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Zinc aspartate suppresses T cell activation in vitro and relapsing experimental autoimmune encephalomyelitis in SJL/J mice

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Abstract Zinc is an essential trace element with a critical role in normal growth and development and in immune homeostasis. Zinc deficiency impairs both the innate and the adaptive immune system and can be normalized by zinc supplementation. On the other end of the spectrum, high dosages of zinc diminish immune cell functions similar to zinc deficiency. Here, we investigated the influence of zinc aspartate on proliferation and cytokine production of stimulated human T cells and mouse splenocytes in vitro. Furthermore, the effect of zinc aspartate was examined in mice with experimental autoimmune encephalomyelitis (EAE), an animal model of Multiple Sclerosis (MS) with a Th1/Th17 T cell-mediated immunopathogenesis. Zinc aspartate suppressed proliferation as well as IL-2, IL-10 and IL-17 production in stimulated human T cells and mouse splenocytes. Importantly, administration of a medium range dose of 30 μg/day zinc aspartate [1.5 mg/kg body weight (BW)] in a therapeutic manner led to a significant reduction of the clinical severity of the EAE during the first relapse of the disease. A lower zinc aspartate dose (6 μg/day, 0.3 mg/kg BW) had no significant therapeutic effect on the severity of the EAE, while administration of higher zinc aspartate amounts (120 μg/day, 6 mg/kg BW) led to more severe disease. Taken together, our data suggest that zinc aspartate can modulate activation, proliferation and cytokine production of effector T cells in vitro and in vivo and that activated autoreactive T cells may be potential therapeutic targets of tightly controlled zinc supplementation in autoimmune diseases like MS.

Keywords Zinc aspartate · IL-2 · IL-10 · IL-17 · T cell proliferation · Experimental autoimmune encephalomyelitis (EAE)

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Abbreviations

BW	Body weight
CFA	Complete Freund's adjuvant
CNS	Central nervous system
EAE	Experimental autoimmune encephalomyelitis
ELISA	Enzyme-linked immunosorbent assay
IL	Interleukin
i.p	Intraperitoneal
MLC	Mixed lymphocyte cultures

MS	Multiple sclerosis
NK	Natural killer
PBMC	Peripheral blood mononuclear cells
PLP	Proteolipid protein
PWM	Pokeweed mitogen
PTX	Pertussis toxin

Introduction

Zinc is an essential trace element, which is required by a large number of structural proteins, enzymes and transcription factors. It is involved in regulation of many biological processes like cell and tissue differentiation, proliferation and apoptosis (Prasad 1998; Wellinghausen et al. 1997; Rink and Haase 2007. Furthermore, zinc acts as an antioxidant and protects cells from destructive effects of oxygen radicals (Prasad et al. 2004; Bray and Bettger 1990. Due to the importance and large functional spectrum, zinc homeostasis is tightly regulated. Disturbance of this homeostasis caused by genetic defects, zinc deficiency, or zinc supplementation affects both the components of the innate and the adaptive immune system (Wellinghausen and Rink 1998; Ibs and Rink 2003). Thus, zinc deficiency leads to decreased chemotaxis and oxidative burst of neutrophils and monocytes as well as to diminished lytic activity of natural killer (NK) cells (Prasad 1998; Wellinghausen et al. 1997; Rink and Haase 2007; Keen and Gershwin 1990). States of zinc deficiency induces thymic atrophy, reduces thymulin activity, and decreases T cell proliferation (Prasad 1998; Cakman et al. 1997). Several studies demonstrated a zinc-deficient modulation of the Th1/Th2-balance towards Th2 with reduction of the Th1 cytokines IL-2 and IFN–γ (Prasad 2000; Uciechowski et al. 2008). Furthermore, zinc is described as intracellular second messenger of immune cells, particularly of T cells, and has been reported to affect the regulation of several cytokine signalling cascades (Yamasaki et al. 2007; Varin et al. 2008; Kaltenberg et al. 2010; Kitabayashi et al. 2010; Yu et al. 2011; Fukada et al. 2011). Thus, zinc deficiency results in immunological dysfunction and increases the susceptibility to bacterial, viral and fungal infections. Clinical manifestations offer a wide spectrum from an impaired rate of growth in children, hyperammonemia and hypogeusia to alopecia, diarrhea and neurosensory disorders (Prasad 1998; Wellinghausen et al. 1997; Rink and Haase 2007). Therapeutical zinc supplementation is capable of normalizing the diminished immune functions due to zinc deficiency (Prasad et al. 2007). In contrast, high dosages of zinc induce pathological effects in immune cells, similar to those observed with zinc deficiency (Wellinghausen et al. 1997; Rink and Haase 2007).

Based on these reports, zinc supplementation is considered as a possible therapeutic option for graft-versus-host diseases and T cell-mediated autoimmunity (Wellinghausen et al. 1997; Rink and Haase 2007; Faber et al. 2004).

