/1954	14/03/1953	23/02/1940
67	66	45	72	48	42	61	62	75
01/12/2013	01/01/2015	01/03/2013	01/12/2014	01/05/2015	01/02/2014	01/11/2014	01/11/2014	01/05/2014
Spinal	Bulbar	Spinal	Spinal	Spinal	Spinal	Spinal	Spinal	Bulbar
01/07/2014	01/09/2015	01/10/2013	01/06/2015	01/07/2015	01/07/2014	01/04/2015	01/05/2015	01/05/2015
41	35	40	12	32	41	37	40	22
11	4	12	9	11	12	11	12	6
12	12	11	1	12	12	12	12	3
.	.	.	YES	.	.	.	.	YES
.	.	.	.	.	.	.	.	.
.	.	.	YES	.	.	.	.	YES

DCALS52	DCALS51	DCALS50	DCALS49	DCALS48	DCALS47	DCALS46
M	M	F	M	M	M	M
24/06/1954	09/08/1956	18/02/1944	31/10/1950	28/04/1966	16/10/1952	23/06/1971
61	59	72	65	49	63	44
01/12/2014	01/12/2014	01/03/2015	01/11/2014	01/04/2015	01/03/2015	01/12/2013
Spinal	Spinal	Bulbar	Spinal	Spinal	Spinal	Spinal
01/04/2015	01/07/2015	01/08/2015	01/07/2015	01/11/2015	01/04/2015	01/12/2014
42	34	37	12	41	14	29
12	11	8	8	11	11	11
12	12	10	3	12	1	10
.	.	.	.	.	.	.
.	.	.	.	.	.	.
.	.	.	YES	.	YES	.

Table 7: ALS patients for functional analysis

Age, sex and disease features of ALS patients enrolled in our study.

ALS patients, CTR and CTRN were also described using a descriptive statistic. (Table 8)

	Case (n=50)	Control (n=25)	Neuro Control (n=25)
Age	63 [58,5 - 71,5]	58 [56,84 - 61,52]	48 [44 - 51,68]
Gender (M/F)	31/19	7/18	12/13
Alsfrs-r T0	32 [23 - 37]	.	.
Disease Progression	1,09 [0,65 - 1,55]	.	.
Alsfrs-r Bulbar T0	9 [6 - 11]	.	.
Alsfrs-r Respiratory T0	11 [6 - 12]	.	.
Time from Onset to Diagnosis	7,13 [5,1 - 9,1]	.	.
Time from Onset to Evaluation	16,2 [11,83 - 22,28]		
Time from Diagnosis to Evaluation	7,8 [6,25 - 12,73]		
NIV (y/n)	20/30		
RIG/PEG (y/n)	17/33		
Tracheo (y/n)	1/49		
Exitus (y/n)	3/47		

Table 8: Descriptive Statistic of ALS, CTR and CTRN population.
Median features of ALS and CTR populations.

We collected 18 mL of peripheral blood from each subject included in the study. The blood was conserved for few hours in EDTA prior to perform FICOLL separation to obtain peripheral blood mononuclear cells (PMBCs). Starting from PBMCs, $CD1c^+$ DC were isolated by taking advantage of Milteny CD1c $(BDCA\text{-}1)^+$ dendritic cells purification kit.

The purification strategy is depicted in Fig. 29.After the depletion of $CD19^+$ B cells $CD1c^+$ DCs are collected by positive selection after a second passage in the columns through a positive selection.

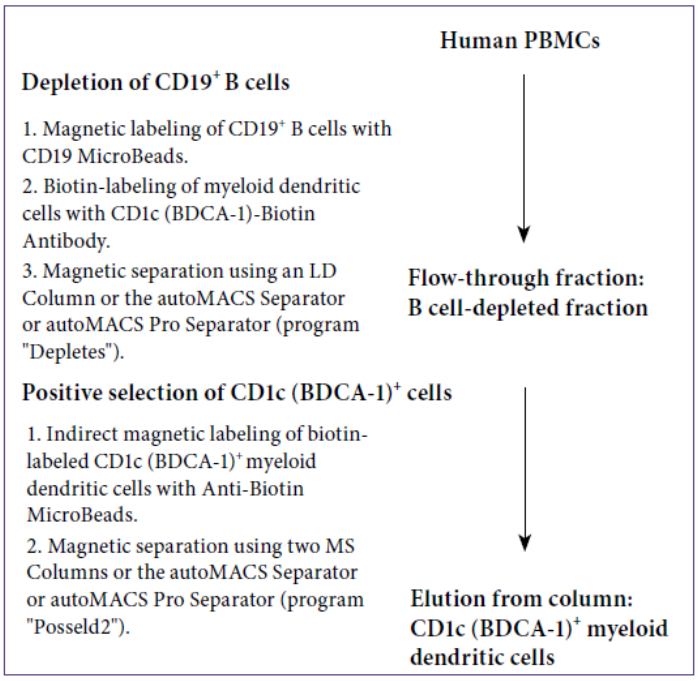


Fig 29: Schematic representation of purification strategy for CD1C$^+$ DC

To obtain a population of CD1c$^+$ DC is essential a first CD19+ B cells depletion. The population of interest are subsequently selected through CD1c magnetic labelling and eluted from the columns.

Using this method we obtain about 100.000 DCs that were plated on 96 well suspension u-bottom plate for 24 hours, in the absence or presence of 1 µg/mL LPS. We then collected surnatants and performed ELISA assay for different cytokines and chemokines.

2.2.1 The efficiency of IL6 production by ALS DCs after LPS stimulation correlates with time from onset to diagnosis.

We evaluated the amounts of pro-inflammatory cytokines such as TNFα (Fig.30), IL-1β (Fig.31), IL-12p40 (Fig 32), IL-6 (Fig. 34) and the amounts of the anti-inflammatory cytokine IL-10, produced by CD1c+ DCs from ALS patients, CTR and CTRN. This analysis did not evidence differences in cytokine production among the three groups both in presence of LPS and not

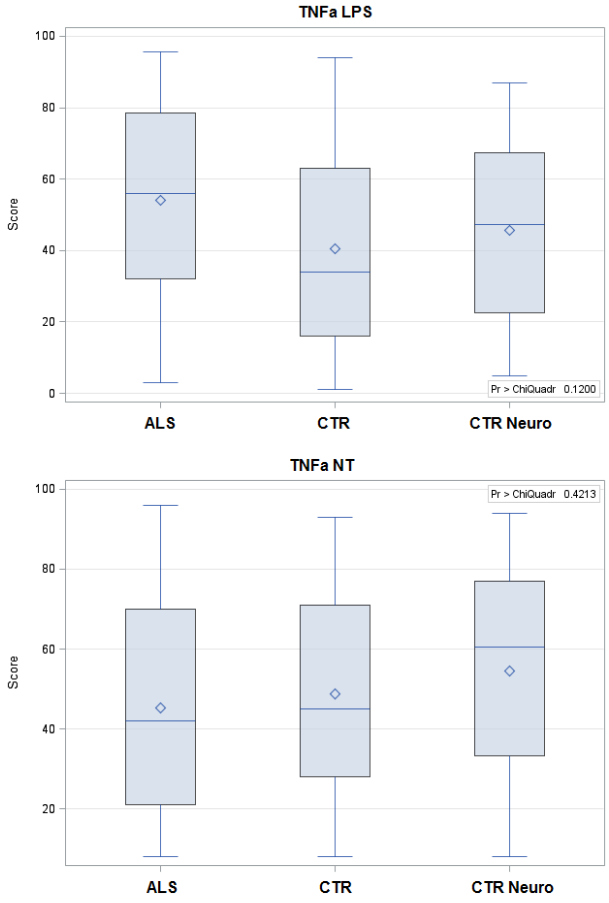


Fig 30: TNFα production by CD1c+ DCs purified from ALS, CTR and CTRN

The amount of TNFα released in the supernatant was measured by ELISA after stimulation (Pr>ChiQuadr 0,4213 – Kruskal-Wallis Test) or not (Pr>ChiQuadr 0,1200 – Kruskal-Wallis Test) with LPS for 24h. No differences in TNFα production were observed. Data were shown in a box and whisker graph with median [Q1-Q3].

