Università degli Studi del Piemonte Orientale “Amedeo Avogadro” and RBM/Merck Serono International S.A

Dottorato di Ricerca in Medicina Molecolare *Ciclo XX*

Relazione III° anno

TITOLO:

TACE (TNFα Converting Enzyme) expression in early and late stage mouse chronic experimental autoimmune encephalomyelitis (EAE)

Candidato: Claudia Patrignani
MerckSerono Tutor: Valeria Muzio
Academic Tutor: Andrea Graziani

SEZIONE 1

RISULTATI SCIENTIFICI

INTRODUCTION

In the first two years of PhD I focused my work mainly on PTPH1 and its role on neuronal functions. It has been recently pointed out that TNFα Converting Enzyme (TACE) is a downstream target of PTPH1 *in vitro* (*Zheng et al., 2002*).
TACE is a sheddase responsible for the cleavage of membrane-bound pro-TNFα into its soluble form *(Plumb J. et al., Mult.Scler. 2006)*. Recently it has been shown that increased expression of TACE in MS PBMC appears to precede blood brain barrier leakage and is also observed in infiltrating T-cells, in active and chronic MS plaques *(Seifert T. et al., Mult.Scler. 2002)*. Furthermore, it is differentially regulated in MS subforms suggesting that different regulatory mechanisms of TACE-TNFα release may be involved in the different clinical subtypes of MS *(Comabella M. et al., J. Neurol., 2006)*. In EAE models, little information is available regarding TACE regulation. Increased TACE expression has been described in astrocytes and invading macrophages in the spinal cords of rat acute EAE at the peak of the disease *(Plumb J. et al., J. Neuroimmunol. 2005)*. Similarly increased TACE expression in the spinal cord of mice relapsing-remitting EAE has been reported during the primary inflammatory phase *(Toft-Hansen H., et al., J. Immunol. 2004)*.
In the last year I worked to better characterize the mouse chronic EAE model in C57Bl/6 mice, investigating TACE endogenous expression in the CNS, brain and spinal cord, in the early (25-26dpi) and late phase of the disease (48-50dpi).
The results allowed us to understand whether in the mouse chronic EAE, as in MS, inflammation reaches the brain, even in the late chronic phase, providing another proof of the validity of this mouse model as preclinical tool.

MATERIALS AND METHODS

Study design
Two mouse chronic EAE experiments were performed using the same immunization procedure. In the first study mice were sacrificed at day 25 while in the second study mice were sacrificed at day 48 post immunization. In the 26 day-long experiment 2 month-old C57Bl/6 mice (n=14) provided by Charles River Italy, were immunized with MOG_{35-55} peptide in CFA; n=10 mice were immunized just with CFA (no MOG peptide) as controls. In addition a naïve group of 8 mice was included. In the 50 day-long experiment 2 month-old C57Bl/6 mice (n=12) were immunized with MOG_{35-55} peptide in CFA and n=4 mice received only CFA (no MOG peptide) as controls.

Immunization procedure
C57Bl/6 mice, 2 month-old provided by Charles River Italy, were immunized as follow:
On day 0 immunization was conducted by injecting s.c. in the left flank 0.2 mL of an emulsion composed of 200 μg MOG_{35-55} peptide (Neosystem, Strasbourg, France) in Complete Freund's Adjuvant (CFA, Difco, Detroit, U.S.A.) containing 0.5 mg of *Mycobacterium tuberculosis*. Immediately after, they received an i.p. injection of 500 ng pertussis toxin (List Biological Lab.,

Campbell, CA, U.S.A.) dissolved in 400 μL of buffer (0.5 M NaCl, 0.017% Triton X-100, 0.015 M Tris, pH=7.5).

On day 2 the animals were given a second i.p. injection of 500 ng pertussis toxin.

On day 7, the mice received a second dose of 200 μg of MOG_{35-55} peptide in CFA injected s.c. in the right flank. Starting approximately from day 8-10, this procedure resulted in a gradually progressing paralysis, arising from the tail and ascending up to the forelimbs. Clinical score and body weight were recorded daily. Mice were scored as follows: 0, no sign of disease; 0.5, partial tail paralysis; 1, tail paralysis; 2, partial hind limb paralysis; 3, complete hind limb paralysis; 4, hind limb and forelimb paralysis; 5, moribund or dead. All the mice were sacrificed by an overdose of intraperitoneal injection of thiopenthal at the time-point indicated in the above paragraph.

Western blot

Brains microdissected in different areas (olfactory bulbs, cerebellum, hippocampus, striatum, cortex pontine region and midbrain) and spinal cords were removed and snap frozen from EAE and CFA mice.

Protein extraction was performed by mechanical homogenation in Cell extraction buffer provided by R&D Systems (α-secretase activity kit #FP001). The method used allowed using the samples for Western blot and for α-secretase activity. Western blot analysis was performed on 30-50 μg of proteins. Lysates were run on an 8% SDS-page and transferred to nitrocellulose membrane (BioRad). Blots were cut at the level of 50KDa. The blots up to 50KDa were incubated in rabbit anti-TACE (1:2000, Sigma) overnight at 4°C with gentle rocking. Following washing, blots were incubated in HRP-linked anti-rabbit IgG (1:1000, Cell Signaling Tech.) for 1 hour, followed by washing and detection by ECL (Pierce). The blots from 50KDa were probed using a rabbit anti β-actin (1:250, Sigma). The bands were detected by the ChemiDoc™ XRS system, PC, an imaging system using a supercooled 12-bit CCD camera with 1.3 megapixel resolution (BioRad, #170-8070). The intensity of the bands were analyzed by the Quantity One® software for PC.

