Erythropoietin Ameliorates Rat Experimental Autoimmune Neuritis by Inducing Transforming Growth Factor-Beta in Macrophages

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Abstract

Erythropoietin (EPO) is a pleiotropic cytokine originally identified for its role in erythropoiesis. In addition, in various preclinical models EPO exhibited protective activity against tissue injury. There is an urgent need for potent treatments of autoimmune driven disorders of the peripheral nervous system (PNS), such as the Guillain-Barré syndrome (GBS), a disabling autoimmune disease associated with relevant morbidity and mortality. To test the therapeutic potential of EPO in experimental autoimmune neuritis (EAN) - an animal model of human GBS – immunological and clinical effects were investigated in a preventive and a therapeutic paradigm. Treatment with EPO reduced clinical disease severity and if given therapeutically also shortened the recovery phase of EAN. Clinical findings were mirrored by decreased inflammation within the peripheral nerve, and myelin was well maintained in treated animals. In contrast, EPO increased the number of macrophages especially in later stages of the experimental disease phase. Furthermore, the anti-inflammatory cytokine transforming growth factor (TGF)-beta was upregulated in the treated cohorts. In vitro experiments revealed less proliferation of T cells in the presence of EPO and TGF-beta was moderately induced, while the secretion of other cytokines was almost not altered by EPO. Our data suggest that EPO revealed its beneficial properties by the induction of beneficial macrophages and the modulation of the immune system towards anti-inflammatory responses in the PNS. Further studies are warranted to elaborate the clinical usefulness of EPO for treating immune-mediated neuropathies in affected patients.

Introduction

Acute inflammatory autoimmune diseases of the peripheral nervous system (PNS) are disabling disorders that - despite advances in treatments of the last decade [1] – are still associated with relevant morbidity and mortality [2]. The most common prototypic acute inflammatory neuropathy is the Guillain–Barré syndrome (GBS) manifesting as a monophasic ascending flaccid tetraparesis with minor sensory deficits [3,4]. Understanding of the underlying pathomechanisms is still incomplete. There is consensus that GBS results from aberrant cellular and humoral immune responses directed to peripheral nerve antigens resulting in demyelination and/or axonal damage of the peripheral nerve.

Most of our immunopathogenic understanding of GBS was gathered in a well established animal model of this disease, experimental autoimmune neuritis (EAN). The model mimics various clinical and paraclinical features of GBS and is inducible by active immunization with peripheral myelin homogenates, appropriated antigen peptides or transfer of neuritogenic T cells [5]. It offers the possibility to study preclinical effects of novel therapies that may exhibit a clinical benefit in GBS.

Erythropoietin (EPO), known for its role in erythropoiesis, revealed remarkable tissue protective properties in different preclinical models [6]. In experimental autoimmune encephalomyelitis (EAE) treatment with EPO reduces the clinical score, reduces the demyelination and protects from axonal loss [7]. Animals with an ischemic stroke developed smaller infarction zones and reduced inflammation with EPO [8]. Some of these preclinical findings could even be translated into clinical phase II studies, in which peripherally administered EPO exhibited beneficial potential in stroke and patients with MS [9,10].

Thus, we explored the anti-inflammatory and neuroprotective properties of EPO in autoimmune disorders of the PNS. Therefore, EPO was applied in EAN in a preventive and a therapeutic paradigm and the effect on clinical, histological and immunological measures was assessed.