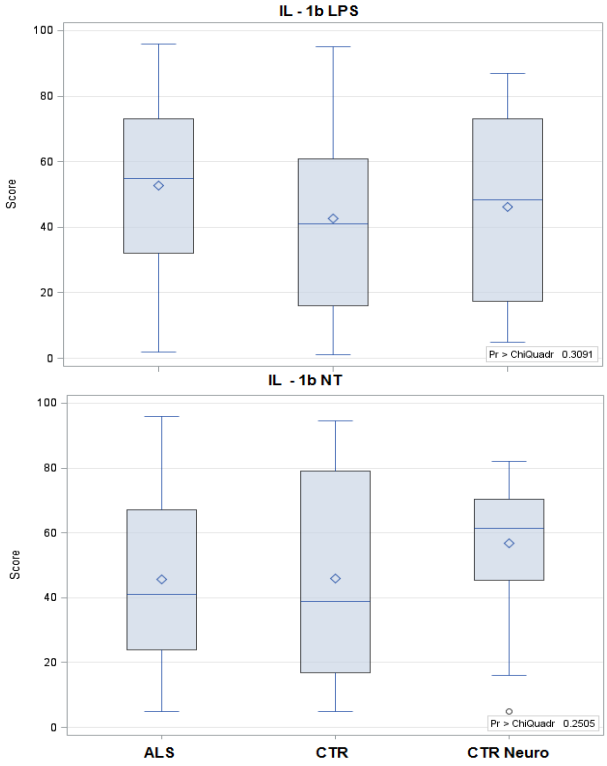


Fig 31: IL1β production by CD1c+ DCs purified from ALS, CTR and CTRN

No differences in IL-1β production by CD1c+ DCs from ALS patients, CTR and CTRN were observed. DCs were left untreated (Pr>ChiQuadr 0,3091 – Kruskal-Wallis Test) or stimulated with LPS for 24h(Pr>ChiQuadr 0,2505 - Kruskal-Wallis Test). Data were shown in a box and whisker graph with median [Q1-Q3].

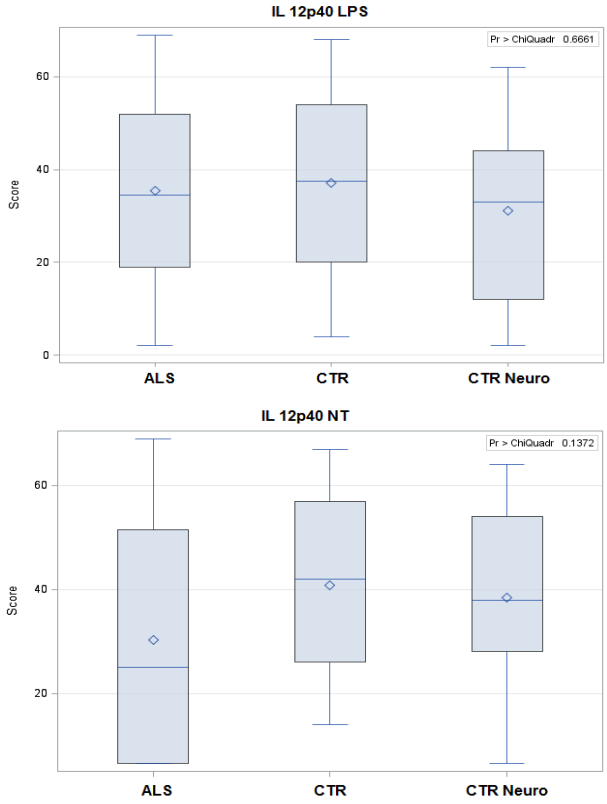


Fig 32: IL12p40 production by CD1c+ DCs purified from ALS, CTR and CTRN

No differences between IL-12p40 production by CD1c+ DCs of ALS patients, CTR and CTRN were observed. DCs were left untreated (Pr>ChiQuadr 0,6661 – Kruskal-Wallis Test) or stimulated with LPS for 24h /(Pr>ChiQuadr 0,1372 - Kruskal-Wallis Test). Data were shown in a box and whisker graph with median [Q1-Q3].

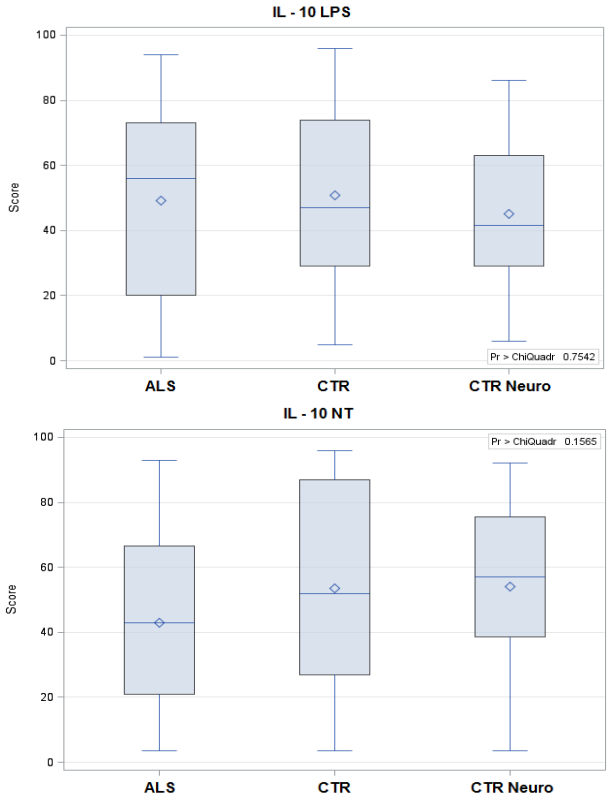


Fig 33: IL-10 production by CD1c+ DCs purified from ALS, CTR and CTRN

No differences in IL-10 production by CD1c+ DCs from ALS patients, CTR and CTRN were observed. DCs were left untreated (Pr>ChiQuadr 0,7542 – Kruskal-Wallis Test) or stimulated with LPS for 24h (Pr>ChiQuadr 0,1565 - Kruskal-Wallis Test). Data were shown in a box and whisker graph with median [Q1-Q3].

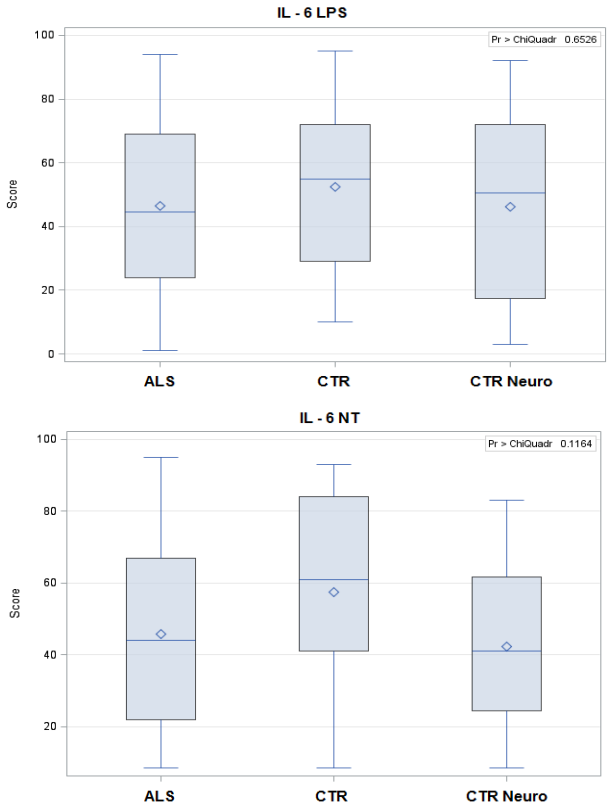


Fig 34: IL-6 production by CD1c+ DCs purified from ALS, CTR and CTRN

No differences in IL-6 production by CD1c+ DCs from ALS patients, CTR and CTRN were observed. DCs were left untreated (Pr>ChiQuadr 0,6526 – Kruskal-Wallis Test) or stimulated with LPS for 24h (Pr>ChiQuadr 0,1164 - Kruskal-Wallis Test). Data were shown in a box and whisker graph with median [Q1-Q3].

