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Prevention of clinical and histological signs of MOG-induced experimental allergic encephalomyelitis by prolonged treatment with recombinant human EGF

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ABSTRACT

Epidermal growth factor (EGF) represents the prototype of the group I EGF family. The pleiotropic effects of the EGF have attracted attention to the possibility that it could be implicated in autoimmune diseases, such as Multiple Sclerosis (MS). We show here that treatment with EGF, as a late prophylactic regime, improved the clinical and histological features of EAE, a preclinical model of MS. In silico analysis further corroborated these findings by demonstrating that EGF receptors are less expressed in CNS from patients with MS as compared to controls. Taken together these data provide clear-cut in vivo proof of concept for a beneficial role of exogenously administered EGF in MS, that may, therefore, represent a novel therapeutic approach.

1. Introduction

Epidermal growth factor (EGF) is a single-chain polypeptide of 53 amino acids that is derived from the cleavage of a large precursor, the prepro-EGF. EGF was discovered by Dr. Stanley Cohen more than half a century ago, and it represents the prototypical member of a family of peptide growth factors that activate the EGF receptors. EGF represents the prototype of the group I EGF family, that also includes transforming growth factor-α (TGF-α), heparin-binding EGF (HB-EGF), amphiregulin, betacellulin, epiregulin and epigen (Zeng and Harris, 2014).

EGF binds to a specific receptor, the EGFR, also known as ErbB1/HER1, that belongs to the EGFR family, that also includes ErbB2/HER2/Neu, ErbB3/HER3, and ErbB4/HER4 (Wee and Wang, 2017). EGFR is a tyrosine kinase-associated receptor, commonly upregulated in different types of cancers, including non-small-cell lung cancer, metastatic colorectal cancer, glioblastoma, head and neck cancer, pancreatic cancer, and breast cancer. Due to its capacity to induce cell proliferation, while inhibiting apoptosis, the EGF/EGFR signaling system has been classically identified as a pro-oncogenic molecule and this has led to the development of specific inhibitors of either EGF, such as mAb, or its receptors, that are currently used for the treatment of certain forms of cancers, such as breast cancer and colorectal cancer.

However, the pleiotropic effects of the EGF have attracted attention on the possibility that an altered production of this peptide and/or an abnormal expression of its receptor could also be implicated in other non-neoplastic conditions, including immunoinflammatory and autoimmune diseases. In particular, much attention has been paid to the role of EGF in the development and progression of multiple sclerosis (MS) that is a prototypical neuroinflammatory disease, characterized by demyelination and often followed by neurodegeneration. This interest stems from the well-known myelinotrophic effect of EGF in mammalian central nervous system (CNS) (Scalabrino et al., 2014). It has been demonstrated that EGF stimulates the oligodendrocytes (ODC) process formation (Chandran et al., 1998; Pfeiffer et al., 1993), especially after a CNS injury (Knapp and Adams, 2004), and that intranasal administration of EGF immediately after neonatal mouse brain injury, enhances new ODC proliferation from progenitor cells (Scafidi et al., 2014). In addition, EGF treatment increases the ODC number derived from subventricular zone type B-cells after lysolecithin-induced demyelination of mouse corpus callosum (Gonzalez-Perez et al., 2009). A key role was also found for endogenous EGF in the processes of CNS repair following Theiler's virus-induced demyelination in rodent models. While FVB/NJ

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(FVB) mice repair damaged myelin spontaneously and completely, B10.D1-H2(q)/SgJ (B10.Q) mice are deficient in this repair process. Two QTL were detected that differentially regulate CNS repair following chronic demyelination in these strains, one on chromosome 3 with and a second on chromosome 9. The mouse genes for EGF and Tyk2 are encoded within the QTL on chromosomes 3 and 9, respectively. Sequence polymorphisms between the FVB and B10.Q strains at both the EGF and Tyk2 loci define functional variations consistent with roles for these genes in regulating myelin repair, with a sevenfold increase in EGF expression in FVB compared to B10.Q mice. Simultaneously, an attenuation of the Tyk2, a Janus kinase that plays a central role in controlling the Th1 immune response, was also observed in FVB mice (Bieber et al., 2010). Finally, EGF-related mRNA synthesis is significantly increased in the white matter of the corpus callosum of mice during remyelination, after cuprizone-induced demyelination (Gudi et al., 2011). These last results emphasize the notion that EGF may be required in the repair phase in two types of experimental models (i.e. the viral-induced and the toxin-induced models) of human demyelinating diseases, resembling MS.

In agreement with these experimental data, we have previously demonstrated that patients with relapsing-remitting (RR) or secondary progressive (SP) MS have significantly lower EGF level in their cerebrospinal fluid (but not in their serum than controls (Scalabrino et al., 2010), and that EGF level is markedly decreased in spinal cord (SC) samples, autoptically taken from MS patients (Scalabrino et al., 2015). More recent data have however, demonstrated that blood levels of EGF are significantly reduced in the patients with secondary progressive forms of MS as compared with those with relapsing remitting forms and that, along with MIP-1β/CCL4, it represents a protective factor for developing a progressive clinical form of MS (Tejera-Alhambra et al., 2015). Differences in sensitivity of the assays may probably account for the different findings from our group and those from of Tejera-Alhambra et al. as regard the detectability of circulating levels of EGF in the blood of MS patients.

We have also demonstrated that EGF synthesis is regulated by cobalamin in rat CNS, and that EGF is a local mediator of the well-known myelinotrophic effect of cobalamin (Scalabrino et al., 1999). In fact, when otherwise normal rats received repeated intra-cerebroventricular injection of anti-EGF antibodies, but not irrelevant immunoglobulins, they developed selective morphological damage of SC myelin (Scalabrino et al., 2000). Viceversa, repeated intra-cerebroventricular administrations of EGF to cobalamin-deficient rats increase the SC levels of normal cellular prions (PrPCs) (Scalabrino et al., 2012) that are abundantly expressed in mammalian CNS, play a key role in CNS myelin maintenance (Linden et al., 2008), and positively influence ODC proliferation and differentiation (Bribián et al., 2012). Hence, EGF seems to play a dual role in CNS myelin maintenance: as effector of cobalamin CNS myelinotrophism and as stimulator per se of CNS PrPC synthesis. It is therefore tempting to speculate that insufficient EGF level in CNS of MS patients could be causally associated with the remyelination failure, often observed in MS patients.

