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Use of the non-peptidic Nerve Growth Factor mimetic MT2 to activate TrkA and TrkB receptors: a therapeutic tool in Multiple Sclerosis

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Alla mia famiglia,
il mio punto di riferimento

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Abbreviations table

Akt = Protein kinase B
BDNF = Brain-derived neurotrophic factor
CNS = Central nervous system
GPCR = G protein coupled receptor
NT-3 = Neurotrophin-3
NT-4/5 = Neurotrophin-4/5
NGF = Nerve growth factor
Trk = Tropomyosin receptor kinase
dpi = Days Post Immunization
MS = Multiple Sclerosis
EBV = Epstein-Barr Virus
HSV = Herpes Simplex Virus
Cpn = Chlamidia pneumonia
RRMS = Relapsing Remitting Multiple Sclerosis
SPMS = secondary progressive Multiple Sclerosis
PPMS = Primary Progressive Multiple Sclerosis
PRMS = Progressive Relapsing Multiple Sclerosis
BBB = Blood Brain Barrier
IFN = Interferon
CSF = cerebrospinal fluid
EAE = experimental autoimmune encephalomyelitis
JCV = John Cunningham Virus
DMF = Dimethyl-fumarate
TMEV = Theiler's murine encephalomyelitis virus
MBP = myelin basic protein
MOG = myelin oligodendrocytes glicoprotein
PLP = proteolipidic protein
MAG = myelin associated glicoprotein
MHC-II = major histocompatibility complex class II
DC = Dendritic cells
APC = Antigen presenting cells
NK = Natural Killer
PML = progressive multifocal leukoencephalopathy
GA = Glatiramer Acetate
MRI = magnetic resonance imaging
NT-3/4 = Neurotrophin 3/4
SC = Spinal Cord
MAPK = Mitogen-activated protein kinase
PCD = Programmed cell death
PARP = poly (ADP-ribose) polymerase

ERK = extracellular signal-regulated kinase
PTX = Bordetella Pertussis Toxin
PBS = Phosphate buffered saline
CXCL = chemokine (C-X-C motif) ligand
CPM = counts per minute
LFB = Luxol Fast Blue
HE = Haematoxylin Eosin
TEM = Transmission Electron Microscope
HRP = horseradish peroxidase
iNOS = inducible nitric oxide synthases
mCC = medial corpus callosum
IL = Interleukin
TCL = T-cell line
SEM = standard error of the mean
SD = standard deviation

Introduction

Introduction

Multiple Sclerosis

Multiple Sclerosis (MS) is a chronic immune-mediated demyelinating and neurodegenerative disease of the central nervous system Noseworthy *et al.* 2000. This disease leads to a slower axonal conduction due to myelin damages and loss of neurons. First traces of MS description was between 1838 and 1842, when Jean Cruveilhier and Robert Carswell illustrated the lesions of MS almost simultaneously in the their respective atlas Compston 1988. In particular, Cruveilhier described symptoms like tiredness and paraplegia that histologically shown scars in spinal cord tissue. Anyway, first full description was written by Jean Charcot in 1868 under the name *"sclerose en plaques"* by Jean Martin Charcot (Charcot, J. M., 1868): he was the first one to link disease symptoms and typical observed features, the sclerotic plaques. Furthermore, he carried on the histological work identifying, in the lesions, reactive gliosis, loss of myelin and phagocytes full of fatty acids, hypothesizing that was due to pathogens such as smallpox, typhoid and cholera infections[i].

Etiopathogenesis

Onset of MS is therefore the results of interplay of many factors, like the genetic and environmental components.

[i] Raine C.D., McFarland H.F., Hohlfeld R., 2008. Multiple Sclerosis: a comprehensive text. Saunders Elsevier Editor

Epidemiology and environmental factors

There are about 2.5-3 million people in the world with MS, 600.000 in Europe and about 114.000 in Italy. MS is usually diagnosed during early adulthood (although people can be diagnosed at any age) and that people are living with this progressive neurological disease for many decades.

Due to its multifactorial nature, its distribution is not uniform: the disorder is more common in areas far from the temperate climate, particularly North Europe, the United States, New Zealand and South Australia. On the other hand, the prevalence of the disease seems to have a progressive reduction getting closer to equator.[ii]

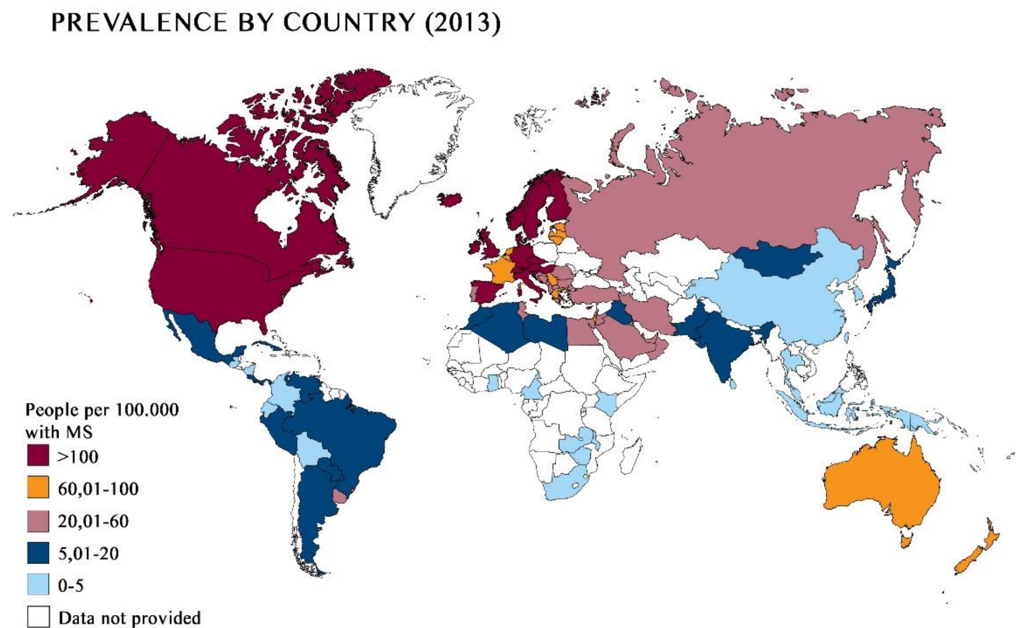


Figure 1. Atlas of MS, 2013, Multiple Sclerosis International Federation (MSIF)

Furthermore, these geographic data could support the "hygiene hypothesis", according to which subjects who are not exposed to infections at early age due to extreme cleaning conditions (as in developed countries), have aberrant responses to infections when affected by these infections during first adulthood.

[ii] *https://www.aism.it/index.aspx?codpage=sclerosi_multipla*, **upd. June 2017**

Examples of diseases which are likely to have a role in MS susceptibility if occurring later in life include poliomyelitis Nathanson and Miller 1978, infectious mononucleosis and measles. Infectious agents like Epstein-Barr virus (EBV), herpes simplex virus (HSV) and Chlamydia pneumonia (Cpn) are often suggested as etiologic factors in MS (Antony et al., 2004; Christensen, 2005; 2010). Anti-EBV antibodies are high in MS patients and, after relapses, these patients often reactivate latent EBV infections. Wandinger *et al.* 2000.
Sex is another factor in MS onset: this disease is twice more common among women than men, but this ratio is variable and local-depending: in fact, ratio of women:men with MS is considerably higher in some regions, such as East Asia where the female-to-male ratio is 3.0, and the Americas where it is 2.6. Nevertheless, usually men that develop MS that worsens more rapidly than women.

Genetic factors

Analogous to other autoimmune diseases, MS tend to cluster in families and several studies showed that monozygotic twins have an increased risk to develop MS in respect to unrelated population (Sadovnick 1993; Mumford *et al.* 1994). In part these studies were conducted in Finland and Denmark – two nations with an high incidence of MS cases - by Kuusisto and Hansen, respectively, on homozygous and heterozygous twins (Kuusisto *et al.* 2008 - Hansen *et al.* 2005). The overall trend is a higher concordance among monozygotic twins (25-30%%) than dizygotic twins (0-3%). In particular, Finnish researchers conducted a two-time study, analyzing how incidence of MS was varied over the years, observing a higher concordance for dizygotic twins during last 20 years, increased up to 15%. Also being siblings, but not twins, represents a risk factor: the risk in developing MS is greater for first degree relatives of patients affected by MS, with an incidence that can go from the 3% among brothers to 2% for parents and sons (Ahlgren *et al.* 2012 - Korn 2008 - Sadovnick 1993). Therefore, scientists support a complex combination of environmental and genetic factors responsible

of MS disease. Since the 1970s MS has been known to be associated with major histocompatibility complex class II (MHC) genes (Olerup and Hillert 1991). This gene is expressed by cells presenting the antigen and is important to induce a mediated CD4+ cell response. The gene encoding for this complex is, together with other genes involved in the functioning of the immune system, in a locus in position 6p21. It has been extensively studied with GWAS, observing that some HLA haplotypes may predispose to the disease. In the Caucasian population, the risk is attributed to the haplotype HLA-DR15: DRB1*15:01 (encoding HLA-DR2b), DRB5*01:01 (expressing HLA-DR2a), DQA1*01:02, DQB2*06:02 (encoding HLA-DQ6) (Ballerini *et al.* 2004 - Hoppenbrouwers and Hintzen 2011). Homozygous DRB1*15:01 seems to be the phenotype correlated to the risk of MS onset, with the risk halved in DRB1*15:01/X heterozygous population. DRB1*15:01 has been also correlated with phenotypic markers of disease severity (Isobe *et al.* 2016). The first association studies showed that the idea of a polygenic model of MS heritability was the most appropriate and following this model research groups in Europe and USA started the assembling of large multicenter data sets in order to investigate by genome-wide association study (GWA study, or GWAS) the genetic correlation to MS occurrence (reviewed in Baranzini and Oksenberg 2017). GWAS studies allowed, for example, to confirm familiarity and to individuate associated genes (i.e. IL7R, CTLA4, and IL2RA between others) showing up to 200 autosomal associated susceptibility variants outside the MHC genes. Of note, there are some rare variants associated with the appearance of MS in certain populations, like in the Sardinian (Cocco *et al.* 2013 - Steri M *et al.* 2017).

MS disease course

As already mentioned, multifocal areas are the hallmark of this disease: this is the result of myelin loss within the central nervous system called plaques or lesions. Clinically, MS has manifestations that are dependent on nerve fibers

affected. The commonest manifestation is a visual disturbance, the optical neuritis, a set of symptoms as vision loss, painful eyes and blurred vision. Other typical manifestations are deficit or lacks in sensory, tremors, scanning speech and coordination defects and they can be attributed to an involvement of the cerebellum. (Stuke *et al.* 2009) Another disease feature is the manifestation of depression, that occurs with a prevalence range from 19 to 45%. (Skokou *et al.* 2012) Besides these signs, inflammatory nature of disease is also reflected by CSF of patients, as higher concentration of total protein, mononuclear cell count and gammaglobulin (IgG) fraction (Compston 2006 – Popescu *et al.* 2013).
There are usually schematically classified in four types of MS (Figure 2 - Kieseier and Hartung 2003):

- Relapsing-Remitting MS (RRMS), that includes 90% of patients affected, secondary progressive MS (SPMS), and primary progressive MS (PPMS). (Manouchehrinia and Constantinescu 2012). Most MS patients (approximately 85%) initially present with this form of the disease, characterized by clearly defined disease relapses with full recovery or with sequelae and residual deficit upon recovery. RRMS is not classified as a progressive form of multiple sclerosis, but residual deficits can be established with each exacerbation.
- Secondary progressive MS (SPMS). At least 50% of patients with RRMS will transition into this sub form, characterized by disease progression with or without occasional relapses, minor remissions, and plateaus.
- Primary progressive MS (PPMS). Approximately 10% of the MS population presents a disease progression from the onset with occasional plateaus and temporary improvements.
- Progressive-relapsing MS (PRMS). The least common form is a progressive disease from onset with acute relapses, with or without full recovery, with periods between relapses characterized by continuous progression.

According to this subdivision, every type of disease is characterized by a specific clinical course: RRMS has well-known interspersed relapses during which there

is no disease progression followed by recovery of varying extent; conversely PPMS form has a progressive course of disease from the onset of illness. Last, SPMS starts as a RRMS showing a progressive pattern that may include relapses and remissions; it can occur after 5–25 years from disease onset, and the pattern often changes: the number of acute exacerbations is greatly reduced and replaced with a slow, steady increase in symptom severity. (Tremlett *et al.* 2009).

Furthermore, Fred Lublin (Lublin 2014) overhauled and redefined the RR and Progressive Disease, taking in consideration either clinical signs and images (MRI). His intentions were to make it easier to characterize the state of the disease, considering the clinic and the instrumental images. Defining the active or inactive state of the disease, especially for the progressive phase, this new subdivision would have helped to track the right disease phase. He considers the Clinically Isolated Syndrome (CIS) as part of RR disease spectrum, and subdivided CIS and RR in active or not active (fig. 2). Moreover, PPMS and SPMS were collected in a generic Progressive Disease voice, subdividing it in (fig. 2):

• *Active and with progression*: individual has had an attack and is also gradually worsening)

• *Active but without progression*: e.g. individual has had an attack within a previous specified timeframe, i.e. 1 year, 2 years

• *Not active but with progression*: e.g. walking speed has decreased)

• *Not active and without progression*: stable disease.

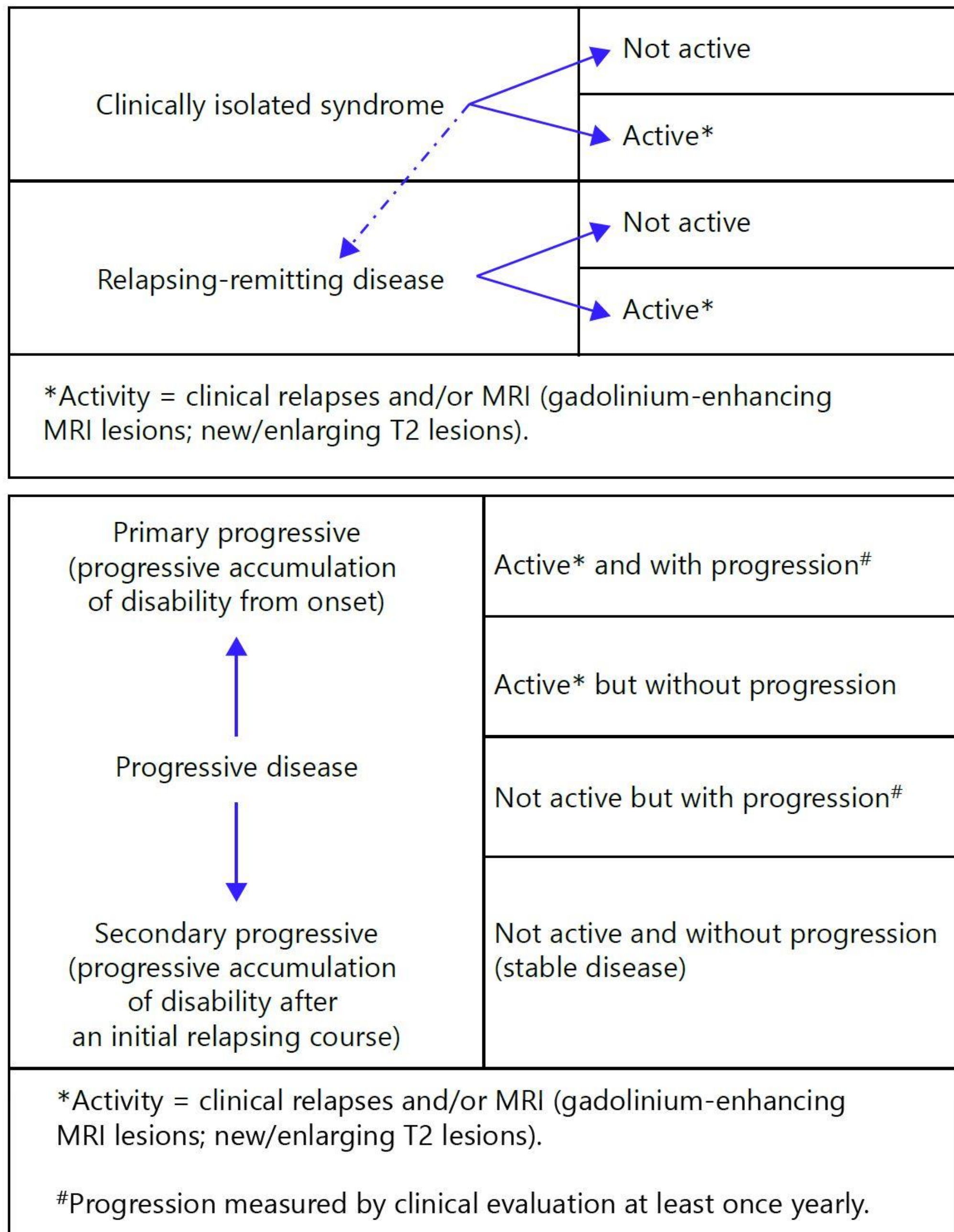


Figure 2. **Illustration of different clinical courses of multiple sclerosis reviewed by Fred Lublin in 2013. Lublin 2014**

MS therapies

Final goal of MS treatment is treating, avoiding and diminishing relapses, then slowing progression of disability. Basically, there are two different strategies: a short-term one to reduce damage effects after an acute relapse, and a long-term one, that aims at stabilizing disease processes. (Spain *et al.* 2009)

Thanks to their potent immunosuppressive and anti-inflammatory potential through induction of T-cell apoptosis, glucocorticosteroids have represented the first lines of treatment since 1960s. Adrenocorticotropic hormone (ACTH) is a melanocortin peptide that was approved by the FDA for use in humans in 1952, only three years after it was first tested in rheumatoid arthritis (RA) (Henche PS et al., 1949), but it caused unselected release of steroids from adrenal glands. Later, methylprednisolone and prednisolone have been the MS therapies were mostly based on their assumption.

Nowadays, these corticosteroids are still used during acute relapses because their anti-inflammatory properties make them perfect to slower disease progression and relapse duration through T-cell lymphocytes apoptosis (Gold *et al.* 2001) and accelerates reconstitution of blood brain barrier (BBB). For relapsing treatment, a high dosage steroid-based therapy (500-1000 mg/die) is recommended. Intravenous methylprednisolone is usually administered for 3-7 days and is followed by prednisolone (60-80 mg/die) if symptoms are particularly severe.

So far, from 1993 when IFN-β1a has been approved by FDA as drug for Relapsing-remitting multiple sclerosis (RRMS) treatment, a lot of therapies and drugs has been developed and approved. During these years, drugs helped the MS understanding, leading to new discoveries and, conseguently, categorisation of MS in relapsing and progressive course (Comi *et al.* 2017).