A correlation analysis between cytokine levels and some disease features was also performed. Disease features taken into account were as follows:

- Gender
- Site of Onset
- Age
- NIV
- RIG/PEG
- Alsfrs-r
- Disease Progression
- Alsfrs-r bulbar
- Alsfrs-r respiratory
- Alsfrs-r spinal
- Time from Onset to Diagnosis
- Time from Onset to Evaluation
- Time from Diagnosis to Evaluation

A significative inverse correlation was observed for IL-6 and the time from onset to diagnosis. (Fig 35) in particular, we can observe that the lowest is the time needed for the diagnosis, the higher is the IL-6 production after LPS stimulation. This observation suggested a detrimental role of this cytokine at the early stages of disease progression. This was in agreement with literature data showing overexpression of IL-6 in CFS and serum of ALS patients. [121] [122]

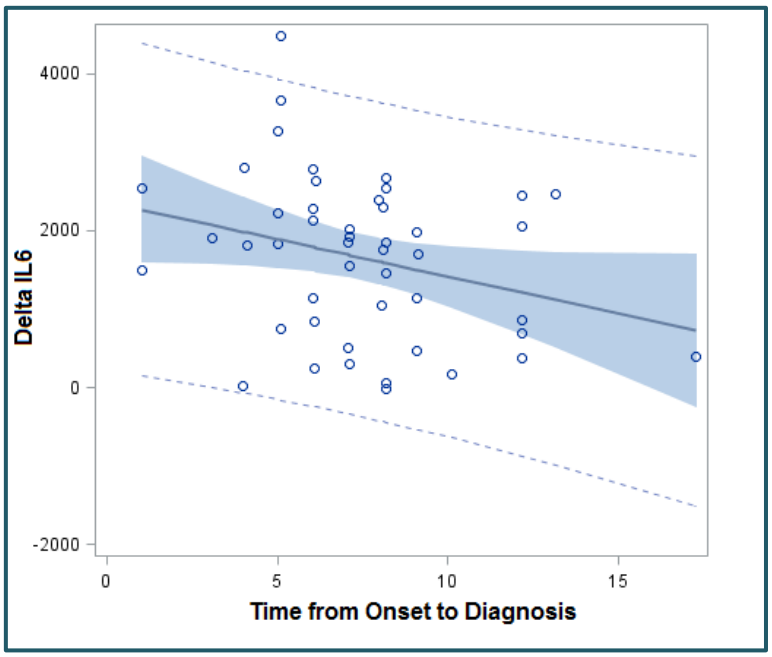


Fig 35: IL-6 and Time from Onset to Diagnosis correlation

An inverse correlation (p=0,048 - Spearman test) between the time from onset to diagnosis and the ΔIL-6 levels (IL-6 LPS – IL-6 NT) is shown.

2.2.2 Focus on IL-6: ALS26 a case report.

Given the potential IL-6 detrimental role in ALS pathogenesis a patients with a high inflammatory profile received an αIL-6R treatment with the purpose to slow down disease progression.

ALS26 is a 62 years old, male ALS patients with a disease onset on November 2014 and a diagnosis made in February 2015.

On 24th of September 2015 he was enrolled into our study with an ALSFRS/48 of 33 and without RIG, PEG or tracheotomy.

We analyzed the cytokines production of his CD1c^{+} DCs, and we found a profile with high levels of IL-6 and inflammatory cytokines. A pharmacological treatment with an anti-IL6R antibody was conducted for approximately three month.

Here we report cytokine values before and after treatment. As shown in Table 9 and in Fig.36 after treatment there was a reduction of the inflammatory state of DCs. Unfortunately we are not able to link this reduction with an improvement in the clinical state (ALSFRS/48 = 32, no RIG or PEG, no tracheotomy)

	NT(pg/mL)	NT after αIL-6R treatment (pg/mL)	LPS (pg/mL)	LPS after αIL-6R treatment (pg/mL)
TNFα	131,17	39,01	259,29	342,11
IL-6	58,18	174,41	1876,41	1180,61
CCL2	669,41	0	3603,53	1668,99
IL-10	160,22	145,21	1766,12	871,24
IL-8	2958	1909,11	10320,3	6280,56
IL-12p40	/	823,59	/	1026,82
IL-1β	2,24	15,91	103,45	795,3

Table 9: Cytokines production before and after αIL-6 treatment

After αIL-6R treatment DCs, taken from ALS26, spontaneously produce a lower amount of TNFα, CCL2, and IL-8. This trend is maintained also after LPS stimulation with lower amounts of IL-6, IL-10, CCL2 and IL-8 produced.

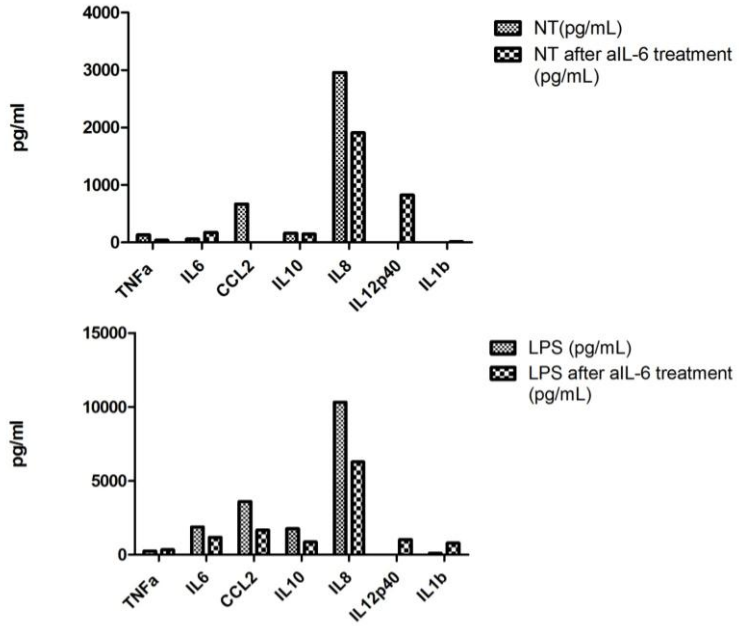


Fig 36: Cytokines production before and after αIL-6R treatment.

2.2.3 A subpopulation of ALS patients expresses higher levels of IL8 and CCL2 compared with healthy controls.

Chemokines production by DCs from ALS patients, CTR and CTRN was also evaluated. The spontaneous and LPS induced production of IL-8 (Fig. 37) and CCL2 (Fig. 38) was analyzed. No statistically differences among the three grouped were observed.