However, in spite of these multiple preclinical and clinical data that appear to support, in a convergent manner, the protective role of EGF during the progression of MS, other preclinical studies have questioned this concept. In fact, exogenously administered EGF was only capable of ameliorating the course of EAE when administered in a combined treatment with growth hormone releasing peptide-6 (Adelmann et al., 1995) and, even more strikingly, the course of MOG-induced EAE was ameliorated by the treatment with specific anti-EGF Abs (Amir-Levy et al., 2014).

To gain further insights into the role of EGF in the development of demyelinating lesions that occur in rodent EAE and human MS, and to evaluate the feasibility of treatment with exogenous EGF to counteract demyelinating lesions and eventually promote remyelination, we decided to undertake this study where we have evaluated a dose-finding analysis of recombinant human EGF in a well- known model of mouse EAE, induced by immunization with MOG. MOG-induced EAE is characterized by progressive course of the disease, that is well-known to be histologically associated with inflammatory infiltrates of the spinal cord and brain, along with marked areas of demyelination (Eugster et al., 1999; Mangano et al., 2014).

We demonstrate here that prolonged treatment with the lowest dose of EGF given i.p., as a late prophylactic regime, from 7 days after immunization to day 50, afforded clinical and histological protection against signs of EAE, that were superimposable to those observed with the positive control drug, dexamethasone (Dex). The effects were associated with an almost complete absence of signs of demyelination. In silico analysis further corroborated these findings by demonstrating that EGF receptors are significantly less expressed in spinal cords from patients with MS as compared to controls.

2. Materials and methods

2.1. Microarray analysis

2.1.1. Analysis of CNS-infiltrating CD4+ T cells from a preclinical model of MS

The GSE57098 microarray dataset was selected to determine the transcriptional levels of EGF and EGFR in encephalitogenic T cells from a preclinical model of MS. Naive CD4+ T cells were isolated from the spleen of B6.2d2 transgenic mice with MOG-specific T cell receptors. Activated encephalitogenic CD4+ T cells were isolated from brain and spinal cords of mice with MOG35–55-induced EAE at the peak of disease. Details on the experimental design can be retrieved from the relative publication (Hoppmann et al., 2015).

2.1.2. Analysis of circulating blood cells from MS patients

Gene Expression data from CD4+ T Cells from MS patients and healthy subjects were obtained from the GSE78244 dataset. Data on EGF and EGFR expression were determined from unstimulated cells and following 24 h incubation with anti-CD3/CD28 antibodies. Data included 14 RR (Relapse Remitting) - MS patients and 14 control subjects. All the patients were women who had not received immunomodulatory or immunosuppressive treatment for at least two months prior to blood collection, with the exception for one patient (Hellberg et al., 2016).

2.1.3. Analysis of oligodendrocyte remyelination processes in spinal cords of EAE mice and brain lesions of MS patients

For the analysis of expression of EGF and EGFR in the CNS during inflammatory conditions, the microarray datasets, GSE60847, GSE48872 and GSE38010 were selected. GSE60847 consisted of data from homogenized lumbar spinal cord tissue isolated from EAE-affected mice at the flare of the disease (16 days post-immunization) and sham control mice (Schmitz et al., 2014). The GSE48872 dataset included gene expression profiles from adult Oligodendrocytes Precursor Cells (aOPCs) isolated from the brain of post-natal (days 1–5) and 2-month-old mice, while adult OPCs in demyelinating conditions (activated aOPCs) were isolated from the brain of mice treated with cuprizone, that induces demyelinating lesions (Moyon et al., 2015). The microarray dataset GSE32645 was interrogated to determine the expression levels of EGF and EGFR in active cortical MS lesions from Secondary Progressive patients, as compared to normal cortex of age matched controls, as well as from patients with other neurological diseases (chronic tuberculous meningitis, neurodegenerative lesions of Alzheimer's disease) (Fischer et al., 2013). The GSE38010 dataset included data from chronic active plaques and chronic plaques from MS brains and white matter from healthy controls (two replicates each)(Han et al., 2012).

2.2. Animal study

2.2.1. Acclimatization, housing and feeding

Six to 7 weeks old female C57BL/6 mice weighing between 16 and 18 g and purchased from Envigo, (San Pietro al Natisone, Udine, Italy) were kept at the animal facility of the Department of Biomedical and Biotechnological Sciences, University of Catania, under standard laboratory conditions (non-specific pathogen free) with free access to food and water and were allowed to adapt for at least one week to their environment before commencing the study.

Animals were housed within a limited-access rodent facility under controlled microbial conditions, which excluded murine pathogens, and were kept in groups of maximum 5 mice, in polycarbonate cages (Tecniplast, Varese, Italy), according to the Italian legislation (each mouse is to have a surface of 180 cm2 with minimum 12 cm height). This regimen does not exclude various species of Helicobacter. Cages are sterilized and filled with wood shavings as bedding material. Automatically controlled environmental conditions are set to maintain temperature at 20–24 °C with a relative humidity (RH) of 30–70%, 10–30 air changes /hr and a natural dark:light cycle.

Protection of animals used in the experiment is in accordance with Directive 2010/63/UE, enforced by the Italian D. Lgs 26/2014. Physical facilities and equipment for accommodation and care of animals are in accordance with the provisions of EEC Council Directive 86/609.

2.2.2. Induction of MOG-induced EAE

Mice were immunized by s.c. injection of an emulsion composed of 200 µg $MOG_{35–55}$ peptide (Genemed Synthesis, San Francisco, CA) in Complete Freund's Adjuvant (CFA, Difco, Detroit, U.S.A.) containing 0.5 mg (per injection) of *Mycobacterium tuberculosis*. Each mouse received subcutaneous injections of 200 ul emulsion divided among two sites, left and right flank, (50% /50%) draining into the axillary lymph nodes. Pertussis toxin (Calbiochem, Nottingham, UK) was used as a co-adjuvant and was administered i.p. at the dose of 200 ng/mouse on day 0 and 200 ng/mouse on day 2 post immunization (Donia et al., 2010).

2.2.3. Study design

Six groups of 10–12 animals each were treated under a late prophylactic regime from 7 days after immunization to day 50, as described in the Table 1. Five additional mice were considered as SHAM. These mice, on the day of EAE induction, were anesthetized as the other mice but were injected only with Freund adjuvant without *Mycobacterium tuberculosis* and without MOG. These untreated mice were considered as healthy mice. Dexamethasone (Soldesan, Dex) was purchased from a local pharmacy and was used as positive control drug as previously described (Donia et al., 2010).