α-secretase activity Test

The same protein extract was tested for TACE activity by using a fluorometric kit of R&D Systems (α-secretase activity kit #FP001), following the kit protocol. Cleavage of the □-secretase/TACE-specific peptide conjugated to the reporter molecules EDANS and DABCYL by TACE physically separates the EDANS and DABCYL allowing for the release of a fluorescent signal. The level of α-secretase enzymatic activity in the cell lysate is proportional to the fluorometric reaction. The experiment was run in duplicates and the results were expressed as fold increases in fluorescence over background controls (reactions without cell lysate or substrate).

Citokine Beads Assay (CBA)

The BD™ CBA Mouse Inflammation Kit (# 552364) was used to quantitatively measure Interleukin-6 (IL-6), Interleukin-10 (IL-10), Monocyte Chemoattractant Protein-1 (MCP-1), Interferon-γ (IFN-γ), Tumor Necrosis Factor (TNF), and Interleukin-12p70 (IL-12p70) protein levels in the serum of healthy and diseased animals at 26dpi.

ELISA

Demyelination and axonal loss were assessed in the 48 day-long mouse chronic EAE experiment by ELISA. The antibodies mouse anti-MBP (Myelin Basic Protein) (1/5000, Chemicon MAB382) and anti-SMI35 (neurofilament) (1/5000, Sternberger) were used to coat the plates. After incubation with 1mg of total protein, a rabbit anti-MBP or anti-NfSMI35 (Zymed 18-0038) was used and amplified by using a tertiary anti-rabbit IgG HRP-linked (Cell Signaling 7074) and revealed with OPD substrate (Sigma P-918).

Statistics

Clinical score of the mouse chronic EAE was analyzed by a one-way ANOVA followed by a Fisher post-hoc test. CBA analysis was performed by one-way ANOVA followed by Tukey's post-hoc test. Data of WB, ELISA and of α–secretase tests were analyzed by T-test.

RESULTS

Disease course

The C57Bl/6 mice immunized with MOG developed clinical signs of paralysis starting at 17-20 dpi (Figs 1 and2) and reached a chronic and stable disease at 25-26 dpi with a score value of about 3 (complete hind limb paralysis) (Figs. 1 and 2). No clinical signs of the disease were recorded in the CFA immunized mice.

Western Blot

Both pro-TACE and TACE mature form are differentially expressed in the CNS in a early and late stage mouse chronic EAE (26 and 50dpi). At sacrifice the spinal cords and the brains of EAE and CFA mice were removed and the brains were dissected, in order to semi-quantify the amount of TACE forms expression in each area (olfactory bulbs, cerebellum, hippocampus, striatum, cortex pontine region and midbrain).

The results of the WB analysis show a differential and regional expression of both TACE forms in the CNS at both time points.

TACE expression at 26 dpi: pro-TACE (catalytically inactive) expression is significantly upregulated in the pontine region ($p_{pro\text{-}TACE}<0.05$) and downregulated in the cerebellum ($p_{pro\text{-}TACE}<0.05$) (Fig. 3b). No differences were recorded in the midbrain and spinal cord (Figs. 3b and c). As for TACE (catalytically active) expression is downregulated in the spinal cord (Fig. 3e), and there is a trend of increase in the pontine region ($p_{TACE}=0.08$) (Fig. 3d).. No significant differences were recorded in the other CNS areas investigated.

TACE expression at 50 dpi

Hippocampus: pro-TACE and mature TACE expression is significantly lower in the EAE mice compared to the controls ($p_{pro\text{-}TACE}=0.0011$; $p_{TACE}<0.05$) (Figs. 5b and c), but not significant differences were recorded in the percentage of activated TACE over the total amount of protein expression (data not shown). These data could suggest a strong inhibition of inflammation in the hippocampus 50dpi.

Cerebellum: there is no significant difference in the expression of both TACE forms in the cerebellum detected by WB, and no difference either in the percentage of activated TACE (Figs. 5b and c).

Cortex: pro-TACE expression is not significantly decreased in the cortex of EAE mice compared to controls. The mature TACE forms expression was not detectable for quantification (Figs. 5b and c).

Pontine region: as for the cerebellum, even in the pontine region no differences in TACE forms expression between diseased and healthy animals were detected, but it is appreciable a trend in the decrease of both forms (Figs. 5b and c). As for the percentage of activated TACE over the total protein content, EAE mice display a trend of increase ($p_{\%active\ TACE}= 0.08$, not significant) in activated TACE form compared to controls (data not shown).

Midbrain: this brain area includes most of the thalamic nuclei. Both TACE forms are upregulated in the midbrain of EAE mice compared to controls ($p_{pro\text{-}TACE}<0.001$; $p_{TACE}<0.05$). Percentage of activated TACE does not differ between the groups.

