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Blockade of tumour necrosis factor-α in experimental autoimmune encephalomyelitis reveals differential effects on the antigen-specific immune response and central nervous system histopathology

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Summary

In various autoimmune diseases, anti-tumour necrosis factor (TNF)-α treatment has been shown to reduce both clinical disease severity and T helper type 1 (Th1)1/Th17 responses. In experimental autoimmune encephalomyelitis (EAE), however, the role of TNF-α has remained unclear. Here, C57BL/6 mice were immunized with myelin oligodendrocyte glycoprotein (MOG) peptide 35–55 and treated with anti-TNF-α, control antibody or vehicle. The clinical disease course, incidence and severity were assessed. On day 20 after immunization the antigen-specific Th1/Th17 response was evaluated by enzyme-linked immunospot (ELISPOT) in spleen and central nervous system (CNS). Also, the extent of spinal cord histopathology was analysed on semi- and ultrathin sections. Our results demonstrate that anti-TNF-α treatment reduced the incidence and delayed the onset of EAE, but had no effect on disease severity once EAE had been established. Whereas anti-TNF-α treatment induced an increase in splenic Th1/Th17 responses, there was no effect on the number of antigen-specific Th1/Th17 cells in the spinal cord. Accordingly, the degree of CNS histopathology was comparable in control and anti-TNF-α-treated mice. In conclusion, while the anti-TNF-α treatment had neither immunosuppressive effects on the Th1/Th17 response in the CNS nor histoprotective properties in EAE, it enhanced the myelin-specific T cell response in the immune periphery.

Keywords: EAE/MS, inflammation, monocytes/macrophages, neuroimmunology, T cells

Introduction

Tumour necrosis factor (TNF)-α is a cytokine with pleiotropic functions and has been implicated in various autoimmune diseases. The main sources of TNF-α are activated mononuclear phagocytes and, to a lesser degree, T cells. TNF-α is synthesized as a transmembrane protein, and cleavage of the extracellular domain results in the release of soluble TNF-α. TNF-α can bind to two receptors, TNF receptor (TNFR) p55 (TNFR I) and TNFR p75 (TNFR II). TNFR p55 is expressed on almost every cell type; TNFR p75, in contrast, is restricted mainly to immune cells, but is also found on oligodendrocytes [1] and neurones [2]. While the extracellular domains of the two receptors are structurally similar, the intracellular domains have little homology. TNFR p55 contains an intracellular death domain, which ultimately activates caspases and triggers apoptosis. In contrast, TNFR p75 lacks a death domain, but activates mitogen-activated protein kinase (MAPK), nuclear factor (NF)-κB and activator protein (AP)-1 pathways, leading to transcription of genes involved in inflammation or cell survival [3]. However, there is thought to be a degree of cross-talk between the two TNF receptors, so that both contribute to gene transcription and apoptosis [3].

Aberrant TNF production [4] and defects in TNF receptor signalling [3] have been detected in various autoimmune diseases. To date, TNF-α inhibitors are licensed for rheumatoid arthritis, psoriasis, psoriatic arthritis, ankylosing spondylitis and Crohn's disease [5–9]. However, in a clinical trial with multiple sclerosis (MS) patients, an anti-TNF agent led to aggravation of the relapse rate [10]. In other patients undergoing anti-TNF treatment, demyelinating side effects and autoantibody production (e.g. against DNA or nuclear proteins) have been observed. Even new onset of MS or of other autoimmune disorders has been reported [11], highlighting the complex role played by TNF-α in autoimmunity.

In-depth analyses of treatment effects in patients are hampered by the difficulty in obtaining cells from lymphoid organs and inflammatory sites. Therefore, research relies largely upon suitable animal models. The most widely used animal model for multiple sclerosis is experimental autoimmune encephalomyelitis (EAE), in which immunization of susceptible animal strains with myelin antigens emulsified in complete Freund's adjuvant induces an autoreactive immune response, spinal cord histopathology and finally clinical symptoms [12].

Collagen-induced arthritis (CIA) is a well-validated model of rheumatoid arthritis that has been used widely to study the effects of anti-TNF-α treatment. Anti-TNF-α treatment reduced paw swelling and the severity of joint histopathology [13]. However, more recent studies showed that TNF-α antibody had a dual effect on the T cell response: while treatment reduced T helper type 1 (Th1)/Th17 infiltration of the joint, it led to increased numbers of Th1/Th17 cells in the lymph nodes [14]. The literature on TNF-α abrogation in EAE presents a controversial picture. While anti-TNF-α antibody treatment in general conveys some clinical disease amelioration, not all studies found the same features to be affected in the same way [15–18]. Data on the antigen-specific Th1/Th17 response in lymphoid organs and the central nervous system (CNS) are heterogeneous [11,12], as is the effect of TNF-α neutralization on axonal damage [11].