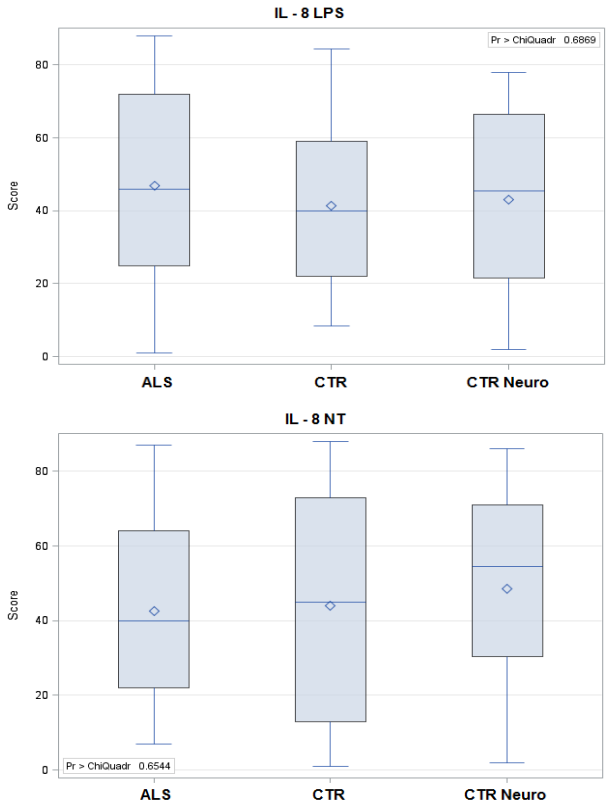


Fig 37: IL-8 production by CD1c+ DCs purified from ALS, CTR and CTRN

No differences in IL-8 production by CD1c+ DCs from ALS patients, CTR and CTRN were observed. DCs were left untreated (Pr>ChiQuadr 0,6869 – Kruskal Wallis Test) or stimulated with LPS for 24h (Pr>ChiQuadr 0,6544 - Kruskal-Wallis Test). Data were shown in a box and whisker graph with median [Q1-Q3].

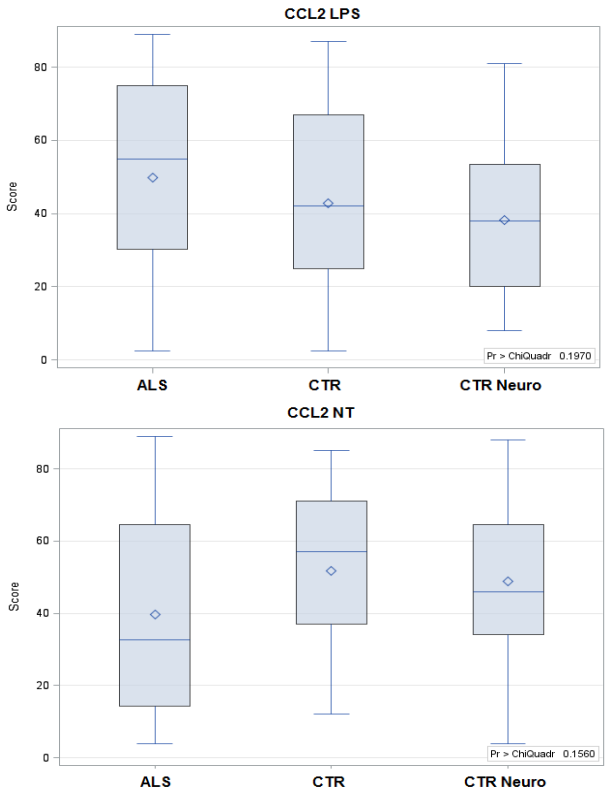


Fig 38: CCL2 production by CD1c+ DCs purified from ALS, CTR and CTRN

No differences in CCL2 production by CD1c+ DCs from ALS patients, CTR and CTRN were observed. DCs were left untreated (Pr>ChiQuadr 0,9170 – Kruskal-Wallis Test) or stimulated with LPS for 24h (Pr>ChiQuadr 0,1560 - Kruskal-Wallis Test). Data were shown in a box and whisker graph with median [Q1-Q3].

We then evaluated the distribution of IL-8 production by ALS patients. We noticed that a subgroup of ALS patients expressed higher levels of IL8 than others after LPS stimulation. (Fig. 39, Table 10)

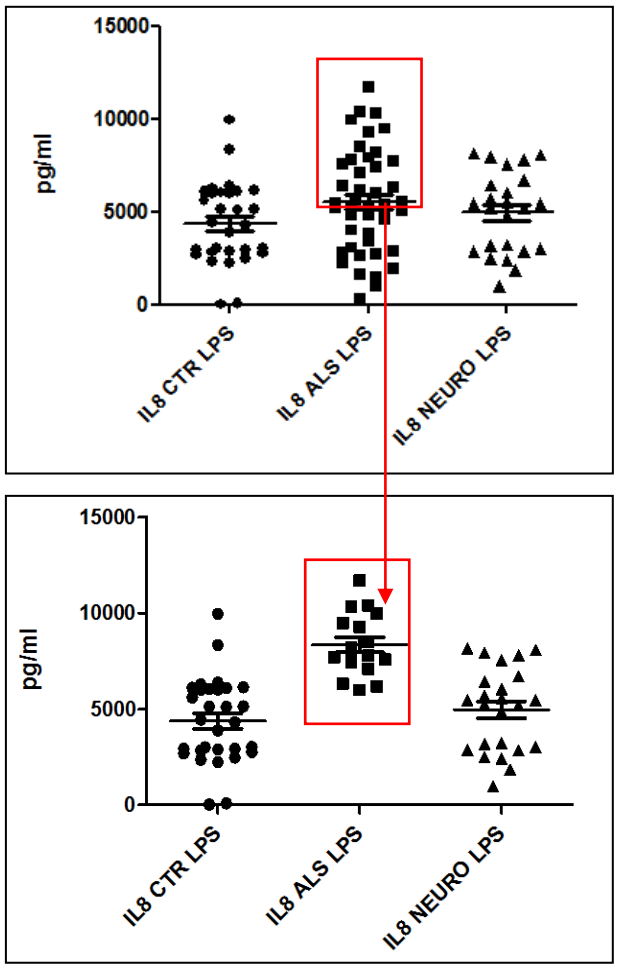


Fig 39: ALS patients could be divided in two groups based on the levels of IL8 by CD1c^{+} DCs.

DCs from 18 ALS patients produce higher amount of IL8 than others. These patients are included in the red square in the graphs below.

DCALS25	DCALS24	DCALS23	DCALS22	DCALS21	DCALS20	DCALS19	DCALS18	ALS
M	F	M	M	M	M	M	F	Sex
02/10/1962	04/05/1939	24/01/1951	13/11/1941	04/05/1978	28/05/1955	06/07/1953	30/08/1944	DOB
52	76	64	73	37	60	62	70	Age
01/04/2014	01/01/2014	01/12/2014	01/07/2013	01/05/2014	01/11/2013	01/10/2014	01/07/2013	Onset
Bulbar	Bulbar	Spinal	Respiratory	Bulbar	Bulbar	Bulbar	Bulbar	Type of onset
01/11/2014	01/01/2015	01/04/2015	01/10/2014	01/01/2015	01/04/2014	01/07/2015	01/12/2014	Diagnosis
11	23	18	31	30	40	37	43	ALSFRS/48
4	5	7	6	9	12	12	12	Bulbar
6	6	6	7	11	12	12	12	Respiratory
YES	YES	.	YES	.	.	.	.	PEG/RIG
.	.	.	.	.	.	.	.	TRACHEO
YES	YES	YES	YES	.	.	.	.	NIV

DCALS44	DCALS43	DCALS42	DCALS39	DCALS38	DCALS36	DCALS34	DCALS32	DCALS26
F	M	M	M	M	M	F	M	M
16/06/1949	06/03/1970	26/03/1943	19/07/1954	14/03/1953	27/02/1944	06/01/1947	20/08/1967	18/08/1953
66	45	72	61	62	71	68	48	62
01/01/2015	01/03/2013	01/12/2014	01/11/2014	01/11/2014	01/06/2014	01/06/2014	01/12/2013	01/10/2014
Bulbar	Spinal	Spinal	Spinal	Spinal	Spinal	Spinal	Spinal	Spinal
01/09/2015	01/10/2013	01/06/2015	01/04/2015	01/05/2015	01/11/2014	01/07/2015	01/01/2014	01/02/2015
35	40	12	37	40	13	33	32	33
4	12	9	11	12	7	9	12	11
12	11	1	12	12	6	12	12	12
.	.	YES	.	.	YES	.	.	.
.	.	.	.	.	.	.	.	.
.	.	YES	.	.	YES	.	.	.

DCALS45	M	02/09/1948	67	01/12/2013	Spinal	01/07/2014	41	11	12	.	.	.

Table 10: ALS patients with higher IL-8 levels after LPS stimulation

Age, sex and disease features of ALS patients with DCs able to produce higher amount of IL8 after LPS stimulation than others.