Spinal cord: pro-TACE expression follows a trend of decrease in spinal cord of EAE mice compared to controls, and the mature TACE expression is significantly higher in EAE mice compared to controls ($p_{TACE}=0.0484$).

α-secretase activity Test

TACE is one of the enzymes involved in the cleavage of APP protein within the αbeta peptide and for this reason is known as □-secretase. The fluorometric kit allowed to quantify TACE activity in the CNS extract already analyzed by WB.

No significant differences have been recorded in TACE activity at 26dpi in the CNS areas investigated (Fig. 4) . A significant decrease in TACE activity was detected in the pontine region at 48dpi ($p<0.05$) and a trend of increase activity in the hippocampus. As for hippocampus and midbrain a mismatch between TACE activity and expression was noticed (Fig. 5 d).

CBA

To verify which proinflammatory cytokines are involved at 26, serum samples from EAE, CFA and healthy animals were collected and analyzed.

At 26 dpi TNF□ level in the serum of EAE mice is significantly increased compared to healthy mice ($p<0.01$) (Fig. 6). Other pro-inflammatory cytokines as IFNγ and IL12p70 are significantly increased in EAE vs. healthy animals ($p<0.05$) (????). A significant increase in IL10 was recorded in EAE mice compared to the CFA and healthy animals ($p<0.05$). No difference in MCP-1 and IL6 profile was detected at this time point in EAE vs control mice.

ELISA

The levels of demyelination and axonal loss were assessed in order to understand the dynamic between demyelination and neurodegeneration in late stage EAE in upper brain regions.

ELISA for myelin basic protein (MBP) was used as measure for demyelination. Lower levels of MBP were detected in the spinal cord of EAE vs control mice ($p<0.05$) at 48 dpi, indicating higher level of demyelination. No differences were recorded in other CNS regions (Fig. 7a).

Axonal damage was measured using an antibody for hypophosphorylated form of neurofilament. No significant differences have been recorded at this stage of mouse chronic EAE in the CNS areas investigated (Fig. 7b).

CONCLUSIONS

In this experimental study the regional inflammation, as TACE expression and activity, in upper CNS areas in early and late stage of a model of MS (mouse chronic EAE) was investigated. Amadio and colleagues described that inflammation leads EAE progress in the early phase of the disease (20 dpi), whereas the paralysis in the chronic phase (from 40 dpi to 120 dpi) is sustained mainly by demyelination and axonal loss. Our data on cytokine profile on the serum of EAE vs control mice confirmed the presence of inflammation at 26 dpi

In particular, TNFα, IL12p70 and IFNγ, pro-inflammatory cytokines involved in the EAE inflammatory phase (Juedes et al., 2000), are significantly increased in the serum of EAE vs CFA mice. An increase in IL10 production was also detected. IL10 is reported to be an anti-inflammatory cytokine, inhibiting the production of other cytokines, such as TNFα and IL1 (Imitola et al., 2005). Its increased in this inflammatory phase could be due to some compensatory events, that we did not further investigated. . These observations on the inflammatory process in the periphery match partially with the data obtained on TACE expression and activity in the CNS. Although no differences in TACE expression and very slight modulation of its activity were detected in the spinal cord of diseased animals at 26 dpi, an increase in pro TACE and TACE expression was recorded in the pontine region at 26 dpi, without any modulation of the activity. The lack of TACE expression and activity in the spinal cord could be explained by the fact that the whole spinal cord was processed for the analysis Since at this stage the most important events occur in the hindlimbs, the alteration in protein expression could be concentrated in the thoracic part of the (lumbar?) spinal cord. Therefore the absence in the modulation in TACE expression and activity might be due to a dilution effect. Interestingly at 26 a modulation of TACE is detectable in the pontine region,

congruent with the consideration of EAE as an ascending paralysis. On the other hand on the same brain region an overall decrease in TACE expression and activity was detected at 48 dpi, In addition, a more significant decrease of PROTACE/TACE expression and activity was recorded in the spinal cord, confirming that the inflammatory events affect the pathogenesis in a minor extent at this late stage than demyelinating events. ELISA test clearly showed a strong demyelination in the spinal cord of EAE vs CFA mice at 48 dpi confirming that his pathological event predominates at this stage..

In the late stage, modulation of inflammatory response (PRO TACE/TACE) was detected also in upper CNS areas.Expression of both TACE forms is repressed at protein level in the hippocampus of EAE mice compared to CFA, whereas TACE activity is slightly increased. Possible post-translational event might occur in the hippocampus in this late stage of the disease. Hippocampal TACE involvement in EAE at almost 50 dpi has not been yet invenstigated, but it is known that other MMPs levels are impaired in the hippocampus at the peak of the disease in other mouse model *(Jovanova-Nesic et al., 2006).*

On the contrary an increase in midbrain TACE protein level was assessed at late stage EAE; this difference in protein expression was not translated into increased activity of the protein, strengthening the hypothesis of an effect of some post-translational events.