Results

Effect of EPO on the clinical course of experimental autoimmune neuritis (EAN)

Treatment of EAN with EPO was studied in two different paradigms (Fig. 1). In a preventive paradigm (blue line) EPO was...
Figure 1. Treatment with EPO ameliorates the clinical course of EAN. EAN was induced in female Lewis rats by subcutaneous immunization with bovine peripheral nerve myelin homogenates (BPNM, 8 mg/animal) in complete Freud adjuvants. Animals were treated daily with rhEPO (5000 IU/kg) via i.p. injections from day 3 (preven: preventive) or day 10 (therap: therapeutic) after immunization or with vehicle alone (n = 10 per group). Clinical score ranging from 0 (healthy) to 10 (death) was assigned daily in a blinded fashion. On day 15, five randomly selected animals from each group were sacrificed for subsequent histological analysis. Both preventive (red triangles) and therapeutic (blue squares) shortened the duration of severe impairments and disability, while preventive treatment also reduced the maximum EAN severity (A). The preventive treatment also significantly protects from weight loss (B). Clinical score and weight was assessed at the indicated time points (mean ± SEM, * p<0.05; ** p<0.01). doi:10.1371/journal.pone.0026280.g001

Histological analysis of EPO treatment

To analyze grade and distribution of endoneurial inflammatory infiltrates, sciatic nerve sections were stained for T cells (Fig. 2A–C) and macrophages (Fig. 2D–F). The number of T cells, determined by CD3 staining at day 15 and day 29 was markedly reduced in the treatment group compared to control (Fig. 2G). The preventive treatment showed an approximately 4-fold reduction in T cell numbers at the peak of disease (PBS: 28.2±4.4; prevention: 5.8±1.1), while in the remission phase the effect on T cells was less pronounced (PBS: 52.3±4.1; prevention: 35.1±5.3). The therapeutic administration of EPO reduced the number of T cells at day 15 (19.8±2.7) as well as at day 29 (14.8±1.9). Quantification of T cell staining correlated with the clinical scores in untreated animals compared to the two treatment groups. Infiltration was less pronounced in cohorts with an ameliorated disease. Surprisingly, the CD68 macrophage staining showed disproportionately higher number of macrophages compared to T cells in EPO-treated animals. The CD68 staining at day 29 displayed a massive induction of macrophages in the target organ after preventive and therapeutic treatment (Fig. 2D–F). The striking differences in macrophage number in the treated and untreated groups were not present in the spleen (data not shown). Quantification of macrophages confirmed the significant differences in the EPO-treated groups compared to controls (Fig. 2 H). Although macrophage numbers were not prominent at the peak of disease (PBS: 25.2±6.2; prevention: 10.7±1.5; therapeutic: 4.9±0.1), the remission phase was characterized by a duplication of macrophage numbers under preventive (PBS: 62.7±9.1; prevention: 114.3±7.8) as well as therapeutic conditions (143.3±13.4).

Integrity of myelin was determined in ultrathin sections of the peripheral nerve. Representative electron micrographs depicted pathological changes in EAN nerves. Control nerves without EPO treatment (Fig. 2I,J) showed more demyelinated fibres without remyelination and axon degeneration. Additionally, more interstitial oedema were present. In contrast, in nerves from EPO-treated animals more remyelinated fibres and less demyelination was observable (Fig. 2 K). Pathology, however, exhibited great intraindividual heterogeneity. To address this variability we performed quantitative histological analyses and detected an amelioration of the PNS pathology in EPO-treated animals. The percentage of demyelinated (Fig. 2 M) and hypomyelinated axons (data not shown) showed a non-significant trend towards reduction, while the percentage of degenerating axons was significantly reduced by preventive EPO treatment (Fig. 2 L).