Recombinant human EGF with EC50: 0.08–0.8 ng/ml (as assessed on the growth on mouse fibroblast cell lines BALB/3 T3) (Sigma Aldrich, Milan, Italy) was dissolved in water for injection and administered by i.p. injection as described in Table 1. Previous experiments evidenced that EGF is active on rat CNS even when it is administered by i.p. route, thus entailing its crossing the BB barrier (Scalabrino et al., 1995).

Table 1
Study design.

Group	Number	Treatment	Dose	Route	Administration frequency
1	12	EGF	2.5 µg/mouse	i.p.	Every other day
2	10	EGF	10 µg/mouse	i.p.	Every other day
3	11	EGF	2.5 µg/mouse	i.p.	Daily, 5 times a week
4	11	EGF	10 µg/mouse	i.p.	Daily, 5 times a week
5	12	Dex	0.6 mg/kg	i.p.	Daily, 5 times a week
6	12	Vehicle	–	i.p.	Daily, 5 times a week
7	5	SHAM			

2.2.4. Endpoints

2.2.4.1. Clinical assessment. Starting from day 7 post-immunization the animals were examined individually for the presence of paralysis according to the following score: 0 = no sign of disease; 0.5 = partial tail paralysis; 1 = tail paralysis; 1.5 = tail paralysis + partial unilateral hind limb paralysis; 2 = tail paralysis + hindlimb weakness or partial hindlimb paralysis; 2.5 = tail paralysis + partial hindlimb paralysis (lowered pelvis); 3 = tail paralysis + complete hindlimb paralysis; 3.5 = tail paralysis + complete hindlimb paralysis + incontinence; 4 = tail paralysis + hindlimb paralysis + weakness or partial paralysis of forelimbs; 5 = moribund or dead (Donia et al., 2010). Clinical signs were monitored daily and body weight was monitored three times a week. Mice reaching a score of 4 and/or losing > 20% of their body weight were monitored daily by the veterinary, subcutaneously treated with saline and, if necessary, ethically euthanized. The clinical readouts for this study include the clinical score, cumulative score, incidence, duration and onset of the disease and variation of the body weight throughout the study period.

2.2.4.2. Histological analysis. Mice were sacrificed and brain and the spinal cords were resected and stored in 4% formaldehyde (Sigma, Milano, Italy) at 4 °C for 24 h. To assess inflammation, serial 5-µm cross sections were stained with hematoxylin and eosin (H&E) (Mangano et al., 2014). Semiquantitative histological evaluation was performed using the following scores based on the severity of inflammation: 0, no inflammation; 1, few cells; 2, moderate, perivascular cuffing 3, dense inflammatory cellular infiltrates, parenchymal necrosis (Mangano et al., 2014). To assess the severity and extent of demyelination. Myelin structure was analysed by Luxol fast blue staining (Bio-Optica) and scored using a semiquantitative grading system: 0 = normal; 1 = demyelinated fibres < 25%; 2 = demyelinated fibres 25–50%; 3 = demyelinated fibres 50–75%; 4 = demyelinated fibres > 75% (Furlan et al., 1998). Cross sections of spinal cord were analysed using a light microscope (LEICA DM 2000 combined with LEICA ICC50 HD camera). Images (n = 20 images from each group) were acquired for the evaluation of the percentage of demyelination using the Leica Application Suite V4.2.0 software. Histological analyses were only performed in sham-treated mice, and the groups of mice treated with vehicle, EGF every other day (E.O.D.) at 2.5 µg/mouse and Dex.

2.3. Statistical analysis

The results are presented as mean ± SD. Statistical differences in the incidence of disease among groups were assessed using the Fisher Exact test for each group comparison. Data have been subjected to a normality test for the assessment of their Gaussian distribution. The Shapiro Wilk, Kolmogorov Smirnov and D' Agostino Pearson omnibus tests were used. Based on the distribution of the data, either the *t*-test test (for parametric data) or the Kruskal-Wallis test, followed by Dunn's multiple comparison test (for non-parametric data) were performed. A p value < .05 has been considered for statistical significance. All analyses have been performed using the Prism GraphPad v5 software or Microsoft Excel.

3. Results

3.1. Microarray analysis

3.1.1. Evaluation of transcriptomic levels of EFG and EGFR in T helper cells

The expression levels of EGF and EGFR were first evaluated on CD4+ T cells isolated from the CNS of mice with MOG-induced EAE. As compared to naive CD4+ T cells, the activated encephalitogenic T cells expressed significantly lower levels of EGF (p < .05) (Fig. 1A). In contrast, no significant differences were observed in the levels of EFGR (Fig. 1A). In CD4 T cells isolated from PBMCs of MS patients and healthy controls, no differences in the transcriptomic levels of EGF and