In this study we characterized simultaneously the effects of anti-TNF-α treatment on clinical, immunological and histopathological disease development in the traditional myelin oligodendrocyte glycoprotein peptide 35–55 (MOG:35–55)-induced EAE model. We analysed whether or not the treatment provoked a dual effect on the T cell response similar to the one observed in collagen-induced arthritis, which would predict increased Th1/Th17 frequencies in the spleen and reduced Th1/Th17 frequencies in the CNS. Additionally, we investigated the effect of anti-TNF-α treatment on axon and myelin integrity. The assessment of histopathology is of particular importance, as neurodegeneration is the morphological correlate of irreversible clinical symptoms in MS patients [19]. Currently, TNF-α blocking agents are not considered as candidate drugs for MS because of the dramatic failure of a clinical trial in 1999 [10]. It is therefore important to elucidate the general effects of anti-TNF-α treatment on immune responses in EAE *versus* its effect on inflammation in the CNS. In this way we may ultimately gain invaluable insights towards successful CNS immune modulation.

Material and methods

Animals

C57BL/6 mice (6–8 weeks old) were purchased from the Harlan Laboratories (Sulzfeld, Germany) and Janvier (Saint Berthevin Cedex, France) and maintained in individually ventilated cages at the animal facilities of the Department of Anatomy of Cologne University. Incomplete Freund's adjuvant (IFA) was prepared as a mixture of paraffin oil (EMScience, Gibbstown, NJ, USA) and mannide monooleate (Sigma, Schnelldorf, Germany). Complete Freund's adjuvant (CFA) was obtained by adding *Mycobacterium tuberculosis H37RA* (Difco Laboratories, Franklin Lakes, NJ, USA) at 5 mg/ml to IFA. Animals were immunized subcutaneously in both sides of the flank with a total dose of 100 μg MOG:35–55 (EZBiolab, Carmel, IN, USA) emulsified in CFA (injection volume = 200 μl). Each mouse received 200 ng pertussis toxin (List Biological Laboratories, Hornby, Ontario, Canada) in 500 μl sterile phosphate-buffered saline (PBS) on the day of immunization and 48 h later. Clinical symptoms were evaluated daily according to the standard EAE scale: 0, no symptoms; 1, floppy tail; 2, hind limb weakness; 3, hind limb paralysis; 4, quadriplegia; and 5, death. Mice were euthanized with CO_2 on day 20 post-immunization. For treatment, mice were injected intraperitoneally every other day, starting from day 3 post-immunization, either with 100 μg Enbrel®, 100 μg Humira® or PBS (injection volume of 500 μl). Enbrel® is a fusion protein between the extracellular domain of the TNFR2/p75 and the Fc fragment of human immunoglobulin (Ig)G1. Humira® is of the same isotype as Enbrel, but does not neutralize murine TNF. All experiments were approved by the German Animal Welfare Act.

Cell preparation

The spleen and spinal column were removed, and the spinal cord was flushed out with Dulbecco's modified Eagle's medium (DMEM) (PAA, Pasching, Austria). Specimens were disintegrated mechanically and filtered through a 70-μm nylon cell strainer (BD Falcon, Heidelberg, Germany). After washing the cells with RPMI-1640 (Biochrom AG, Berlin, Germany) and counting them with acridine orange (0·1%, Sigma)/ethidium bromide (0·1%, Serva, Heidelberg, Germany), cells were resuspended in HL-1 (Lonza, Cologne, Germany) supplemented with 1% glutamine (Sigma) and 1% penicillin/streptomycin (Sigma).

ELISPOT assays

Low-volume Unifilter Whatman plates (Whatman Inc., Florham Park, NJ, USA) were coated overnight with the capture antibodies rat anti-mouse interferon (IFN)-γ (final concentration 3 μg/ml, clone AN-18; eBioscience, San Diego, CA, USA) and rat anti-mouse interleukin (IL)-17 (final concentration 4 μg/ml, clone TC-11-18H10; BD Biosciences, San Diego, CA, USA) in PBS as follows: first, plates were precoated with anti-IFN-γ, after 10 min anti-IL-17 was added. Plates were washed with PBS, and blocked