The selected patients showed statistically significant higher levels of IL-8 production compared to CTR and CTRN (Fig 40a). The same subject furthermore expressed also statistically significant higher levels of CCL2 (Fig 40b). Accordingly patients with low levels of IL-8 production showed also low levels of CCL2 (Fig.41).

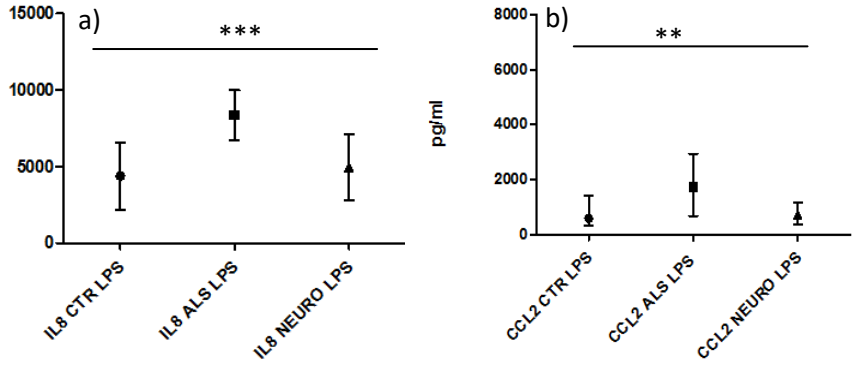


Fig 40: ALS patients characterized by high IL8 production have also a CCL2 high production.

The subpopulation of IL-8^{high} ALS patients has statistically significant higher levels of IL-8 production than CTR and CTRN ($p<0,0001$ - Kruskal-Wallis Tests) (a). The same subjects has also statistically significant higher levels of CCL2 ($p=0,0059$ - Kruskal-Wallis Tests) (b). Data are shown with median [Q1-Q3].

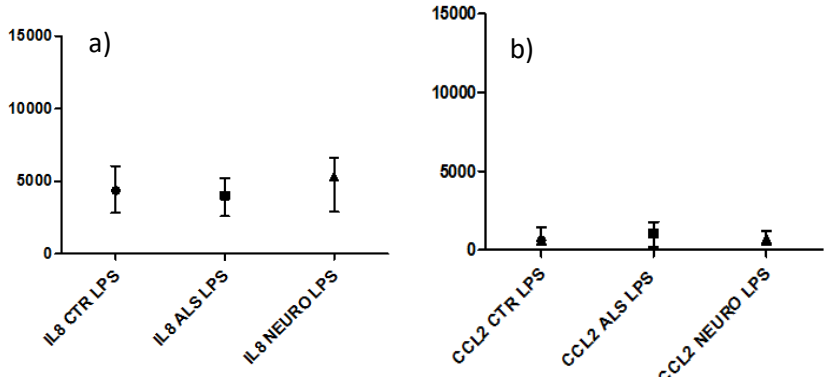


Fig 41: ALS patients characterized by low IL8 production have also a low CCL2 production.

The subpopulation of IL-8^{low} ALS patients has no differences in the levels of IL-8 production in respect of CTR and CTRN (a). The same subjects has also no differences in CCL2 production (p=0,0059 - Kruskal-Wallis Tests) (b). Data are shown with median [Q1-Q3].

2.2.4 Longitudinal analysis

We also considered the possibility that cytokines values changed during the course of the disease. To investigate this aspect we performed some longitudinal analysis of some ALS patients at T0, T3 (three month after the first blood sample collection) and T6 (six month after the first blood sample collection). Table 11 shows we report the clinical data of ALS patients analyzed during these three time points.

ALS	ALSFRS/48 T0	ALSFRS Bulbar T0	ALSFRS Respiratory T0	ALSFRS/48 T3	ALSFRS Bulbar T3	ALSFRS Respiratory T3	ALSFRS/48 T6	ALSFRS Bulbar T6	ALSFRS Respiratory T6
DCALS1	26	4	7	.	.	.	17	0	9
DCALS2	31	6	12	20	4	5	.	.	.
DCALS5	16	5	3	15	4	5	exitus	Exitus	Exitus
DCALS8	43	9	12	.	.	.	41	6	12

DCALS26	DCALS23	DCALS22	DCALS19	DCALS18	DCALS15	DCALS14	DCALS12	DCALS9
33	18	31	37	43	40	42	20	36
11	7	6	12	12	10	10	5	11
12	6	7	12	12	12	11	6	11
32	18	.	40	.	35	38	.	.
11	5	.	12	.	9	7	.	.
12	11	.	12	.	12	10	.	.
.	.	21	35	35	32	31	12	33
.	.	4	11	12	8	7	3	11
.	.	3	10	12	12	10	2	11

DCALS28	37	12	12	28	4	8	.	.	.
DCALS33	37	12	11	32	12	11	.	.	.
DCALS34	33	9	12	26	7	9	.	.	.
DCALS35	36	10	11	28	8	10	.	.	.
DCALS39	37	11	12	35	11	12	.	.	.

Table 11: ALS patients selected for longitudinal analysis

Disease features at T0, T3 (n=12) and T6 (n=9) of ALS patients selected for longitudinal analysis.

We did no observe differences in cytokines production by $CD1c^+$ DCs at the three different time points before or after LPS stimulation. These data are in agreement with the absence of correlation between cytokines and chemokines production and disease progression. (Fig.42-48)

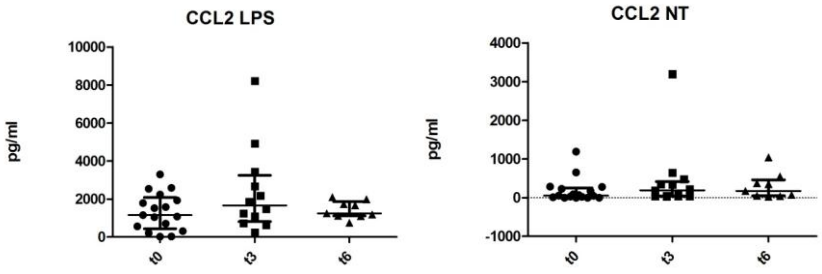


Fig 42: Longitudinal analysis of CCL2 production.

CCL2 production by CD1c+ DCs of ALS patients at t0, t3 and t6 before (p=0,1876 - Kruskal-Wallis Test) and after stimulation with 24h of LPS (p=0,4054 - Kruskal-Wallis Test). Data were shown in a dot plot with median and first third percentile.

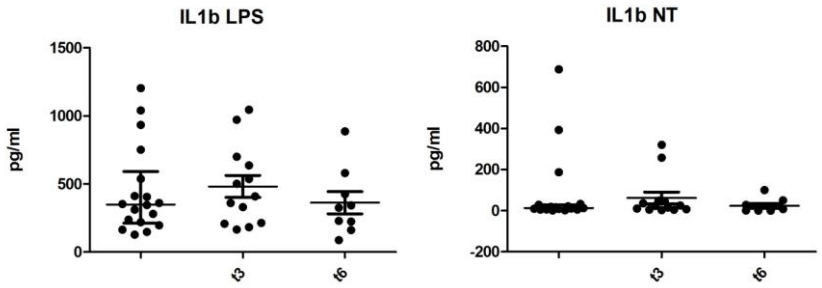


Fig 43: Longitudinal analysis of IL-1β production.

IL-1β production by CD1c+ DCs of ALS patients at t0, t3 and t6 before (p=0,4855 - Kruskal-Wallis Test) and after stimulation with 24h of LPS (p=0,5990 - Kruskal-Wallis Test) . Data were shown in a dot plot with median and first third percentile.