Proliferation and cytokine profile of EPO treated lymphocytes in vitro

The disproportionate numbers of infiltrating T lymphocytes compared to macrophages in the peripheral nerves of EPO-treated animals prompted us to examine the immunomodulatory effects of EPO in leukocyte cultures in vitro. Allogenic T cell proliferation of a mixed leukocyte reaction was strongly reduced to approximately 50 percent at higher concentration of EPO (Fig. 3A). In line with this observation, the proinflammatory cytokine IFN-gamma was almost reduced to half in collected cell-supernatants, while the anti-inflammatory cytokines IL-10 and TGF-beta were slightly increased after 72 h of T cell proliferation (Fig. 3B). Comparable results were observed in an antigen specific T cell proliferation and the concomitant supernatant analysis (data not shown). To analyze the effect of EPO on macrophages intraperitoneal cells of naïve rats were cultivated in the presence of EPO and the amount of IL-
Figure 2. Treatment with EPO improves the peripheral nerve histology. Sections of sciatic nerves obtained at day 29 after immunization were stained for CD3\(^+\) T lymphocytes in non-treated (A) and EPO-treated animals (B and C). Scale bar = 50 μm. Immunohistochemistry developed with 3,3'-diaminobenzidine as peroxidase substrate. Insets: Higher magnification of stained T cells showing brown DAB staining in close proximity to blue EPO in EAN.
and TGF-beta was assessed (Fig. 3C). In control cultures modest levels of IL-10 were detected but these were not altered by EPO. Altogether the cytokines was expressed in low quantities (below 100 pg/ml). In contrast, a higher TGF-beta level was even present in unstimulated controls (323.3±6.8 pg/ml) and this was further increased by EPO treatment (437.6±2.6 pg/ml at highest concentrations of EPO). To validate our findings in intraperitoneal cells from naïve animals we analyzed peritoneal macrophages from three rats with clinically active EAN independently (Fig. 3D). IL-10 levels were either slightly induced or reduced by EPO in different animals, but in summary not substantially altered. Again, the high levels of TGF-beta were further significantly elevated by EPO stimulation to a comparable amount in all animals. In contrast to T cell proliferation, proliferation of different macrophage subpopulations was not influenced by the presence of EPO, even when cells were prestimulated with IFN-gamma or LPS (data not shown). Taken together, the stimulation with EPO reduces the proliferation as well as inflammatory cytokines in vitro, while anti-inflammatory cytokines were induced to a greater extend.

Detection of TGF-beta in the peripheral inflamed nerve

Staining for TGF-beta in sciatic nerves was performed at day 15 and 29 p.i. to assess the possible mechanisms underlying the beneficial effect of EPO (Fig. 4A–F).

At the peak of the clinical disease only a few TGF-beta positive cells were detectable in the sciatic nerve sections of control animals (Fig. 4A,G; PBS: 92.7±9.2). However, even at this early time point the

![Figure 3](https://example.com/figure3.png)

**Figure 3.** EPO reduces the inflammatory profile of macrophages in vitro. Splenocytes from Lewis rats were cultivated with irradiated allogenic Wistar splenocytes and T cell proliferation was measured by 3H-thymidine incorporation in the presence of increasing concentrations of EPO (0–200 IU/ml) in quadruplicates. One representative experiment is depicted out of three (A). Supernatants of the proliferation were collected after 72 hours and levels of IFN-gamma, IL-10 and TGF-beta were determined using ELISA (B). Peritoneal macrophages from 2 naïve rats were plated in triplicates in the presence of EPO (0–200 IU/ml) and cytokines were measured via ELISA. Depicted is one representative experiment out of three (C). Peritoneal macrophages from EAN rats at the peak of the disease were cultivated with EPO (0–100 IU/ml) and cytokines were measured. Dots represent mean cytokine levels of single animals determined in duplicates (D). Asterisks indicate significance (mean±SEM, *p<0.05; **p<0.01; ***p<0.001); student’s t test.

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number of TGF-beta positive cells was significantly increased to two folds in EPO-treatment groups (Fig. 4 B,C,G, prevention: 175.3±20.8; therapeutic: 187.1±13.1). In the remission phase at day 29 p.i., numbers of TGF-beta positive cells were increased in all three groups. While in control and preventive regimens a comparable amount of cytokine positive cells were counted at day 29 p.i. (PBS: 427.0±38.2; prevention: 378.2±35.2) the increase in the therapeutic group was not as prominent (270.3±32.1). Costaining of TGF-beta and CD3 (Fig. 4H) or CD68 (Fig. 4I) showed colocalisation of TGF-beta with some CD3
+ T lymphocytes. However, most of the TGF-beta staining colocalized with CD68
+ cells, indicating that macrophages are the major source of TGF-beta.