Multiple Sclerosis (MS) is an inflammatory autoimmune disorder and the most frequent demyelinating disease of the central nervous system (CNS). MS can affect all functional systems of the CNS. Most frequent symptoms are sensory deficits, weakness of one or several limbs, optic neuritis, cerebellar or brainstem dysfunction, and cognitive impairment (Noseworthy et al. 2000; Dyment et al. 2004; Hemmer et al. 2006). Based on findings in MS patients and animal models, a Th1/Th17 T cell-mediated immunopathogenesis, together with additional pro-inflammatory mediators is widely believed to play a key role in the development of the symptoms and signs of MS (El-Behi et al. 2011; Codarri et al. 2011).

In order to study whether T cells could be potential targets of zinc supplementation in autoimmune diseases like MS, we investigated here the effect of zinc aspartate on T cell activation in vitro and on T cell-mediated autoimmunity in vivo. Specifically, the influence of zinc aspartate, a commercially available zinc supplement, on DNA synthesis as well as IL-2, IL-10 and IL-17 production was measured in human stimulated T cells and in activated mouse splenocytes. Furthermore, the effect of preventive and therapeutic application of zinc aspartate was examined in vivo in experimental autoimmune encephalomyelitis (EAE), the animal model of MS.

Materials and methods

Materials

Zinc aspartate (Unizink®), a registrated pharmaceutical infusion solution with good bioavailability was

purchased from Köhler Pharma GmbH (Alsbach-Hähnlein, Germany). Mouse anti-human CD3 (OKT3) hybridoma supernatants were produced in our institute. Proteolipid protein (PLP) peptide (p)139–151, corresponding to mouse sequence (HSLGKWLGHPDKF) was synthesized on a peptide synthesizer and purified by HPLC.

Mice

Female SJL/J and C57BL/6 mice, age 10–12 weeks, were purchased from JANVIER (LE GENEST-ST-ISLE, France) and housed in the animal facilities of the medical faculty of the Otto-von-Guericke-University, Magdeburg. All procedures were conducted according to protocols approved by the Institutional Animal Care and Use Committee.

Cells

Human peripheral blood mononuclear cells (PBMC) were isolated by Ficoll gradient (Biochrom, Berlin, Germany) centrifugation of heparinized blood collected from healthy volunteers. Human T cells were further purified by non-T cell depletion using the "Pan T cell isolation kit II" (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Cells were washed twice and resuspended in serum-free AIM-V culture medium (Invitrogen, Eggenstein, Germany). The study was approved by the local ethics committee. Blood donors gave written informed consent.

Splenocytes were obtained from naïve C57BL/6 mice by separation of the spleen using cell strainers (Falcon, Heidelberg, Germany). Erythrocytes were removed by lysis with a hypotonic ACK-buffer containing 10 mM $KHCO_3$, 150 mM NH_4Cl and 0.1 mM EDTA (pH 8.0). Cells were washed twice and resuspended in AIM-V medium supplemented with 10 μM 2-β-mercaptoethanol (Merck, Darmstadt, Germany).

Proliferation and cell viability assays

For proliferation assays, human T cells (10^5 cells/well) were incubated in quadruplicate cultures in flat-bottom 96-well microtiter culture plates (Falcon, Heidelberg, Germany) with pokeweed mitogen (PWM, 2 μg/ml, Sigma, Taufkirchen, Germany) or plate-bound anti-CD3 antibodies (clone OKT3) in the presence of increasing concentrations of zinc aspartate or AIM-V culture medium as vehicle control. Cell cultures were incubated for 96 h at 37°C.

Mouse splenocytes (10^5 cells/well) were incubated in quadruplicate cultures in round-bottom 96-well microtiter culture plates (Falcon, Heidelberg, Germany) with PWM (1 μg/ml, Sigma, Taufkirchen, Germany) or plate-bound anti-mouse CD3ε antibodies (clone 145-2C11, BD Biosciences, Heidelberg, Germany) in the presence of increasing concentrations of zinc aspartate. Cell cultures were incubated for 72 h at 37°C.

Proliferation was assessed by measuring [^{3}H]thymidine [^{3}H-TdR] incorporation. [^{3}H]thymidine was added at 0.2 μCi/well for the last 6 h (human T cells) or 16 h (mouse splenocytes) of the incubation. At the end of the incubation period, cells were harvested and radioisotope incorporation was measured as an index of lymphocyte proliferation using a betaplate liquid scintillation counter (MicroBeta, Wallac, Turku, Finland).

Cell viability was determined in parallel cell cultures using the "CellTiter-Blue" cell viability assay (Promega, Madison, USA).

Cytokine measurements

For determination of IL-2, IL-10 and IL-17 secretion, human T cells (10^6 cells/ml) were cultured in AIM-V medium with PWM (2 μg/ml, Sigma, Taufkirchen, Germany) in presence of different concentrations of zinc aspartate or AIM-V culture medium as vehicle control. Cell culture supernatants were harvested after 72 h (IL-2, IL-10) or after 6 days (IL-17) and stored at −70°C until cytokine determination. Cytokine concentrations of cell culture supernatants were determined with specific enzyme-linked immunosorbent assays (ELISA; R&D Systems, Wiesbaden, Germany) according to the manufacturer's instructions.