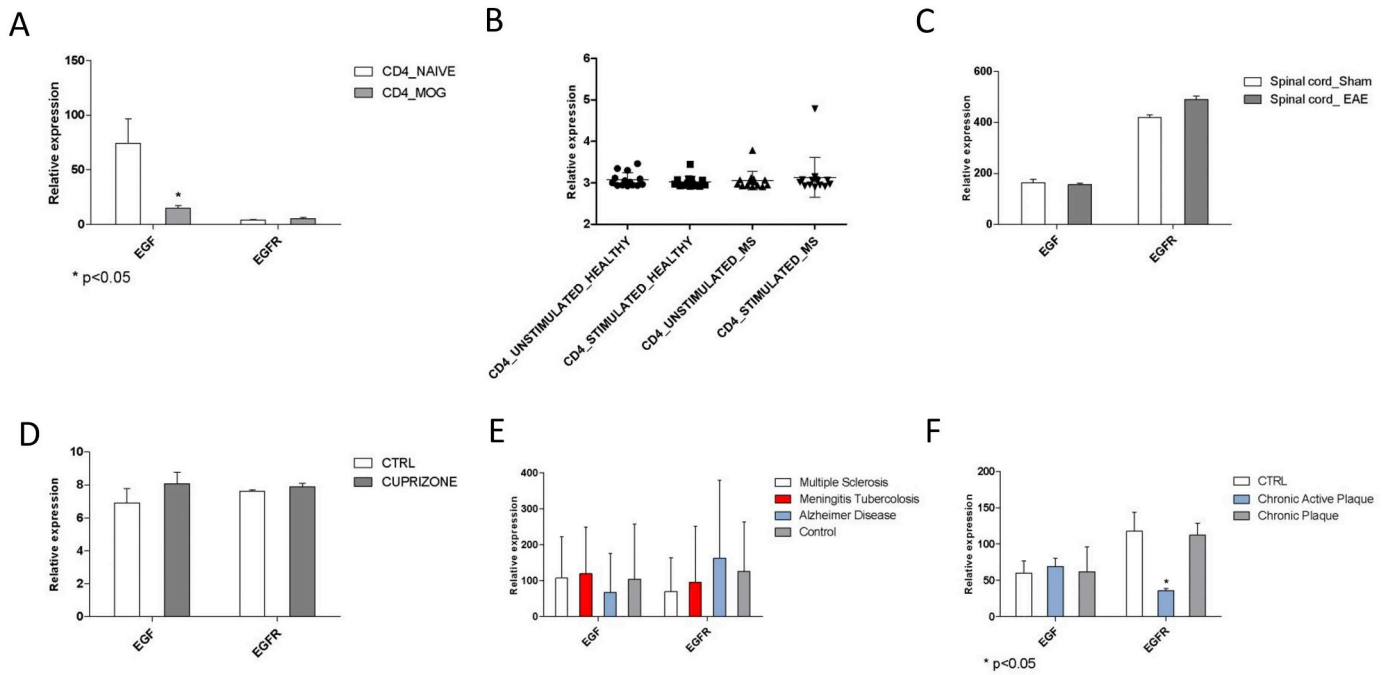

Fig. 1. EGF and EGFR expression in preclinical and clinical Multiple Sclerosis. A. The GSE57098 microarray dataset was selected to determine the transcriptional levels of EGF and EGFR in encephalitogenic T cells from a preclinical model of MS. B. Gene Expression data from CD4 + T Cells from MS patients and healthy subjects were obtained from the GSE78244 dataset. Data on EGF and EGFR expression were determined from unstimulated cells and following 24 h incubation with anti-CD3/CD28 antibodies. Data included 14 RR (Relapse Remitting) - MS patients and 14 control subjects. C. GSE60847 consisted of data from homogenized lumbar spinal cord tissue isolated from EAE-affected mice at the flare of the disease (16 days post-immunization) and sham control mice. D. The GSE48872 dataset included gene expression profiles from adult Oligodendrocytes Precursor Cells (aOPCs) isolated from the brain of post-natal (days 1–5) and 2-month-old mice, while adult OPCs in demyelinating conditions (activated aOPCs) were isolated from the brain of mice treated with cuprizone. E. The microarray dataset GSE32645 was interrogated to determine the expression levels of EGF and EGFR in active cortical MS lesions from Secondary Progressive patients, as compared to normal cortex of age matched controls, as well as from patients with other neurological diseases. F. The GSE38010 dataset included data from chronic active plaques and chronic plaques from MS brains and white matter from healthy controls.

its receptor were observed between the two groups of people, neither under basal nor under anti-CD3/CD28 stimulated conditions (Fig. 1B).

3.1.2. Evaluation of transcriptomic levels of EGF and its receptors in MS-associated CNS lesions

We next wanted to determine the expression levels of EGF and its receptor in the CNS from patients and animal models of MS. To this aim, we analysed the microarray datasets, GSE60847, GSE48872 and GSE38010. In the spinal cord of EAE-affected mice, no significant modulation in either EGF or EGFR expression was found, in comparison to healthy mice (Fig. 1C). Along the same lines, no differences were found for EGF and EGFR in adult oligodendrocyte precursors from cuprizone-challenged animals, as compared to control adult oligodendrocyte precursors (Fig. 1D). In cortical lesions of MS patients, a trend of reduced expression was observed for EGFR as compared to healthy control cortex (Fig. 1E) and, accordingly, significantly lower levels ($p < .05$) were found in chronic active plaques of MS patients, as obtained from the GSE38010 dataset (Fig. 1F) On the other hand, no modulation was observed for EGF neither in cortex of MS patients, nor in the chronic or chronic active plaques from MS brains (Fig. 1E, F).

3.2. Animal study

The EAE model worked well, although the clinical course was more aggressive compared to our previous studies (Donia et al., 2010; Mangano et al., 2014) and this must be taken into consideration when examining the effects of EGF on the course of the disease.

The treatment with EGF administered E.O.D. from day 7 post immunization at the dose of 10 μg /mouse and mostly at the lower dose of 2.5 μg /mouse induced an amelioration of the clinical course of the disease. The mice treated with Dex also exhibited a strong amelioration of clinical readouts. Sham mice, injected only with Freund adjuvant without MOG, as expected, didn't develop disease.

3.2.1. Toxicity

The i.p. treatment with test compounds appeared to be well tolerated throughout the entire study, as judged by the clinical status of the mice and by body weight (BW) variation (Fig. 2A).

3.2.2. Disease incidence

Disease incidence was determined by summing up the number of animals per group that ever showed a score ≥ 0.5 throughout the whole experiment.

The determined incidences showed that the MOG-EAE model worked well. No significant effects were observed in the incidence of the disease in groups of mice treated with both doses and both regimens of EGF and with Dex, as compared to vehicle-treated mice (not shown).

3.2.3. Disease duration

The duration of the disease was calculated for each animal by counting the number of days on which the animal showed a clinical score higher than 0. Finally, the mean for each treatment group was calculated.

The treatment with EGF at the dose of 2.5 μg/mouse both administered daily and E.O.D., significantly reduced the duration of the disease. The treatment with Dex also showed a statistically significant reduction in the disease duration as compared to vehicle-treated control mice (Fig. 2B).