In conclusion we have demonstrated that TACE expression and activation are differentially regulated in the CNS of early and late stage chronic EAE both at translational and post-translational levels. Furthermore, we have shown that inflammation, measured by TACE function, reaches upper CNS areas in late stage chronic EAE. However this is not translated into significant demyelination in brain areas besides the spinal cord. Possible pathways involved in the translational and post-translational regulation are:

- zinc chelators and TIMPs (inhibition of TACE activity);
-TACE/TNFα feedback loop (modulation of proteolitic cleavage);
- unknown factors and/or compensatory events (Fig.8).

These results provide the evidence for a regional expression and modulation of TACE in the early and late stage of a mouse chronic EAE and provide further knowledge on the pathophysiology of the animal model. Further studies could be designed to understand which are TACE-modulating factors at trascriptional, translational and post-translational level, in earlier and late phase of the disease.

REFERENCE

- Zheng Y., Schloendorff J., Blobel C.P., (2002) J.Biol Chem. 277: 42463-42470;
- Seifert T., Kieseier BC, Ropele S, et al., (2002) Multiple Sclerosis; 8: 447 –451;
- Plumb J.,.McQuaid S, Cross AK, Surr J, et al., (2006) Multiple Sclerosis; 12(4):375-85;
- Comabella M., Romera C., Camina M., Perkal H. et al., (2006) J. Neurol, 253: 701-706;
- Toft-Hansen H., Nuttal R.K., Edwards D.R. et al., (2004) J Immunol. 173: 5209-5218;
- Plumb J., Cross A.K., Surr J., Haddock G. et al., (2005);
- Vann SD, Aggleton JP, (2002) Behav. Neurosci.; 116 (1): 85-94;
- Mirò-Bernié N., Ichinohe N., et al., (2006) Neurosci. 138 (2): 523-535;
- Zhang Z., Oliver P., Lancaster J.R. et al., (2001) FASEB J. 15(2): 303-5;
- Zhang Z., Kolls J-K., Oliver P. et al., (2000) J Biol Chem. 26;275:15839-44;
- Imitola J., Chitnis T., Khoury S.J., (2005) Pharmacology & Therapeutics 106: 163– 177;
- Juedes A.E., Hjelmstro¨m P., Bergman C.M. et al., (2000) J Immunol. 1;164(1):419-26;
- Jovanova-Nesic K., Shoenfeld Y., (2006) Journal of Neuroimmunology 181: 112–121;
- Amadio S., Pluchino S., Brini E. et al, (2006) Muscle&Nerve 33: 265-273;
- Madrigal J.L.M., Hurtado O., Moro M.A., et al., (2002) Neuropsycoph. 26: 155-163.