Mouse splenocytes (10^6 cells/ml) were stimulated with PWM (1 μg/ml, Sigma, Taufkirchen, Germany) in presence of different concentrations of zinc aspartate. Cell culture supernatants were harvested after 72 h (IL-2, IL-10) or after 6 days (IL-17) and stored at -70°C until cytokine determination. IL-2, IL-10 and IL-17 concentrations of cell culture supernatants were determined with specific ELISA (R&D Systems,

Wiesbaden, Germany) according to the manufacturer's instructions.

Induction of active EAE

Female SJL/J mice were immunized subcutaneously (s.c.) in depots distributed over four spots across the flanks with 200 µg PLP (p)139–151 in 0.2 ml emulsion consisting of equal volumes of PBS and complete Freund's adjuvant (CFA; Sigma, Taufkirchen, Germany), containing 4 mg/ml of *Mycobacterium tuberculosis* H37Ra (Difco, Detroit, MI). 200 ng pertussis toxin (PTX; List Biological Laboratories, Campbell, CA) was administered intraperitoneally (i.p.) at days 0 and 2 (Reinhold et al. 2011).

Treatment of EAE

6 µg/day [0.3 mg/kg body weight (BW)], 30 µg/day (1.5 mg/kg BW), or 120 µg/day (6 mg/kg BW) zinc aspartate were injected i.p. from day 1 to 10 after immunization (preventive treatment) or from day 11 to day 19 (therapeutic treatment). Mice injected with equal amounts of PBS (vehicle) served as controls.

Clinical evaluation of EAE

Mice were scored daily for clinical signs of EAE according to the following increasing severity scale: 0: no disease; 1: tail weakness (tail plegia); 2: hindlimb paraparesis and/or weak rightning-reflex; 3: hindlimb paraplegia; 4: paraplegia with forelimb weakness or paralysis; 5: moribund animals. Mice with intermediate clinical signs were scored in 0.5 increments. For reasons of animal welfare, mice were killed when reaching a score of 3 or above. Mean clinical scores at each day were calculated by adding disease scores of individual mice divided by the number of mice in each group (Reinhold et al. 2011).

Statistical analysis

Statistical comparison of EAE disease severity was accomplished by performing a Wilcoxon analysis as previously described (Fleming et al. 2005; Steinbrecher et al. 2001). Statistical analyses of cell proliferation, viability and cytokine production assays were performed using the ANOVA test.

Results

Zinc aspartate suppresses DNA synthesis as well as IL-2, IL-10 and IL-17 production of stimulated human T cells

In order to study the role of zinc in T cell activation in vitro, we first examined the influence of different concentrations of zinc aspartate on proliferation (DNA synthesis) in human PWM- and anti-CD3-stimulated T cells. As shown in Fig. 1a, zinc aspartate suppresses the DNA synthesis of these cells in a dose-dependent manner, with IC_{50} values in the range of 125 µM for PWM-stimulated T cells and 40 µM for anti-CD3-stimulated T cells. To exclude possible cytotoxic

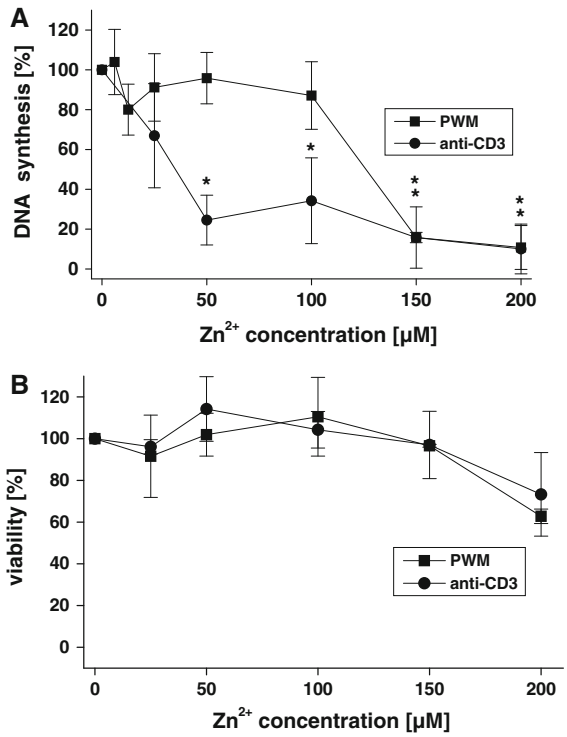

Fig. 1 Zinc aspartate suppresses DNA synthesis in stimulated human T cells. Human T cells were stimulated with PWM or anti-CD3 antibodies in the presence of different concentrations of zinc aspartate or vehicle. **a** Cells were cultured for 96 h and DNA synthesis determined by standard 3H-thymidine uptake. [3H-TdR] incorporation is shown as mean percentage ± SD of DNA synthesis in relation to control cultures (PWM: 84,785 ± 36,685 cpm, anti-CD3: 85,622 ± 78,052 cpm) set as 100%. Data plotted represent the means from eleven (PWM) and six (anti-CD3) independent experiments. **b** Cell viability was determined in parallel cell cultures using the "CellTiter-Blue" cell viability assay (mean ± SD, $n = 4$, $^*p < 0.05$)

effects of zinc aspartate, we measured the viability of cell cultures using the "CellTiter-Blue" cell viability assay and observed that the viability of PWM- and anti-CD3-stimulated T cells was not impaired by zinc aspartate concentrations up to 150 μM under the experimental conditions used (Fig. 1b).