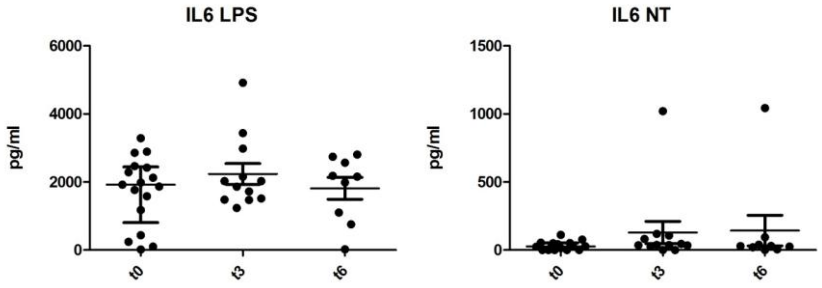


Fig 44: Longitudinal analysis of IL-6 production.

IL-6 production by CD1c+ DCs of ALS patients at t0, t3 and t6 before (p=0,3410 - Kruskal-Wallis Test) and after stimulation with 24h of LPS (p=0,8497 - Kruskal-Wallis Test). Data were shown in a dot plot with median and first third percentile.

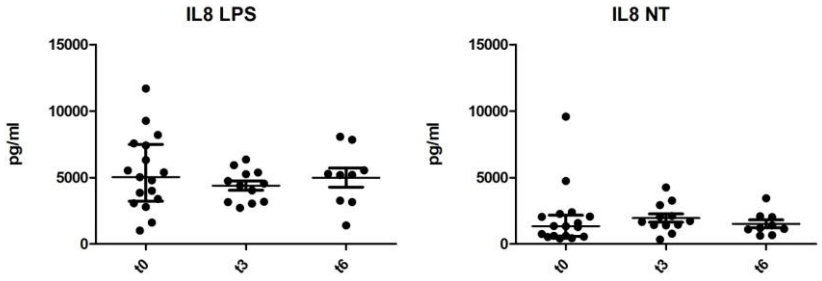


Fig 45: Longitudinal analysis of IL-8 production.

IL-8 production by CD1c+ DCs of ALS patients at t0, t3 and t6 before (p=0,3736 - Kruskal-Wallis Test) and after stimulation with 24h of LPS (p=0,6034 - Kruskal-Wallis Test). Data were shown in a dot plot with median and first third percentile.

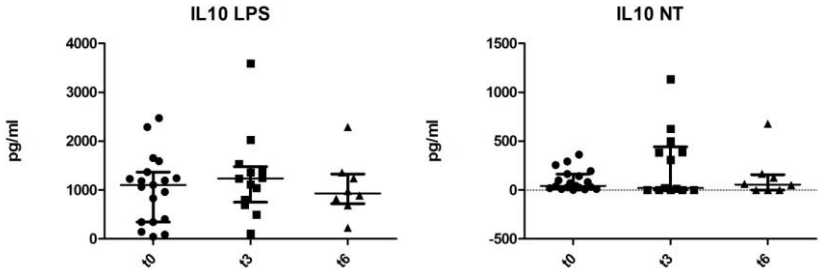


Fig 46: Longitudinal analysis of IL-10 production.

IL-10 production by CD1c+ DCs of ALS patients at t0, t3 and t6 before (p=0,9542 - Kruskal-Wallis Test) and after stimulation with 24h of LPS (p=0,66 - Kruskal-Wallis Test). Data were shown in a dot plot with median and first third percentile.

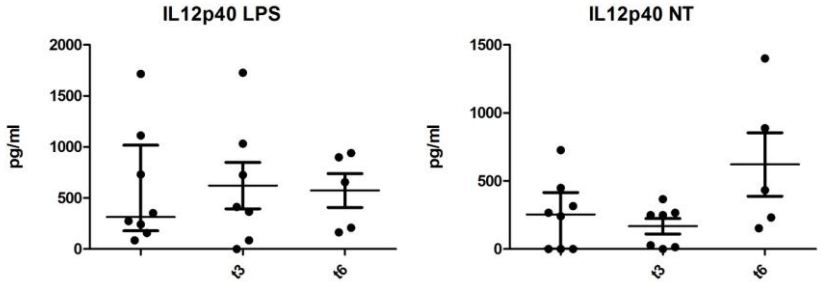


Fig 47: Longitudinal analysis of IL-12p40 production.

IL-12p40 production by CD1c+ DCs of ALS patients at t0, t3 and t6 before (p=0,2952- Kruskal-Wallis Test) and after stimulation with 24h of LPS (p=0,9753- Kruskal-Wallis Test). Data were shown in a dot plot with median and first third percentile.

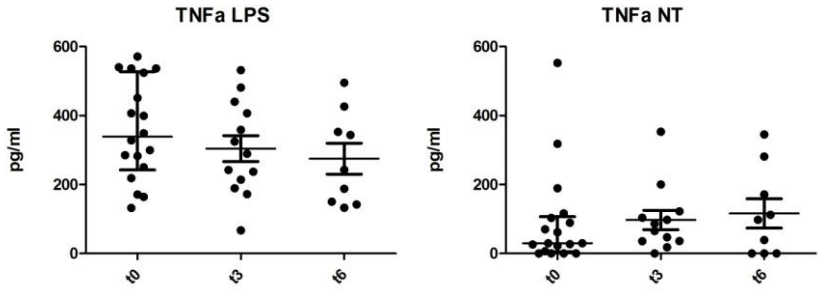


Fig 48: Longitudinal analysis of TNFα production.

TNFα production by CD1c+ DCs of ALS patients at t0, t3 and t6 before (p=0,5634 - Kruskal-Wallis Test) and after stimulation with 24h of LPS (p=0,3232- Kruskal-Wallis Test). Data were shown in a dot plot with median and first third percentile.

2.3 Material and methods

Patients enrollment

72 ALS patients were enrolled in this study, selected at NEMO (NEuroMuscular Omnicentre). Patients with definite or probable ALS diagnosis according to EL-Escorial criteria haves been selected with an age between 20 and 75 and disease duration no longer than 24 month. Patients have also been characterized taking into account their level of disability and rate of rapidity evaluate by ALSFRS-R scale and Disease Progression Index [calculated as (48-ALSFRS-R score)/disease duration)] respectively and by their phenotypic expression (i.e. Classical ALS, predominant upper motor neuron or lower motor neuron, etc). 37 Healthy Donors and 25 Neurological Patients stratified for age and sex were also enrolled

Flow cytometry

Blood sample from ALS patients and healthy donor was treated with red blood cells lysis buffer, than single-cell suspensions were pelleted and resuspended with the appropriate amount of antibody in 200 µl of PBS, and incubated for 20 minutes on ice in the dark. The cells were than washed once with 1 ml of PBS and resuspended in a final volume of 270 µl of PBS. To this final solution 30 µl of Beckman Coulter Flow-Count Fluorospheres were added in order to determinate absolute cells number.

For FACS analysis, the following antibodies were used: anti-CD1c PE, anti-CD19 APC, anti-CCR5 alexa700, anti-HLA DR/DP/DQ FITC, anti-HLA A/B Pacific Blue, anti-CCR7 APC Cy7, anti-CCR2 PECy7, anti-CD80 FITC, anti-CD86 Pacific Blue, anti-CD62L Alexa700, anti-CD40 PECy7 . Data were acquired using a Beckman-Coulter FACS Gallios and analyzed with FlowJo (TreeStar) software.

CD1c (BDCA1)$^{+}$ Dendritic cells purification and culture

18 mL of peripheral blood were drawn and cells purification were performed using CD1c (BDCA-1)$^{+}$ Dendritic Cells Isolation kit purchased from Miltenyi Biotech .

30.000 CD1c DCs were plated in RPMI complete medium and were cultured in presence or absence of 100 ng/mL LPS for 24 hours. The supernatants were than collected.

ELISA assay

Concentrations of TNFα, IL-1β, IL-6, IL-12p40, IL-8, CCL2 and IL-10 in BDCA1 dendritic cells supernatants were assessed by ELISA kits purchased from R&D Systems.

Antibodies and chemicals

Antibodies for flow cytometry were purchased from Biolegend and TRL4-grade smooth LPS (E. coli,O55:B5) was purchased from Enzo Life Sciences.