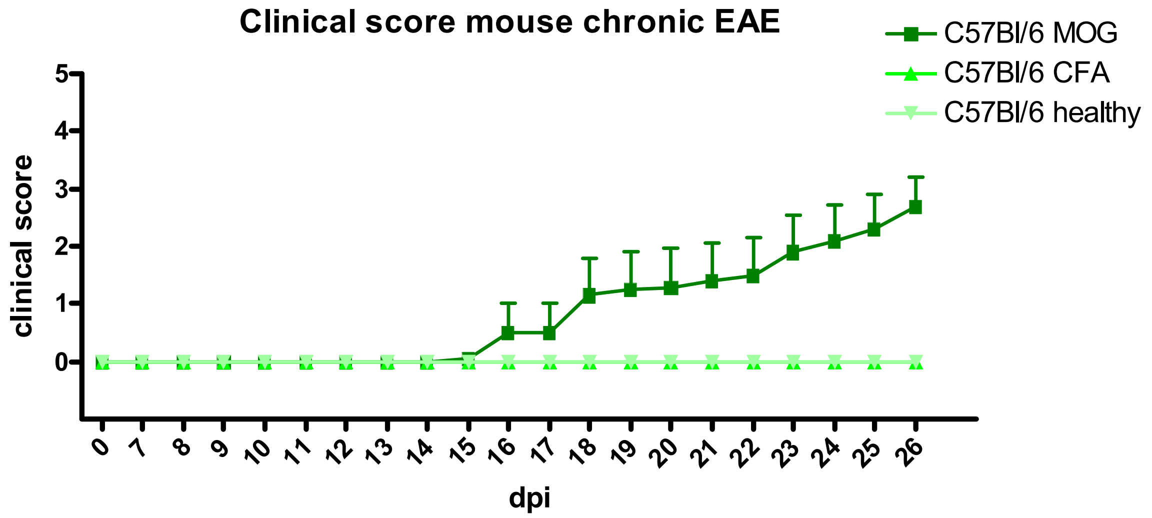

Fig.1.: Daily clinical score of the 26 day-long mouse chronic EAE

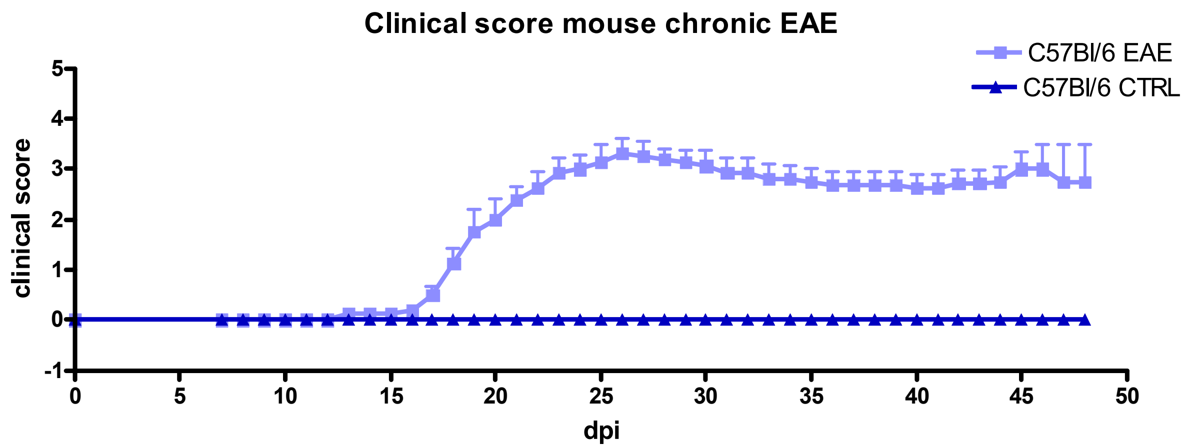

Fig.2.: Daily clinical score of the 50 day-long mouse chronic EAE

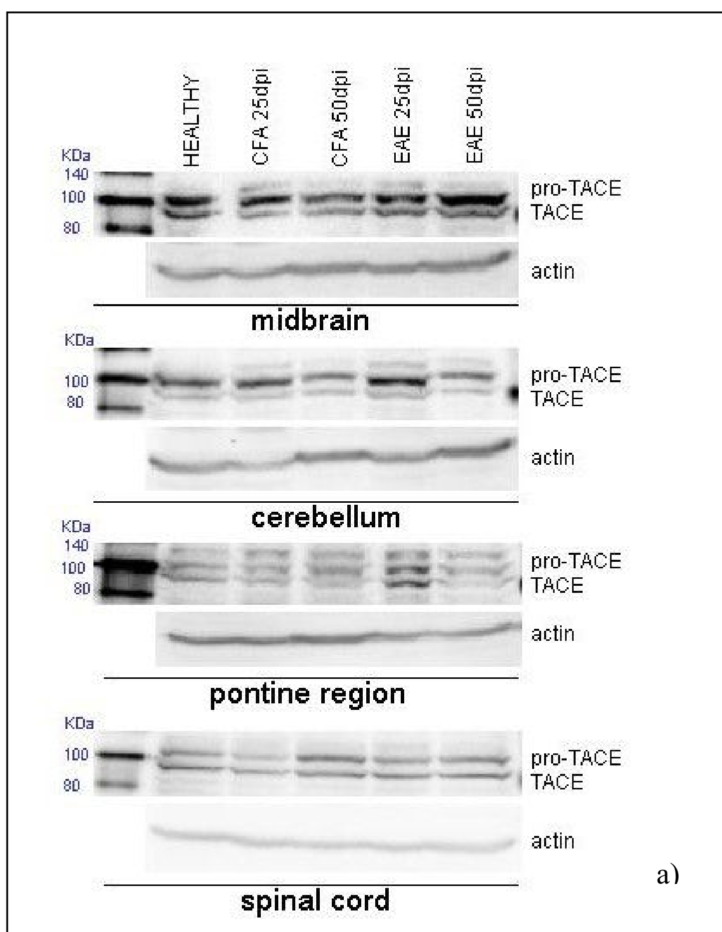

Fig.3.: a):Representative Western blots of TACE subforms in control and diseased CNS regionally expressed and modulated in disease condition. **b-e)** pro-TACE and TACE bands quantification at 26dpi**.**