In further experiments, the influence of zinc aspartate on IL-2, IL-10 and IL-17 production in PWM-stimulated T cells was investigated. We found that zinc aspartate concentrations higher than 100 μM were capable of suppressing IL-2 and IL-10 as well as IL-17 production of PWM-stimulated T cells (Fig. 2a–c).

Zinc aspartate suppresses DNA synthesis as well as IL-2, IL-10 and IL-17 production of stimulated mouse splenocytes

Next, we studied the influence of zinc aspartate on DNA synthesis in PWM- and anti-CD3-stimulated splenocytes from C57BL/6 mice. As shown in Fig. 3a, zinc aspartate suppresses the DNA synthesis of these cells in a dose-dependent manner, with IC_{50} values in the range of 60 μM (PWM) and 70 μM (anti-CD3). The viability of mouse splenocytes was not impaired by concentrations up to 100 μM under these experimental conditions (Fig. 3b). Like in human T cells, we measured the effect of different concentrations of zinc aspartate on IL-2, IL-10 and IL-17 production in PWM-stimulated mouse splenocytes. As expected, incubation with zinc aspartate results in significant suppression of the production of all three cytokines in these cells in a dose-dependent manner (Fig. 4a–c).

Based on these in vitro findings, we decided to study the effects of zinc aspartate treatment in vivo in the EAE, an established effector T cell-mediated autoimmune model of MS.

Preventive and therapeutic administration of zinc aspartate led to a significant reduction of the clinical severity of the EAE

Active EAE in SJL/J mice was induced by immunization with PLP (p)139–151 and the effect of zinc aspartate was studied after i.p. administration in both a preventive and therapeutic manner (Figs. 5, 6). For investigation of preventive administration, treatments started on day 1 after immunization of mice. Mice were treated daily with 6, 30, or 120 μg zinc aspartate, or vehicle control from day 1 to day 10.

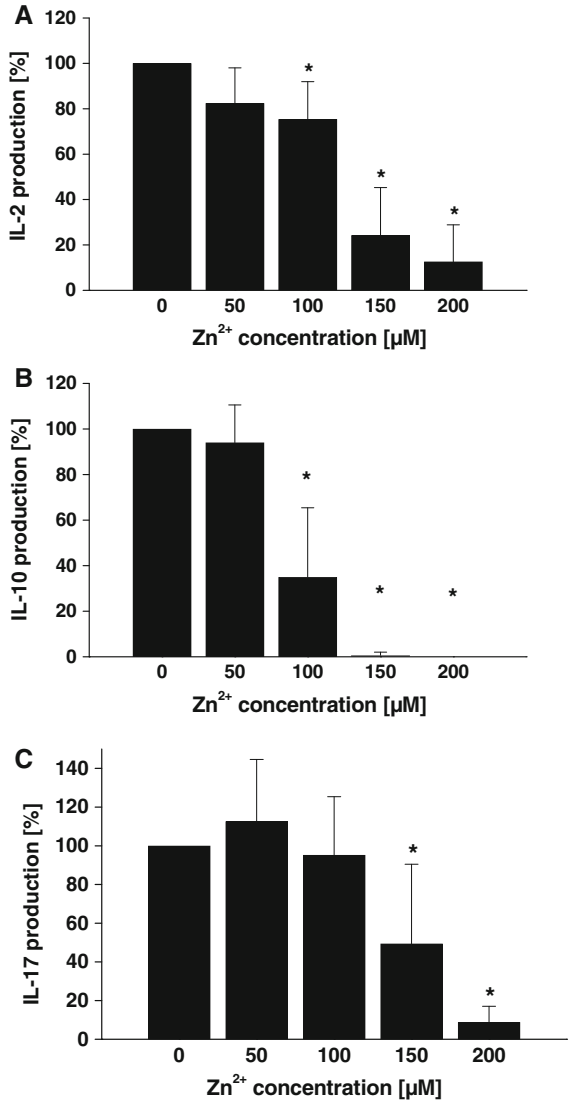

Fig. 2 Zinc aspartate suppresses IL-2, IL-10 and IL-17 production in stimulated human T cells. Human T cells were stimulated with PWM in the presence of different concentrations of zinc aspartate or vehicle. Cell culture supernatants were harvested after 72 h (IL-2 and IL-10) or after 6 days (IL-17) and the cytokine concentrations were determined with specific ELISA. IL-2 (**a**), IL-10 (**b**) and IL-17 (**c**) production are shown as mean percentage + SD of cytokine production in relation to control cultures (PWM: 2,843 ± 1,762 pg/ml IL-2; 1,562 ± 888 pg/ml IL-10; and 117.8 ± 28.5 pg/ml IL-17) set as 100%. Supernatants from unstimulated cultures served as background controls (medium: 3.3 ± 4.6 pg/ml IL-2; 1.8 ± 2.6 pg/ml IL-10; and 7.2 ± 10.1 pg/ml IL-17). Data plotted represent the means from eight (IL-2), four (IL-10) and six (IL-17) independent experiments; $*p < 0.05$