Discussion
Erythroid precursors were believed to be the only cells responsive to EPO, however, an emerging body of evidence reports the EPO receptor (EPOR) to be expressed on a number of different cell types. In contrast to erythrocytes using an EPOR homodimer, in non erythroid tissues the binding of EPO assembles a heteroreceptor complex composed of EPOR and the common beta chain which is also used by other cytokine and growth factor receptors [11]. Hereby, EPO exerts antiapoptotic and proliferative effects on cells of various tissues including the nervous system, kidney, heart and liver (Shaheen and Broxmeyer, 2009).
安徽省的高校也支持将中国列为“发展中国家”。其中，除了《中国教育发展白皮书》外，还有一份最新的《中国教育发展报告》。该报告探讨了如何在经济和教育的背景下，促进中国的教育发展。报告强调，教育的发展需要政府、企业和学校共同参与。此外，还建议政府在教育政策和改革方面，要注重和地方政府的沟通和合作。
with 100 µl complete Freund's adjuvant (CFA, Difco) containing 1 mg/ml heat inactivated Mycobacterium tuberculosis (H37Ra). A modified EAN score [34] was applied: 0 no impairments, 1 reduced tone of the tail, 2 limp tail, 3 absent righting reflex, 4 gait ataxia, 5 mild paraparesis, 6 moderate paraparesis, 7 severe paraparesis or paraplegia, 8 tetraparesis, 9 moribund, 10 death due to neuropathy.

**EPO treatment**

EPO (Epoetin Alfa, Ratiopharm, Germany) in 2,000 U/ml vial stock was used for treatment. Administration of EPO was either starting at day 3 after immunization (preventive) or starting at day 10 after immunization (therapeutic). Animals were treated daily with intraperitoneal (i.p.) EPO at a dose of 5000 IU/kg/day, as established before [7] or as controls with an equal volume of PBS.

**Histology**

At peak of clinical disease activity, day 15 post immunization (p.i.), and at day 29 p.i. a randomly chosen half of all experimental groups of animals was sacrificed and perfused with PBS followed by 4% paraformaldehyde. Spleens and sciatric nerve were dissected, post-fixed with paraformaldehyde overnight and paraffin embedded. 10 µm sections (standard microtome HM355S, Microm, Walldorf, Germany) were stained with haematoxylin/eosin (HE) or rabbit anti-CD3 antibody (DAKO, Glostrup, Denmark) or mouse anti-CD68 antibody (Serotec, Duesseldorf, Germany) using matching biotinylated secondary antibodies (Vector, Peterborough, UK) followed by an avidin-biotin-horseradish peroxidase complex (DAB Kit, DAKO) using 3,3'-diaminobenzidine (DAB) as peroxidase substrate according to manufacturer's instructions. Transforming growth factor (TGF)-beta staining (rabbit anti-TGF-beta antibody, Santa Cruz, Heidelberg, Germany) was performed without HE staining. For fluorescent staining FITC and Alexa Flour 633 conjugated secondary antibodies were applied (Invitrogen, Darmstadt, Germany) and slices were covered using Vectashield (Vector) mounting medium with or without 4,6-diamidino-2-phenylindole (DAPI). For quantitative analysis of positive cells infiltrating the nerve three entire nerve longitudinal sections from each animal were photographed with a high magnification (Axioplan 2, Zeiss), the area covered by the tissue was determined and the number of positive cells per mm² was counted using ImageJ software (v1.44, NIH).