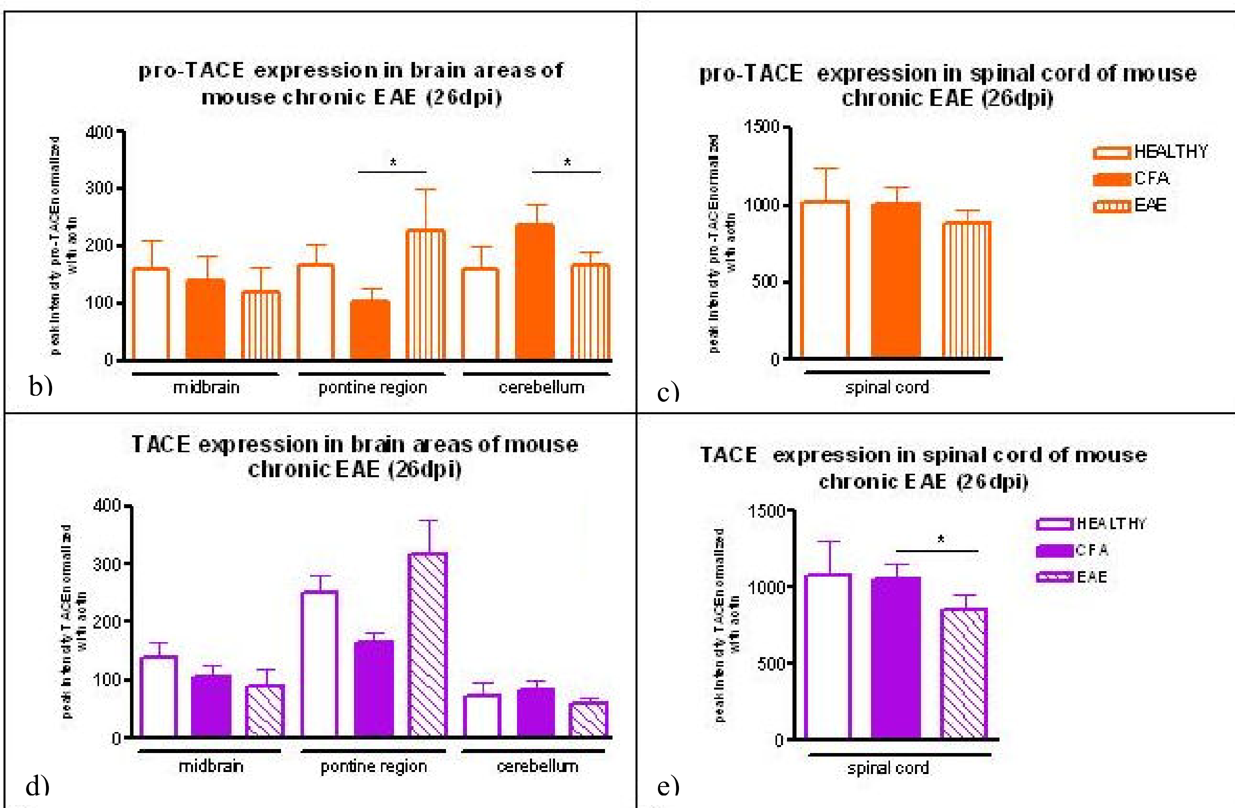

Fig.4.: Regional TACE activity in CNS areas at 26 dpi

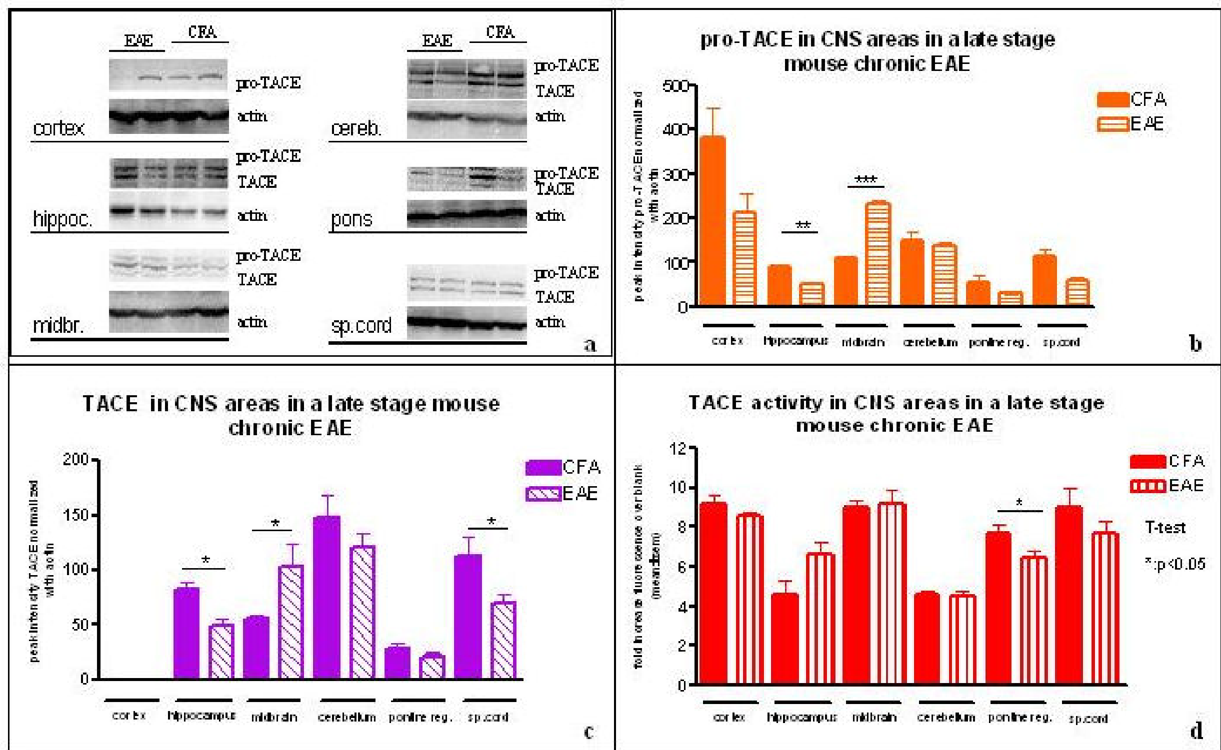

Fig.5.:a) Representative Western blots of TACE subforms in control and diseased CNS regionally expressed and modulated in disease condition at 48dpi. **b) Pro-TACE bands quantification:** significant regional regulation: up-regulation in midbrain while down-regulation in hippocampus. **c) TACE bands quantification (catalytic active form):** significant regional regulation with up-regulation in midbrain while down-regulation in hippocampus and spinal cord. **d) Differential and regional TACE activity in CNS areas at 48 dpi:** mismatch between TACE activity and expression in some CNS diseased areas (hippocampus and midbrain).