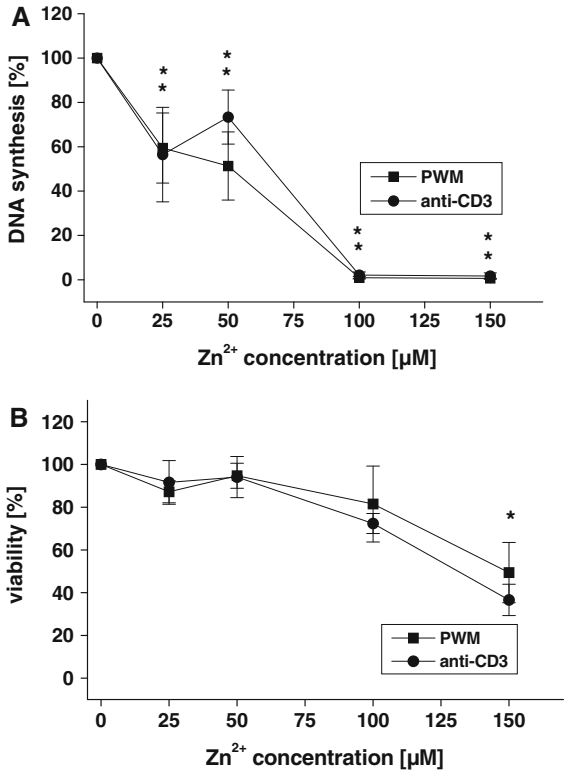

Fig. 3 Zinc aspartate suppresses DNA synthesis on stimulated splenocytes of C57BL/6 mice. **a** Mouse splenocytes were stimulated in 96-well microtiter culture plates (10^5 cells/well) with PWM or anti-CD3 antibodies in the presence of different concentrations of zinc aspartate for 72 h and DNA synthesis determined by standard 3H-thymidine uptake. [3H-TdR] incorporation is shown as mean percentage ± SD of DNA synthesis in relation to control cultures (PWM: 41,117 ± 13,703 cpm, anti-CD3: 66,722 ± 42,242 cpm) set as 100%. Data plotted represent the means from four independent experiments. **b** Cell viability was determined in parallel cell cultures using the "CellTiter-Blue" cell viability assay (mean ± SD, $n = 4$, $^*p < 0.05$)