with 1% bovine serum albumin (BSA) in PBS for 2 h at room temperature. Spleen cells were plated at 5×10^5 cells/well and spinal cord cells at 1×10^5 cells/well. Antigen-presenting cells were obtained by irradiating spleen cells from naive C57BL/6 mice with 26 Gy and added to the spinal cord cells at a concentration of $2{\cdot}5 \times 10^5$ cells/well. Cells were incubated with either medium or MOG:35–55 (final concentration: 15 μg/ml) at 7% CO_2 and 37°C for 24 h. Plates were washed and incubated with fluorescein isothiocyanate (FITC)-conjugated anti-IFN-γ (0·5 μg/ml; gift from M. Tary-Lehmann, clone R4-6A2) and biotin-conjugated anti-IL-17 (0·5 μg/ml; Pharmingen, San Diego, CA, USA; clone TC-11-8H4·1) overnight at 4°C. After washing, plates were incubated for 2 h with anti-FITC-labelled alkaline phosphatase (1/500; Dako, Glostrup, Denmark) and streptavidin-conjugated horseradish peroxidase (1/1000; Dako). Plates were developed with Vector Blue (Vector Laboratories, Burlingame, CA, USA) and AEC (Vector Laboratories) solution according to the vendor's instructions. Plates were air-dried overnight and spots were counted with an ImmunoSpot Series 5 UV Analyzer (Cellular Technology Limited, Shaker Heights, OH, USA). All results were medium-subtracted and normalized to 10^6 cells per well.

Histology

In order to investigate the effects of anti-TNF-α treatment on spinal cord histopathology, semi- and ultrathin sections of the lumbar spinal cord of PBS-, Enbrel®- and Humira®-treated mice were obtained as described previously [20]. Briefly, mice were perfused intracardially on day 20 after EAE induction with a 4% paraformaldehyde/4% glutaraldeyhde/PBS solution. The lumbar spinal cord was post-fixed, rinsed in cacodylate buffer and treated with 1% osmium tetroxide (Chempur, Karlsruhe, Germany). Tissues were treated with 1% uranyl acetate (Plano GmbH, Wetzlar, Germany) in 70% ethanol for contrast enhancement overnight. Subsequently, specimens were embedded in epon (Fluka, St Louis, MO, USA) and polymerized at 60°C for at least 72 h. Eighty nm-thick ultrathin and 500 nm-thick semithin sections of each plastic-embedded spinal cord sample were cut on a Leica Ultracut UCT ultramicrotome (Leica Microsystems, Wetzlar, Germany). For light-microscopic analysis, sections were stained with methylene blue and for electron microscopy with 1% aqueous uranyl acetate solution for 20 min and Reynold's lead citrate solution (Merck KGaA, Darmstadt, Germany) for 7 min. Specimens were examined with a Zeiss EM 902 A transmission electron microscope at 80 kV acceleration voltage and images were taken with an EM digital camera system (MegaView III; Olympus Soft Imaging Systems GmbH, Münster, Germany) at ×7000 magnification. Images of score-matched Enbrel®- ($n = 4$), Humira® ($n = 3$)- and non-immunized control mice ($n = 4$) (10 images per mouse) from the ventrolateral tract were analysed for the extent of myelin and axonal pathology. The numbers of physiological, demyelinated and axolytic nerve fibres were counted with ImagePro Plus software (Media Cybernetics, Inc., Rockville, MD, USA). Methylene blue-stained sections were observed using a Leica DM LB2 microscope and digital images were acquired at ×20 magnification with an AxioCam camera (Zeiss, Oberkochen, Germany) and Zeiss software (AxioVision 40 4·7). The degree of inflammation was determined semiquantitatively according to the following scoring system: 0, no infiltrates (resembling naive mice); 1, partial meningeal and perivascular infiltration; 2, pronounced meningeal and perivascular infiltration; 3, pronounced meningeal and perivascular infiltration and some parenchymal infiltration; and 4, pronounced meningeal and perivascular and widespread parenchymal infiltration.

Statistical analyses

SigmaPlot Software version 12·0 (Chicago, IL, USA) was used for all statistical analyses. For evaluation of differences in the clinical score, disease onset, antigen-specific IFN-γ and IL-17 production by enzyme-linked immunospot (ELISPOT) and CNS histopathology the Wilcoxon–Mann–Whitney test was used. Fisher's exact test was used for evaluating differences in disease incidence. Spearman's rank-order correlation was used for correlating antigen-specific cytokine production and clinical score. The limit of statistical significance was $P \leq 0{\cdot}05$.