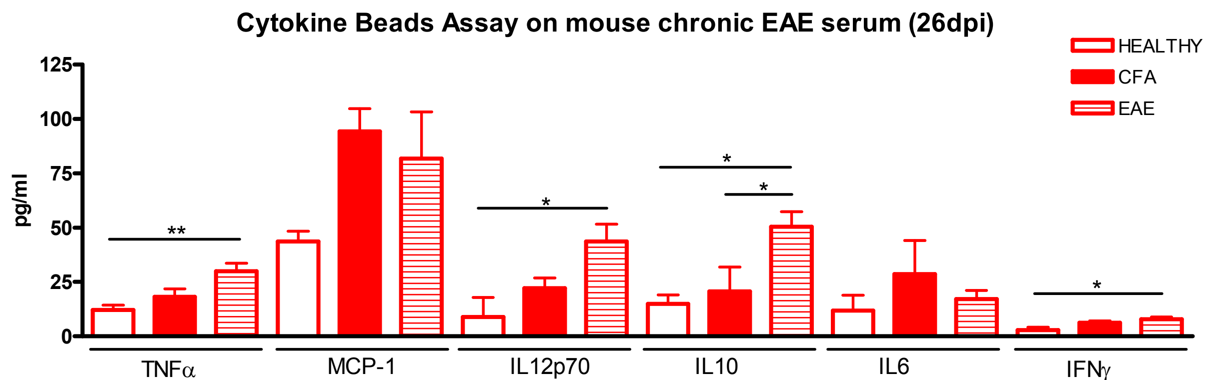

Fig.6.: CBA profile of EAE mouse serum at 26dpi

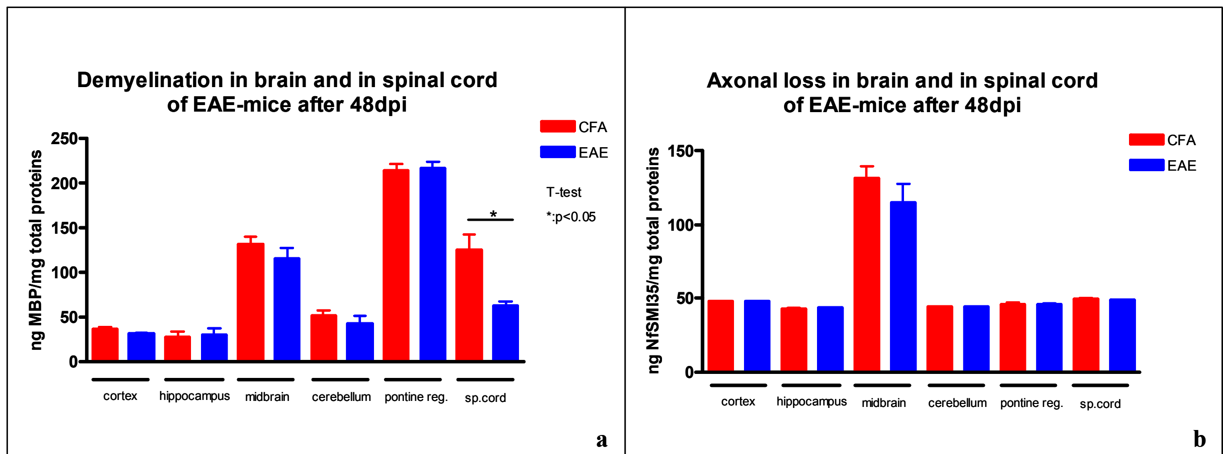

Fig.7.: a)Regional demyelination and b) axonal loss in EAE mice

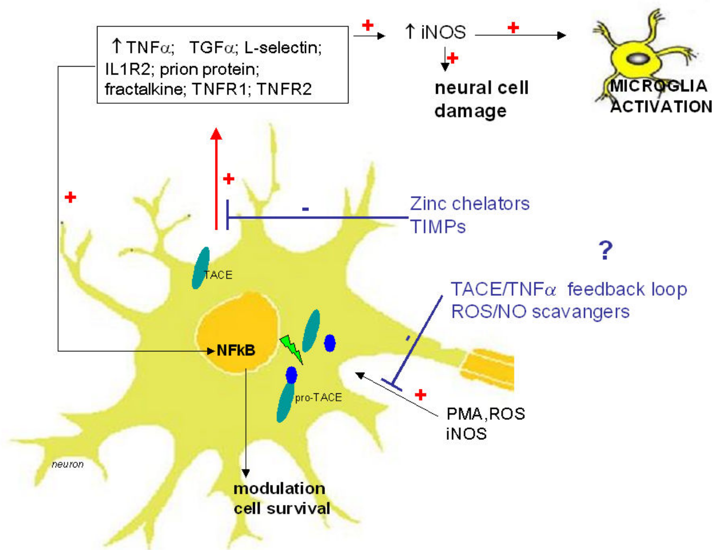

Fig.8. Representation of differential TACE involvement and regulation in CNS inflammation.