Ransohoff et al. in 2015 outlined history of disease-modifying drug for MS in three distinguished temporal eras:

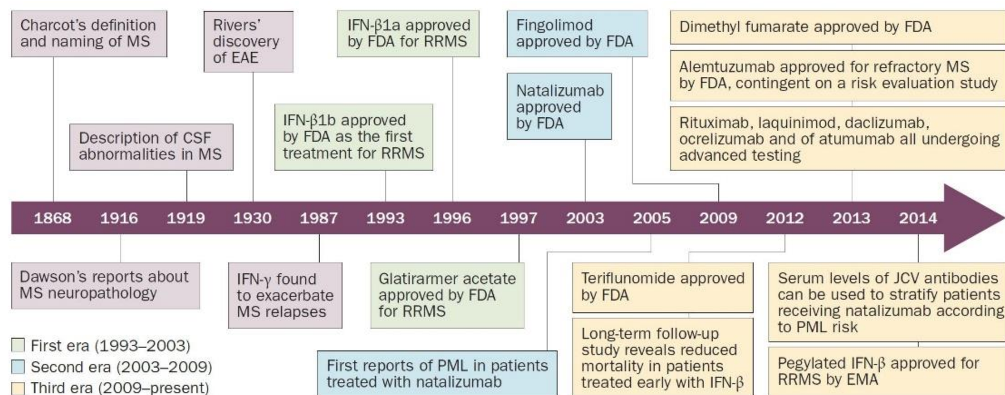


Figure 3. **Schematic timeline for the development of disease-modifying drugs for MS**. Abbreviations: CSF, cerebrospinal fluid; EAE, experimental autoimmune encephalomyelitis; JCV, polyomavirus JC; MS, multiple sclerosis; PML, progressive multifocal leukoencephalopathy; RRMS, relapsing–remitting. (Ransohoff *et al.* 2015)

The representative drugs of the first era were IFN-β1 and glatiramer acetate, two immunomodulator drugs. The approval of IFN as drug for RRMS treatment by FDA in 1993 opened the first era (Hauser *et al.* 1983). Until then, strategies were intravenous methyl-prednisolone-based.[iii] IFN-β1 was demonstrated to have a positive effect in reducing inflammatory cells migration in CNS, in inhibiting T lymphocytes proliferation, proinflammatory cytokines production and in stimulating the production of anti-inflammatory ones (Yong *et al.* 1998).

IFN-β is marketed in three different formulations: Avonex (IFN-β-1a; 30 mg I.M. 1/wk), Rebif (IFN-β-1a; 22 or 44 MG S.C. 3/wk) and Betaferon (IFN-β-1b; 250 mg S.C. every other day). Developed for RR-MS form patients, IFN-β-1b showed to be effective also in the SP-MS forms (SPECTRIMS Study Group, 2001). Randomized and controlled double-blind multicenter clinical trials versus placebo, such as prisms-4 and OWIMS, demonstrate that the use of IFN-β reduced the risk of relapsing of about 30% and it reduced the emergence of new MRI lesions of about 70% (prisms Study Group, 2001; OWIMS Study Group, 1999).

iii Interferon β-1b is effective in relapsing-remitting multiple sclerosis. Part I and II. Clinical results of a multicenter, randomized, double-blind, placebo-controlled trial. The IFNB Multiple Sclerosis Study Group. Neurology 43, 655-661 and 662–667 (1993)).

Glatiramer acetate (GA) is a random polypeptide made up of four amino acids (L-glutamic acid, L-lysine, L-alanine, and L-tyrosine) in a specific molar ratio. GA causes an immune deviation from a Th1 to a Th2 phenotype, induces antigen-specific T-suppressor cells that cross-react with putative autoantigens in the CNS, and inhibits antigen presentation. (Yong *et al.* 1998 – Neuhaus *et al.* 2001). It also represents the best safety profile for pregnant women with not very severe disease.

The second era is marked by the introduction of monoclonal antibodies and small molecules in therapies: MS therapeutic development further evolved, and it is characterized by approvals of natalizumab (monoclonal antibody, 2003) and fingolimod (a sphingosine-1-phosphate receptor modulator, 2009). Natalizumab is a monoclonal antibody to integrin-alfa4-beta1 in leukocytes that showed its efficacy in disease suppression in the rodent model of MS, EAE. (Yednock *et al.* 1992) Natalizumab can be used as monotherapy in RRMS cases, it demonstrated its efficacy in reducing clinical relapses, disability progression, and magnetic resonance imaging (MRI) disease activity measures. (Polman *et al.* 2006). It interferes with T cells entrance in the brain and avoiding inflammation and reducing and retarding relapses. (Barten *et al.* 2010). Despite the good clinical results, this therapy has been linked with a progressive multifocal leukoencephalopathy (PML), a rare, demyelinating opportunistic infection of the CNS caused by the John Cunningham virus (JCV) (Langer-Gould *et al.* 2005 - Buck and Hemmer 2011 – Clifford *et al.* 2010).

Fingolimod (FTY720) is a pro-drug derived from Isaria Sinclairii and that is converted *in vivo* to sphingosine S1P analog, becoming a S1P receptor modulator. The latter is downregulated on leukocytes and the endothelium, trapping naïve and central memory T lymphocytes in lymph nodes (Brinkmann 2009). Although it has a high therapeutic index with a 55-60% lower relapse rates and MRI-activity reduction, this medication has a potential safety problem due to its low target specificity. Indeed, fingolimod is able to bind four of the five known S1P receptor and it can affect other cells then lymph-node, as vascular endothelium, arterial smooth muscle cells, atrial myocytes, bronchial smooth

muscle, and CNS astrocytes and oligodendrocytes (Pelletier and Hafler 2012). For this reason, this therapy requires a very strict safety-monitoring protocol.

The third era (2009 to present) includes small molecules, antibodies and biologics drugs. Examples are teriflunomide, dimethyl-fumarate and Alemtuzumab. The first one is a pyrimidine synthesis inhibitor, exploiting its function with inhibition of the mitochondrial enzyme dihydro-orotate dehydrogenase (DHODH), an enzyme expressed at high levels in proliferating lymphocytes (Ruckemann *et al.* 1998). Dimethyl-fumarate (DMF) is an immunomodulator drug that seems to have the activation of the transcription factor nuclear (erythroid-derived 2) related factor (Nrf2) as mechanism of action. (Linker and Gold 2013) By activating this transcription factor, DMF enhanced neuronal survival and protected glial cells against oxidative stress. (van Horssen *et al.* 2010) Alemtuzumab is a monoclonal antibody that has as target CD52, a highly expressed protein on monocytes, CD4+ and CD8+ T cells. It promotes complement and cell-mediated lyses of targets. Patients who use it show reduction of inflammatory activity and decreased CNS lesions due to some immune cell subsets reduction, shifting the lymphocyte profile. (Compston Compston 2006).
Present days link to a more rational approach, based on effectiveness versus safety and tolerability. (Ransohoff *et al.* 2015)

Use of animal models for MS research

As said, MS is a very complex disease that involves several genetic and environmental risk factors and, based on this, some therapies have been developed to modify disease course. Animal models surely played and will play an important role to improve therapies for MS. Taken the complexity of this disease and its unknown etiology, a single animal model can't cover the entire spectra of all aspects of pathology and clinical feature of human MS.
Three are the main commonly studied animal models for MS (Denic *et al.* 2011):

- purely Experimental Autoimmune/Allergic Encephalomyelitis (EAE)
- toxin-induced models of demyelination, including the cuprizone model.

Experimental Autoimmune Encephalomyelitis

Experimental autoimmune encephalomyelitis (EAE) is, in general, a good model to study the inflammation in the brain (Baxter 2007) and one of the most used animal model for MS, with over 6000 publications listed on PubMed. It can be induced by active or passive immunization of different animal species such as mice, rats and monkeys (marmoset) or genetically modified animal models such as the mouse strain C57BL/6 mice, that express 1-9 peptide-T cell receptors specific for myelin basic protein (MBP) (Zujovic *et al.* 2012). As said, in general there's no model which reproduces all aspects of MS and, in particular, there's no a unique EAE model that allows to emulate one or more characteristics of the disease (Schmidt 1999 - Van Epps 2005 - Hafler *et al.* 2005).

Historical notes

The birth of EAE model dates to 1925, when Koritschoner and colleagues found that rabbits immunized with homogenate of human spinal cord developed paralysis (active induction) (Koritschoner, R. 1925). In 1933 Rivers and colleagues (Rivers *et al.* 1933) explained that the paralysis, that arose in monkeys immunized with brain tissue homogenate, was associated with perivascular infiltrates and tracked to demyelination in central nervous system (CNS) (brain and spinal cord). Freund's adjuvant introduction in 1942 (Freund Freund *et al.* 1947) boosted immune response improving and facilitating the immunization. The first passive induction of EAE has been proposed by Paterson in 1960 (Paterson 1960), when he demonstrated that EAE could be induced transferring lymph node cells isolated from rats immunized with spinal cord homogenate, to naive animals. The addition of pertussis toxin in 1973 improved efficiency in the induction of disease. Nowadays, it has become clear that the clinical outcome of

immunization depends on genetic background of animals studied, the type of antigen and following immunization protocol (Hartung *et al.* 2005 - *Olsson et al. 2000*; Wekerle *et al.* 1994)

EAE models

Over the years, the material used for the immunization protocol changed (passing through the homogenate of CNS towards the immunodominant myelin proteins and peptides) allowed to increase the reproducibility of EAE models, more faithful to the different aspects of MS (Wekerle *et al.* 1994). Among myelin proteins, MBP is the most used (Martenson *et al.* 1969):

- MOG, myelin oligodendrocytes glicoprotein (Lebar and Vincent 1981)
- PLP, proteolipidic protein
- MAG, myelin associated glicoprotein (Poduslo 1983)

and among encephalitogenic small peptides usually this is the list of the most used (Eylar *et al.* 1970):

- MBP_{1-37}
- MBP_{1-11}
- MBP_{1-9}
- MBP_{83-99}
- MOG_{35-55}
- $PLP_{139-151}$.

Each mouse strain is susceptible for specific antigen protein or only for certain synthetic peptides. The combination mouse strain/antigen determines the type of illness, EAE, which will be received. For example, immunizing mice of C57BL/6 strain with syngeneic MOG_{35-55}, Freunds' complete adjuvant (CFA) and pertussis toxin (PTX), it will be obtained an EAE presenting an acute phase followed by a brief remission until the onset of a chronic form characterized of, especially in the spinal cord, demyelination and axonal loss with macrophage and $CD4^+$ T-lymphocytic infiltrates.

Main MS animal models are resumed in

Table 1.

DISEASE COURSE	ANIMAL	STRAIN	ANTIGEN
RELAPSING-REMITTING	Mouse	SJL/J	PLP: 139-151
RELAPSING-REMITTING	Mouse	C57BL/6	Low dose MOG_{35-55}
RELAPSING-REMITTING	Rat	DA	MOG_{1-125}
CHRONIC	Mouse	C57BL/6	MOG_{35-55}
CHRONIC	Mouse	C57BL/6	$PLP_{178-191}$
CHRONIC	Mouse	C57BL/6	MP4
CHRONIC	Rat	Lewis N1	MOG
MONOPHASIC	Mouse	B10.PL	MBP
MONOPHASIC	Mouse	PL/J	MBP
MONOPHASIC	Rat	Lewis	MBP

Table 1. ***Classical models of di EAE.*** Abbreviations: **DA**, Dark agouti; **EAE**: Experimental Autoimmune Encephalomyelitis; **MBP**: Myelin Basic Protein; **MOG**: Myelin Oligodendrocyte Glycoprotein; **PLP**, Proteolipid protein. *(Adapted from Batoulis et al. 2011);* **MP4**: a chimeric recombinant fusion protein of myelin-basic and proteolipid protein.

Choice of EAE model

The variability of EAE models is due to species susceptibility and strain immunized with different myelin antigens. The combination strain-antigen and choice immunization protocol are important for the success of immunization and for the development of a specific EAE form (Steinman 1997). Each animal species and each strain of each species has a different genetic background that gives it a different susceptibility to antigens (Luckey *et al.* 2011). In the Decade 1960-70 it was discovered that the MHC genes were essential for all immune responses to protein antigens. Baruj Benacerraf, Hugh McDevitt and other researchers found that strains of rats or mice differed in their ability to produce antibodies against simple synthetic polypeptides and responsiveness was

inherited as a Mendelian dominant character. Genetic predisposition is particularly important for the possible development of the disease. The combination strain/peptide has a great relevance for the assignation of the type of inflammatory infiltrate in the CNS (Berger *et al.* 1997; Kuerten *et al.* 2010). Myelin antigens generate inflammatory infiltrates of several different cellular subtypes in CNS, allowing to evaluate in distinct patterns the high variability of lymphocytic infiltrates in MS. For example, the EAE model, induced with $MOG_{35\text{-}55}$ peptide in C57BL/6, especially studies Th1 and Th17 lymphocytic infiltrates and generates a chronic course. Lymphocytic response-antigen-specific causes lesions in CNS and a wide variety of clinical signs and different types of EAE: acute (for example in Lewis rat), chronic (C57BL/6 or mice Biozzi ABH) or relapsing-remitting MS (SJL mice).
The choice of a EAE model depends on disease aspects of interest.

EAE pathogenesis

Naïve T cells present in animal blood can be potentially autoreactive: once escaped the immune tolerance, these cells could be specific to myelin proteins. This mechanism is exploited with animal immunization strategy: it usually provides the use of a myelin-associated antigen and an adjuvant, and leads to an autoimmune disease arising from a breakdown of immune tolerance in T cells specific for myelin antigens (Seamons *et al.* 2003). The presentation of the antigen to T-cells by antigen presenting cells (APCs), such as dendritic cells (DC). Once activated, T lymphocytes reach CNS, via bloodstream, penetrating the blood brain barrier (BBB - Wekerle *et al.* 1994): immunization compromises BBB integrity encouraging lymphocytes, DC and activated macrophages to migrate toward the CSF. T-cells specific to myelin antigens are reactivated in CNS by infiltrated and residents DCs and consequently they produce proinflammatory cytokines that activate infiltrated macrophages, astrocytes and microglia. The release of proinflammatory cytokines (IFN-γ, TNF-α, IL-17, TGF-β, NO, IL-6) causes oligodendrocyte loss inducing demyelination and axonal injury (Figure 4). Immune-mediated tissue damage or hypersensitivity

can be mediated by auto-specific IgG antibodies. Pathology results from activation of complement and antibody-dependent cellular cytotoxicity (ADCC), mediated by inflammatory effector leukocytes that include macrophages, natural killer cells and granulocytes. Antibodies and complement have been associated to demyelinating pathology in MS lesions, whereas macrophages predominate among infiltrating myeloid cells (Khorooshi *et al.* 2015). The presence of anti-aquaporin 4 (AQP4) antibodies is another important immunological feature implicated in demyelination. AQP4-IgG can cause ADCC when effector cells are present and complement-dependent cytotoxicity (CDC) when complement is present (Saadoun *et al.* 2010). Although researchers believed for many years that Th1 lymphocytes were responsible of the disease, more recent studies have highlighted the role of another lymphocyte subpopulation: Th17. The differentiation of T helper cells into Th1 required cytokines such as IL-12 and INF-γ, that induces the expression of specific transcription factors as T-bet. Differentiation in Th-17, instead, is induced by IL-6, IL-23 and TGF-β cytokines that induce the expression of RORC transcription factor. Th1 and Th17 populations can have a phenotypic plasticity that converts Th1 in Th17 and vice versa. Such plasticity comes from rapids epigenetic modifications that silence T-bet or RORC gene depending on the received stimulus. EAE development is an alteration of balance between inflammatory responses mediated by Th1 and Th17 and function of regulatory T cells that promote tolerance to self. Initially, in EAE, regulatory role was attributed to T CD8+ effector cells (Koh *et al.* 1992) and later also to natural Killer cells (Zhang *et al.* 1997). More recent works recognized the regulatory role to T cells expressing CD4+CD25+ and Foxp3 transcription factor. Disease progression takes place by a process known as " epitope spreading ": within a few weeks of onset of EAE, begins a tissue damage that results in the release of new epitopes, which are processed and presented by APC found in the CNS, which express high levels of MHC II and costimulatory molecules. Then activation of brain tissue induces activation of autoreactive T cells which recognize myelin proteins other than antigen that initially triggered the disease. Clinical significance of this phenomenon is the fact that, at a time

when the disease is advanced, it is likely that multiple antigens are involved in disease progression. Another cell population involved in MS and EAE immunopathogenesis is represented by B lymphocytes. These cells play a dual role, i.e. they act both as antigen-presenting cells that as autoantibody producing cells (Franciotta *et al.* 2008). The antibody production against MOG is crucial because this phenomenon increases disease severity causing extensive demyelination. The sequence of events that lead to EAE pathogenesis is the same in both active and passive induction (Goverman *et al.* 1993). However, inducing passive autoreactive T cells are isolated from lymphoid tissue of an animal previously immunized with myelin antigen, stimulated *in vitro* and transferred to a naïve receiver.

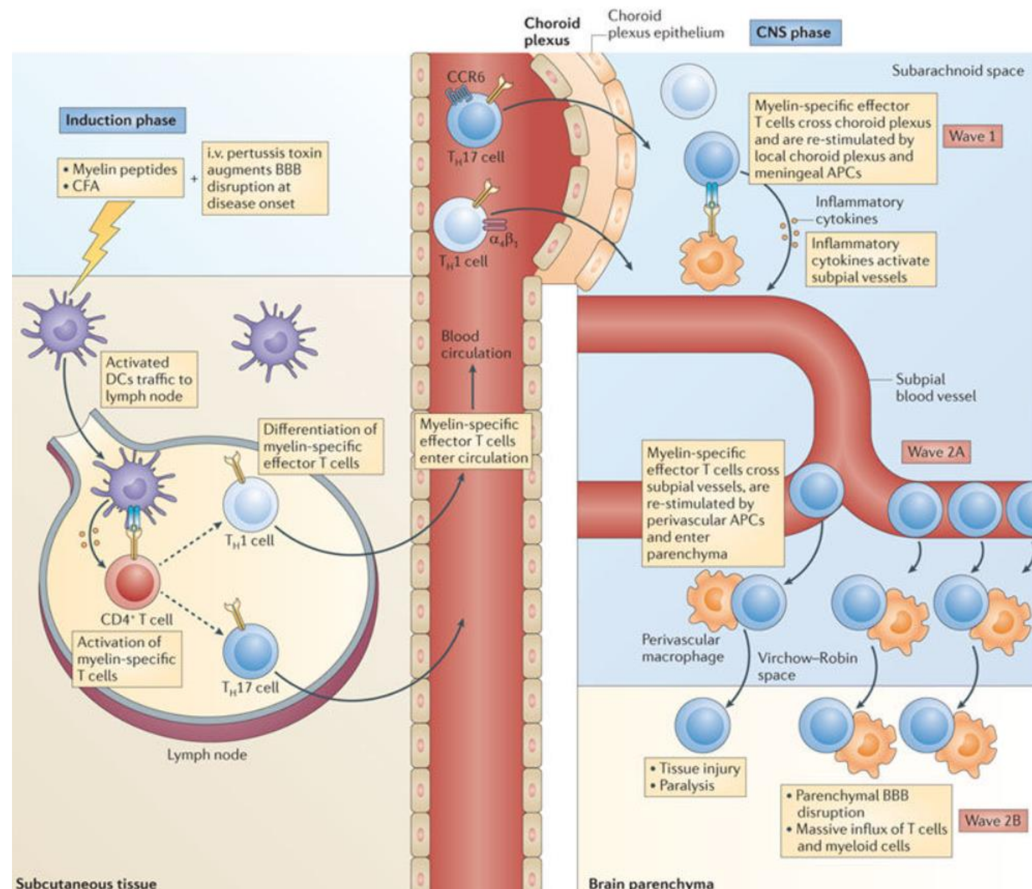


Figure 4. **Pathogenic cascade in the periphery and central nervous system (CNS) during experimental autoimmune encephalomyelitis (EAE).** *From Dendrou et al. 2015.*

EAE pathogenesis induced with MOG_{35-55} peptide. After immunization with MOG_{35-55} peptide, peripheral lymph nodes, dendritic cells (DC) and naïve T cells are activated aganst the antigen. Activated T-cells (Th1 and Th17) enter into bloodstream and reach central nervous system (CNS) crossing the blood-brain barrier (BBB). T-cells in CNS are recruited by periphery inflammatory molecules that activate microglia and APCs (antigen presenting cells). T-cells expand and continue to produce inflammatory molecules that destroy myelin resulting in axonal damage. (Fletcher *et al.* 2010)

EAE, limits and application

EAE mouse models can be used in order to associate clinical manifestations with data derived by histopathology and inflammatory mechanisms, that are involved in MS. The model is one of the most studied for new MS therapeutic approaches but with obvious limitations. The first limitation is that to date the model is not a spontaneous model of disease, it is always necessary an induction of disease by immunization, although there are some models of transgenic mice that express T cell receptors specific to myelin, which partly reproduce a spontaneous onset of illness (Goverman *et al.* 1993). The second limitation of EAE is that myelin synthetic peptides don't undergo post-translational modification (PTM) along the production pathway as with *in vivo* endogenous myelin proteins (Zhou *et al.* 1995). Schematically, the ribosome translates a mRNA sequence into a protein and releases it through the endoplasmic reticulum; here, the protein is cut, folded and held in shape by disulfide (-S-S-) bonds. Consequently, the protein passes through the Golgi apparatus, where it is packaged into a vesicle. Here the protein is subjected to last proteolysis modifications and it turns into mature protein (Potelle *et al.* 2015). So, it is possible that lymphocyte populations activated by synthetic peptides could be different from those triggered by processed peptides. The third limitation is in the exclusive role of $CD4^+$ T in mediating EAE, where human disease sees an important role of $CD8^+$ T lymphocytes (Lassmann and Ransohoff 2004). Finally, we must always remember the phylogenetic distance that exists between man and mouse.