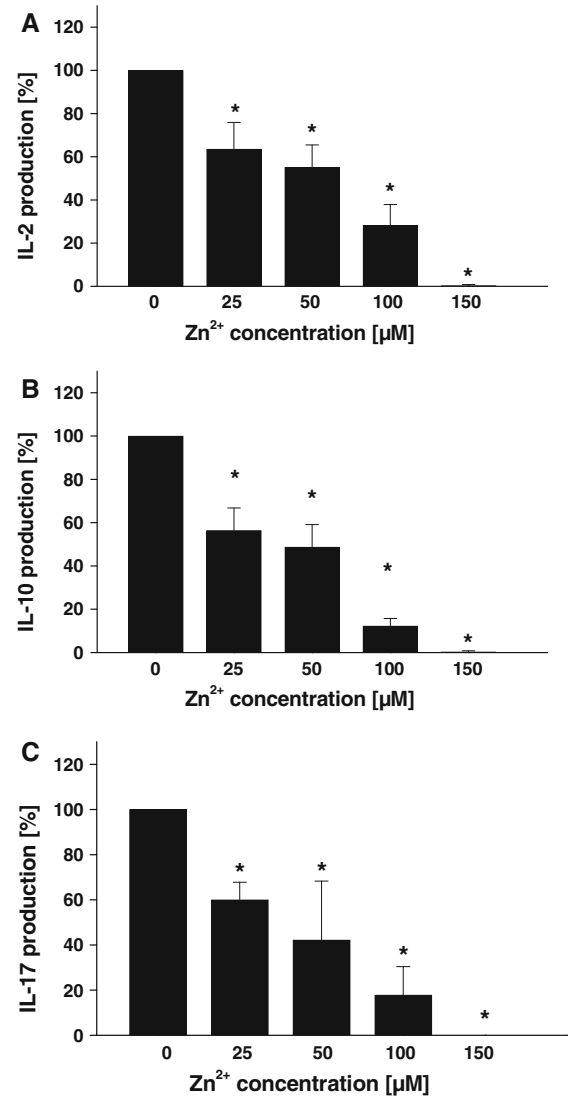

Fig. 4 Zinc aspartate suppresses IL-2, IL-10 and IL-17 production in stimulated splenocytes of C57BL/6 mice. Mouse splenocytes were stimulated with PWM in the presence of different concentrations of zinc aspartate. Cell culture supernatants were harvested after 72 h (IL-2 and IL-10) or after 6 days (IL-17) and concentrations of these murine cytokines were determined with specific ELISA. IL-2 (**a**), IL-10 (**b**) and IL-17 (**c**) production is shown as mean percentage + SD of cytokine production in relation to control cultures (PWM: 1,429 ± 223 pg/ml IL-2; 1,453 ± 1,064 pg/ml IL-10; and 596 ± 232 pg/ml IL-17) set as 100%. Supernatants from unstimulated cultures served as background controls (medium: 1.58 ± 3.17 pg/ml IL-2; 5.47 ± 6.56 pg/ml IL-10; and 0.2 ± 0.4 pg/ml IL-17). Data plotted represent the means from four independent experiments; $^*p < 0.05$

As shown in Fig. 5b, administration of 30 µg zinc aspartate per day led to a significant reduction of the clinical severity of the EAE during the first relapse of the disease. The differences in the mean clinical scores between both groups were statistically significant between day 36 and day 66 ($p < 0.05$; Wilcoxon test). In contrast, lower zinc aspartate concentrations (6 µg/day) and higher zinc aspartate concentrations (120 µg/day) had no significant influence on the clinical severity of the EAE (Fig. 5a, c).

In next experiments, the effect of zinc aspartate was studied in a therapeutic manner. Treatments started after onset of first clinical signs, at the beginning of acute clinical disease, on day 11. Mice were treated also with 6, 30, or 120 µg/day zinc aspartate, or vehicle control from day 11 to day 19 (Fig. 6). As

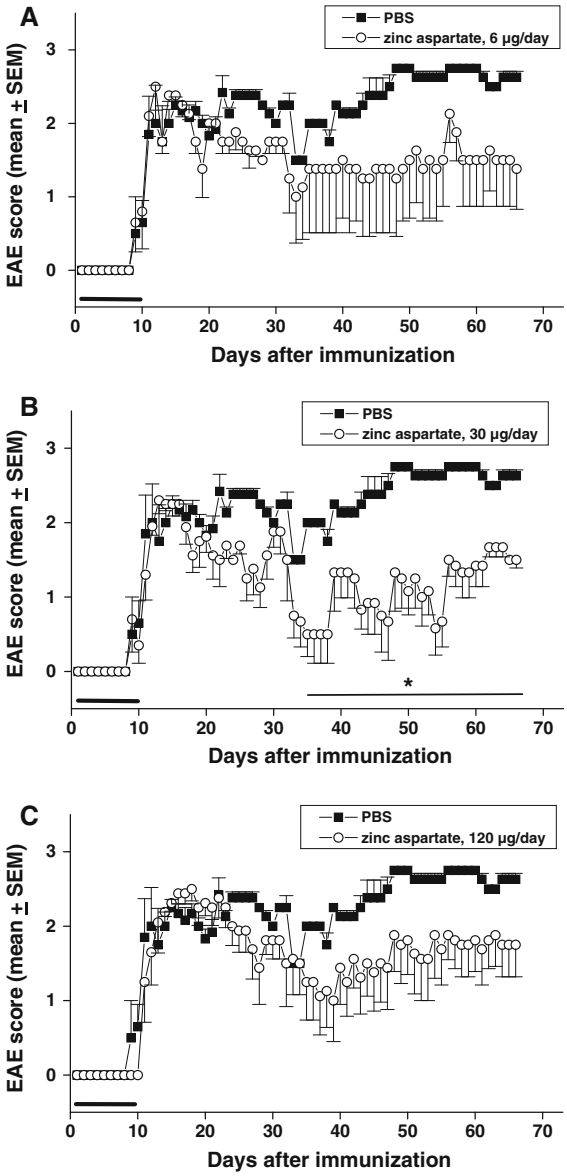

Fig. 5 Effects of preventive application of zinc aspartate on clinical signs of EAE. EAE was induced by immunization of SJL/J mice with $PLP_{139–151}$ as described in "Materials and methods". Mice ($n = 5$ per group) were treated i.p. from day 1 to day 10 with 6 µg/day (0.3 mg/kg BW; **a**), 30 µg/day (1.5 mg/kg BW; **b**), or 120 µg/day zinc aspartate (6 mg/kg BW; **c**). PBS treatment served as vehicle control. Treatments are indicated by the *horizontal bar*. Clinical disease scores were recorded daily. Data are presented as daily averages of disease scores ± SEM; $^*p < 0.05$