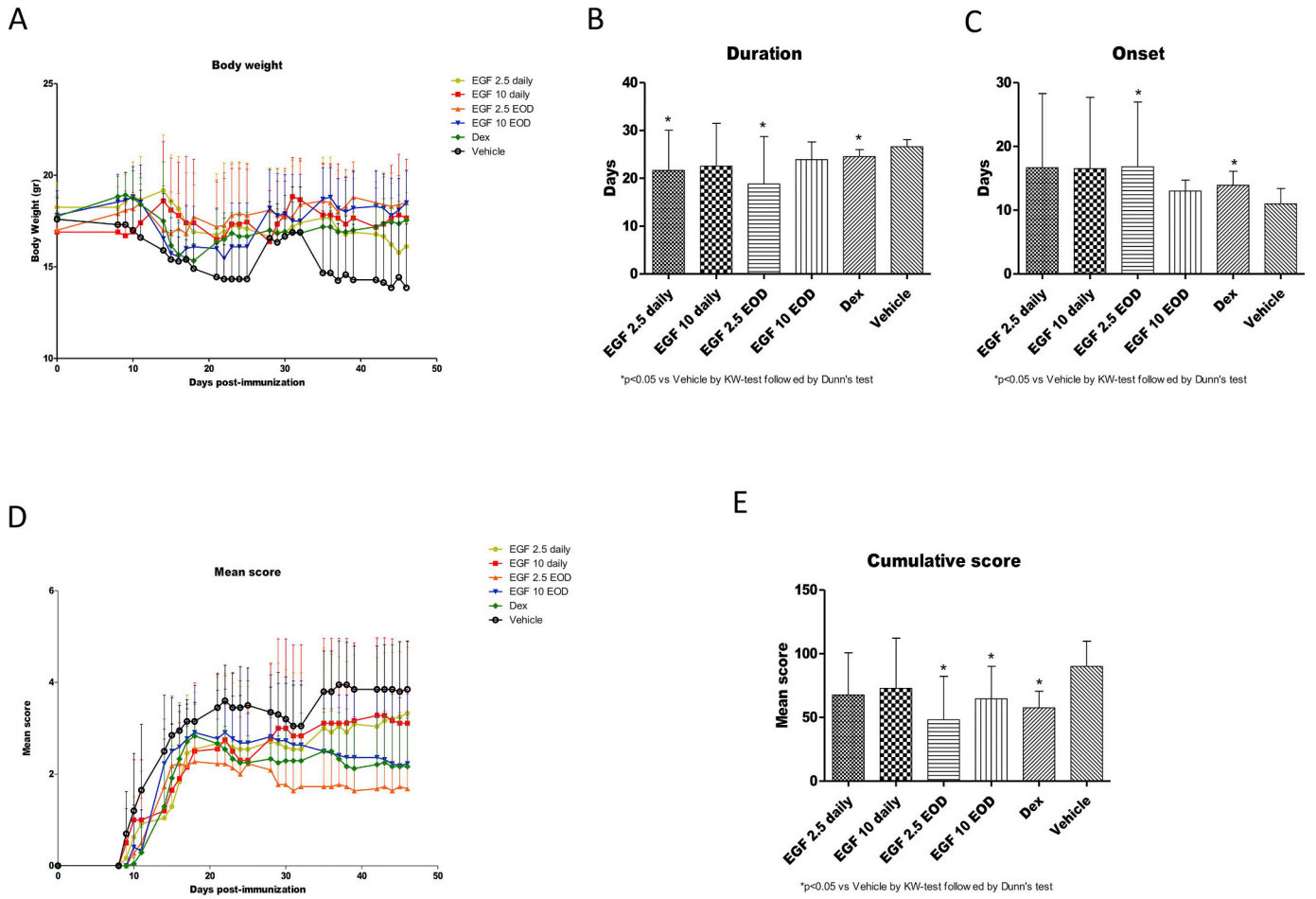

Fig. 2. Effects of late prophylactic treatment with EGF in MOG-induced EAE. A. Body weight variation in MOG-induced EAE upon EGF administration. Statistical differences among groups were assessed using one-way ANOVA B. Effects of late prophylactic treatment with EGF on duration of disease in MOG-induced EAE. Statistical differences among groups were assessed using Kruskal-Wallis followed by Dunn's post-hoc test. C. Effects of EGF treatment on the onset of the disease in MOG-induced EAE. Statistical differences among groups were assessed using Kruskal-Wallis followed by Dunn's post-hoc test. D. Effects of late prophylactic treatment with EGF on clinical score in MOG-induced EAE. Mean ± S.D. is shown. Statistical differences among groups were assessed using Kruskal-Wallis followed by Dunn's post-hoc test. E. Effects of late prophylactic treatment with EGF on the cumulative score in MOG-induced EAE. Statistical differences among groups were assessed using Kruskal-Wallis followed by Dunn's post-hoc test.

3.2.4. Disease onset

Disease onset was considered as the first day on which an animal showed a clinical score ≥ 0.5. For animals that never showed symptoms, onset was considered to be the last day of observation.

The onset of the disease was significantly delayed in the group of mice treated with EGF 2.5 g/mouse E.O.D. and with the positive control drug Dex, as compared to vehicle-treated mice (Fig. 2C).

3.2.5. Clinical score

Classical signs of EAE started to appear in the vehicle-treated control animals 9 days after the immunization (see Fig. 2D). The daily treatment with EGF at the dose of 2.5 μg/mouse, significantly reduced the clinical score between day 14 to 16 and, at the dose of 10 μg/mouse, from days 14 to 16 and at days 24 and 25 post-immunization. Stronger effects were observed when EGF administered E.O.D., in particular the dose of 2.5 μg/mouse, significantly reduced the clinical score from day 9 to 11 and from day 21 until the end of the study. The higher dose of 10 μg/mouse significantly reduced the clinical score from day 9 to 11 and from 34 until the end of the study (Fig. 2D and Table 2). Dex-treated animals exhibited a significant lower clinical score through the entire treatment period (Fig. 2D and Table 2) compared to vehicle-treated mice.

3.2.6. Cumulative score

The cumulative clinical score was calculated for each mouse by adding the daily score from the day of onset (score of disease ≥0.5) until the end of treatment. The late prophylactic treatment with EGF at the dose of 2.5 and 10 μg /mouse administered E.O.D. as well as with Dex showed a reduction in the cumulative score compared to vehicle, which was statistically significant (Fig. 2E). No significant effects were observed in the animal treated daily with EGF, at both doses.

3.2.7. Histology

The inflammation and the demyelination score for each mouse is presented as the rounded mean of the score of three sections from the respective mouse. No cell infiltrate was found in the brains of mice from all experimental groups. The H&E sections of the spinal cord showed that the mice treated with the vehicle underwent infiltration of lymphocytes into the white matter of SC. In contrast, the histological scores of damage were significantly reduced in the EGF 2.5 μg/mouse group of animals in comparison to vehicle-treated mice. The treatment with EGF induced a significant reduction in the SC infiltration of leukocytes similar to the positive control drug Dex. EGF-treated mice, in comparison to DEXA-treated group, also exhibit a greater reduction of the edema of the white matter score (Fig. 3). Severe demyelination was detected in the group treated with the vehicle (Fig. 4). The administration of EGF

Table 2
Kruskal-Wallis analysis of daily score of mice treated under late prophylactic regimen.