For all these considerations, while recognizing the value of this model, it is still difficult to exploit the results obtained in EAE in order to predict the efficacy of treatments in human disease and over time there have been both important successes that serious failures (Sriram and Steiner 2005).

Toxic model of demyelination: Cuprizone

Cuprizone [bis–cyclohexanone-oxaldihydrazone] is a copper chelating reagent, which supplemented to normal rodent chow, directly or indirectly causes oligodendroglial cell death with subsequent demyelination. Once demyelination is complete new oligodendrocytes, generated from the pool of oligodendrocyte progenitors (OPC), begin to form new myelin sheaths (Gudi *et al.* 2014). Toxins, such as cuprizone, provokes demyelination in the absence of inflammatory infiltrates. Lacking lymphocytic inflammatory component this model is adopted mainly to evaluate the demyelination and re-myelination. Instead, intracerebral inoculation of murine encephalomyelitis virus is used to study possible mechanism involved in autoimmune reactions triggered by viral infection, as it is thought to occur as a result of EBV infection (Abbas *et al.* 2007). The mechanism by which the virus acts has not yet been clarified: for example, it can be hypothesized that active viral infects resident APC, leading to an increase of costimulatory molecules expression, such as to break tolerance and encouraging autoimmune diseases reactions to self-antigens; or there could be a phenomenon of molecular mimicry, so through mechanisms of cross reactivity-specific T cells recognize myelin self-antigens, viral antigens. From an epidemiological point of view viral infections have been associated with a clinical exacerbation of the disease, so it seems unlikely that these factors are the etiologic factors of demyelination.

Neurotrophins and NGF

Neurotrophins are a class of cytokines able to rule development, growth and survival of a large number of cells, not exclusively from nervous system

(Reichardt 2006 – Caporali and Emanueli 2009). In CNS these molecules are necessary for the development and maintenance of the vertebrate nervous system, neural regeneration and remyelination (Kerschensteiner *et al.* 2003).
Nerve Growth Factor (NGF) was the first and best characterized member, discovered by Rita Levi Montalcini and Cohen in 1956 during the study of chick embryonal development. Levi-Montalcini clearly demonstrated that in chick embryos the production of this diffusible factor in the target organ was responsible for the survival of peripheral neurons during development (Levi-Montalcini and Hamburger 1951). The activity of NGF is not restricted to embryonal life: during adulthood there is a basal production of NGF in the innervation field that is essential in regulating neuronal plasticity: experiment based on antibodies that neutralized the growth factor caused a selective depletion in sympathetic chains, leading to hypothesize the central model of neurotrophic factor action. (Cohen 1960 - Levi-Montalcini and Booker 1960).
Later, other members of neurotrophic factors and member of NGF family of growth factor was discovered in mammals: Brain Derived Neurotrophic Factor (BDNF, Barde *et al.* 1982), Neurotrophin 3 (NT-3, Hohn *et al.* 1990) and Neurotrophin 4/5 (NT-4/5, Berkemeier *et al.* 1991) and 6 (NT-6, Gotz *et al.* 1994). A schematic potential activity of neurotrophins with their receptor is reported in **Figure 5**: mature neurotrophic factors binds their high affinity receptors (NGF-TrkA, BDNF and NT-4-TrkB and NT-3-TrkC) and induce an heterodimerization with $p75^{NTR}$ receptor.

NGF, a cross-talk molecule between neuronal and immune cells

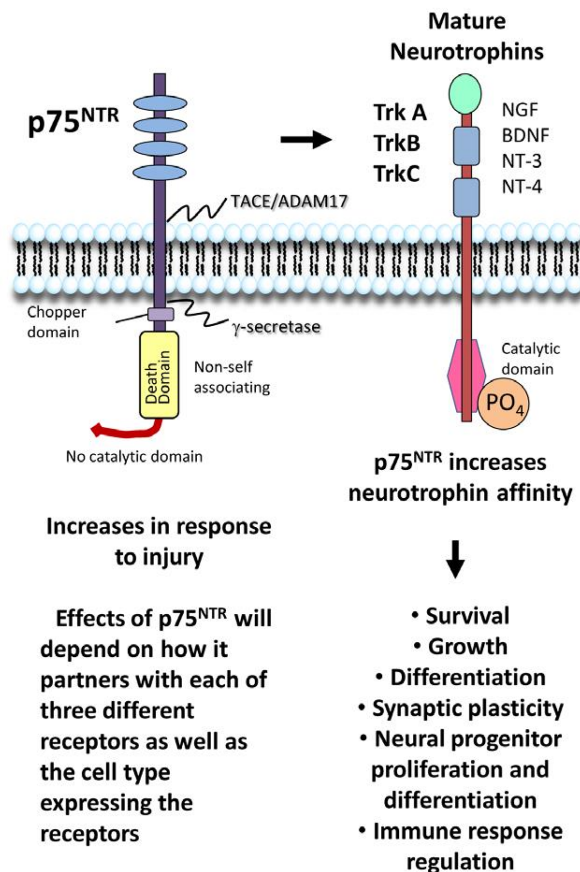


Figure 5. **Schematic representation of the effect of interaction of neurotrophins with their receptor**. Adapted from *Meeker and Williams 2015.*

The Nerve Growth Factor has a structure that is composed by three subunits, named α, β and gamma: their interaction leads to constitute an high molecular weight 7S complex, with a final weight of 130-140 kDa. (Vega *et al.* 2003). NGF interacts with two distinct receptor chains, a high-affinity receptor belonging to the Tropomyosin receptor kinase A (TrkA, 140 kDa) and a low affinity one, $p75^{NTR}$ (NTR stands for Neurotrophin Receptor) that binds all neurotrophins (Villoslada and Genain 2004).

These receptors are transmembrane proteins that are made up of three parts, that comprises transmembrane, extracellular and intracellular domains. Once bound with these membrane receptors through their extracellular domains, NGF induces the dimerization, exerting trophic and differentiation functions. Trophic

function involves the apoptotic events inhibitions, while the latter one triggers growth and modeling of axons and dendrites.

High-affinity receptors are not exclusively expressed on nerve cells: they're also present on deep-tissue and lymphoid and epithelial cells. As matter of the fact, one of the most increasing evidences is that NGF can also exert specific effects on immune functions. For example, treatment of young rats with NGF before and after immunization with sheep erythrocytes results in an enhancement of T lymphocyte-dependent Ab synthesis (Manning *et al.* 1985); NGF can also induce shape changes in platelets as demonstrated in rabbit model (Gudat *et al.* 1981); growth factor is able to enhance vascular permeability in the rat skin (Otten *et al.* 1984), and causes degranulation of rat peritoneal mast cells, suggesting that NGF is involved in acute inflammatory responses (Bruni *et al.* 1982). Furthermore, NGF promotes colony growth and differentiation of myeloid progenitor cells (Matsuda *et al.* 1988) and induces lymphocyte proliferation of both B and T cell populations (Thorpe and Perez-Polo 1987). Functional NGF receptors have also been demonstrated on human B lymphocytes (Brodie and Gelfand 1992) implying that NGF may act as a B cell growth factor.

In summary, NGF can induce proliferation and differentiation of B cells into Ig-secreting cells and the presence of functional NGF receptors on human blood monocytes (Kimata *et al.* 1991 - Ehrhard *et al.* 1993) suggests that NGF, in addition to its neurotrophic functions, can act as an immunomodulator, mediating the cross-talk between nervous and immune systems.

NGF pre-clinical studies

The biological effects of NGF are primarily mediated via its high affinity receptor-TrkA. In 2003 B. Oderfeld-Nowak and colleagues (Oderfeld-Nowak *et al.* 2003) examined the effect of EAE on the expression of TrkA in neuronal and non-neuronal cells of spinal cord (SC) of Lewis rat during the acute (14 days postimmunization, dpi) and chronic (12 months postimmunization, mpi) phases of disease. Under EAE conditions, they noticed an upregulation of TrkA on astroglia associated with an immunoreactivity; moreover, the researchers

verified their hypothesis according to which in the acute phase of disease there was an up-regulation of this receptor in the population of oligodendroglia in the white matter. On the contrary, TrkA was not seen in the chronic phase of the disease. These results suggested that both neuronal and glial TrkA expression changes depend on inflammation, indicating that during the acute phase of EAE the glial cells become more receptive for NGF, marking glia cells as an important target for pharmacological manipulation, particularly for exogenously administered NGF.

NGF clinical studies

Studies on inflammatory and autoimmune diseases showed an abnormal activation of immune cells and an increased production of cytokines and revealed a localized increase in NGF levels at the sites of inflammation. Enhanced NGF levels were initially found in the CSF of MS patients, and it was shown that an increase in NGF closely follows the course of the disease (Bracci-Laudiero, L. et al.; 1992). The list of inflammatory diseases characterized by an enhanced production of NGF in the inflamed tissues or in the blood is fairly long and includes diseases with different pathogenic mechanisms (Bonini *et al.* 1996; Stanzel *et al.* 2008). Inflammatory cytokines can induce the synthesis of NGF in neuronal and glial cells, as well as in epithelial, endothelial, connective and muscle cells (Figure 6) (von Boyen *et al.* 2006) and there are also other molecules that can up-regulate the basal production of NGF in tissue as prostaglandin (Bullo *et al.* 2005) and histamine (Lipnik-Stangelj and Carman-Krzan 2004).

Recently, NGF was associated to autoimmune disease and inflammation: Prencipe et collegues (Prencipe *et al.* 2014), demonstrated that NGF downregulates inflammatory response in human monocytes via TrkA pathway, interfering with the response of TLR-activated monocytes and inducing the downregulation of pro-inflammatory cytokines.

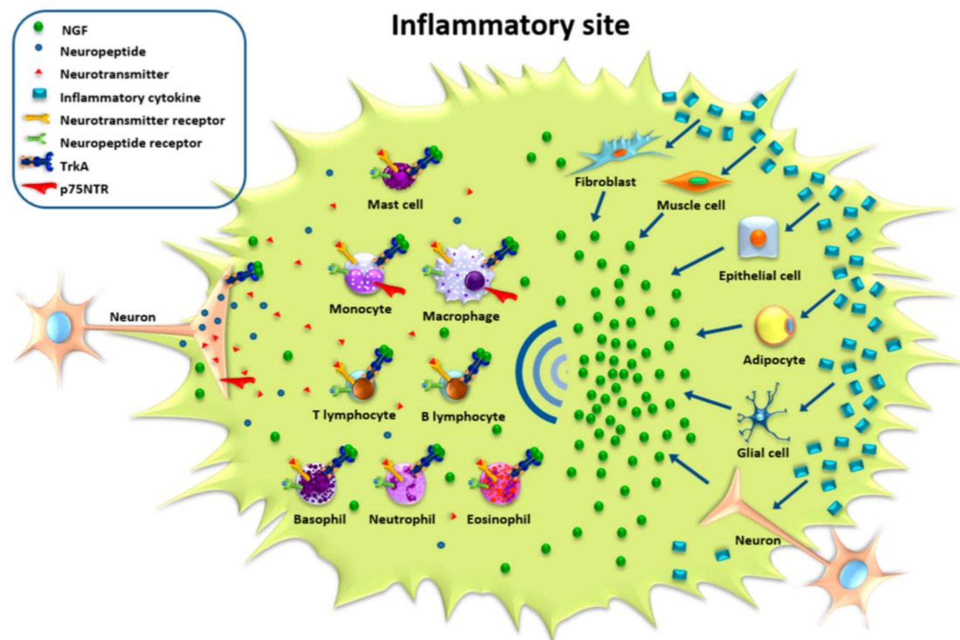


Figure 6. **Direct and indirect effects of NGF on inflammatory responses.** At the site of inflammation, inflammatory cytokines induce (blue arrows) the production of NGF in different cell types, such as muscle cells, epithelial cells, fibroblasts, adipocytes, neurons, glia, and immune cells. The enhanced local production of NGF influences nerve fiber distribution and neuronal activity, inducing the synthesis and release of neuropeptides and neurotransmitters that have immunomodulatory effects. NGF receptors are also expressed on the membrane of immune cells and NGF can directly modulate the activity and functions of immune cells (Minnone *et al.* 2017).

BDNF

The brain-derived neurotrophic factor (BDNF) is a 27-kDa basic protein and is one of the most potent factors that support neuronal survival, regulating neurotransmitter release and dendritic growth (Kerschensteiner *et al.* 1999) binding TrkB and p75NTR receptors.

This NGF family member is mainly produced by immune cells, neurons and activated astrocytes present in inflamed areas of neurodegenerative disease (Stadelmann *et al.* 2002); it has also been found in thymus and spleen. Furthermore, *in vitro* studies show that T and B cells and monocytes expressed BDNF in neurodegenerative lesions.

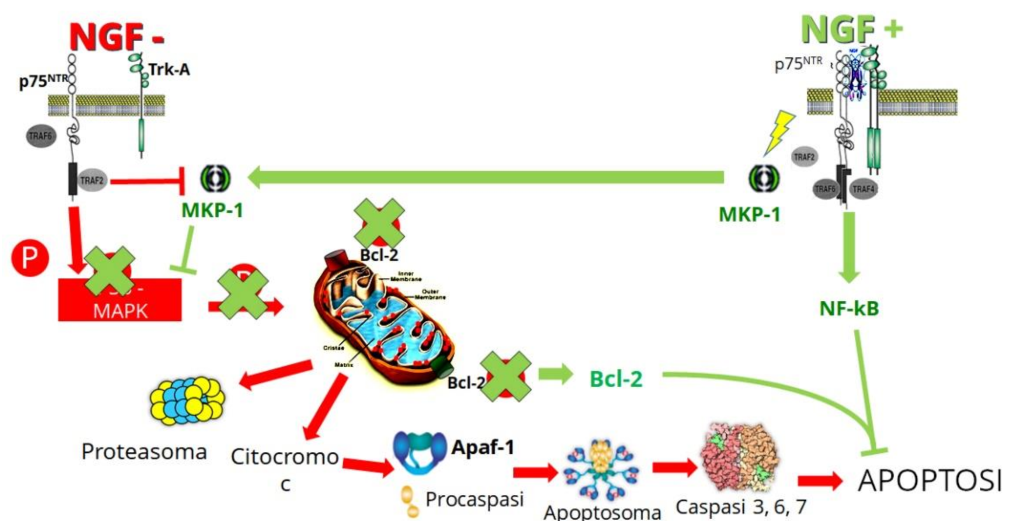
Several studies highlighted the BDNF ability in preventing neuronal degeneration and inducing oligodendrocyte proliferation and myelination in some forms of neuronal injury (McTigue *et al.* 1998), making of this neurotrophic factor a possible therapeutic target.

NGF and its role as anti-apoptotic agent

NGF is a potent pro-survival factor

The interaction of NGF with TrkA activates molecular circuits mediated mainly by PI3K-Akt and MAP kinases that block both the intrinsic and apoptotic pathways (Ichim *et al.* 2012), thus maintaining cell survival under various stress conditions. It has been described that NGF is able to deactivate p38 MAPK and JNK, two enzymes involved in the induction of the apoptotic process (Weston and Davis 2007) in nerve cells undergoing metabolic stress. In memory B cells, neurotrophin acts as an autocrine survival factor contributing to maintaining the structural and functional integrity of Bcl-2 protein. This function is exerted through the continuous inactivation of p38 MAPK, an enzyme able to phosphorylate Bcl-2 and induce functional deficiency (cytochrome c release and caspase activation) [Torcia *et al.* 2001].

Here we particularly describe, in detail, molecular mechanism of apoptosis that are inhibited by NGF-TrkA triggered pathway. The schematic representation of apoptosis pathway in presence or not of NGF are schematically resumed in figure below.



Schematic representation of the pathway triggered by the presence of NGF: binding its High-affinity receptor and p75^{NTR}, it induces their heterodimerization and triggering a kinase cascade that culminates with the suppression of apoptosis.

Molecular mechanism of apoptosis

Cell death is a process that can be triggered by many stimuli. The main mechanisms of cell death are necrosis and apoptosis (Krysko *et al.* 2008). Necrosis is a pathological process, unleashed in response to an acute injury such as hypoxia, hypothermia, radiation, viral infections, immune response, chemicals and drugs (Israels and Israels 1999). These events lead to a loss of integrity of the membranes of organelles including nucleus, resulting in the release of their contents (ATP, protease and lysozymes), up to the failure of the whole cell and DNA degradation in non-specific ways. This phenomenon, which the cell undergoes passively, triggers an inflammatory response with release of pro-inflammatory cells including macrophages.

In contrast, apoptosis is a term that can be used to describe the programmed cell death (Programmed Cell Death; PCD), a cellular mechanism genetically controlled and highly conserved during evolution (Metzstein *et al.* 1998). Described in all multicellular eukaryotes (Ellis *et al.* 1996), apoptosis is a physiological event that occurs during the early stages of development and plays a key role in morphogenesis (Jezek and Kozina 2009), in sexual differentiation and epigenetic processes of auto immune and nervous system organization. Furthermore, balancing the relationship life/death of damaged cells allows tissue homeostasis in adults (Orrenius *et al.* 2003). Therefore, apoptosis is a finely regulated process, in which the cell is actively involved with many morphologically and biochemically well-defined events. Cellular changes begin with a cell rounding, which entails the loss of contact with surrounding cells and the disappearance of their membrane structures like microvilli and specific regions of contact with other cells. At the same time, the loss of water and ions reduces cell volume resulting in cytoplasmic condensation (Kerr 2002). At nuclear level some events happen: the loss of the nucleolus and the chromatin condensation in dense and circumscribed clusters which accumulate on the nuclear membrane (pyknotic core).

Subsequently, the nuclear matrix is solubilized, leading to the DNA exposure to the enzymatic attack by nucleases, which result in breakage of the nucleic acid in high molecular weight fragments. At the end of chromatin condensation, the nucleus looks destroyed in fragments that contain even cellular organelles (mitochondria and endoplasmic reticulum portions), surrounded by the nuclear membrane or casted in protuberances, which are formed at the surface of the cell (blebs). The plasma membrane that surrounds these protuberances welds, giving rise to "apoptotic bodies", quickly absorbed and digested by macrophages (Krysko *et al.* 2008).

Two have been identified: the extrinsic apoptotic pathways, receptor-mediated (Ashkenazi and Dixit 1998), and the intrinsic pathway, also known as mitochondrial pathway (Green and Reed 1998). Although these two pathways are distinct, are not clearly separated from each other.

A key phenomenon of cell apoptotic cell death is an exclusive class of proteases known as caspases aspartate-specific. Between these 3 classes have been identified, 2 of which play important roles in apoptosis: one can distinguish the initiators caspases (Caspase-2, -8, -9 and -10) and performers caspases (Caspase-3, -6 and -7); the third group is the inflammatory caspases (Caspase-1, -4, -5, -11 and -12). The caspase activation is a process that provides for the activation of Caspase initiators through auto-cleveage and, the proteolytic cleavage of Caspase performers by these enzymes. Once activated, caspases performers induce the activation of other substrates such as cytokeratins and poly (ADP-ribose) polymerase (PARP). The intrinsic and extrinsic activation pathway of apoptosis intersect at a common point represented by the activation of effector caspases -3, -6 and -7.