Results

TNF-α blockade reduces the incidence and delays the onset of EAE

For the induction of EAE, C57BL/6 mice were immunized with 100 μg MOG:35–55 in CFA. Pertussis toxin was administered at 200 ng per mouse on days 0 and 2 post-immunization. Mice were scored daily for the occurrence of clinical symptoms. Starting on day 3 post-immunization, $n = 15$ mice were treated with Enbrel® every other day, while $n = 19$ mice received sham injections (PBS). Ten mice were treated with Humira® as control antibody. Figure 1a shows the clinical course of a representative cohort of Enbrel®-, Humira®- or PBS-treated mice. In comparison to PBS- or Humira®-treated mice, mice that received Enbrel® showed reduced EAE incidence (Fig. 1b). While 95% of the PBS- and 100% of the Humira®-treated mice exhibited symptoms, only 53% of the Enbrel®-treated mice developed EAE ($P < 0{\cdot}05$, Fisher's exact t-test). Enbrel® also caused delayed EAE onset (Fig. 1c). In PBS-treated mice, EAE developed on day 10 (± 1 day) and mice that received Humira® showed an EAE onset on day 12 (± 0·6 days), whereas in Enbrel®-treated mice EAE onset was on day 17 (± 3 days) ($P < 0{\cdot}001$ and $P < 0{\cdot}01$, respectively, rank sum

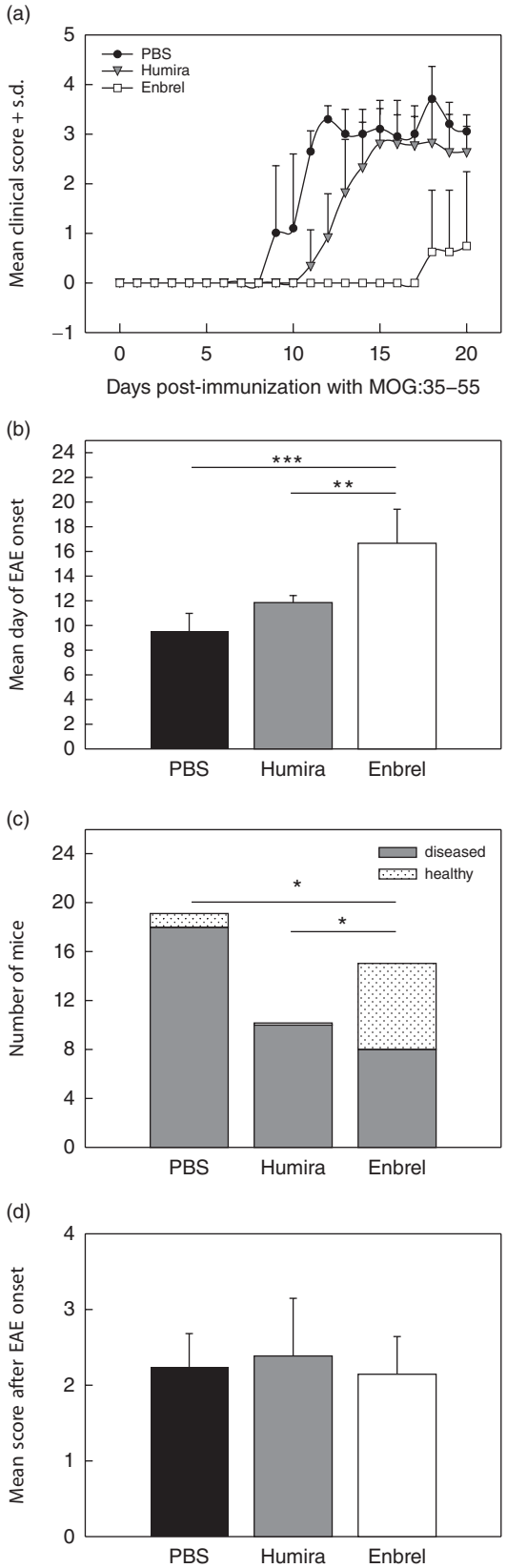

Fig. 1. Tumour necrosis factor (TNF)-α blockade reduces the incidence and delays the onset of experimental autoimmune encephalomyelitis (EAE). (a) Clinical course of a representative cohort of Enbrel®-treated mice (white squares) and mice that received phosphate-buffered saline (PBS) (black circles) or Humira® injections (grey triangles). The Enbrel® and PBS cohort included mice that did not develop EAE. (b) Mean day of EAE onset (***$P < 0{\cdot}001$, rank sum test). (c) EAE incidence with solid bar segments referring to diseased mice and dotted segments referring to healthy mice 20 days post-immunization (*$P < 0{\cdot}05$, Fisher's exact test). (d) Mean score after EAE onset of all diseased mice. The data refer to $n = 15$ Enbrel®-, $n = 10$ Humira®- and $n = 19$ PBS-treated mice.

t-test). However, once clinical EAE had been established, there was no significant difference in the mean clinical scores between the three groups (Fig. 1d).