Statistical Analysis

All data were statistically analyzed. Medians were compared by Wilcoxon Test or Kruskal-Wallis Tests. Correlation between parameters were analyzed with Spearman test. Data are expressed and plotted as median [Q1-Q3]. Sample sizes for each experimental condition are provided in the figures and the respective legends.

Chapter 3: Final consideration

3.1 Summary

Several published data highlight the importance of chronic inflammation for neurodegeneration in ALS with an increased spinal cord recruitment of peripheral proinflammatory monocytes, DCs and T cells found in patients and animal models. T lymphocytes infiltrates the brains of ALS patients and by interacting with glial cells could play a role in motor neuron degeneration. [99]

In order to become activated and infiltrate target organs, T cells need to interact with DCs, professional antigen presenting cells capable of priming T cells and skewing their responses toward an inflammatory or anti-inflammatory phenotype. [14] Because of their unique capacity to shape immune responses, DCs play important roles in the pathogenesis of several inflammatory diseases including neuroinflammatory diseases. [100] Studies on human DCs demonstrate that in the peripheral blood of patients with progressive neurodegeneration these cells are functionally altered. [101]

To date no clear data are available regarding the functional state of DCs in peripheral blood of ALS patients.

In this study we examined the phenotype and functionality of circulating DCs in a large cohort of ALS patients selected on the basis of their clinical phase. The goal of this work was to understand how these cells contribute to disease progression. To do this, we performed ex vivo analyses of the frequency and expression of costimulatory, MHC and chemokines molecules of $CD1c^+$ DC subsets and we investigated the capacity of purified DCs to spontaneously produce inflammatory cytokines and to respond to LPS.

We enrolled 72 ALS patients (52 to test cytokine production and 20 for phenotypic analyses), 47 healthy donors (37 to test cytokine production and 10 the phenotype), and 25 Patients affected by neurological disorders unrelated with ALS stratified for age and sex.

DC numbers and their phenotype were investigated by cytofluorimetric analyses. We found that ALS patients have much lower number of circulating DCs (identified as $CD1c^+$ and $CD19^-$) compared with healthy donor. ALS DCs showed an increased expression of the integrin CD62L (Table 12). Since this integrin is required for the recruitment of leukocytes to secondary lymphoid organs or to inflammatory sites, these observations confirmed that in ALS patients DCs are actively recruited in the central nervous system with a mechanism presumably involving the CD62L molecule.

	ALS	CTR	*p-value*
CD62L, Median [Q1 - Q3]	**5247,50 [3741 – 6532,50]**	**2857 [2149 - 4664]**	**0,0190**
CD86, Median [Q1 - Q3]	295,5 [267 - 377]	299 [264 - 319]	0,6284
CD80, Median [Q1 - Q3]	189,50 [161 - 202]	183 [173 - 189]	0,8875
CD40, Median [Q1 - Q3]	561,50 [421,50 - 717]	588 [445 - 699]	0,5975
CCR5, Median [Q1 - Q3]	1156 [896 - 1693]	1225,50 [1070 - 1532]	0,8603
CCR7, Median [Q1 - Q3]	2424 [1714 - 3267]	2035 [1201 - 2669]	0,2178
CCR2, Median [Q1 - Q3]	90165,50 [72264,50 – 101655]	62475 [40282 - 99442]	0,1405
MHC I, Median [Q1 - Q3]	2288,50 [1725,50 – 2770]	2285,50 [1508 - 2813]	1,0000
MHC II, Median [Q1 - Q3]	18778,50 [11162,50 – 23045]	18070 [7218 – 23518]	0,8431

Table 12: Summary of cytofluorimetric analysis

The only significative difference we observed between ALS patients and CTR is in CD62L expression; in particular this integrin is more expressed in ALS patients than in healthy controls.

We then analysed the spontaneous or LPS-induced production of inflammatory cytokines such as TNFα, IL1β, IL6, IL8, IL10 and CCL2 (Table 13). We noticed a subpopulation of ALS patients with a higher spontaneous and LPS-induced IL8 production. These patients, interestingly, also showed higher efficiency of CCL2 secretion. Although we could not define a correlation between the higher efficiency of these inflammatory cytokine production and disease progression, high levels of CCL2 have been shown in the spinal cord of SOD mice, a mouse model of a subclass of ALS disease, and in some ALS patients. According with our results, DCs can be a source of CCL2 in the spinal cord in a subpopulation of ALS patients. This

observation suggests that a simple peripheral blood analysis can be sufficient to identify subgroups of ALS patients.

ALS	NT	LPS	LPS-NT
TNFa, Median [Q1 - Q3]	62,20 [14,4 - 131,07]	385,33 [254,65 - 524,25]	293,01 [178,80 - 417,08]
IL6, Median [Q1 - Q3]	33,84 [12,04 - 58,18]	1923,57 [1176,59 - 2516,42]	1832,77 [751,57 - 2386,61]
IL8, Median [Q1 - Q3]	1312,63 [622,69 -2209,08]	5357,65 [3232,36 - 7638,71]	4112,72 [1993,26 - 5534,6]
IL1b, Median [Q1 - Q3]	13,30 [5,925 - 30,45]	322,41 [202,44 - 459,57]	285,22 [165,08 - 427,27]
IL12p40, Median [Q1 - Q3]	181,46 [0 - 641,76]	388,09 [251,65 - 758,17]	148,68 [21,035 - 422,52]
IL10, Median [Q1 - Q3]	92,11 [22,34 - 198,09]	1068,96 [375,64 - 1610,55]	977,21 [314,86 - 1338,27]
CCL2, Median [Q1 - Q3]	93,91 [20,95 - 366,58]	115,97 [524,9 - 2535,98]	996,99 [215,01 - 1938,81]

CTR	NT	LPS	LPS-NT
TNFa, Median [Q1 - Q3]	70,17 [46,40 - 123,69]	267,71 [198,79 - 336,91]	198,70 [152,63 - 237,87]
IL6, Median [Q1 - Q3]	48,93 [35,43 - 107,53]	2148,24 [1586,59 - 2528,54]	1749,15 [1238,59 - 2055,31]
IL8, Median [Q1 - Q3]	1443,85 [820,70 - 1901,48]	5124,3 [3531,13 - 6004,99]	2937,49 [2367,74 - 3915,49]
IL1b, Median [Q1 - Q3]	12,99 [4,44 - 48,13]	268,23 [175,75 - 302,65]	261,00 [143,95 - 287,62]
IL12p40, Median [Q1 - Q3]	386,85 [312,93 - 531,76]	404,05 [281,49 - 652,92]	60,17 [-77,37 - 207,59]
IL10, Median [Q1 - Q3]	135,79 [60,37 - 317,68]	912,73 [701,27 - 1383,29]	739,32 [420,65 - 909,74]
CCL2, Median [Q1 - Q3]	278,21 [182,47 - 449,59]	837,18 [504,73 - 1200,80]	441,70 [184,38 - 1001,39]

NEURO CTR	NT	LPS	LPS-NT
TNFa, Median [Q1 - Q3]	122,99 [75,31 - 131,43]	327,49 [247,40 - 426,53]	214,33 [160,98 - 269,41]
IL6, Median [Q1 - Q3]	33,71 [22,67 - 43,80]	2061,03 [1301,69 - 2271,56]	2022,05 [1278,02 - 2192,55]
IL8, Median [Q1 - Q3]	1720,01 [1016,72 - 2200,03]	5345,99 [3208,71 - 5744,01]	2585,99 [1833,81 - 4051,85]
IL1b, Median [Q1 - Q3]	27,50 [19,05 - 30,42]	301,13 [171,58 - 462,18]	283,05 [148,88 - 390,13]
IL12p40, Median [Q1 - Q3]	376,89 [303,48 - 530,09]	378,26 [234,11 - 452,69]	21,4 [-53,99 - 141,39]
IL10, Median [Q1 - Q3]	151,43 [109,52 - 187,49]	781,75 [635,13 - 924,54]	623,46 [471,33 - 768,20]
CCL2, Median [Q1 - Q3]	191,45 [134,47 - 266,29]	738,50 [431,49 - 904,53]	469,56 [299,76 - 706,08]

Table 13: Summary of CD1c^{+}DCs cytokines production

There are no significative differences in cytokines production between ALS patients, CTR and NEURO CTR.