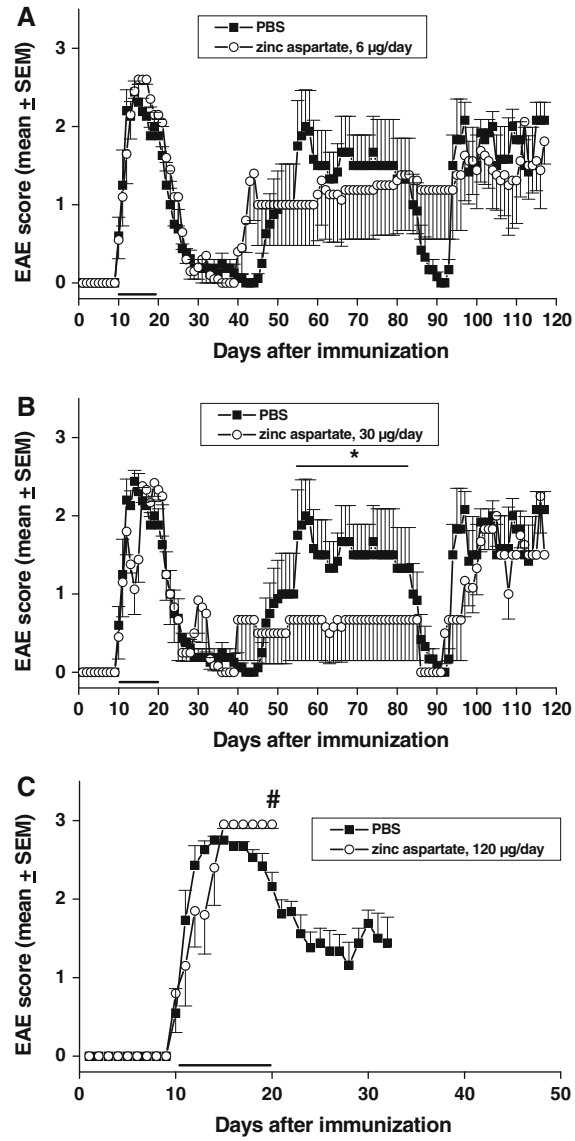

Fig. 6 Effects of therapeutic application of zinc aspartate on clinical signs of EAE. EAE was induced by immunization of SJL/J mice with $PLP_{139–151}$ as described in Materials and methods. Mice ($n = 5$ per group) were treated i.p. from day 11 to day 19 with 6 µg/day (0.3 mg/kg BW; **a**), 30 µg/day (1.5 mg/kg BW; **b**), or 120 µg/day zinc aspartate (6 mg/kg BW; **c**). PBS treatment served as vehicle control. Treatments are indicated by the *horizontal bar*. Clinical disease scores were recorded daily. Data are presented as daily averages of disease scores ± SEM; $^*p < 0.05$

observed in the preventive treatment (Fig. 5b), administration of 30 µg zinc aspartate per day led to a significant reduction of the clinical severity of the EAE during the first relapse of the disease (Fig. 6b). The differences in the mean clinical scores between both groups were statistically significant between day 55 and day 82 ($p < 0.05$; Wilcoxon test). In contrast, lower zinc aspartate concentrations of 6 µg per day had no significant therapeutic effects on the severity of

the EAE (Fig. 6a). The application of higher amounts of zinc aspartate (120 μg/day) led to an strong increase of the severity of the EAE. All animals of this group were sacrificed for ethical reasons in line with the rules of the ACC protocol (Fig. 6c). In the other experimental groups with preventive (Fig. 5) or therapeutic application of zinc aspartate (Fig. 6a, b) mice had not to be sacrificed because of to high EAE scores. The variance in severity and course of clinical EAE in vehicle (PBS) treated mice is frequently observed in this disease model and reflect the biological variability of disease induction in vivo (Brocke et al. 1996; Steinbrecher et al. 2001; Biton et al. 2011; Reinhold et al. 2011).

Discussion

Work of different groups has clearly established that zinc is an essential regulator of the immune system and capable of stimulating or suppressing different immune cells in vitro and in vivo. These effects are dose- and cell type-dependent (Wellinghausen et al. 1997; Rink and Haase 2007). In the present study, we determined several previously unrecognized effects of zinc aspartate (Unizink®), a commercially available zinc supplement with a good bioavailability, on proliferation and cytokine production of stimulated human T cells and mouse splenocytes in vitro and in mouse EAE in vivo.

First, we analysed the influence of zinc aspartate on the viability of the immune cells used in our experiments. We observed that the viability of human stimulated T cells was not impaired by zinc aspartate concentrations up to 150 μM and of stimulated mouse splenocytes by concentrations up to 100 μM. This is in line with the observations of other authors, who showed previously that the viability of stimulated PBMC or of PBMC in mixed lymphocyte cultures (MLC) is not impaired by 100 μM $ZnSO_4$ (Chang et al. 2006; Campo et al. 2001).

In further experiments, we could show that zinc aspartate is capable of suppressing both, the proliferation (DNA synthesis) as well as the production of IL-2, IL-10 and IL-17 of stimulated human T cells and of stimulated mouse splenocytes dose-dependently. Interestingly, in mouse splenocytes the IC50 value of inhibition of DNA synthesis was two times lower as in human T cells (70 and 150 μM, respectively). These data confirm our previous observations that concentrations of several zinc derivatives ($ZnCl_2$, ZnO and $ZnSO_4$) higher than 100 μM suppress the proliferation and cytokine production (IL-2, IL-6 and IL-10) of mitogen-stimulated human PBMC (Reinhold et al. 1997, 1999). Furthermore, these results are in support of data from Campo and coworkers, who showed that $ZnSO_4$ is capable of decreasing the production of IFN-γ of human PBMC in MLC dose-dependently (Campo et al. 2001).