TNF-α blockade increases the antigen-specific Th1/Th17 response in the spleen, but has no effect on the antigen-specific Th1/Th17 response in the spinal cord

In order to determine if the clinical amelioration observed with anti-TNF-α treatment was associated with changes in the antigen-specific T cell response, mice were killed on day 20 post-immunization, after the onset of full-blown EAE. ELISPOT assays were performed with spleen and spinal cord cells. Figure 2 shows the number of antigen-specific IFN-γ- and IL-17-producing cells of PBS-, Humira®- and Enbrel®-treated mice. With Enbrel® treatment, there was a significant increase in the splenic antigen-specific Th1 ($P < 0{\cdot}01$, Wilcoxon's rank-sum test) and Th17 ($P < 0{\cdot}05$, Wilcoxon's rank-sum test) response in comparison to PBS or Humira® treatment (Fig. 2a). However, we observed no difference in the antigen-specific Th1/Th17 response with Enbrel® treatment in the spinal cord (Fig. 2b).

In a next step, we analysed the extent to which the antigen-specific Th1/Th17 response correlated with disease severity. While there was a significant correlation between the antigen-specific Th1/Th17 response in the spinal cord and the clinical score ($r_s = 0{\cdot}668$, $P < 0{\cdot}05$ for IFN-γ; $r_s = 0{\cdot}6$, $P < 0{\cdot}05$ for IL-17; Spearman's rank-order correlation), no such correlation could be found in the spleen ($r_s = 0{\cdot}567$, $P = 0{\cdot}051$ for IFN-γ; $r_s = 0{\cdot}251$, $P = 0{\cdot}415$ for IL-17; Spearman's rank-order correlation).

These findings indicate that inhibition of TNF-α increased the numbers of antigen-specific Th1/Th17 cells in the spleen, although these cells appeared not to be associated with the disease process. In the CNS, however, no protective effects of Enbrel® treatment could be observed, which was mirrored by the similarity of disease severity in the various treatment groups. To validate the lack of a neuroprotective effect of TNF-α blockade, we performed in-depth histopathological analysis of the spinal cord.

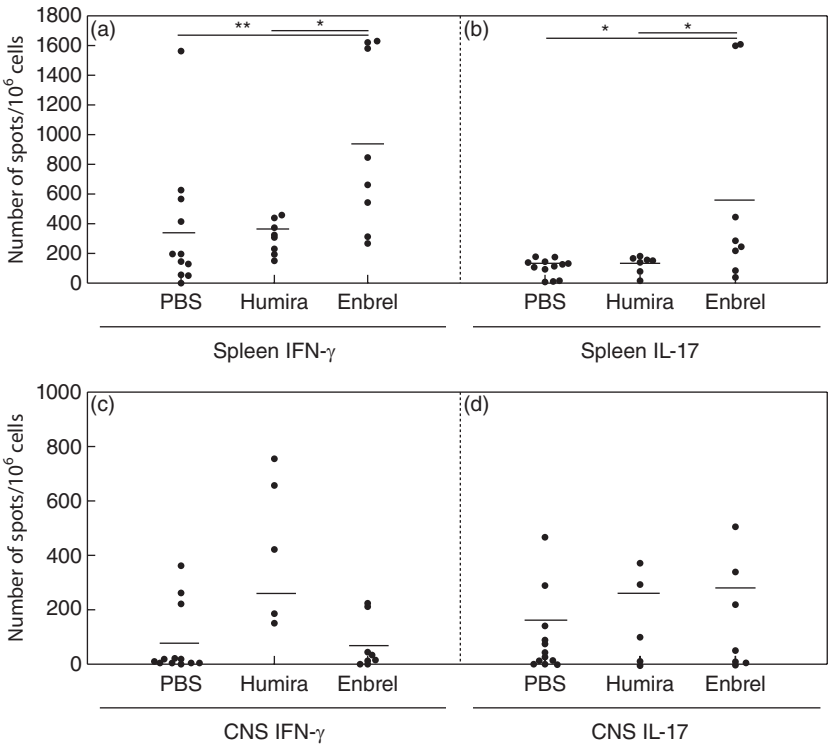

Fig. 2. Effects of tumour necrosis factor (TNF)-α blockade on the frequencies of antigen-specific Th1/Th17 cells in spleen and central nervous system (CNS). (a) Results of enzyme-linked immunospot (ELISPOT) assays in phosphate-buffered saline (PBS) ($n = 12$)-, Humira® ($n = 8$)- and Enbrel®-treated mice ($n = 8$) for interferon (IFN)-γ (**$P < 0{\cdot}01$, rank sum test) and interleukin (IL-17 (*$P < 0{\cdot}05$, rank sum test) in the spleen. (b) Results of IFN-γ/IL-17 ELISPOT assays in PBS ($n = 13$)-, Humira® ($n = 5$)- and Enbrel®-treated mice ($n = 8$ IFN-γ; $n = 7$ IL-17) in the spinal cord.