SEZIONE 2:

CORSI FREQUENTATI (per ciascun anno del corso)

1st year PhD

- Summer course: "Cell Culture and Cellular Model Systems", organized by EMIL: European Network of Excellence, Milan 18-22/07/2005;

2nd year PhD

- *"Inflammatory Mechanisms in Neurodegenerative Disease"*, organized by the MS Centre ErasMS and Erasmus Postgraduate School for Molecular Medicine, held in Rotterdam, 30-31 March 2006;
- *"English course"*, organized by RBM and by the M.I.T. Center of Turin.

3rd year PhD

- Baltic Summer School 2007-"Analysis of models for multiple sclerosis. Analysis of cells in the central nervous system", organized by Shohreh Issazadeh-Navikas, Lund 17-21/09/07.

SEMINARI FREQUENTATI (during 3rd year PhD)

- "Center of Multiple Sclerosis" held by Paola Cavalla, Ospedale Molinette-Dipartimento di Neuroscienze, Torino;
- "Role of Pax2 in Apoptosis Resistance and Proinvasive Phenotype of Kaposi's Sarcoma Cells" held by Stefano Buttiglieri, Dipartimento di Scienze Biomediche e Oncologia, Università di Torino, Torino;
- "Multiple sclerosis, parasites and the hygiene hypothesis" held by Stefano Sotgiu, Istituto di Clinica Neurologica Universita' di Sassari;
- "Revealing disease-relevant molecular mechanisms: The example of myosin VI" held by Sarah Vreugde.
- "Phenotypic and functional study of human neural cells" held by Francesca, Istituto Vita e Salute San Raffaele, Milano;
- "Disease heterogeneity in EAE" held by Robert Weissert, MerckSerono Research Center of Geneva;
- "Animal models of stroke" held by Johan Van Beck, MerckSerono Research Center of Geneva.

CONGRESSI FREQUENTATI (elenco completo: denominazione congresso, sede, data)

1st year PhD

- "Le Biotecnlogie per progettazione, sviluppo e produzione dei farmaci", organized by Charles River Lab., Milan 20-21/05/2005;
- "20th Biennial Meeting of the International Society For Neurochemistry", organized by European Society for Neurochemistry, Innsbruck 21-26/08/2005;

2nd year PhD

- *"Neurodegenerative Diseases: Molecular Mechanisms in a Functional Genomics Framework",* organized by the Max Delbruck Center for Molecular Medicine, held in Berlin, 6-9 September 2006.

3rd year PhD

- Baltic Summer School 2007 – "Inflammation: A Key to Common Complex Diseases" Organized by the Faculty Members of the Baltic Summer School, coordinator Prof. Rikard Holmdahl and course-leader Dr. Bo Nilson, Lund 2-13/09/07.

COMUNICAZIONI A CONGRESSI (elenco completo: autori, titolo, denominazione congresso, sede, data)

A) poster1: C. Patrignani[1,2], P. Tavano[1], A. Graziani[2] , R. Hooft [3], C. Rommel[3] and M.C. Magnone[1]
[1] LCG-RBM/ Serono Research, Turin, Italy; [2] Università del Piemonte Orientale, Novara, Italy Serono Pharmaceutical Research Institute, Geneva, Switzerland: **"PTPH1 in central nervous system: possible involvement in neurological functions"** presented at the conference *"Neurodegenerative Diseases: Molecular Mechanisms in a Functional Genomics Framework",* organized by the Max Delbruck Center for Molecular Medicine, Berlin, 6-9 September 2006.

A) poster2: C. Patrignani[1,2],S. Carboni[1], V. Muzio[1], B. Greco[1], P. Zaratin[1]
[1] :RBM/Merck Serono International S.A. (an affiliate of Merck KGaA, Darmstadt , Germany,) Turin, Italy; [2] :Università del Piemonte Orientale, Novara, Italy: **"TACE expression in late stage mouse chronic experimental autoimmune encephalomyelitis"** presented at the Baltic Summer School 2007 – "Inflammation: A Key to Common Complex Diseases" Organized by the Faculty Members of the Baltic Summer School, Lund 2-13/09/07.

ARTICOLI SCIENTIFICI PUBBLICATI NEL CORSO DEL DOTTORATO

1) submitted to JBC: **Control of Growth Hormone Receptor Signaling by Protein Tyrosine Phosphatase H1 (PTP-H1/PTPN3)** by Iwona Pilecka[1,3], Claudia Patrignani[2], Rosanna Pescini[1], Marie-Laure Curchod[1], Dominique Perrin[1], NN[5] , Ann Clark[6], Maria Chiara Magnone[1,4], Paola Zaratin[2], Christian Rommel[1] and Rob Hooft van Huijsduijnen[1,7]

[1] Merck Serono International S.A., 9, chemin des Mines, 1211 Geneva, Switzerland
[2] Merck Serono Istituto di Ricerche Biomediche "Antoine Marxer" (RBM); Via Ribes, 1 Colleretto Giacosa(TO)10010, Italy
[3] Present address: International Institute of Molecular and Cell Biology, Ks. Trojdena 4, 02-109 Warsaw, Poland
[4] Present address: Novartis Pharma AG Lichtstrasse 35 Basel CH-4002 Switzerland
[5] Regeneron Pharmaceuticals, Inc. 777 Old Saw Mill River Road, Tarrytown, NY 10591-6707, USA
[6] Serono, Inc. One Technology Place. Rockland, Massachusetts 02370, USA