The exposure of the death domain (DD) molecular adapter (FADD, *Fas-Associated protein with Death Domain;* TRADD, TNFR *Associated protein with Death Domain*) is the main event of extrinsic pathway. DD domains exposure occurs upon activation of its membrane receptors as FAS, TNFR. The exposure of those domains DD induces the procaspase-8 and -10 recruitments. From the assembly of these proteins come out a complex

called *Death-Inducing Signaling Complex* (DISC) in which the molecules of proenzyme can activate itself, thanks to partial coupling and intrinsic protease activity (Boatright and Salvesen 2003). The initiators caspase activates the effector ones, that affect specific substrates as cytokeratins, cytoskeletal proteins, PARP and nuclear protein NuMA, responsible for the biochemical and morphological changes observed in apoptotic cells (Elmore 2007).

The intrinsic pathway of apoptosis is mainly triggered by events that affect mitochondrial membranes homeostasis. Bcl-2 family proteins plays a key role in maintaining proper mitochondrial membrane permeability. In the family of Bcl-2 in fact recognize both anti-apoptotic proteins such as Bcl-2, Bcl-xL, Bcl-1 and Bcl-w, both pro-apoptotic proteins like Bax, Bad, Bid or Bim (Elmore 2007; Cory and Adams 2002). The Bcl-2 family is controlled by p53 in turn involved in inhibiting neoplastic transformation. The first consequence of the intrinsic pathway of apoptosis activation is the increased permeability of mitochondria through mitochondria permeability transition pore (MPTP), induced by inner mitochondrial membrane changes and, consequently, the release of proapoptotic proteins such as cytochrome c and SMAC/DIABLO, containing space to the cytosol (Saelens *et al.* 2004). Cytochrome c is involved the apoptosome formation, binding and activating Apaf-1 and procaspase-9 (Chinnaiyan 1999). Bcl-2 family proteins control a network of protein interactions that regulate all the apoptotic process. In turn, some protein Bcl2 family undergo translational modifications (phosphorylation) by enzymes that play an important role in the regulation of apoptosis. The family of MAP (Mitogen-Activated Protein) kinase are a good example: in this family there are protein with both antiapoptotic kinase enzymatic function (ERK, extracellular signal regulated kinase) and proapoptotic as JNK kinase (JUN-KINASE) or p38MAPK. The ERK are preferentially activated by a mitogen stimulation (Matsuda *et al.* 1998), while JNK and p38 MAPK are activated by stimuli as oxidative stress, hypoxia ischemia due to UV radiation, and various cytokines such as Interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) [Raingeaud *et al.* 1995]. Literature data show that because of cellular stress, p38 MAPK activity is associated with the

apoptotic intrinsic pathway induction (Ghatan *et al.* 2000) and one of the molecular mechanisms is the phosphorylation of two Bcl-2 family proteins, Bcl2 and Bax (Rosini *et al.* 2000), a very important event in maintaining mitochondrial membrane integrity. Conformational changes of these two proteins alter their function and allow the release of cytochrome c (Mandal *et al.* 2008). The inhibition of phosphorylation of p38MAPK is a key event in trophic function of neurotrophins.

Map kinase

The MAPK (Mitogen-Activated Protein Kinase) are a family of serine/threonine kinase activity enzymes that can regulate the functional activity of target proteins and control fundamental cellular processes as development, proliferation, differentiation, survival, response to stress and apoptosis. MAPK activity target proteins are present in all subcellular compartments, including nuclear. They are very heterogeneous molecular and functional point of view (kinases, phosphatases, transcription factors, transcription, chromatin structure regulator suppressors).

So far, it has been described four distinct pathways of MAPK: ERK1/2 kinases way (extracellular signal-regulated kinase) and ERK5, p38 MAPK and JNK. ERK1/2 pathway is typically activated by G-protein coupled receptors or activation with intracellular tyrosine kinase domain. ERK 1/2 activation is therefore common occurrence of molecular pathways activated by growth factors, hormones and inflammatory cytokines. p38 MAPK and JNK is activated in response to stimuli, intracellular, such that both extracellular growth factors, cytokines, microbial substances (LPS, viral proteins, etc.) and products of cellular stress such as oxygen free radicals.

MAPK activities and functions are finely regulated by tyrosine and threonine residues phosphorylation/dephosphorylation: these residues are highly preserved in *Thr-Xaa-Tyr* pattern, typical in dual specificity kinase MAPKK family (Wancket *et al.* 2012). Each MAPK differs from the other members of the

family for the amino acid present in the *Xaa* position (Su and Karin 1996). Phosphatases that regulate the phosphorylation and activity of MAPK belong to the superfamily protein tyrosine phosphatase (PTP).
In humans, the PTP are divided into four classes, based on amino acid sequence of the catalytic domain. The PTP class I proteins are classified as PTP Dual specificity tyrosine-specific phosphatase (DUSP).

MKP-1, a DUSP subfamily member

One of DUSP subfamily are the MAPK phosphatases (MKP). Targets of MKP family activity are the "consensus sequence" *Thr-Xaa-Tyr* in phosphorylated MAPK. The MKP can have specificity for one or several MAPK and the expression of certain MKP may be modulated significantly by extracellular signals (growth factors, cytokines or LPS) or from micro-environmental conditions (as could be a stressed environment).
Among family members DUSP, MKP1 is one of the most involved phosphatase in apoptosis inhibition activated by cellular stress.
MKP-1 has been originally described as a non-receptor tyrosine phosphatase induced by growth factors or by cellular stress. Many evidences show that MKP-1 preferentially dephosphorylates p38 MAPK and JNK (Franklin and Kraft 1997), although his task doesn't seem restricted to only these two proteins. (Boutros *et al.* 2008, Shipp *et al.* 2010).
Structurally, MKP-1 contains a highly conserved catalytic unit and Cdc25/rhodanese homology domain, that includes the KIM pattern (53-55 Arg) involved in substrate specificity (Kwak *et al.* 1994). N-terminal fragment contains a nuclear localization sequence, while the phosphatase catalytic domain is located near the C-terminal end.
The expression of DUSP1 gene, coding for MKP-1 human, increases in response to LPS, cytokines, growth factors, but also in response to cellular stress factors such as hypoxia and thermal shock (Zhang *et al.* 2012). MKP-1 promoter contains not only CRE elements (cAMP responsive elements), E-Box, GRE (glucocorticoid responsive elements), VDRE (vitamin D receptor responsive

element), but also binding sites for transcription factors (Ryser *et al.* 2004, Wang *et al.* 2008) that are able in modulating expression (Ryser *et al.* 2001).
The stability of the protein is regulated by the function of ERK1/2 through phosphorylation on residues Ser-359 and Ser-364, which prevents the proteasome-mediated degradation (Scimeca *et al.* 1997).

Functional mimetics of neurotrophins acting at Trk receptors

Despite the therapeutical potential that neurotrophins could have, their peptidic nature gives them drawbacks typical of polypeptides applied as drug. Taken together, *in vivo* instability, activation of undesired pathways, the difficulty in crossing the blood brain barrier, a weak diffusion and their vulnerability to proteases draw a picture that leads to renounce to their usage as drugs (Poduslo et al., 1996). Anyway, a good way to exploit their potential as drugs can be given by synthetizing smaller molecules that have chains useful to mimic peptides, as the peptidomimetic could be. As well as of lack of immunogenicity, low molecular mass and good pharmacokinetic, peptidomimetic are cheaper than peptide. (Saragovi *et al.* 1992)
As reviewed by Skaper, structure based alignment was performed to have a wide idea about the effector and activating site of NGF, BDNF and NT-3. The most used strategy was to enrich small molecules with functional chains useful to bind Trk family receptors. Based on this idea, several compounds were developed, trying to find the so-called “hot spots” at ligand-receptor surfaces, that resulted to be mostly constituted by β-turn conformations (Skaper 2011).
As a matter of fact, LeSauteur et al developed a mimetic antagonist of NGF, a beta-loop NGF monomeric cyclic peptide analogs able to block the signal triggered by TrkA (LeSauteur *et al.* 1995). Further studies based on mimicry concept of pre-existent NGF-mimetics, (Zaccaro *et al.* 2005), developed a library of small molecules peptidomimetics and agonist of TrkA and TrkC, basing the chains structure on β-turns of NT-3 and NGF.

More recently, a novel compound, called MT2, was selected among over one hundred molecules with bicyclic three-dimensional scaffold, taking into account the spatial conformation of the binding site for NGF in TrkA and their affinity for the NGF receptor.

MT2

MT2 is a patented non-peptidic compound and NGF-mimetic (Figure 7). It is the result of a selection from a chemical library based on the study of NGF receptor, and in particular its active domain, TrkA- domain 5 (TrkA-d5). In fact, Scarpi et al. took in consideration the spatial conformation of the binding site of NGF and TrkA and selected only molecules that could fit in the domain 5 of receptor.
From the *in vitro* dose-dependent effect experiments on PC12 cells, MT2 resulted the compound with the highest NGF-like activity of four compounds.

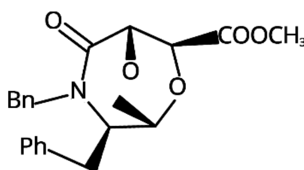


Figure 7. Structural formula of MT2 (Scarpi et al., 2012)

In serum deprivation conditions, it has been demonstrated that MT2 lead to the same NGF-TrkA bound effects, increasing phosphorylation of ERK1/2 and Akt proteins and production of MKP-1 phosphatase, modulating p38 mitogen-activated protein kinase activation. It has also been demonstrated that corrects biochemical abnormalities and maintains cell survival *in vitro* model of Alzheimer's disease (AD) based on NGF deprivation of rat hippocampal neurons. (Scarpi *et al.* 2012) The drug is also able to counteract the onset of toxin induced dopaminergic neuronal cell death and synaptic alterations in *in vitro* models of Parkinson disease (PD) (data not shown).
Thanks to its non-peptidic nature and small dimensions, MT2 has a favored pharmacokinetic if compared to a grow factor, being able to cross the blood brain barrier (BBB) and not being attacked by proteases.

Aims

Aims

MT2 is a novel compound, kindly given by laboratory of Prof. Cozzolino, that showed to be able to bind TrkA and TrkB, mimicking the effect of the NGF and BDNF (Scarpi *et al.* 2012). Unlike growth factors, MT2 can exploit a better pharmacokinetic, with a better half-life and a potential efficacy due to its better diffusion through blood brain barrier than a polypeptide. MT2 showed to activate the same pathway driven by the bound between NGF and TrkA the anti-apoptotic pathway in condition of starvation (deprivation of growth factor). Binding the domain-5 of TrkA (TrkA-d5), MT2 triggers the survival pathway through activation of ERK1/2 and p-Akt proteins and production of MKP-1 phosphatase, modulating the p38 mitogen-activated protein kinase activation.

Principal aim of our work was to test efficacy of MT2 treatment on inflammatory, neurodegenerative damage in central nervous system. Furthermore, we wanted to dissect MT2 mechanism of action investigating its role in nervous tissue protection and repair together with the possible modulation of immune response.

To achieve this aim we exploit three different experimental models:

- Mouse organotypic spinal cord culture challenged with a proinflammatory cytokines cocktail, this model permitted us the study of *in vitro* CNS microenvironment and synaptic function during the early phases of inflammation.
- MOG induced experimental autoimmune encephalomyelitis in C57Bl/6 mice. We analyzed MT2 protective effects on neuronal tissue and on autoimmune reaction during acute and chronic phases of the disease.
- Cuprizone induced demyelination in C57Bl/6 mice. We investigated MT2 treatment effects during toxic demyelination, in order to isolate

demyelinating events from immune reaction, mimicking late neurodegenerative phases of MS.

Methods

Methods

Animals

In the present work Experimental autoimmune encephalomyelitis (EAE) was induced in 6 weeks-old female C57BL/6 mice (a total of 18, obtained from ENVIGO RMS s.r.l., Italy), both for acute and chronic phase of disease. We also used this strain for Cuprizone model. Animals had a mean of ~20 gr weight were kept in a standard housing condition, with a light/dark cycle of 12 h (08.00–20.00 hours), at temperature of 23°C and free access to food and water. Animals were usually subdivided in 4-5 animals per cage.

MOG_{35-55}-induced EAE in C57BL/6 mice

Active EAE was induced in 6-weeks-old female mice through an active MOG_{35-55} peptide emulsion immunization strategy. Emulsion subcutaneous injections are composed by 1:1 ratio of MOG_{35-55} peptide (MEVGWYRSPFSRVVHLYRNGK, EPK1, 5 mg, purity >85%; Espikem, Florence, Italy) and Mycobacterium Tuberculosis (prod. #231141, BD Difco, Detroit, MI) in Incomplete Freund's Adjuvant (IFA – Sigma Aldrich F5881).

MOG_{35-55} was diluted in ddH_2O to a final concentration of 1,33 mg/ml; Mycobacterium Tuberculosis was added to CFA, obtaining a final 4 mg/mL concentration. 100 µl of antigen/CFA emulsion was injected subcutaneously into two different sites on each hind flank; another injection was performed closer to the base of the tail; Bordetella Pertussis Toxin (PTX - 516560-50UG) was administered by intraperitoneal (ip) injections on days 0 and 2 post immunization (days post immunization, dpi). (Hofstetter *et al.* 2002) Food and water were available ad libitum, and mice were weighed daily.

EAE's clinical course assessment

Mice's clinical course was judged daily, giving them an assessment as following (Figure 8): 0, no detectable signs; 0.5, partial limp tail; 1, complete limp tail; 1.5, limp tail with hind limb weakness or gait abnormality; 2, limp tail with unilateral hind limb paralysis; 3, bilateral hind limb paralysis; 4, bilateral hind limb paralysis and forelimb weakness; and 5, bilateral forelimb and hind limb paralysis (endpoint). Furthermore, animal's weights were daily evaluated.

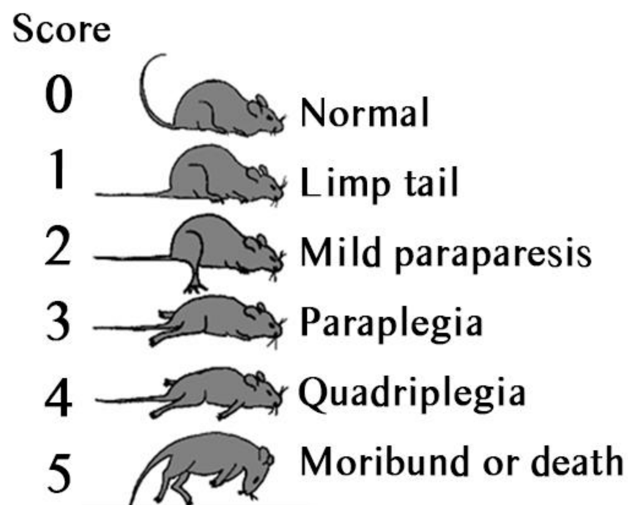


Figure 8. ***Schematic representation of EAE severity assessment.*** Clinical signs of the EAE are assessed with a grading scale with an arbitrary score of 0 to 5.

Cuprizone model

Cuprizone model allowed us to investigate of remyelinating mechanisms in young adolescent animals following toxin-induced demyelination. Demyelination was induced by 0.2% cuprizone [N,N'-

bis(cyclohexylideneamino)oxamide; *Sigma-Aldrich*] into the food over a period of 35 days starting on day 0 to day 35. Food and water were available ad libitum. In this experiment were used 7 mice for vehicle; 8 mice for treated group. In detail: 7 mice (3 for vehicle and 4 for treated group) were sacrificed at 49th day during demyelination phase, 8 mice (4 for each group) were sacrificed at 56th day during remyelinating phase.

MT2 treatment

MT2 (Figure 9) is a NGF mimetic molecule, MT2 that is a patented non-peptide compound, agonist of the high affinity Nerve Growth Factor Receptor (NGFR) Tropomyosin receptor kinase A (TrkA) and of TrkB. Mice were treated daily, 2 mg/Kg per mice through intraperitoneal injections, 150 µL. MT2 is solved in a 9% ethanol solution.

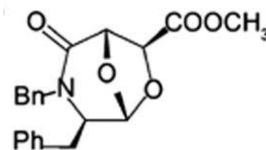


*Figure 9. **The MT2 structure*** (Scarpi et al., 2012)

In EAE experiments, mice were treated for 10 days both acute phase and chronic phase. For the acute phase treatment, we started treatment from 14 dpi, when usually EAE acute phase starts (Figure 10A), ending administration at day 23 dpi. Treatment during chronic phase of disease was experimented treating animals with MT2 or vehicle from 22 dpi to 31 dpi. During cuprizone experiments, MT2 was given daily from day 21 to day 42 (Figure 10B). During both experiments, vehicle mice received i.p. the solution without drug in the same period of treated ones.

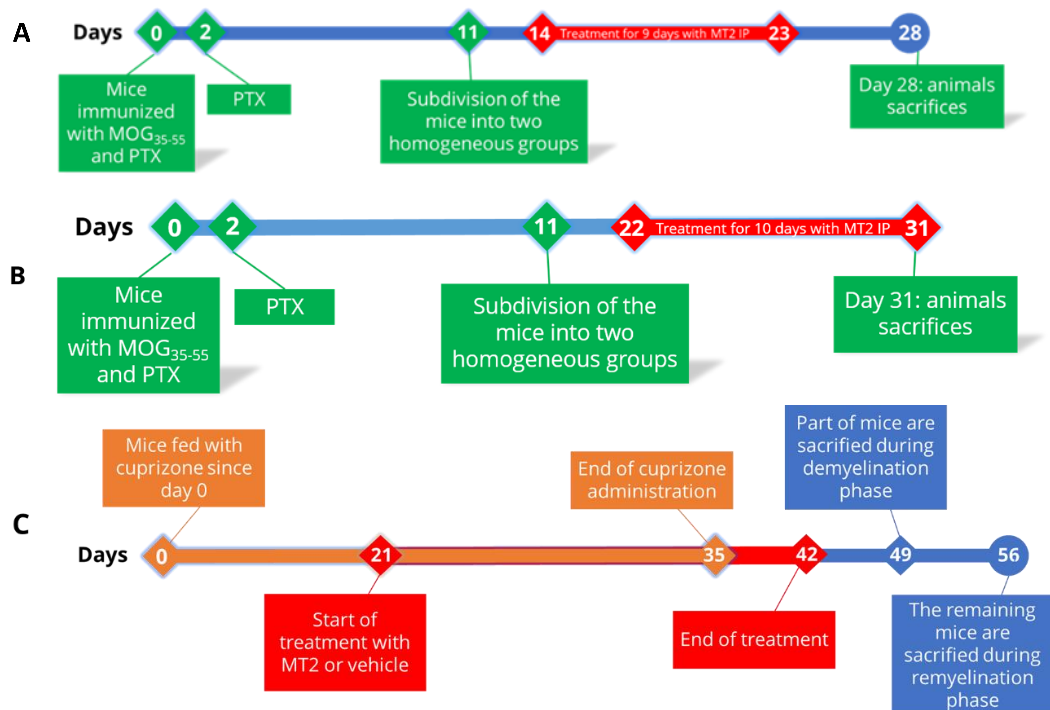


Figure 10. ***MT2 treatment during EAE and cuprizone experiments.*** Experimental scheme of acute (A) and chronic (B) EAE with 10-days MT2 treatment scheduling. (C) shows the experimental course of MT2 treatment during Cuprizone model.

After the mice were sacrificed, lymph nodes, spleens, spinal cords and brains were collected.

Cells isolation from lymphoid and CNS organs

Isolation of cells from lymph node and spleen

Cells were isolated from the cervical and drainage lymph nodes or spleen by mechanical dissociation of the tissues by using sterile nylon filters with 70 µm pore (BD Falcon). Cells derived from spleen were, subsequently, separated by centrifugation in Ficoll gradient (Biochrom AG) obtaining the mononucleated fraction (MNCs).