TNF-α blockade has no protective effect on inflammatory activity, myelin pathology and axonal damage in MOG:35–55-induced EAE

Twenty days after EAE induction, $n = 4$ PBS-, $n = 3$ Humira®- and $n = 4$ Enbrel®-treated mice were killed. The spinal cord was epon-embedded and semi- and ultrathin sections were obtained. Representative images of semithin sections are shown in Fig. 3a–d. In these sections, we quantified the mean lesion size per total white matter. Table 1 shows that there was no difference in the overall lesion size between PBS-, Humira®- and Enbrel®-treated mice. In addition, the extent of inflammation was assessed semiquantitatively. Again, no difference in the two inflammatory parameters was detected between the treatment groups (Table 1).

Light-microscopic analysis can give a general overview of the pathological traits, while falling short of providing

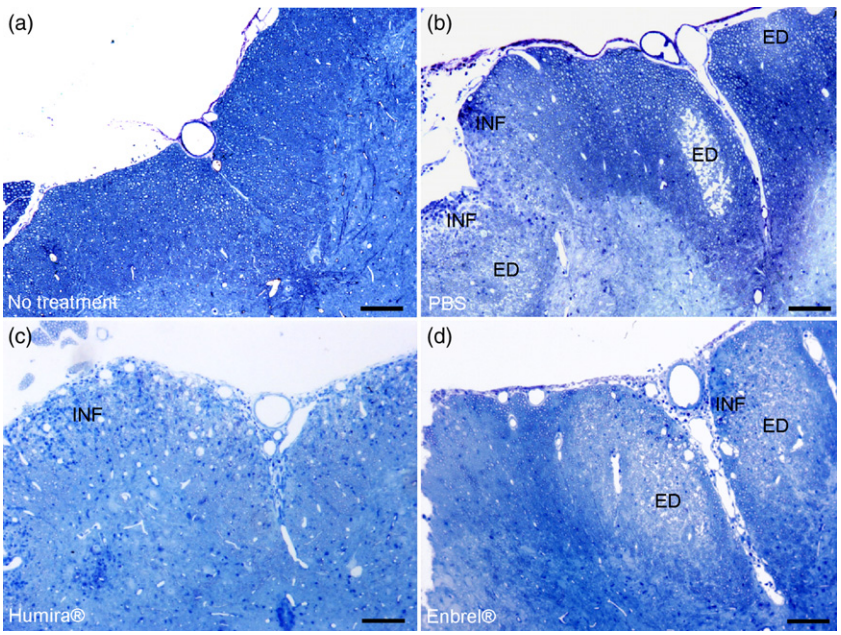

Fig. 3. Light-microscopic analysis of spinal cord histopathology in anti-tumour necrosis factor (TNF)-α-treated mice. C57BL/6 mice were immunized with MOG:35–55, treated with phosphate-buffered saline (PBS) ($n = 4$), Humira® ($n = 3$) or Enbrel® ($n = 4$) and killed on day 20 post-immunization. Spinal cords were removed carefully from the vertebral canal, epon-embedded and semi-thin sections of the lumbar part were obtained. (a–d) Representative images of non-immunized control mice (a) and PBS- (b), Humira®-(c) or Enbrel®-treated MOG:35–55-immunized mice (d). The scale bar denotes 100 µm. ED = oedema; INF = blue dots represent immune cells infiltrating the central nervous system (CNS).

Table 1. Central nervous system (CNS) histopathology in phosphate-buffered saline (PBS)-, Humira®- and Enbrel®-treated mice.

Treatment	No. of mice	Clinical score	Degree of inflammation	Lesion size [%]	No. of intact axons/mm^2	No. of axolytic axons/mm^2	No. of demyelinated axons/mm^2
PBS	4	2·50 ± 0·41	2·00 ± 1·08	31·92 ± 20·37	$2{\cdot}17 \times 10^5 \pm 2{\cdot}75 \times 10^4$	$3{\cdot}39 \times 10^4 \pm 3{\cdot}51 \times 10^3$	$6{\cdot}17 \times 10^3 \pm 6{\cdot}64 \times 10^3$
Humira®	3	2·92 ± 0·14	2·33 ± 0·94	29·73 ± 10·36	$1{\cdot}40 \times 10^5 \pm 1{\cdot}40 \times 10^5$	$5{\cdot}21 \times 10^4 \pm 2{\cdot}83 \times 10^3$	$5{\cdot}58 \times 10^5 \pm 9{\cdot}33 \times 10^5$
Enbrel®	4	2·69 ± 0·13	2·88 ± 0·85	55·76 ± 25·47	$1{\cdot}60 \times 10^5 \pm 1{\cdot}11 \times 10^5$	$4{\cdot}06 \times 10^4 \pm 3{\cdot}72 \times 10^4$	$4{\cdot}96 \times 10^3 \pm 5{\cdot}02 \times 10^3$