Interestingly, zinc has been reported to affect the regulation of IL-6/STAT3 signalling cascade, resulting in development of IL-17-producing CD4 cells (Th17 cells) (Kitabayashi et al. 2010). These Th17 cells have been suggested as major contributors to the pathogenesis of autoimmune inflammation (Weaver et al. 2006, 2007; Bettelli et al. 2007; Steinman 2007) although their enecphalitogenicity was shown to be dependent on other pro-inflammatory cytokines (El-Behi et al. 2011).

Here, we could show for the first time that zinc aspartate is capable of suppressing the IL-17 production of stimulated human T cells and of stimulated mouse splenocytes in dose-dependent manner. Kitabayashi et al. described that zinc suppresses Th17-mediated autoimmune diseases at least in part by inhibiting the development of Th17 cells via attenuating STAT3 activation (Kitabayashi et al. 2010). While IL–17 was shown to play an important role in the development of EAE (Hofstetter et al. 2007; Komiyama et al. 2006), other reports have challenged these findings (Haak et al. 2009; Codarri et al. 2011). Nevertheless, the therapeutic efficacy of IL-17 neutralization and IL-17 vaccination has been demonstrated by various groups (Hofstetter et al. 2005; Röhn et al. 2006; Uyttenhove and Van Snick 2006.

Based on this knowledge and on our in vitro observations, zinc supplementation in vivo may provide a potent approach to modulate T cell functions and tissue-specific autoimmunity in the CNS.

Thus, in further experiments, we studied the application of zinc aspartate in EAE. SJL/J mice with chronic EAE were treated preventively and therapeutically with different concentrations of zinc aspartate. Preventive treatments started on the first day after immunization of mice. While administration of zinc aspartate suppressed EAE at all doses tested (6 μg–120 μg/day), we found that only the dose of 30 μg zinc aspartate per day given from day 1 to day 10 led to a

significant reduction of the clinical severity of the EAE during the first relapse of the disease. These data show that zinc aspartate treatment has profound effects during the sensitization phase of EAE which mainly occurs within the peripheral immune system.

In a treatment paradigm more relevant for the clinical management of human disease, we next tested the therapeutic application of zinc aspartate initiated at the peak of the first episode of acute clinical disease—between days 11 and day 19. At this stage in CNS inflammation, the major targets of immune therapy are T cells infiltrating the brain and spinal cord as well as resident CNS cells and recruited inflammatory leukocytes. Our results show that administration of 30 μg/day (1.5 mg/kg BW) zinc aspartate led to a more profound reduction of the clinical severity of EAE as compared to lower zinc concentrations (6 μg/day) or the use of PBS as control substance. Importantly, therapeutic application of higher amounts of zinc aspartate (120 μg/day) led to an increase of the severity of the EAE. It is unclear whether the increase in EAE severity is solely based on the toxicity of high zinc doses as would be suggested by our in vitro data, or whether as yet unknown specific effects of higher zinc doses on the immune system are responsible for the observed exacerbation.

To the best of our knowledge this report is the first description of a therapeutic effect of zinc application in the EAE model. The effect of preventive zinc supplementation in the EAE model has previously been studied by several groups. Preventive supplementation of $ZnSO_4$ in EAE disease of SJL/J mice (Schiffer et al. 1990) and of $ZnCl_2$ in EAE of Lewis rats (Penkowa and Hidalgo 2000) had no effect on the disease. Recently, a significant effect of preventive zinc supplementation on the severity of disease was shown in MOG (35–55) peptide-induced active EAE in C57BL/6 mice (Kitabayashi et al. 2010). Conceivably, the different bioavailability of the zinc supplements, and the different application protocols used are responsible for these contradictory results.

Taken together, the data of the present paper support the hypothesis that zinc can suppress activation, proliferation and Th1/Th17 cytokine production of T cells in vitro. Moreover, our in vivo results suggest that controlled zinc aspartate supplementation could be indicated and helpful in patients with MS. Interestingly, mild zinc deficiency is observed in sera of patients suffering from MS (Leopold 1981). It is important to note that zinc supplementation with high concentrations of zinc in terms of immunosuppression has to be under control of serum zinc levels. Long-time administration of high dosages of zinc can not only induce suppression of immune cells, but can cause copper or iron deficiency, anemia and growth retardation (Prasad et al. 1978; Porter et al. 1977; Fosmire 1990). Collectively, our data indicate that an optimal level of serum zinc is associated with efficient immunoregulation in vivo, while low or very high zinc levels can disturb tightly controlled autoreactive immune responses and lead to an exacerbation of autoimmune disease. In conclusion, our and other studies strongly suggest that further preclinical and clinical studies should be initiated to investigate the effect of zinc supplementation in T cell-mediated autoimmune diseases like MS.

Conflict of Interest Statement The authors declare that there are no conflicts of interest.

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