Isolation of cells from nervous system

Spinal cord and cerebellum were directly lysed with Collagenase (5 mg/ml, Sigma Aldrich) for 15 minutes at 37°C. Tissues were then mechanically dissociated by sterile Falcon™ Cell Strainers with nylon mesh with 70 µm micron pores (Fisher Scientific). Cells were separated by centrifugation in Percoll gradient (Biocoll) following the manufacturer instruction.

Ex-vivo proliferation test of specific antigen cells

Cells isolated from the lymph nodes or spleen were distributed in 96-well plates at a concentration of $1,5x10^5$ cells / 200 µl / well in the presence or absence of peptide MOG_{35-55} (10 or 50 µg/ml). Cells were cultured for 72h at 37° C in a humidified atmosphere containing 5% CO2. After this incubation time, 1 µCi of 3H-Timidine (PerkinElmer) was added to each well. Cells were then collected on glass fiber filters using the appropriate Cell Harvester instrument (Mach III Cell Harvester, Tomtec, Inc.). Thymidine incorporation was measured as count per minute (*cpm*) using the MicroBeta® TriLux 1450 (Wallac) radiation counter. The extent of proliferative response was expressed as a stimulation index (SI):

$$SI = \frac{\text{cpm of cultured cultures}}{\text{cpm of non-stimulated cultures}}.$$

Cytokines assay

For treated-MT2 group and vehicle one (4 or 3 mice for each experimental setting) cells isolated from lymph-node or spleen were plated (1×10^5 cell/well) and activated or not with MOG_{35-55} for 72 h. Supernatants were collected and stored at -20°C for determination of IL-10, IL-4, IL-6, IL-17, IFN-γ, TNF-α, CXCL1, CXCL2 and CXCL10 through Bio-Plex® Multiplex Immunoassay System assay (Bio-Rad) using ProcartaPlex kit (Thermo Fisher). This technique involves the use of the multiplex immunoassay that enables the simultaneous detection and quantification of different biomolecules. Bio-Plex apparatus allows the multiplex analysis, according to manufacturer's recommendations.

Flow cytometry analysis

Phenotype analysis focused on the characterization of dendritic cells (DC) and T cells producing IFN-γ or IL10. For DC and T cells characterization cells were isolated from lymph node. For IFN-γ and IL10 production analysis, cells were isolated from spinal cord. After isolation from the lymph nodes, cells were suspended in PBS containing 0.05% BSA (bovine serum albumin) and EDTA 2 mM, counted and subdivided into $1x10^5$ cells each. Cell suspensions were labelled with fluorescent monoclonal antibodies, specific for membrane molecules expressed by DCs and T cells. Cells were incubated for 15 minutes in the dark and at room temperature with the following fluorescent monoclonal antibodies: CD3 FITC (1:100), IL-10 APC (1:100) and IFN-gamma PE (1:100). (eBioscience). Subsequently, cells were washed and suspended in 500 μl of PBS. Cells were then analyzed by flow cytometry. Simultaneously cells were isolated from spinal cord and cerebellum with Percoll gradient solution (Biocoll) and labelled with fluorescent monoclonal antibody, specific to membrane molecules expressed by T cells. The cells were incubated for 15 minutes in the dark and at room temperature with CD3 FITC (1:100) (eBioscience). Then, cells were washed and fixed with formaldehyde 0,01% (SIGMA) for 20 minutes. After the incubation time cells were washed and left overnight at 4° C. The day after membranes were disrupted using Saponin (0.5% in PBS) permeabilization buffer, enable antibodies to go through pores without dissolving plasma membrane. Follow antibody staining: IFN-γ and IL10 (eBioscience) antibodies were prepared in permeabilization buffer to keep cells permeable. For the detection of secreted proteins Brefeldin (R&D System) was added to antibodies mixture, preventing protein release from the Golgi apparatus and enabling the detection of cells expressing the protein.

As last step cells were ready for flow cytometry analysis by a 4-color Cytofluorimeter with Argon 488 nm laser (Partec).

Histopathology

Spinal cords and brains were fixed in 4% paraformaldehyde for 48h, washed in PBS, dehydrated with a growing alcohol concentration and xilen, changing every solution twice. Last step was to embed organs in paraffin. 5 µm-cross sections were then stained to evaluate the presence of inflammatory infiltrates, demyelination and axonal loss. Pictures were taken with Olympus BX40 (Olympus, Milano, Italy).

Hematoxylin and eosin stain

To detect the presence of inflammatory infiltrates, slides with spinal cord samples were stained with hematoxylin-eosin (HE). Hematoxylin is a basic dye that stains in purple blue the negatively charged cellular components such as nucleic acids, membrane proteins and cell membranes that are thus called basophils. Eosin is a stain contrast agent of hematoxylin. It stains basic components loaded positively like many cellular proteins, mitochondrial proteins and collagen fibers that are thus called eosinophils or acidophilic. Binding these components, it gives dark red or pink and it is used in histology for staining cytoplasm.

The staining protocol provides the following steps:

- Samples were de-paraffined in xylene (6' for 2 times);
- Samples were rehydrated by a decreasing sequence of alcohols: 100% ethanol (3', 3 times), 95% (3') ethanol, 75% (3') ethanol, distilled H_2O;
- At this point the samples were stained: haematoxylin (2'), current H_2O (10'), eosin (20-30”);
- The samples were rinsed in running water and dehydrated by means of an ascending alcohol sequence: 75% (3') ethanol, 95% (3') ethanol, 100% (3') ethanol, xylene (3')
- Slides are fitted with a drop of upright balm and the slide covers the object, then dry in the stove.

Luxol fast blue stain

Luxol fast blue (LFB) is a chemically derived dye-tetrabenzotetrazo-porphyrin dye, which is used in histology for myelin colouring, allowing to evaluate the extent of demyelination. The selective affinity of porphyrins for myelin has been demonstrated and the electivity of the dye for the central nervous system is mainly due to the bonds that form with phospholipid structures such as lecithin and sphingomyelin.

The staining steps are as follows:

- xylene 15’ for 2 times
- ethanol 4’ for 2 times
- Luxol Fast Blue 0,1% overnight at 50-60° C (Prepared by solubilizing 0,50 g of the Luxol Fast Blue powder in 0,5 l of absolute ethanol and adding 2,5 ml of glacial acetic acid)
- in distilled H_2O washing 15” lithium carbonate
- ethanol 70% 30" for 2 times
- in distilled H_2O washing
- Periodic acid 0,8% 10”
- in distilled H2O washing Schiff reagent (at room temperature) 20’
- washing solution 2’ for 3 times (Consisting of: 20 ml of 10% potassium bisulfite, 5 ml of 37% HCL and 500 ml of distilled water)
- water running in the running water 15’
- ethanol 50% 1’ for 2 times
- ethanol 100% 1’ for 3 times
- butylacetate/xylene 1’ for 2 times

Furthermore, two operators blindly evaluated infiltrates through a semiquantitative method: we adopted a specific assessment, giving score 0 for no infiltration, score 1 for foci of subarachnoid cell infiltration, score 2 for subarachnoid infiltration, score 3 for foci of parenchymal infiltration, score 4 for diffuse and widespread parenchymal infiltrates. As well, we performed a demyelination assessment through Luxol Fast Blue staining, judging with a

score of 0 no demyelination; 1 refers to foci of demyelination that is superficial and proximal to the subarachnoid space and that involves less than 25% of the lateral columns; 2 represents foci of deep parenchymal demyelination and that involves over 25% of the lateral columns; while a score of 3 denotes diffuse and widespread demyelination. (Giuliani *et al.* 2005)

Bielschowsky stain

The Bielschowsky technique is a silver staining method used in histochemistry for the visualization of nerve fibers. It includes these following steps:

1. Deparaffinize and hydrate sections to dH_2O. Wash 3 times for 3 min. in dH_2O.
2. Place sections in 50 ml 10% silver nitrate in dark at 37ºC for 30 min. Keep this solution after incubation (for use in step 4).
3. Wash 3X for 3 min in dH_2O.
4. Add concentrated ammonium hydroxide dropwise with stirring to the silver nitrate solution reserved from step 2. Add only enough to dissolve the dark initial precipitate but not more.
5. Incubate sections in this solution for 15 min at 37° C. Again, save this solution for use in step 6.
6. Wash sections in 0.1% ammonium hydroxide 3X for 2 min at room temperature.
7. Add 350 µl developer solution (0.2 ml 37% formaldehyde, 12 ml dH2O, 12.5 µl 20% nitric acid and 0.05 g citric acid) to the silver hydroxide solution saved from step 4.
8. Stain sections in this solution for 10 min until they turn black.
9. Wash in 0.1% ammonium hydroxide 3X for 2 min and dH2O 3X for 2 min.
10. Tone in 0.2% gold chloride for 5 min.
11. Fix in 5% sodium thiosulfate for 1 min.
12. Wash in dH2O, dehydrate in alcohols then xylene and mount.

Immuno-fluorescence on paraffin sections

In 5 µm-cross sections paraffin was removed by xylene, absolute ethanol and 95% ethanol, then rinsed in distilled water. Slices were imaged using a Leica DM6000B microscope with appropriate excitation and emission filters for each fluorophore was used to acquire representative images. The slice details were imaged at 4x magnification. The analyses were then performed using the ImageJ software (http://rsbweb.nih.gov/ij/).

De-waxing and dehydration steps were followed by a step of blocking for non-specific staining between the primary antibodies and the tissue, by incubating in blocking buffer (1% horse serum in PBS) for 30 minutes at RT.

Spinal cords and brains sections were incubated with primary antibodies overnight, 4°C. Primary antibodies used are: mouse monoclonal anti-glial fibrillary acidic protein, GFAP (Sigma G3893, 1:200 dilution); Anti-Myelin Basic Protein Antibody, MBP (Millipore MAB386, clone 12, 1:100); Anti-Iba1 (Wako, cat. #019-19741). Subsequently slices were washed 3 times with PBS and incubated with secondary antibodies for 2h at RT.

The secondary antibodies we used are: Alexa 488 donkey anti-rat (Invitrogen A21208, 1:200 dilution); Alexa 635 goat anti-mouse (Thermo Fisher A31574, 1:400 dilution); Alexa 488 goat anti-rabbit (Thermo Fisher A11008, 1:400 dilution.

Immunohistochemistry on PFA-fixed cells

Cells were seeded (10^5 cells/spot) on positive charged glass slides, fixed with PFA 4% (Bioptica) 15 minutes at room temperature, then washed with PBS and permeabilized with PBS/FBS 5%/Triton 0.3% solution for 30' RT in the dark. TrkA (1:300), TrkB (1:300) CD3 (1:400) CD4 (1:300) were diluted in permeabilization solution and slides immunostained drop by drop overnight a 4°C. The day after, after

three washes, we added the secondary antibodies diluted in permeabilizing solution for 2 hours at room temperature. Slides were then washed three times in PBS and closed with Prolong Diamond mounting medium containing DAPI (Life Technologies). Once dried, slides were observed at a fluorescence microscope (Olympus BX600, Japan). For each slide we analyzed four fields, 6 slides per groups, vehicle and MT2-treated each.

Transmission electron microscopy

After sacrifices, brains were taken from animals and fixed in Karnovsky solution (paraformaldehyde 2.0 gm, 1M sodium hydroxide 2 - 4 drops, 50% glutaraldehyde 5.0 ml (25% glut - 10 ml), 0.2M cacodylate buffer, pH 7.4.), then 3 mm section were first dehydrated, then embedded on Epon 812 Luft resin. 60-70 um sections were stained with uranyl-acetate and bismuth, then view with JEM 1010 electron microscope (Jeol, Tokyo, Japan) at 80 kV and acquired by MegaView III (Soft Image System Gmbh, Germany).

SDS-PAGE and Western-Blot

At the days of sacrifice, a piece of spinal cord were stocked in a tube containing RIPA buffer 1x (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 2 mM EDTA; 1 mM NaF; 1 mM sodium orthovanadate, 1% NP-40) in the presence of phosphatase inhibitor cocktail 2 and 3, protease inhibitor cocktail (Sigma Aldrich), then stocked at -20°C. At the moment of experiment, samples were thaw on ice, added stainless steel beads in the tubes and then mechanically lysed through Tissue Lyser (Qiagen). Samples were lysed after 2 cycles, with an oscillation frequency of 30 Hz. Lysed samples has been

recovered and centrifuged at 13000 rpm at 4°C for 15 minutes, then upper phase containing proteins was collected. After proteins quantification through Pierce™ BCA Protein Assay Kit (Thermo Scientific) in order to load 35-40 ug of proteins onto SDS-PAGE. Subsequently hand-casted 12% Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis polyacrylamide (SDS-PAGE) was prepared following Bio-Rad protocol. Gel was included in specific support for electrophoresis system that was assembled in the specific tank. Once filled with running buffer (TBA 10% in PBS), 35-40 μg of samples were loaded onto gel wells and ran at constant voltage of 200 V for 60 minutes. After proteins separation, gel was blotted onto methanol-activated nitrocellulose filters (GE Healthcare, Fairfield, CT, USA). After a 15 seconds-treatment, nitrocellulose filters were rinsed in ddH_2O for 1 minute and then immersed in transfer buffer. In order to transfer proteins, a sandwich made by sponges/Whatman cellulose filter paper (GE Healthcare Life Sciences)/gel/nitrocellulose/Whatman cellulose filter paper/sponges was made and used in the blotting system filled with transfer buffer. Transferring operation lasted overnight at room temperature at constant voltage of 100 V. Once transferred, membrane with proteins was washed with PBS-Tween 0,05% and then stained with Ponceau solution to verify the correct protein transfer. Nitrocellulose membrane is then incubated for 1 hour with BSA 3% in PBS for blocking.

PVDF membrane is then incubated overnight with primary antibodies in a 3% BSA in PBS solution, keeping it on a shaker at 4°C.

Following primary antibodies were used:

- Rabbit anti-MKP-1 1:1000 dilution,
- Rabbit anti-P-p38 1:1000 dilution,
- Mouse anti-α-Tubulin 1:2000 dilution.

After overnight incubation, membrane was washed 3 times for 5 minutes with PBS-Tween, and immediately afterwards it was incubated in agitation for 1 hour at room temperature with secondary antibodies conjugated with horseradish peroxidase. This last incubation was followed by another 3 astir washes of 5 minutes with PBS-

Tween 0,1%. Washes are made for the purpose of removing antibodies bound not specifically that could give fake positive signals.

Secondary antibodies are:

- Anti-rabbit for MKP-1 e P-p38, dilution 1:10000;
- Anti-mouse α-tubulin, 1:10000.

Chemiluminescence was revelated through Chemidoc (Biorad) machinery, using Luminata Forte Western HRP (Merck) as substrate for horseradish peroxidase.

Organotypic culture preparation

See attached manuscript draft.

Results

Results

Organotypic culture

See paper in press on Molecular Brain Journal titled: "**Neuroinflammation and synaptopathy: bridging pro-inflammatory signals, synaptic transmission and protection in spinal motor circuits *in vitro*"** by Medelin M, Giacco V, Aldinucci A, Sibilla A, Bonechi E, Ballerini L, Cozzolino F and Ballerini C. The submitted document is attached to thesis.

Effect of MT2 treatment on preclinical, acute and chronic phase of EAE

Given the neuroprotective and anti-inflammatory role of NGF, we tested the possible therapeutic effect of our NGF mimetic molecule MT2 on EAE, treating MOG_{35-55} immunized C57Bl/6 mice both during the acute and the chronic phase of the disease.

MT2 treatment during the acute phase of EAE

Effect of MT2 on EAE clinical course

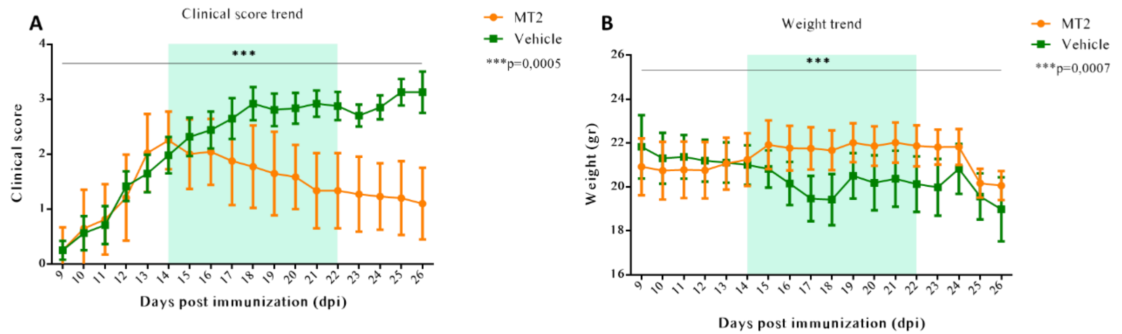


*Figure 11. **MT2 treatment of EAE mice during acute phase of the disease: effect on clinical score and body weight.*** Animals (N=9 per group, two independent experiments) were treated with MT2 or vehicle from 14 dpi (treatment period is highlighted in pale blue in graphs A and B. A) clinical score (mean values±SEM of two independent experiments, 9 animals/ group each) of treated animals (orange curve) and controls (green curve) (***p=0,005, unpaired t-test). B) Body weight trend (mean values±SEM of two independent experiments, 9 animals/group each) of treated animals (orange curve) and controls (green curve) (****p=0,0007, unpaired t-test).

First, we investigated the effect of MT2 treatment during the acute phase of EAE, that usually arise at 14 dpi. We treated animals from 14 to 23 dpi, monitoring clinical score and body weight trend daily. We treated a total of 18 animals per group (MT2 and vehicle), in independent experiments. As reported in Figure 11, we found that MT2 significantly (***p=0,005) improved the clinical score (1,42±0,12 vs 2,18±0,2, mean±SEM) and body weight (21,34±0,15 vs 20,45±0,185 gr).

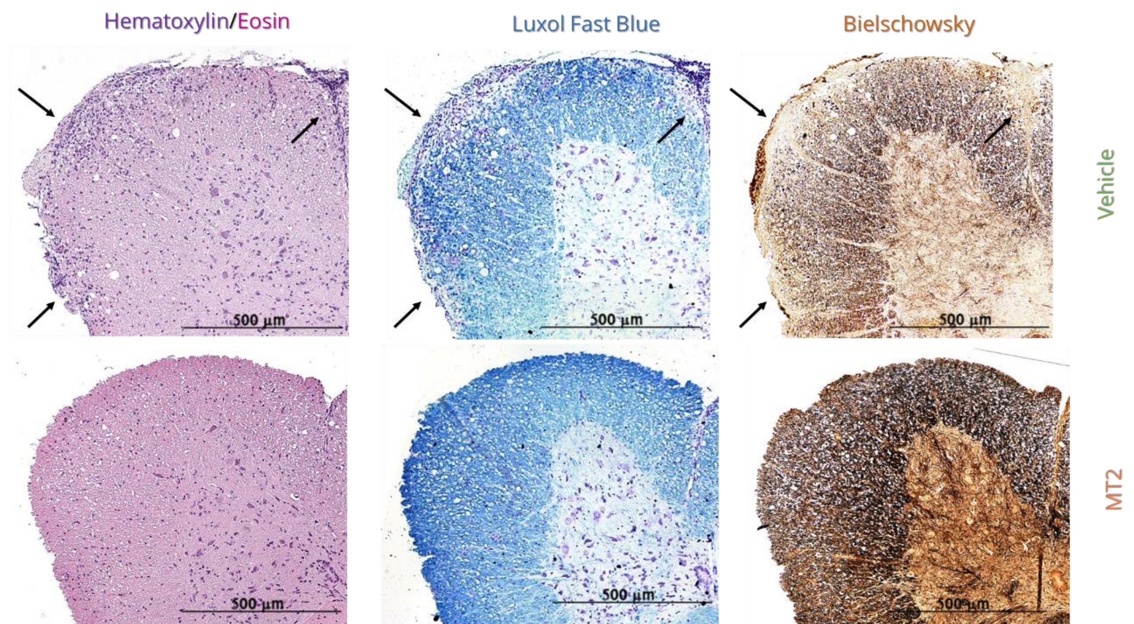
Histopathological analysis of spinal cord

The therapeutic effect of MT2 on EAE was verified through histopathological analysis of spinal cord samples from mice sacrificed at 26 dpi: we evaluated the number of immune cell infiltrates, the degree of demyelination and the axonal loss, by hematoxylin and eosin (H&E), Luxol Fast Blue (LFB) and Bielschowsky stains, respectively (Figure 12A, 12B and 12C).