We, thus, analysed the correlation between the levels of single cytokines in ALS patients before and after LPS exposure and some disease parameters, including site of onset, NIV, RIG/PEG, alsfrs-r, disease progression, alsfrs-r bulbar, alsfrs-r respiratory, alsfrs-s spinal, time from onset to diagnosis, time from onset to evaluation and time from diagnosis to evaluation.

We observed a significant inverse correlation between the time from onset to diagnosis and the ΔIL6 levels (IL6 LPS – IL6NT), suggesting that an increased efficiency of IL-6 production in ALS patients may accelerate the initial phases of the disease. We have also a case report of a patient with high, baseline, inflammatory state treated with αIL-6R. This treatment was able to reduce inflammation but it had no effect on ALS clinical course.

We also performed longitudinal analysis following some ALS patients at T0, T3 and T6. Unfortunately we observed no differences between the three time points for all the cytokines we have taking in exams.

3.2 Discussion and future perspective

In our study we have demonstrated that DCs are one of the major cell subset recruited to the central nervous system at least in some ALS patients. Data from Henkel and colleagues confirm our results showing that there are mature and immature DCs in the spinal cord of ALS patients but not in the same tissue from controls. In particular they found a significant increase of CD83 and CD40 (mature/activated dendritic cells markers) mRNA in spinal cord of sALS and fALS patients and also an increase of CD1a and DEC-205 (expressed on DCs before maturation) in fALS patients. This increased expression of DCs transcripts correlated also with the more rapidly progressing ALS. They also showed, with an immunoistochemical staining, that both parenchymal and perivascular CD1a- and CD83-positive cells are present in the ventral horn of ALS patients, whereas mostly perivascular-positive cells are in the degenerating corticospinal tract. The same cells are, furthermore, absent in control tissue. [51]

The presence of immature DCs in ALS tissue, which can take up and process antigen as well as stimulate other cellular constituents of immune system, such as microglia or natural killer cells, suggest an involvement in the ongoing immune/inflammatory reaction. It is also relevant that among the factors acting on immature DCs there are ROS, IgG aggregates and cytokines, all element that could be found in ALS patients tissues. [123]

As DCs mature, they lose their phagocytic properties and their ability to capture antigen, but express MHC class II as well as costimulatory molecules and become potent APCs. [124] The presence of immature and mature DCs in ALS, and their absences in control specimens, argues for their involvement in accelerating of motor neuron injury in ALS.

CCL2-CCR2 axis could be involved in the disease since we found an increase, even if it is not statistically significant, in the levels of this chemokine ad receptor in ALS patients. One more time the work of Henkel and colleagues supports our findings, showing that there is a 23-fold increase of CCL2 mRNA in ALS spinal cord compared with controls and also and higher level of this chemokine in CFS of sALS compared with controls.

CCL2 is a potent chemoattractant and activating peptide that is expressed mostly in astrocytes, but also in neurons, microglia and macrophages after ischemia, hypoxia or excitotoxicity. Excessive CCL2 can exacerbate the injury. It attracts CCR2-expressing myeloid DCs and activates T-cells regulating both Th1 and Th2 responses. [47] Thus CCL2 is important in the recruitment of immune/inflammatory cells into CNS, and more specifically the recruitment of DCs and monocytes.

The increase in DCs transcript and in CCL2 expression is seen also in mSOD1 mice at 110 and 15 days respectively.

The pathology observed in these mSOD1 transgenic mice resembles the one seen in ALS patients. [126] All of the mice appear normal at birth and begin to show motor dysfunction later, although the age of onset depends on the number of copies of the mSOD1 gene, on the specific mutations and on the genetic background of the mouse. [125] Their symptoms progress from hind limb weakness to muscle atrophy, paralysis and death. Evidence indicates an immune inflammatory activation in these mice and a response present early in disease prior to any evidence of dysfunction.

The CCL2 increased expression is seen before any signs of disease and may indicate the presence of injured cells as early as 15 days of age. However the upregulation of CCL2 could be by or in response to the injured cells and providing a protective function. Later in the disease, when generalized inflammation is present, it is not clear if CCL2 is protective or injurious. [125]

Our results show also that, although the majority of activated DCs may migrate to the central nervous system, some differences are still observable in the peripheral blood. In particular peripheral blood DCs analyses can be useful to stratify patients in those that have a high inflammatory response versus those that do not show an altered inflammatory pathway. Given the high heterogeneity of ALS disease we could not observe for the moment significant correlations with disease parameters, excluding the case of IL-6 that inversely correlated

with the time between onset to diagnosis, nevertheless a more refined analysis based on specific criteria is likely to be informative on some particular disease aspects.

In this regard, we decided to follow a patients treated with αIL-6R, in accord with his clinicians. What we can see is a strongly reduction of his inflammatory state, but, unfortunately this reduction didn't have a positive effect on disease outcome.

The role of IL-6 signaling in neurological diseases is not clear and the roles of IL-6/sIL-6R may vary in different stages of the diseases, and may depend upon the ratios of free IL-6 and sIL-6R or other factors specific to each disease. [127]

The humanized IL-6R antibody called tocilizumab (Actemra^{R}) inhibits IL6 signaling through both IL-6R and sIL6-R. Tocilizumab has shown favourable long term effects in patients with rheumatoid arthritis [128] and is under study in patients with Castleman's disease, juvenile rheumatoid arthritis and inflammatory bowel disease. [129]

Fiala, Mizwicki and collegues performed study in vitro and in vivo on Tocilizumab efficacy in ALS treatment. They studied in vivo baseline inflammatory gene transcription in PBMC of 10 sALS patients and the effect of tocilizumab infusions after the demonstration in a previous work that this drug are able to attenuate in vitro inflammatory activation in ALS PBMCs in comparison to control PBMCs [130]. They showed that, at baseline, one half of ALS subjects studied have strong

inflammatory activation (Group 1) and the other half have weak activation (Group 2) and that the effect of tocilizumab infusion have a different response in this two categories of patients. In fact In Group 1 patients, tocilizumab infusion results in a dramatic down regulation of inflammatory cytokines (in particular IL6 and IL1) and chemokine mRNAs, whereas in Group 2 IL1 and IL6 were upregulated. In comparison to the rate of neurological decline before treatment, three patients are showed to have an attenuation in the progression, but this study was not a double blind trial and a clinical efficacy could not be settled down. Moreover the effects are individual and time- and dose dependent, fact that underline one more time the heterogeneity of ALS disease and patients. [131]

In conclusion, we can argue about the high levels of CD62L expression by peripheral blood DCs, that we observed, suggesting that this molecule could be a possible target for in vivo treatment. To this regard, we are planning to perform a preclinical study in mSOD1 mice to verify if a treatment with a blocking anti-CD62L antibody could interfere with disease progression.

To date there are no authorized clinical treatment with anti-CD62L but it has been developed a monoclonal antibody against this L-selectin named Aselizumab that was used in a clinical trial in a multiple traumatized patients. Unfortunately in the trial were found no differences between the treatment with the drug and with the placebo. [132]

Another humanized antibody was developed to contrast the migration of peripheral blood leukocytes to the brain via the blood brain barrier, named Natalizumab. This monoclonal antibody was yet in clinic and it is used in the treatment of relapsing and remitting multiple sclerosis[133] . It is direct against the α4 subunit of the α4 integrins (α4β1 and α4β2) and it has shown its efficacy in the multiple sclerosis treatments by reducing the annual relapse rate in two clinical trials by 68%. [134] [135] It is also shown in literature that Natalizumab treatment is able to reduces CD62L in $CD4^+$ T cells so it is possible that this drug could also be used to try to manage ALS disease. [136]

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