	Day 9	day10	day11	day14	day15	day16	day17	day18	day21	day22	day23	day24	day25	day28
SHAM-VEHICLE	,028	,014	,005	,001	,000	,000	,000	,000	,000	,000	,000	,000	,000	,000
DEX-VEHICLE	,005	,005	,009	,011	,048	,205	,110	,213	,061	,011	,006	,003	,002	,022
EGF 2.5 DAILY-VEHICLE	,075	,225	,191	,008	,006	,013	,281	,348	,248	,151	,165	,125	,136	,367
EGF 10 DAILY-VEHICLE	,844	,405	,149	,024	,034	,044	,055	,400	,227	,160	,122	,041	,036	,407
EGF 2.5 EOD-VEHICLE	,006	,026	,023	,145	,316	,213	,056	,078	,027	,013	,014	,007	,012	,021
EGF 10 EOD-VEHICLE	,006	,034	,007	,422	,273	,231	,420	,757	,228	,159	,181	,123	,062	,259

	day29	day30	day31	Day32	day35	day36	day37	day38	day39	day42	day43	day44	day45	day46
SHAM-VEHICLE	,000	,000	,001	,001	,000	,000	,000	,000	,000	,000	,000	,000	,000	,000
DEX-VEHICLE	,031	,062	,129	,130	,016	,016	,007	,003	,004	,006	,007	,006	,006	,005
EGF 2.5 DAILY-VEHICLE	,381	,405	,556	,553	,201	,147	,148	,098	,137	,175	,264	,320	,403	,433
EGF 10 DAILY-VEHICLE	,569	,719	,591	,582	,364	,318	,252	,237	,270	,381	,396	,307	,288	,265
EGF 2.5 EOD-VEHICLE	,012	,022	,023	,026	,002	,001	,002	,001	,001	,001	,002	,001	,002	,002
EGF 10 EOD-VEHICLE	,304	,420	,573	,573	,039	,025	,019	,016	,024	,024	,026	,016	,014	,016

and Dex significantly reduced the degree of demyelination compared to the group of vehicle-treated mice.

4. Discussion

The present findings demonstrate for the first time that in vivo treatment with EGF is able to ameliorate the clinical and histological signs of MOG-induced EAE, when given under late prophylactic treatment to C576/Bl6 mice, i.e. after 7 days of immunization with MOG. Although at the beginning of the treatment no clinical signs of EAE were observed, both the literature data from ourselves and others (Donia et al., 2010; Eugster et al., 1999; Mangano et al., 2014) and the initial observation of EAE occurrence in control mice already from day 9th post immunization indicated that immunoinflammatory encephalytogenic events were fully ongoing at the time the treatment with EGF was started. This indicates that EGF has reverted an actively ongoing autoreactive process that has led to aggressive development of EAE and associated to its prototypical histological signs that include inflammatory infiltration of spinal cords and demyelination. Accordingly, the mice treated with the lowest dose of EGF on alternate days showed significant amelioration of the clinical and histological signs of EAE, entailing reduced duration and onset of the disease clinical score and cumulative score, as well as almost absent signs of inflammatory infiltrates and areas of demyelination. The effects of this treatment regime were almost superimposable to those observed with the positive control drug, Dex.

While, relative to vehicle-treated controls, some beneficial effects on the clinical course of EAE could be observed also with the higher dose of EGF administered on alternate days, neither of the 2 doses of EGF administered daily ameliorated the development of the disease. The reason for the lack of dose-dependent effects of EGF in this model remains to be established but could be due to the facilitated production of neutralizing antibodies in mice exposed to larger doses of recombinant human EGF. However, lack of dose-dependent effects with bell shaped curve has often been reported for biological drugs, including monoclonal antibody and cytokines. For example, inverse dose-dependent effects have been observed when a recombinant dimeric tumor necrosis factor receptor (Fc) was administered to healthy volunteers who received endotoxin i.v., with high doses being less immunosuppressive than low doses on secondary cytokine levels, leukocyte margination, and neutrophil migration (Suffredini et al., 1995)

Our present data of the efficacy of EGF in halting the development of MOG-induced EAE is in contrast with previous findings from Del Barco et al. indicating that only the combined treatment with EGF + growth hormone releasing peptide-6, but not EGF alone, reduced the clinical score in monophasic EAE of Lewis rats immunized with spinal cord homogenate (del Barco et al., 2011). The discrepant results may be explained with the different animal model they used and with the different treatment regimens they employed, that consisted of daily i.p. administration with 200 μg/Kg b.d. with EGF from day 10 to day 20, after the first immunization. When translated into dosing as mg/kg, in our study the golden dose of EGF administered (2.5 μg/mouse E.O.D.) corresponds roughly to 1000 μg/kg. In addition, we have started EGF earlier during the course of the disease and have prolonged the treatment much longer than Del Barco et al. did. In addition, monophasic Lewis rat EAE and murine MOG-induced EAE differ in several aspects including the remitting form of the disease in the former versus a progressive and severe form in the latter and, more importantly, the fact that monophasic Lewis rat EAE does not exhibit demyelination, whereas MOG-induced EAE does. Nonetheless, demyelination was not studied either in the Lewis rat EAE or in the MOG-induced EAE in the study by Del Barco et al.(del Barco et al., 2011).

Apparently even more striking is the contrast of our data with another study that proved that an anti-EGF antibody ameliorated clinical and histological course of MOG-induced EAE in C57/Bl6 mice (Amir-Levy et al., 2014). The reason for these discrepant results remains to be