For morphological studies and analysis of axonal degeneration the brachial plexus was dissected from perfused animals (n = 5 per group), post-fixed in 4% paraformaldehyde over night and embedded in epoxy resin, as previously described [35]. Toluindine blue stained semi-thin (1 µm) and ultrathin (200 nm) sections were examined by light and electron microscopy, respectively. For statistical analysis of axonal pathology, semi-thin sections of plexus nerves were photographed and the photographs were photomerged using Photoshop CS3 (Adobe). The total number of normally myelinated, hypomyelinated, fully demyelinated and degenerating axons was manually counted by an investigator blinded towards previous treatment using the CellCounter plugin of ImageJ. Each individual axon was manually marked and automatically counted. Physiologically unmyelinated axons (diameter < 1 µm) and Remak-bundle fibres were not included. Degenerating axons were morphologically defined as axonal remnants with partially intact Schwann cell ensheatment, but with lost axonal interior structure. The percentage of intact and abnormal axons was calculated and compared between groups.

**T cell proliferation assay**

Spleens of rats were dissected under sterile conditions and passed through a 40 µm cell strainer followed by ammonium chloride based erythrocyte lysis (BD Bioscience, Heidelberg, Germany). Derived splenocytes were cultured in flat bottom 96-well plates in standard T cell medium (IIMDM with 5% FCS, 2 mM L-glutamine and 50 µM 2-ME, Invitrogen). Responder cells from Lewis rats (1 x 10^7/well) were cocultured with irradiated (1000 rad) allogenic splenocytes of Wistar rats as stimulator cells (1 x 10^7/well). EPO was added during the culture period with increasing concentration from 0.3 to 200 IU/ml. For antigen specific T cell proliferation spleens of EAN rats were dissected at day 15 p.i. under sterile conditions cultivated as described above in the presence of BPNM (10 µg/ml). T cell proliferation was measured via [3H] thymidine incorporation during the last 24 h of a four day incubation. Liquid scintillation counting (Beta-Plate1205, Perkin Elmer, Rodgau, Germany) given as counts per minute (cpm) of quadruplicate test cultures ± SEM was measured. Stimulation index was calculated as ratio of the cpm at the indicated EPO concentrations to the proliferation of cells in the absence of EPO.

**Macrophage culture**

Peritoneal macrophages were prepared as described previously [36]. Briefly, 2–3 month old Lewis rats were injected i.p. with ice-cold PBS and fluids were recollected. Macrophages were isolated from untreated rats as well as EAN rats at day 15. Obtained cells (2 x 10^6) were cultivated in 96-well plates with DMEM (10% FCS, 2 mM L-glutamine, 100 U/ml Penicillin und 100 µg/ml Streptomycin) with increasing concentrations of EPO (0–200 IU/ml).

**Cytokine quantification**

Supernatants were collected from T cell proliferation assays after 72 hours or from macrophage cultures after 48 hours. Interleukin (IL)-10 and interferon (IFN)-gamma enzyme linked immunosorbtant assays (ELISA) were performed due to the manufacturer's protocol (BD Bioscience). TGF-beta ELISA was derived from R&D Systems (Wiesbaden, Germany). In all analyzed samples latent TGF-beta was activated according to the manufacturers' protocol. Supernatants as well as standard curve were measured in duplicates on a Rainbow Photometer (Tecan, Crailsheim, Germany) using easyWIN software. Concentrations are given as mean ± SEM.

**Data analysis**

Data were statistically analyzed using GraphPad Prism 5.0 (GraphPad Software). The Wilcoxon-Mann-Whitney was used to test for statistically significant differences in clinical score values. Student's t-test for unrelated samples was used to test for statistically significant differences in all other analyses. Differences were considered significant at p-values<0.05.

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**Author Contributions**

Conceived and designed the experiments: AKM MS KAS BCK. Performed the experiments: AKM GM TD MS. Analyzed the data: AKM GM TD MS KAS BCK. Contributed reagents/materials/analysis tools: AKM GM TD MS KAS. Wrote the paper: AKM GM TD MS KAS BCK.
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