The semiquantitative analysis of the number of immune infiltrates, marked with HE staining (Figure 12A, arrows) showed a significant (****p<0,0001) reduction of the number of infiltrates foci in MT2 samples with respect to controls (0,85±0,18 vs 3,81±0,20, mean±SEM) (Figure 12D), corresponding to a higher histological score (Figure 12E) in vehicle mice (1,2±0,07, mean±SEM) than in treated group (0,375±0,06, mean±SEM).

Then we evaluated the extent of demyelination on treated and control mice through LFB staining (Figure 12B). Semiquantitative analysis of demyelination showed that MT2 significantly (****p<0,0001) reduces the number of demyelination areas (0,34±0,08 vs 3,94±0,19, Figure 12F) and the demyelination score (0,40±0,08 vs 1,65±0,11, G).

Bielschowsky staining (Figure 12C) proves that axonal loss is widely present in controls (arrows), but not detectable in treated mice. Since HE, LFB and Bielschowsky staining were performed on consecutive slices (as described in Methods), we found a “geographic” match between presence of infiltration foci, demyelinated areas and axonal loss.



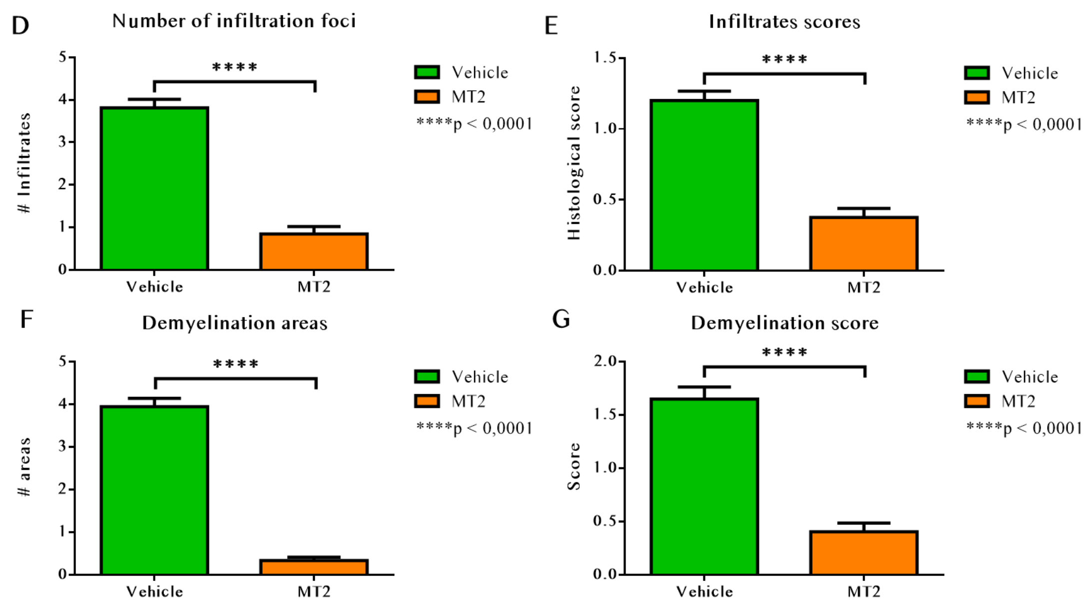


Figure 12. ***Histopathological evaluation of spinal cord of EAE mice treated with MT2 during the acute phase of the disease.*** A) Haematoxylin and Eosin (HE), B) Luxol Fast Blue (LFB) and C) Bielschowsky staining of paraffin embedded spinal cord slices (5μm), of EAE mice treated with MT2 compared to controls (vehicle), to evaluate the presence of inflammatory infiltrates, demyelination and axonal loss, respectively. D-G) Semiquantitative evaluation of number of infiltration foci (D), infiltrates score (E), number of demyelination areas (F), demyelination score (G); each column in graphs represents the mean value±SEM of 6 animals.

As shown in Figure 13, the spatial correspondence between the presence of infiltrates (HE) and lack of myelin (LFB), evident in control mice (upper panel) and almost absent in MT2 treated ones (lower panel), was confirmed by immunofluorescence labelling of myelin basic protein (MBP).

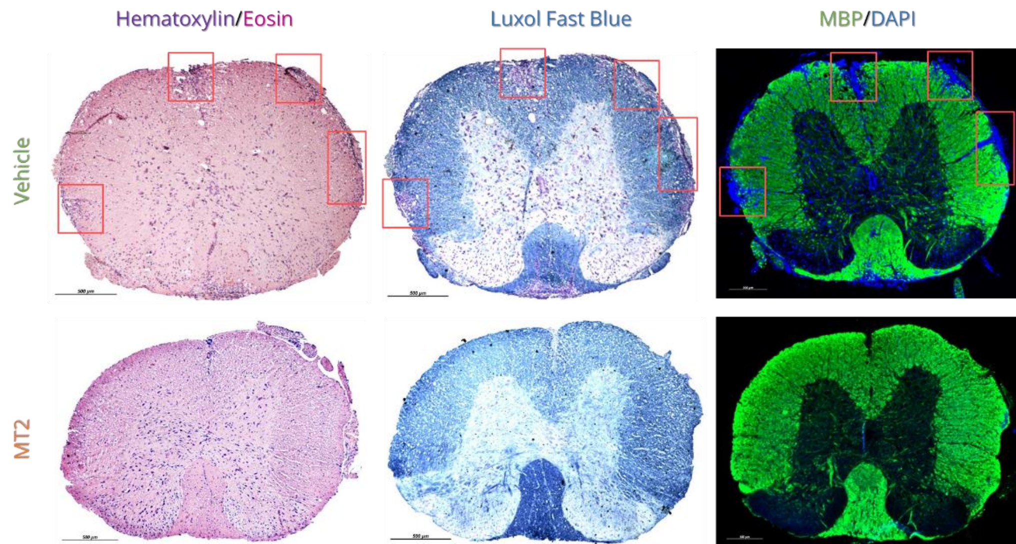


Figure 13. ***Histological analysis of immune infiltrates and demyelination in spinal cord of EAE mice treated with MT2 during the acute phase of the disease.*** Infiltrates were detected by Haematoxilin/Eosin staining (H&E); Luxol Fast Blue/cresyl violet staining (LFB) and MBP immunofluorescence labelling were used to visualize myelin. Upper panel: EAE animals treated with vehicle (CTRL); lower panel: EAE mice treated with MT2 (MT2). Red squares in the upper panel show the correspondence between the presence of infiltrates and lack of myelin.

MT2 ability on mimicking classical NGF anti-apoptotic pathway in CNS

To understand if the beneficial effect of MT2 on EAE pathology could be exerted on CNS cells through its known anti-apoptotic function via TrkA receptor (Scarpi *et al.* 2012), we investigated the expression of proteins involved in TrKA mediated anti-apoptotic pathway. Experiments were performed on spinal cord homogenates by western blot techniques.

As shown in Figure 14, we observed a trend towards an increased MKP-1 expression in MT2-treated animals compared to controls (2,4±0,28 vs 1,4±0,28 ratio MKP-1/β-actin - 15A), accompanied by a decreased level of phosphorylated p-38 (0,79±0,26 vs 2,29±0,81 ratio p-p38/α-tubulin- 15B). Consistently with

these findings, we observed a trend towards an increased expression of Pro-caspase-3 (15C) in MT2 treated mice compared to controls (0,56±0,26 vs 0,17±0,11 Procaspase/α-tubulin).

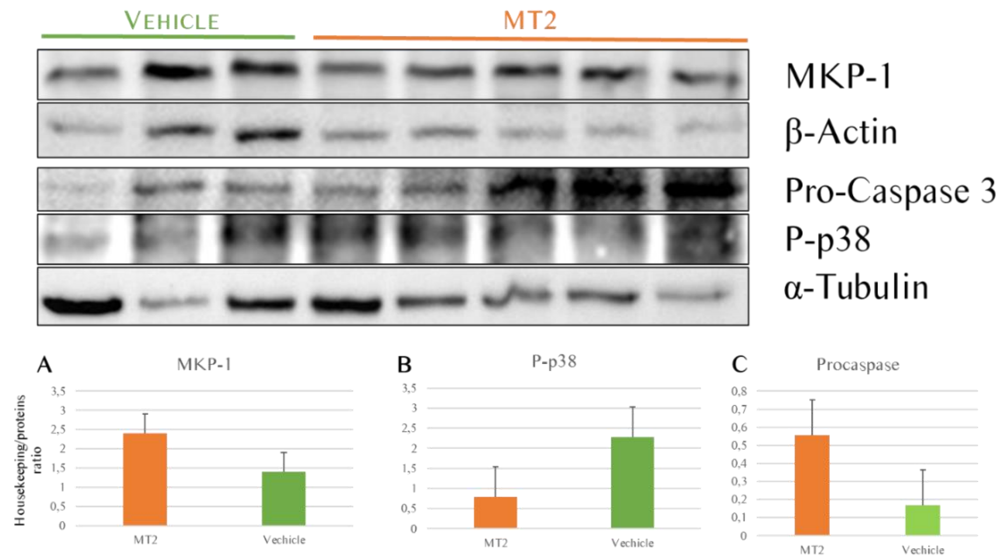


Figure 14. ***Effect of MT2 treatment during EAE acute phase on NGF anti-apoptotic pathway in CNS.*** Upper panel: Western blot analysis of MKP-1, P-p38 and Pro-caspase 3 expression in spinal cord samples of mice treated with MT2 (orange) or vehicle (green) during the acute phase of the disease and sacrificed at 26 dpi. Actin and α-tubulin were used as housekeeping proteins. Each lane represents a single sample. Here we show one experiment representative of 3 independent ones. A-C) Densitometric analysis of MKP-1 (A), P-p38 (B) and Pro-caspase 3 (C) expression reported as ratio versus the optical density of the corresponding housekeeping protein. Each column in the graphs report mean value±SEM of 3 experiments. Green columns: vehicle treated mice (controls): orange columns: MT2 treated mice.

The same samples were analyzed for the expression of the inflammatory marker inducible nitric oxide synthases (iNOS) (Figure 15). We didn't detect any

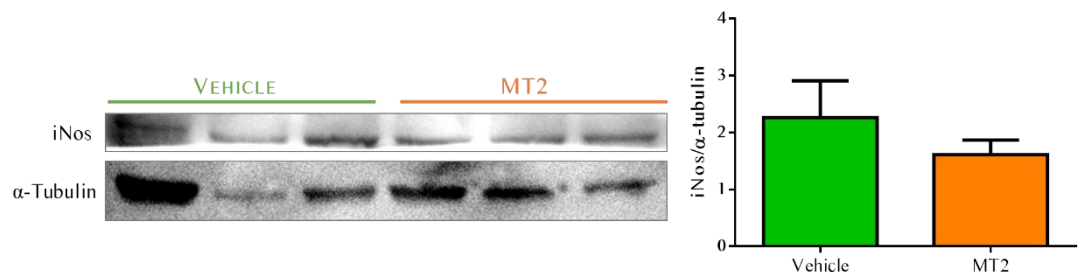


Figure 15. ***Effect of MT2 treatment during EAE acute phase on iNOS expression in CNS.*** A) Western blot analysis of iNOS expression in spinal cord samples of mice treated with MT2 (orange) or vehicle (green) during the acute phase of the disease and sacrificed at 26 dpi. α-tubulin: housekeeping protein. Each lane represents a single sample. One experiment representative of 3 independent ones. B) Densitometric analysis of iNOS expression: graph shows the ratio between iNOS and α-tubulin optical density (mean value±SEM of 3 experiments).

significant variation about iNOS production between MT2 and vehicle treated mice.

Effect of MT2 treatment on immune response

We investigated the possible effect of MT2 on *ex vivo* immune cells collected from lymph nodes, evaluating MOG_{35-55}-driven proliferation and cytokine production. For this purpose, cells were stimulated with MOG_{35-55}: results

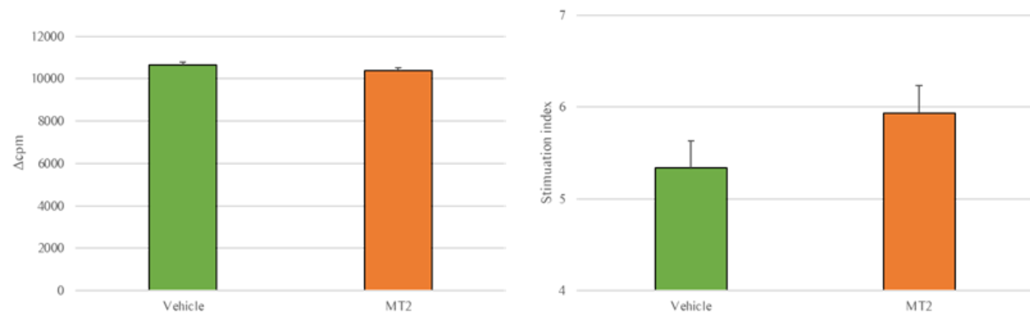


Figure 16. ***Ex vivo proliferation assay of lymph nodes cells from EAE mice treated during the acute phase of the disease.*** Proliferative response to MOG_{35-55} peptide of lymph nodes from EAE mice sacrificed at 26 dpi upon treatment with MT2 (orange columns) or vehicle (green columns) during the acute phase of the disease. Proliferation was tested by ^{3}H-Thymidine incorporation. Left: Δcpm (mean cpm of antigen stimulated cells-mean cpm of not stimulated cells). Right: stimulation index (SI= mean cpm of antigen stimulated cells/mean cpm of not stimulated cells). Mean values±SEM of two independent experiments (N=9 per group).

showed that treatment with MT2 didn't modify the proliferative response of lymphoid cells to the antigen (Figure 16). Afterwards, we assessed the presence of cytokines of inflammatory response in supernatant of MOG_{35-55}-stimulated cells assaying with LUMINEX technology the level of IFN-γ, TNF-α, IP10, MIP2, KC, IL4, IL10, MCP1, IL17 and IL6 (Figure 17). As for proliferative response, MT2 didn't change cytokine profile of MOG_{35-55} stimulated cells.

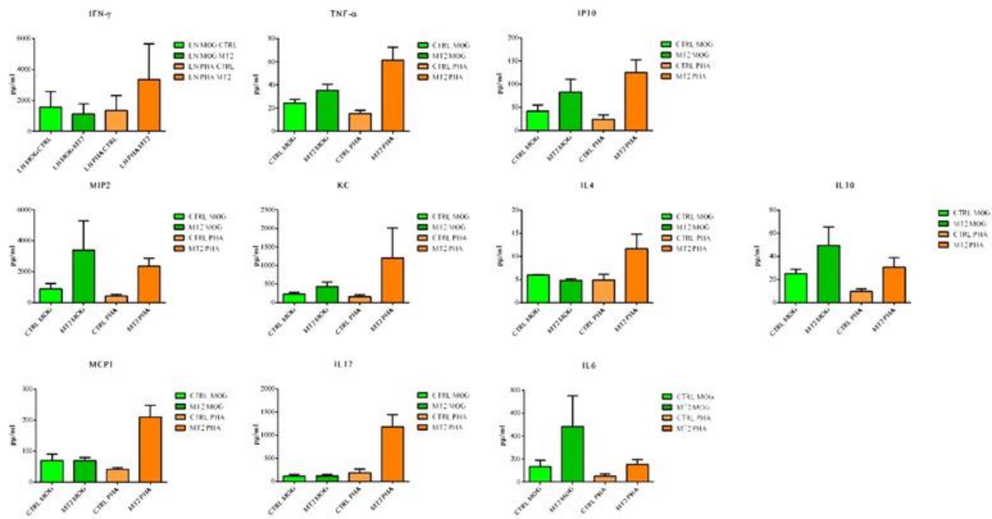


Figure 17. ***Cytokines quantification on acute phase of EAE from lymph nodes.*** The inflammation state was investigated by LUMINEX technology in supernatant of T-cell line MOG35-55 -immunized. We considered IL-6 (A), TNF-α (B), IP10 (C), MIP2 (D), KC (E), IL-4 (F), INF-g (G), MCP1 (H), IL-17 (I), IL.10 (J) as immuno-response markers. Measurements were made in 4 different experimental conditions: MOG-stimulated (CTRL MOG, light green), stimulation with MOG and treated with MT2 (MT2 MOG, green), PHA stimulated (CTRL PHA, light orange) and upon MT2 treated and PHA stimulated (orange, MT2 PHA). The column graphs show total protein levels in supernatant, expressed as mean pg/mL of two experiments ± SEM (N=9 animals). ****p < 0,0001

MT2 treatment during the chronic phase of EAE

1.2.1 Effect of MT2 on EAE clinical course

We then evaluated the possible MT2 therapeutic efficacy in EAE if administered during the chronic phase of the disease. To this aim we treated 12 EAE mice, 6

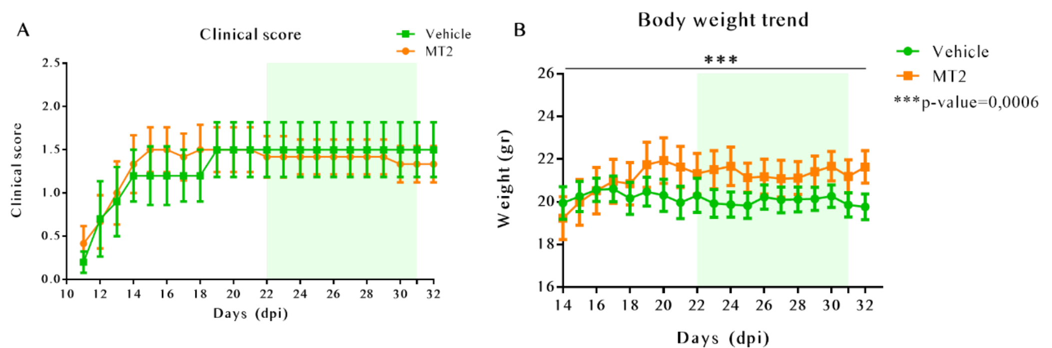


Figure 18. ***MT2 treatment of EAE mice during chronic phase of the disease: effect on clinical score and body weight.*** Animals (N=6 per group) were treated with MT2 or vehicle from 23 to 31 dpi (treatment period is highlighted in pale blue in graphs). A) Clinical score (mean values ±SEM, 6 animals/ group) of MT2 treated animals (orange curve) and controls (green curve). B) Body weight trend (mean values ±SEM, 6 animals/ group) of MT2 treated animals (orange curve) and controls (green curve) (***p=0,0006, unpaired t-test).

with MT2 and 6 with vehicle, from 23 to 31 dpi. As shown in Figure 18B, MT2 was able to avoid the body weight drop observed in control mice (***p=0,0006). Despite the significant result, the difference in body weight that already existed between the two groups prevents the determination of whether the effect during treatment is entirely or partially operable by MT2. On the other hand, the drug is not effective in modifying the clinical score (Figure 18A).

Histological analysis of spinal cord

The clinical findings were supported by the histological analysis of spinal cord isolated at 32 dpi, upon 10 days of MT2 treatment. We evaluated the presence of immune cell infiltrates, the degree of demyelination and the axonal loss, by hematoxylin and eosin (H&E), Luxol Fast Blue (LFB) and Bielschowsky staining, respectively (Figure 19). According to the clinical score observation, the histological analysis didn't reveal any difference between MT2 treated mice and controls.