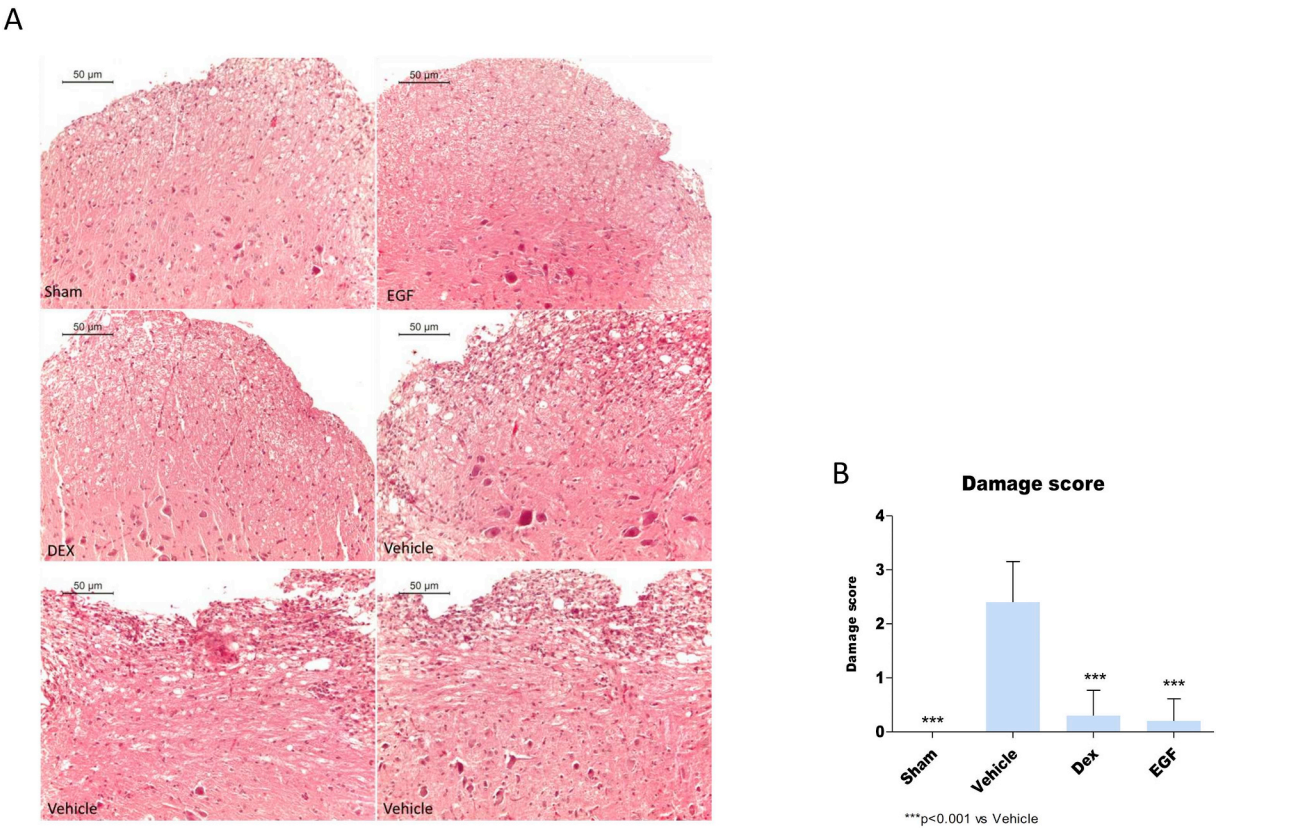

Fig. 3. Effects of late prophylactic treatment on the histological score in a mouse model of MOG-induced EAE. A. Representative images of H&E stained sections of SC. Treatment with EGF improved inflammatory cell infiltration caused by the EAE. The severity of the damage was assessed by hematoxylin/eosin staining. Sham mice did not show histological changes in spinal cord tissues. Significant damage was observed in EAE mice, as demonstrated by the presence of numerous inflammatory foci in the white matter of the spinal cord (indicated by the arrows). In particular, a complete reduction of inflammatory cell infiltration, such as T lymphocytes and monocytes, was observed in samples taken from EAE mice treated with EGF. Magnification: 10×; scale bar: 50 µm. B. Histological score is expressed as means ± SEM and calculated in 20 spinal cord sections per mouse.

established but it should be underlined that the pleiotropic nature of the cytokines and growth factors makes it difficult to compare the in vivo action of a neutralizing mAb with that of the exogenously administered cytokines. Cytokines and growth factors have pleiotropic effects and their systemic administration often at supra-physiological doses may activate pathways that may differ from those that one may expect to be exactly abrogated from negating the action of the endogenous molecule with a specific inhibitor such as mAb or the soluble receptors. Several years ago we demonstrated that autoimmune diabetes in diabetes-prone BB rats could be prevented both from anti-IFN-gamma antibody and exogenously administered recombinant IFN-gamma. However, the mechanism of protection were different as, upon treatment withdrawal, the protection induced from anti-IFN-gamma Ab was reversible whereas those induced from systemic treatment with IFN-gamma conferred long lasting protection (Nicoletti et al., 1997, 1998).

The period of treatment with either EGF or the anti-EGF antibody in the two studies may also be crucial as the effects produced by anti-cytokine Ab and exogenous cytokines on the in vivo modulation of autoimmune responses has been shown to be dependent also on the age of the mice, and hence the period of the disease. For example, treatment of newborn female NOD mice with TNF-alpha E.O.D. for 3 wk., accelerated the onset and the onset of type 1 diabetes (10 versus 15 wks of age in control mice) and augmented the incidence. The effects mediated by TNF-alpha appear to be highly age dependent, as treatment of animals either from birth or from 2 wks of age had a similar effect whereas, if treatment was initiated at 4 wks of age, TNF-alpha delayed disease onset (Jacob et al., 1990). We have also demonstrated that IL-12 only protect NOD mice from diabetes development if administered early during the course of the disease (Nicoletti et al., 1999). In particular, the complexity of the cytokine network in EAE is well exemplified by a study that shows the receptor-dependent dichotomic role of endogenous TNF-alpha in the course of EAE. Interestingly, it has been shown that TNFR1 cell signaling defines a critical pathway responsible for demyelination and the severity of clinical symptoms of MOG-induced EAE, while TNFR2 cell signaling was found to be protective (Eugster et al., 1999). It has also been shown that oligodendroglial TNFR2 mediates membrane TNF-alpha-dependent repair in EAE, by promoting oligodendrocyte differentiation and remyelination (Madsen et al., 2016). If this complex network of pathogenic vs protective interactions of TNF-alpha with TNFR1 vs TNFR2, respectively, may also be applied to human MS, it may provide an explanation of the deleterious effects of TNF-alpha inhibitors in MS ("TNF neutralization in MS: results of a randomized, placebo-controlled multicenter study. The Lenercept Multiple Sclerosis Study Group and The University of British Columbia MS/MRI Analysis Group.," 1999).