details about the fine characteristics of nerve fibre damage. Previous studies have reported changes in the extent of axonal damage and demyelination after anti-TNF-α treatment [16,18]. However, these studies relied upon light-microscopic and immunohistochemical techniques. In order to provide more profound information about axon and myelin pathologies, we performed ultrastructural analysis of PBS-, Humira®- and Enbrel®-treated mice. Representative images of non-immunized control mice and of PBS-, Humira®- and Enbrel®-treated mice are depicted in Fig. 4a–d. To begin with, we determined the number of demyelinated nerve fibres per mm^2 lesion area. There was no statistically significant difference in the number of demyelinated nerve fibres between PBS-, Humira®- and Enbrel®-treated mice (Table 1). We next determined the extent of axonal loss by counting the numbers of axons per mm^2 lesion area. There was no statistically significant difference in axonal density between the treatment groups (Table 1). The same applied to gross axonal damage as characterized by axolysis (Table 1).

Discussion

Here we explored the clinical, immunological and histopathological consequences of treatment with a TNF-α inhibitor in EAE. The results of this study suggest that anti-TNF-α treatment may be helpful in delaying or even preventing EAE outbreak, but once the disease has been established, TNF-α blockade provides no further clinical benefit. More importantly, anti-TNF-α treatment led to the accumulation of antigen-specific Th1/Th17 cells in the spleen, which could have deleterious consequences when the anti-TNF-α antibody is withdrawn.

TNF-α is known to be required for the functioning of regulatory T cells (T_{reg}), which suppress Th1/Th17 cells via IL-10 secretion [21–24]. Anti-TNF-α treatment may therefore have impaired T_{reg} function, thereby reducing their suppressive capacity towards Th1/Th17 cells. This may explain the increased splenic antigen-specific Th1/Th17 response in our study. Along these lines, it has been reported that TNF-signalling is particularly important for natural, but not inducible, T_{regs} [25]. This offers a possible explanation as to why anti-TNF-α treatment has detrimental effects in some human diseases, but not in others: conceivably, the diseases differ in the relevance of natural and/or inducible $T_{regs.}$ However, it has also been shown that TNF-α inhibits the expression of IL-12/IL-23 p40 [14], and this provides an equally plausible explanation for the increased numbers of Th1/Th17 cells observed. It is interesting to note that increased numbers of Th1/Th17 cells were observed in TNFR1$^{-/-}$ mice with reactive arthritis, which was associated with enhanced p40 expression [26]. In

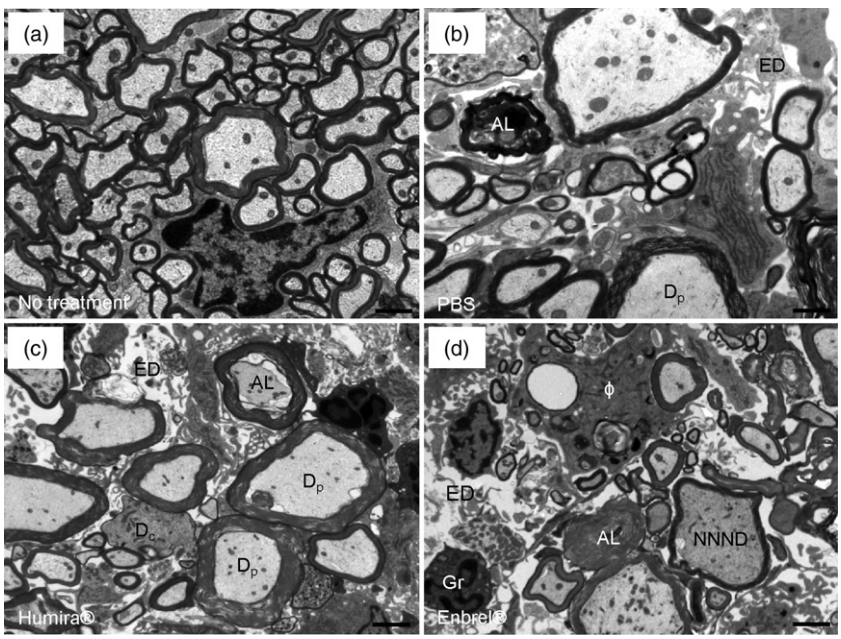

Fig. 4. Ultrastructural analysis of spinal cord histopathology in anti-tumour necrosis factor (TNF)-α-treated mice. Mice were immunized and treated as described in Fig. 3. After epon-embedding, ultrathin sections of the lumbar spinal cord of non-immunized control, phosphate-buffered saline (PBS)-, Humira®- and Enbrel®-treated mice were obtained and assessed by electronmicroscopy. Representative images are shown in (a–d). The scale bar depicts 1 μm. AL = axolysis; ED = oedema; D_c = demyelinated nerve fibre; D_p = nerve fibre in the process of demyelination; Gr = granulocyte; Φ = macrophage with myelin debris in the cytoplasm; NNND = nerve fibre with decreased nearest-neighbour neurofilament distance indicating axonal pathology.