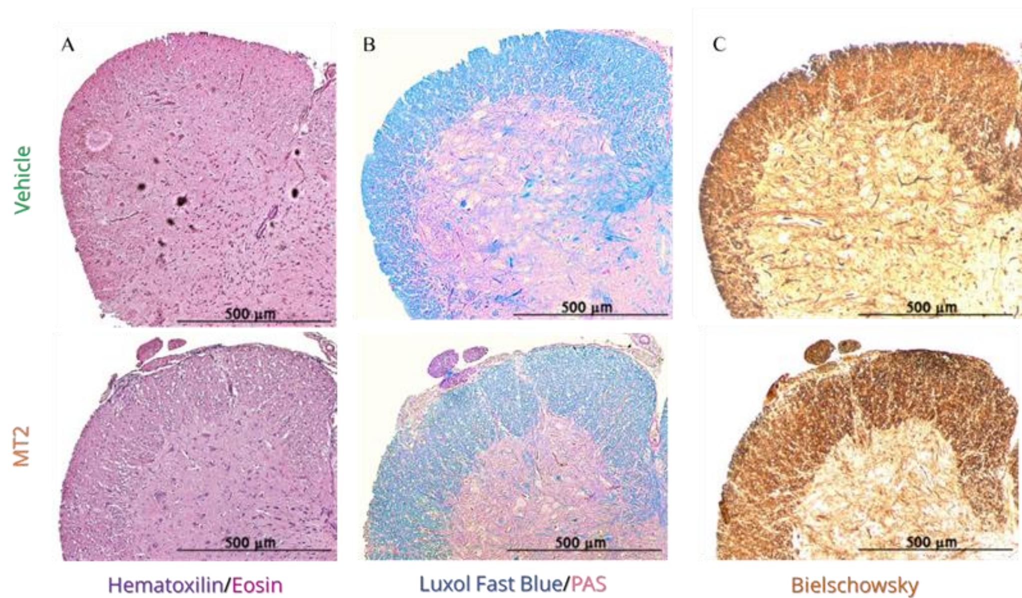


Figure 19. ***Histopathological evaluation of spinal cord of EAE mice treated with MT2 during the chronic phase of the disease.*** A) Haematoxylin and Eosin (HE), B) Luxol Fast Blue (LFB) and C) Bielschowsky staining of paraffin embedded spinal cord slices (5µm), of EAE mice treated with MT2 compared to controls (vehicle), to evaluate the presence of inflammatory infiltrates, demyelination and axonal loss, respectively.

EAE clinical course was further investigated by Rotarod test. Although vehicle and MT2 treated animals showed a similar riding time (Figure 20A), in MT2 group we found a significant (****p<0,0001) lower number of falls during the

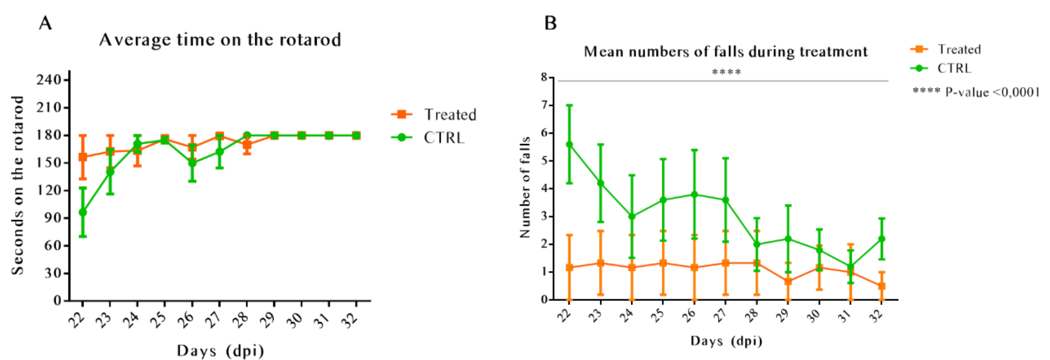


Figure 20. ***Effect of MT2 treatment during the chronic phase of EAE on motor coordination and balance.*** Animals (N=6 per group), during the treatment period, were daily subjected to a walking of 180 seconds on a rotating rod at speed of 30 rpm. In A) we show the average time on Rotarod, Orange curve: MT2 treated mice; green curve: controls (mean values±SEM, 6 animals/ group). In B) graph represents the mean number of falls during the exercise (mean values±SEM, 6 animals/group each) (****p<0,0001 -Mann-Whitney test).

exercise than in controls (1,11±0,08 vs 3,02±0,39 falls, mean±SEM, 6 animals per group) (Figure 20B).

MT2 treatment during immunization and preclinical phase

In order to understand if the ability of MT2 to ameliorate the disease course of EAE when administered during the acute phase could be due to an anti-inflammatory effect, we explored the impact of MT2 on immune system during active immunization, focusing our attention on T cells. To this end we carried out *in vivo* and *in vitro* experiments.

In vivo treatment of MOG $_{35\text{-}55}$ immunized mice during the preclinical phase of the disease

First, we decided to treat with MT2 MOG$_{35\text{-}55}$-immunized mice before the onset of the clinical signs, from 2 dpi to 13 dpi. At 14 dpi mice were sacrificed and we isolated lymphomonocytes from lymphoid organs and spinal cord to characterize phenotype and cytokine production of both peripheral and CNS infiltrating T cells.

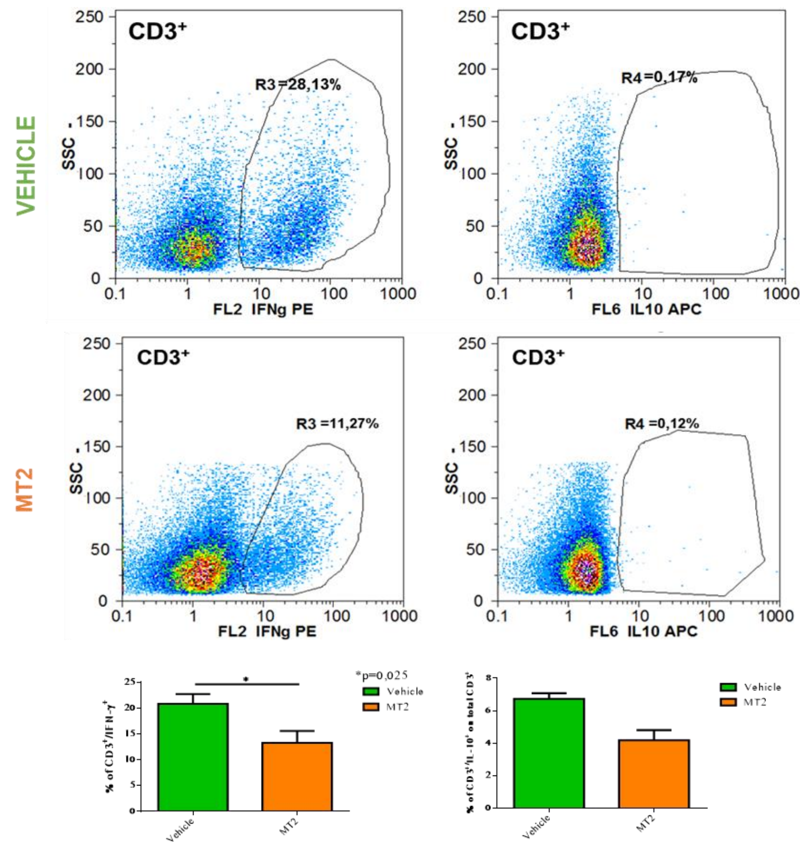


Figure 21. **Effect of MT2 treatment during EAE preclinical phase on IFNg and IL10 production by lymph node T cells.** Lymph nodes cells from MOG$_{35\text{-}55}$ immunized mice treated or not with MT2 in the preclinical phase of EAE were collected at 14 dpi and labelled with anti CD3/anti IFNg/anti IL10 antibodies. Upper panel: representative dot plots showing percentage of IFN-g^{+} (left) and IL-10^{+} (right) positive cells gated on total CD3^{+} cells. Lower panel: average percent of CD3^{+}/IFN-g^{+} (left) and CD3^{+}/IL-10^{+} (right) cells resulting from 6 mice per group analyzed in 2 different experiments (mean±SEM; Unpaired t-test). Green columns: vehicle treated mice; orange columns: MT2 treated mice.

In particular we evaluated the percentage of T cells producing IFNg or IL10 by intracellular staining and flow cytometry analysis. As shown in Figure 21, MT2 *in vivo* pre-treatment significantly reduced (*p=0.025, Unpaired t-test) the percentage of IFN-γ positive cells in draining lymph nodes (20,87%±3,8% in

MT2 vs 13,3%±4,3% in controls, mean±SEM of 6 animals analyzed in two different experiments).

According with these findings, the percentage of IFN-γ positive T cells within the spinal cord in MT2 treated mice was also strongly reduced (Figure 22), dropping to 6,42±0,07% (mean±SEM of 2 different experiments, carried out pooling cells from 3 samples in each one) versus 14,87±4,36% observed in control mice.

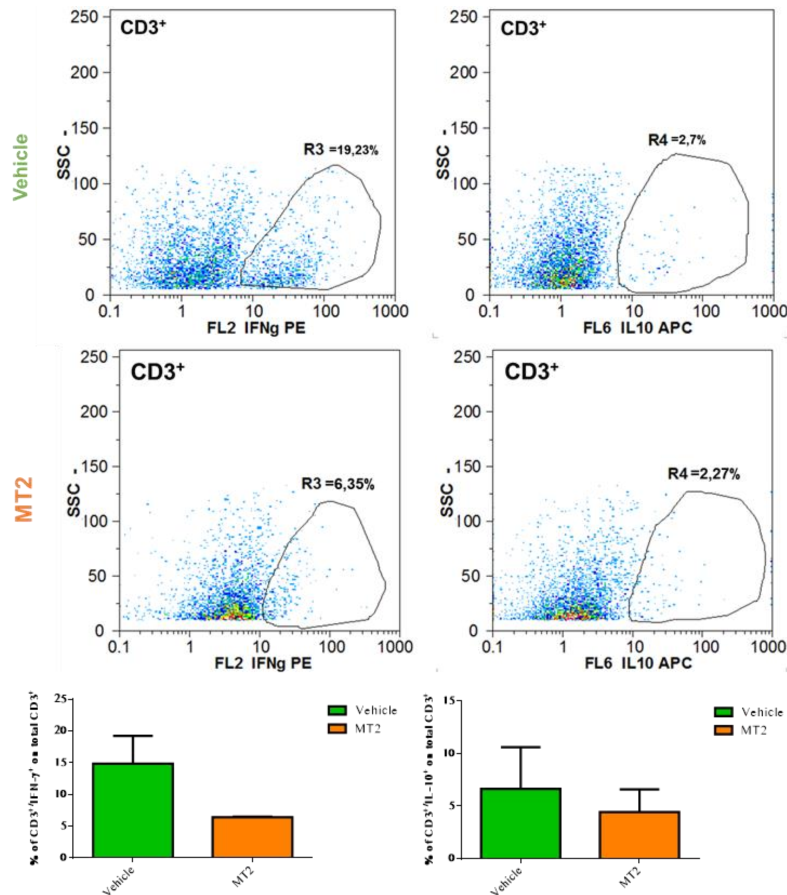


Figure 22. ***Effect of MT2 treatment during the EAE preclinical phase on phenotype of CNS infiltrating T cells.*** Lymphomonocytes isolated at 14 dpi from spinal cord of MOG_{35-55} immunized mice treated or not with MT2 in the preclinical phase of EAE were labelled with anti CD3/anti IFN-g/anti IL10 antibodies and analyzed by flowcytometry. Upper panel: representative dot plots showing percentage of IFN-g^+ (left) and IL-10^+ (right) positive cells gated on total $CD3^+$ cells. Lower panel: average percent of $CD3^+$/IFN-g^+ (left) and $CD3^+$/IL-10^+ (right) positive cells resulting from 2 independent experiments, pooling 3 animals per group in each experiment (mean±SEM). Green columns: vehicle treated mice; orange columns: MT2 treated mice.

Regarding the production of IL-10, this cytokine resulted unchanged by MT2 treatment both in lymph node and spinal cord T cells (Figure 21-22).

In vitro effect of MT2 on MOG 35-55 specific T cell proliferation and cytokine production

To better understand the effect of MT2 on T cells, we tested the effect of the drug *in vitro* on MOG 35-55 specific T cells (TCL), evaluating antigen driven proliferation and cytokine production. To this end TCL were stimulated with MOG 35-55 peptide in the presence or absence of MT2. We found that MT2 didn't modify the proliferative response of TCL to MOG 35-55 peptide (Figure 23).

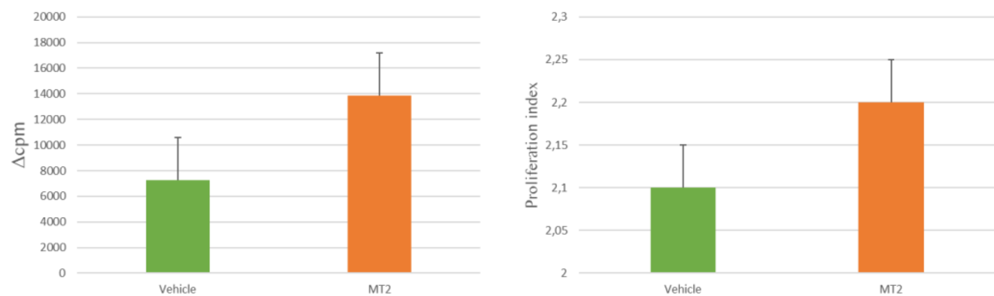


Figure 23. ***In vitro effect of MT2 on antigen specific T-cell proliferation.*** **MOG 35-55 specific T cells**. were stimulated with antigen in the presence or absence of MT2, added in culture medium. Proliferation was assessed by 3H-Thymidine incorporation. Left: Δcpm (mean cpm of antigen stimulated cells-mean cpm of not stimulated cells). Right: stimulation index (SI=mean cpm of antigen stimulated cells/mean cpm of not stimulated cells). Mean values±SEM of two independent experiments.

Then, we investigated the inflammatory state of MT2 treated culture and controls by measuring level of IFN-γ, TNF-α, IL4, IL10, IL6 in TCL supernatant by Luminex assay. As for proliferative response, MT2 didn't change cytokine profile of MOG35-55 stimulated cells (Figure 24).

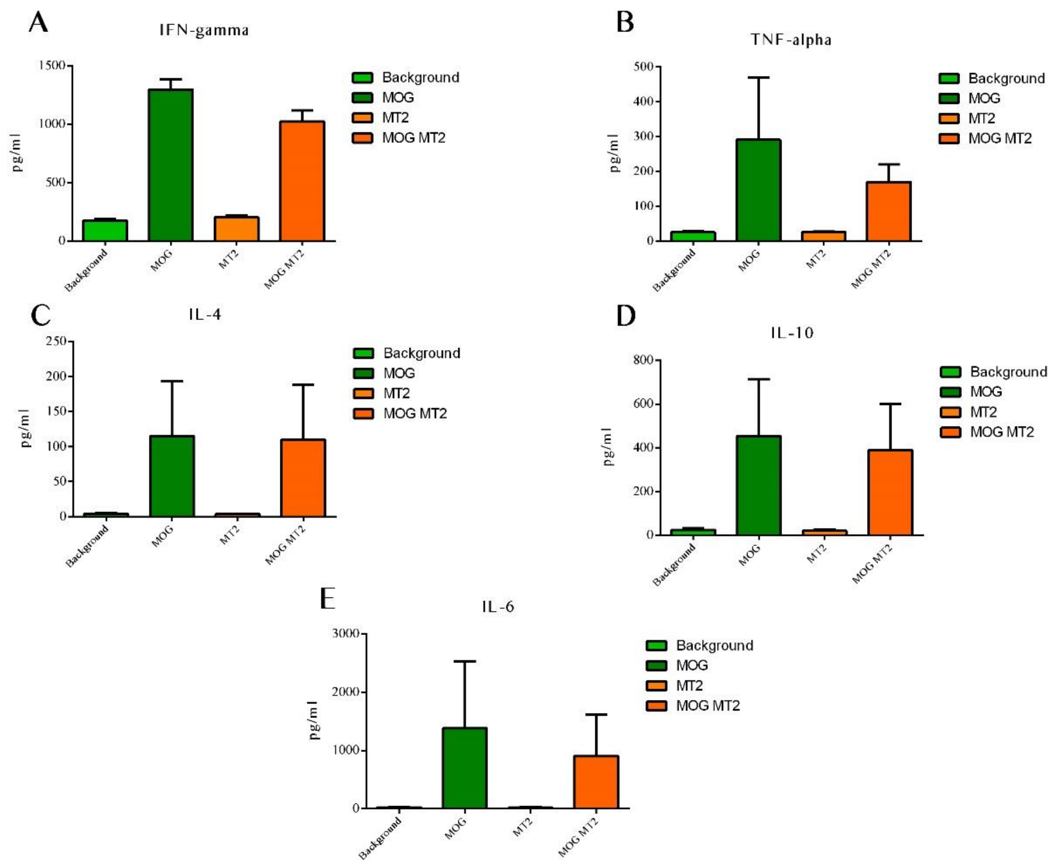


Figure 24. ***In vitro*** **effect of MT2 on cytokine production by MOG35-55 TCL.** *IFN-g (A), TNF-α(B), IL-4 (C), IL-10 (D) and IL-6 (E) were measured on TCL supernatant under basal condition (Background) and upon 48 hours stimulation with MOG35-55 (MOG), in the presence or absence of MT2 added in culture Mean pg/mL±SEM of 3 independent experiments.*

Effect of MT2 treatment on Cuprizone induced demyelination

To verify a potential role of MT2 in protecting CNS during demyelination and inducing remyelinating processes, we tested our drug on a toxic non-autoimmune demyelination model, induced by feeding C57BL/6 mice with the copper chelator cuprizone.

We fed animals for 35 days (0 – 35 experimental days, *e.d.*) and treated animals with MT2 or vehicle from day 21 to day 45 e.d.. Animals were sacrificed at days

49 (7th week, during demyelinating phase) and 56 (8th week, during remyelinating phase).

Microscopic analysis of myelin content

We obtained sagittal sections from cerebellum and corpus callosum (CC), as shown in Figure 25. These slices were stained with GFAP and MBP antibodies in two different timing, using slices from demyelination phase (day 49) and remyelinating phase (day 56). In first phase slices, we noticed how myelin loss was more evident in MT2 tissue (arrow) compared to vehicle one (control).