We have not determined in this study the mechanistic mode of action of EGF in halting EAE development. The literature data that we have previously discussed are strongly supportive for an action of EGF on the remyelination process and this is also consistent with the histological data observed. However, treatment with EGF may have also modulated EAE development by interfering with the cytokine secretory capacity of mice in response to MOG-immunization. MOG-induced EAE has been regarded as Th1 and Th17-dependent disease (Luchtman et al., 2014). Hence, if EGF has downregulated the production of IL-17, IFN-gamma or TNF-alpha, this might have resulted in beneficial effects on EAE development. That EGF may at some extent modulate immune response during EAE development is in agreement with the notion that the autoreactive T cells generated from immunization and that have

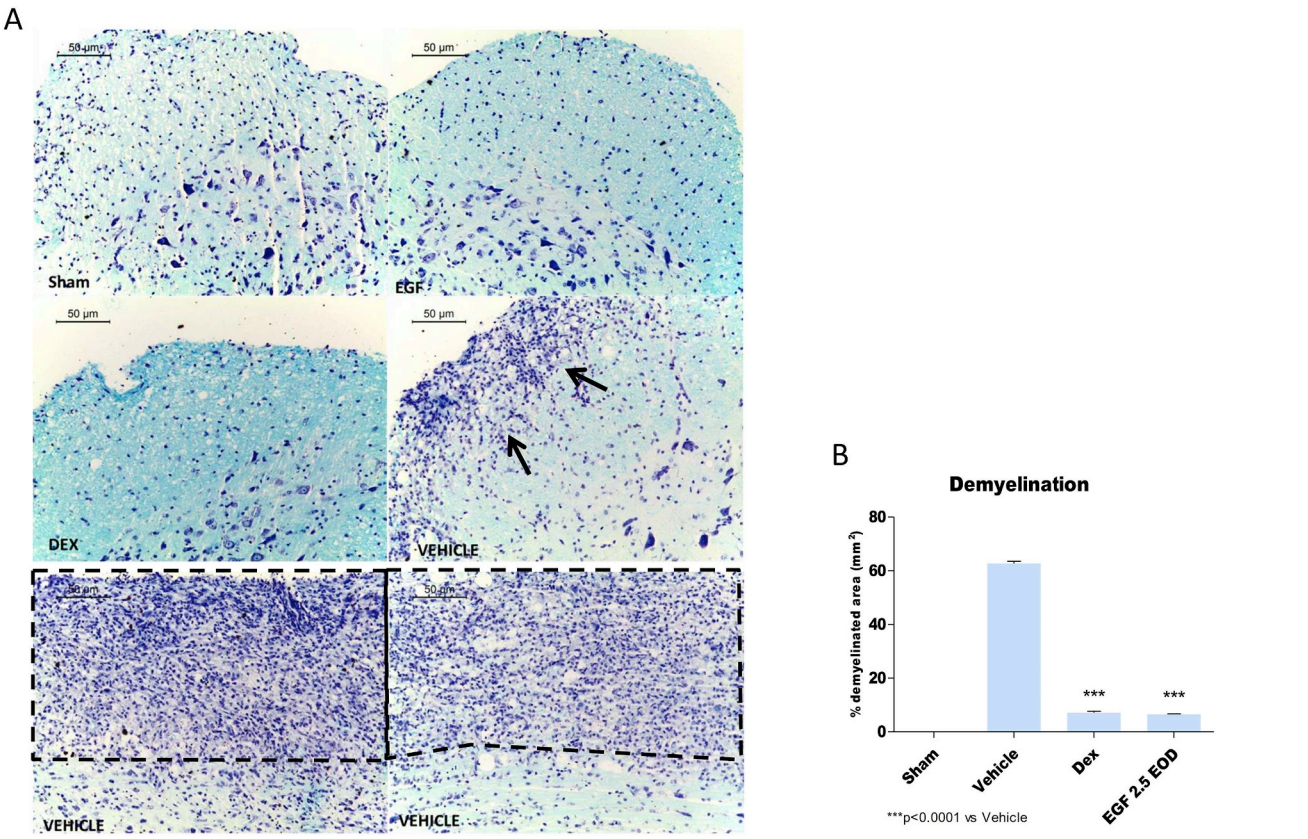

Fig. 4. Effects of EGF late prophylactic treatment on the demyelination score in MOG-induced EAE. A. Representative images of Luxol fast blue stained sections of SC. EGF attenuated the degradation of myelin caused by the EAE. The sections of the spinal cord collected at the end of the treatment on the day of sacrifice were analysed with LFB staining to detect demyelination. In sham and DEX control groups, they do not show demyelination plaques. The staining with LFB shows the formation of demyelination plaques occurred in the EAE group (as shown by the arrows). Myelin degradation was completely attenuated in EAE mice treated with EGF. Magnification: 10×; scale bar: 50 µm. B. Histopathological score is expressed as mean ± SEM and calculated in 20 spinal cord sections per mouse. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

provoked some initial signs of the disease in the mice protected from the "golden "dose of EGF would have continued the destruction of the regenerated myelin unless downregulated from a yet undiscovered action of EGF on the immune system or on antigenic differences in myelin epitopes exposed from the regenerated myelin upon EGF impulse. While studies are in progress to elucidate the immune-pharmacological mode of action of EGF in counteracting EAE, it is interesting to observe that another member of EGF family name HB-EGF has been shown to ameliorate enterocolitis in mice by polarizing macrophages toward ant anti-inflammatory type 2 phenotype (Wei and Besner, 2015). This cell type that produces anti-inflammatory cytokines such as IL-10 and IL-13 has been shown to play protective role in EAE as also shown by the ability of lenalinomide to suppress the development of this disease by induction of M2 macrophages (Weng et al., 2018).

That EGF may have also acted on proinflammatory action of macrophages is also consistent with our observation that in vitro culture with EGF significantly reduces IL-1 beta production from macrophage, like RAW264.7 cell line (data not shown).

The role of EGF in autoimmunity is not largely investigated. However, some studies seem to point out that EGF may rather play a pathogenic role in Th1 and Th17 mediated autoimmune diseases such as type II collagen induced arthritis, that serves as a model for human rheumatoid arthritis, where the disease was ameliorated by an inhibitor of EGF receptor (Chen et al., 2015).

It has also been demonstrated that topical administration of EGF suppresses immune responses and protects skin barrier in DNCB-induced atopic dermatitis in NC/Nga mice and this was associated with a skewing of the immune response from a Th2 to a Th1 phenotype. This phenotype induced by EGF should have a pathogenic role in MOG-induced EAE (Kim et al., 2018).

On the contrary, endogenous EGF seems to play an anti-inflammatory role in an immunoinflammatory disease, such as Sjogren syndrome (SS), as salivary levels of EGF are reduced in SS and correlate with intraoral manifestations of the disease (Azuma et al., 2014).

Taken together these data provide clear-cut in vivo proof of concept for a beneficial role of exogenously administered EGF in a well-known model of human MS, such as MOG-induced EAE. The combined finding of improved clinical development of the disease with absent demyelination suggests that treatment with EGF may represent a novel approach for treatment of some forms of MS, in particular with the aim to promote remyelination.

Declaration of competing interests

None to declare.

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