experimental colitis, IL-17 has been shown to suppress T-bet and limit T_H1 activity [27]. While an increase in Th17 immunity may therefore have beneficial effects, in the present study the increased splenic Th1/Th17 response appeared to have no impact on the clinical EAE outcome. Our results show that, in MOG:35–55-induced EAE, only the antigen-specific Th1/Th17 response in the spinal cord (but not in the spleen) was associated with the clinical disease severity. While, in CIA, lower Th1/Th17 frequencies were observed in the target organ (i.e. the joint) after anti-TNF-α treatment and clinical disease was ameliorated, we observed no difference in the antigen-specific Th1/Th17 response in the spinal cord. In accordance, clinical EAE severity was unaltered in our study. On this basis we hypothesize that TNF-α blockade has the dual effect of inhibiting inflammation, but enhancing pathogenic T cell responses, such that the net effect on pathology is minimal.

One of the possible explanations as to why TNF-α blockade had no immunosuppressive effect on the Th1/Th17 response in the CNS could be that TNF-α may not be required for Th1/Th17 function in this target organ. While T cell co-stimulation is certainly necessary for T cell effector functioning, this co-stimulation may be mediated by molecules other than TNF-α during MOG:35–55-induced EAE, e.g. CD28 (compare with [28]). In addition, TNF-α may have no unbalanced proinflammatory properties in the CNS during EAE. As described above, TNF-α binding can induce apoptosis. In a recent report oligodendrocyte apoptosis was identified to be a crucial event occurring during early EAE, and to contribute to axonal degeneration and neuronal loss [29,30]. The assumption that oligodendrocyte apoptosis paves the way for axonal pathologies suggests that anti-TNF-α treatment may reduce the amount of neurodegeneration. Because the effect of anti-TNF-α treatment on neurodegeneration has not been delineated clearly in the past, we have performed ultrastructural analysis of PBS-, Humira®- and Enbrel®-treated mice. Having analysed various parameters of neurodegeneration, we observed no reduction in axonal pathology after TNF-α blockade. We therefore conclude that inhibition of TNF-α conveys no histoprotective effect in MOG:35–55-induced EAE. We further postulate that the amount of neurodegeneration is independent of apoptosis, and that TNF-α is not involved directly in axonal damage. Rather, TNF-α might function as an inflammatory mediator, stimulating chemokine production, which indirectly attracts more phagocytes into the CNS [31].

Although disease severity of established EAE was unaltered after TNF-α blockade, onset and incidence of the disease were reduced significantly despite the enhanced Th1/Th17 responses observed in spleen cells. The enhanced splenic response implies that TNF-α blockade could have an indirect anti-inflammatory effect that was not due to a reduced response to immunization. Rather, TNF-α blockade may have retained the pathogenic Th1/Th17 cells in the spleen and prevented their egress towards the CNS. It is tempting to speculate that a treatment stop leads to a sudden egress of these pathogenic T helper cells from the spleen into the CNS, and thus causes clinical deterioration. Indeed, our preliminary results indicate a significant reduction in the Th1 response in the spleen after treatment stop, which appeared to be accompanied by an increase in the clinical score (data not shown). However, further experiments are needed to confirm this notion.

In conclusion, although TNF-α blockade exerts protective effects in some autoimmune diseases, it is not capable of down-regulating neither the antigen-specific Th1/Th17 response nor histopathology in the CNS in EAE. Furthermore, the expansion of Th1/Th17 cells observed in Enbrel®-treated mice may have deleterious consequences when the TNF-α inhibitor is withdrawn, providing one possible explanation for the failure of TNF-α inhibition in human MS.

Author contributions

H. B. and M. S. R. designed the study, performed experiments, analysed the data and wrote the manuscript. F. O. H. and F. T. performed experiments. R. O. W. helped to design the study and write the manuscript. S K. designed the study, performed experiments, analysed data and wrote the manuscript. All authors have read and approved the final manuscript.

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Disclosure

The authors declare that they have no competing interests.

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