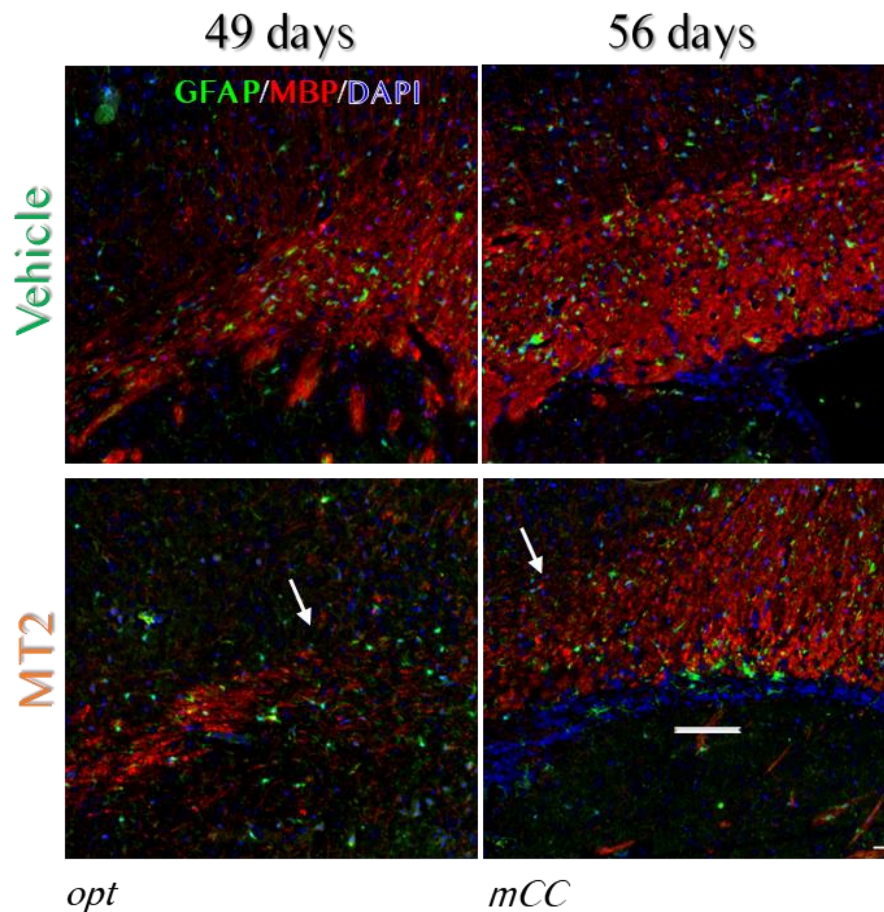


Figure 25. ***Effect of MT2 treatment on myelin loss in Cuprizone demyelinating model.*** We investigated the myelin loss in Cuprizone model in two different phases: the demyelinating phase, typical at 49th experimental day and the remyelinating one, usually visible after 56 days of experiment. First column is referred to the optical region at 49th experimental day, second column to the medial corpus callosum after 56 experimental days. Pictures were taken at immunofluorescence microscope (20x magnification). First line is for vehicle samples, second line is for MT2-treated animals. Green: GFAP; Red: MBP; Blue: DAPI. opt: optic region; mCC: medial corpus callosum. Scale bar 200 μm.

Furthermore, the figure shows how, during remyelinating phase, loss of MBP^+ cells was less broad but still evident (arrow). GFAP staining was similar in both tissues.

Further investigation using transmission electron microscope (TEM) techniques (Figure 26) highlighted how myelin sheets were more plentiful in control sections of corpus callosum compared to MT2 ones.

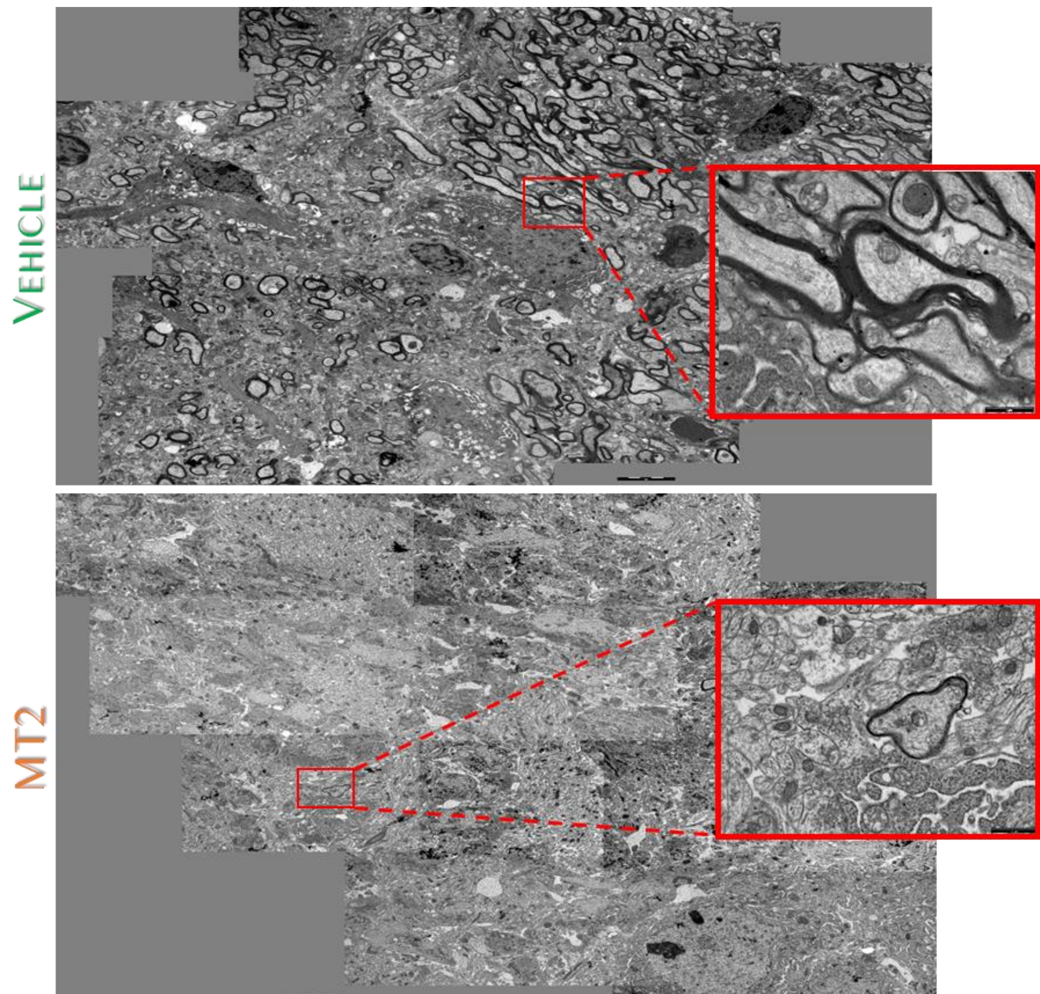


Figure 26. ***Transmission electron microscope micrographs of corpus callosum of cuprizone fed mice treated or not with MT2.*** Scale bar 1 µm. Electron micrograph magnification: x5000, x20000. Red square: particular of myelin sheets.

Electron microscope and fluorescence data were further confirmed by Luxol Fast Blue staining on corpus callosum sections, as shown in Figure 27.

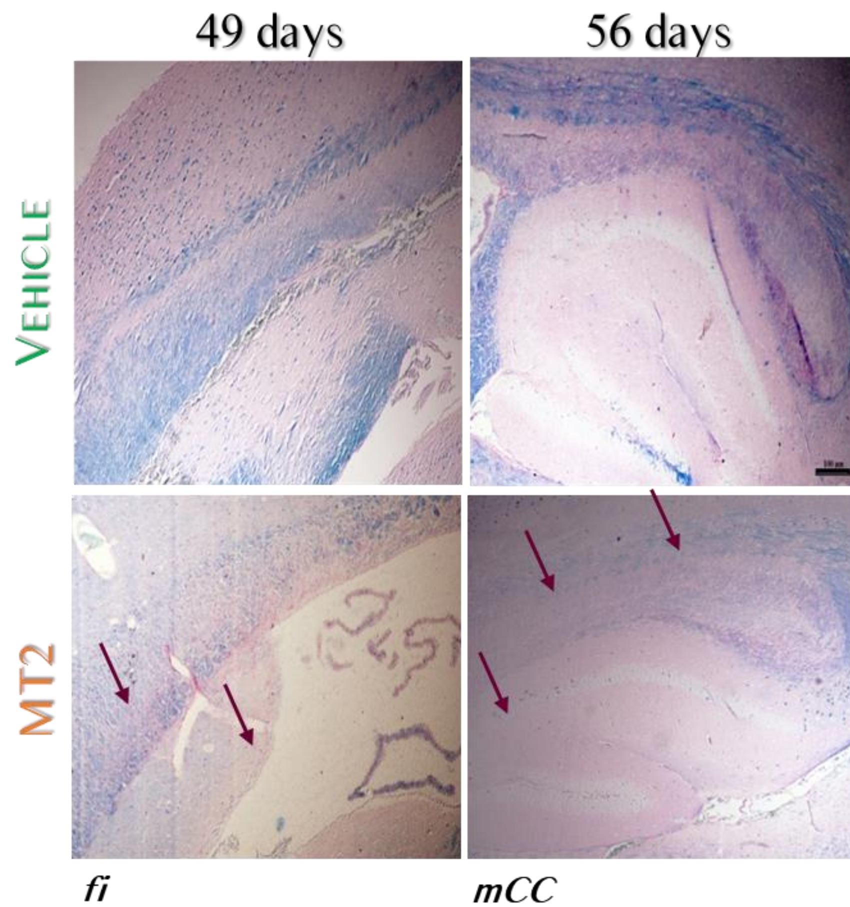


Figure 27. ***Luxol fast blue staining of hippocampal fimbria and corpus callosum of cuprizone fed mice after treatment with MT2.*** Blue: myelin Scale bar 100 µm; abbreviations: fi: hippocampal fimbria; mCC: medial corpus callosum.

Effect of MT2 treatment on serum inflammatory markers

Together with the evaluation of myelin content, we investigated the inflammatory state of MT2 treated animals and controls by measuring serum level of IFN-γ, IL10, IL17, IL6, TNF-α, CXCL1, CXCL2 and IL4 in at 49 and 56 experimental days. We didn't find any significant differences between two groups (Figure 28).

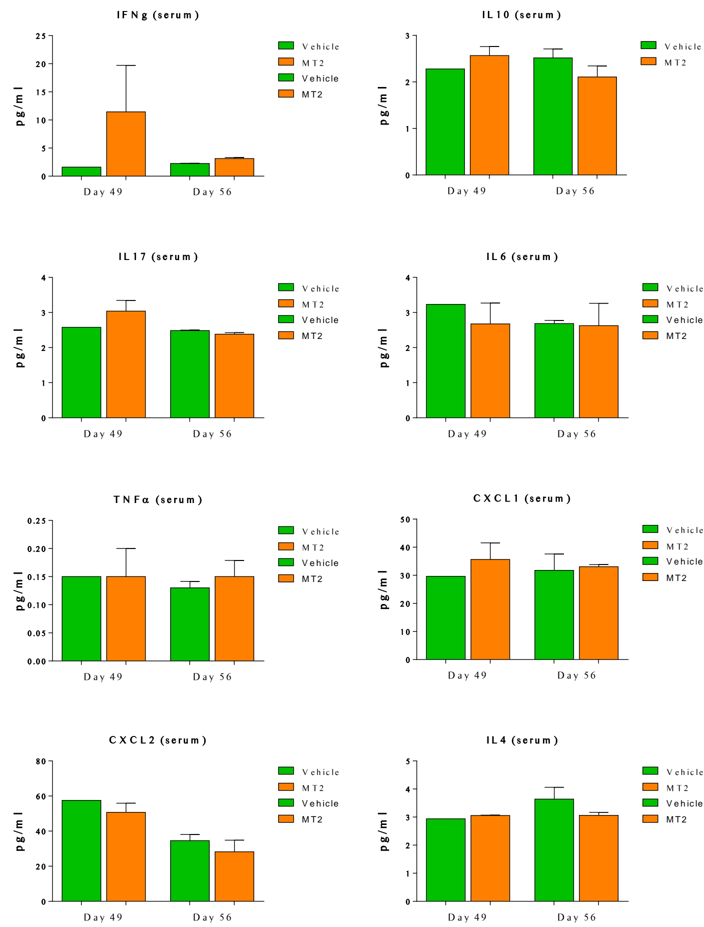


Figure 28. ***Cytokine and chemokine content in serum of Cuprizone fed mice under treatment with MT2.*** A panel of cytokines and chemokines were measured by Luminex assay on serum samples collected at *day* 49 (demyelinating phase) and day 56 (remyelinating phase). Green: vehicle treated mice; orange: MT2 treated mice. Mean±SEM of 6 mice per group.

Discussion

Discussion

Neurotrophins, such as NGF and BDNF, are a family of proteins that rules neuronal survival, synaptic function and neurotransmitter release. Furthermore, they can elicit the plasticity and growth of axons within the adult central and peripheral nervous system, as reviewed by Keefe *et al.* 2017. In particular, their trophic feature leads to think of them as good molecules for therapeutic treatments, mostly for counteract inflammation induced tissue damage in pathologies on CNS. Despite this, their protein nature makes them unfavourable in a pharmacokinetic point of view, especially for central nervous system disorders, which involve the resistance to peptidase enzymes, the crossing of the BBB and poor diffusion in tissues. With these premises, we tested MT2, a patented non-peptidic NGF and BDNF mimetic that potentially had the same neuroprotective beneficial without pharmacological adverse feature of a neurotrophins, but with a better bioavailability and metabolic stability.

We tested MT2 in a rodent model of MS, the EAE MOG $_{35-55}$-induced, to understand if the drug could be a tool to treat animals during acute and chronic phases of the disease.

In order to evaluate MT2 possible beneficial effect during the acute phase of EAE, the molecule was administered via i.p. from day 14th to day 22th. Results showed that the molecule significantly improved the clinical sign of the disease in MT2 treated animals comparing them to vehicle-treated group. Histopathological data were coherent with the improvement related to the clinical course of the disease. In particular, histopathological images, related to the animals observed during the acute phase of the disease, showed that in the spinal cord, considered the main representative site of EAE model, the number of lymphocytes and macrophages

infiltrates, principal responsible of neuronal damage, were reduced in MT2-treated animals compared controls ones. Meantime, the number of demyelinated areas was significantly lower in treated mice compared controls ones. In line with the drug ability (Scarpi et al 2012) in triggering early expression of MKP-1 and, then, leading to the phospho-p38 pathway activation, MT2 showed anti-inflammatory function: we observed a higher axonal integrity in the SC of treated mice compared to controls. This result is coherent with the work by Villoslada and colleagues (Villoslada *et al.* 2000), that showed that intracerebroventricular cannula administration of human NGF (hNGF) in common marmoset, improved the severe clinical sign of EAE. Our data lead us to think that MT2 acted as hNGF, improving clinical signs of EAE, and suppressing both the inflammatory and demyelinating components of CNS pathology. The effect mediated through MT2, that we observed in the acute phase of EAE, was similar to the one exerted by Sildenafil, a phosphodiesterase-5 (PDE5) inhibitor, as explained in a recent work (Pifarre *et al.* 2014). Spanish researchers found that daily i.v. treatment with Sildenafil during peak of disease, rapidly ameliorated clinical symptoms and neuropathology that was related to BDNF up-regulation in immune and neuronal cells, suggesting its implication in the beneficial effects of the drug.
Positive MT2 effect, observed on EAE clinical course and SC histopathology during acute phase, led us to think that MT2 can modulate both neuronal cell function and immune cell activity. The effects on neurons were evidenced in the *in vitro* model of organotypic spinal cord cell culture (developed in laboratories of professor Laura Ballerini, SISSA-ISAS, Trieste Italy). In this work, we demonstrated (see manuscript attached) that MT2 is able to counteract the effect of pro-inflammatory cytokines cocktail preventing the synaptic alteration described without affecting the inflammatory response due to non-

neuronal cells present in the culture. The drug was also able to confirm its ability to increase MKP-1 expression through TrkA pathway.
In order to understand if MT2 directly act on immune system, we investigated T cells function in EAE MT2 treated mice during the acute phase. We observed that MT2 didn't alter T cells proliferation ability versus MOG antigen, indicating that peripheral T cell responses were not affected by i.p. administration of MT2. We analyzed cytokines produced by T cells that influence the course of autoimmune disorders (Charlton and Lafferty 1995). Through Luminex technology data, we noticed a lower IFN-γ, pro-inflammatory cytokine, production by T cells isolated from lymph nodes and spinal cord in MT2-treated mice compared to vehicle one, indicating that the protective effect of MT2 was possibly in part mediated through modulation of the peripheral and central network of cytokines produced by inflammatory cells. Regarding IL-10, an anti-inflammatory cytokine, we noted a comparable production in treated and vehicle mice. This result agreed, again, with the work of Villoslada (2000), confirming that MT2 may play a role in ameliorating the disease mimicking hNGF. The neurotrophin can modulate cytokine expression by inflammatory cells in EAE, underlying its pivotal attitude of acting as an autocrine and anti-inflammatory cytokine. In diseases like EAE and MS, the presentation of myelin antigens to T cells is dependent on the expression of class-II HLA molecules on astrocytes and macrophages/microglia, which is upregulated by IFN-γ (Fierz *et al.* 1985). Thus, a possible effector mechanism for the protective action of MT2 could be exerted decreasing the HLA class-II expression within the CNS, either indirectly through its effects on IFN-γ production or by a direct effect on astrocytes (Neumann *et al.* 1998). As a matter of fact, further investigations are needed in this mouse model, to determine whether the

effects of MT2 on CNS cytokine production provide long-term benefit in a chronic experimental design.

Literature data reported that neurotrophins bind the pan-neurotrophin receptor (p75NTR) and tropomyosin receptor kinase (Trk) receptors, in particular NGF binds with high affinity TrkA and BDNF with the high affinity TrkB (Keefe *et al.* 2017). As widely demonstrated by molecular modeling studying by Scarpi et collegues, MT2 is a TrkA domain-5 activator (TrkA-d5), NGF pathway mediator. Such domain shares a high sequence homology with the secondary structure of binding domain of TrkB, useful for the binding with BDNF (Wiesmann *et al.* 1999 and Ultsch *et al.* 1999). Furthermore, pharmacokinetic and binding studies made in prof. Cozzolino laboratory demonstrated the efficacy of peptidomimetic in activating a TrkB-mediated reaction (unpublished observations). Several works reported that TrkA and TrkB receptors were expressed by neuronal and immune system as reviewed by Minnone G. and Keefe KM in two recent works. In order to understand the cellular targets of MT2 we investigated MT2 signaling pathway on T cells and spinal cord of EAE mice during the acute phase. We showed that MT2 triggered that pathway, increasing MKP-1 expression, phospho-p38 direct phosphatase. This latter protein is a member of MAP-Kinase family, that is deactivated through dephosphorylation, resulting not able to trigger the apoptotic pathway, as shown by the higher pro-caspase 3 presence in treated group than the vehicle one. This pathway, then, seems to be activated by the drug ameliorating the clinical picture, but further analysis was needed to understand if that happened in an exclusive way.

We administered MT2 during chronic phase of EAE, aiming to understand if there was any therapeutic effect after the first inflammatory insult. Clinical observations consistent with histopathological investigations did not showed any amelioration, despite the recovery on the body weight.

This discrepancy between MT2 treatment during acute and chronic phase could be due to variable TrkA and TrkB expression level on immune cells or CNS cells or both. Actually, Oderfeld et al. showed that in rat during EAE TrkA and p75NTR expression in CNS, neuron and glia, was drastically reduced after acute phase (Oderfeld-Nowak *et al.* 2003). Therefore, a lower expression of TrkA and TrkB may affect the protective synergistic mechanism that is physiologically activated near MS lesions during the early stage of the disease (Song, 2013). The positive effects of MT2 treatment on Rotarod test may be due to a specific benefic action on cerebellum, not evidenced in our histopathological analysis, restricted to spinal cord. The same discussion may be extended to immune system cells, la Sala and colleagues (2000) demonstrated that, during *in vitro* monocyte differentiation into dendritic cell, TrkA expression was progressively lost (la Sala *et al.* 2000). This interpretation is further supported by our results during toxic demyelination, where MT2 induced tissue damage with an evident increase of demyelinated areas in comparison to vehicle group. In the same model, Cinthia Farina and colleagues (data presented at ISNI Congress 2013) showed that TrkB expression alone was strongly increased and its activation worsens astrocyte mediated damage. On the other hand, Bonetto and collegues showed that only selective binding of TrkA may determine protection against toxic demyelination (Bonetto *et al.* 2017).
Taken together, our data suggest that MT2 is a anti-inflammatory and neuroprotective drug: its effect in acute phase of EAE is probably due to protection from pro-inflammatory cytokines expression by triggering the classical TrkA pathway. Our results on chronic EAE and Cuprizone led us to think that the double specificity for TrkA and TrkB could turn out to be a double-faced feature, activating pathways that were not intentionally targeted, causing damage in absence of ongoing inflammation events.

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