Efficacy of vitamin D in treating multiple sclerosis-like neuroinflammation depends on developmental stage

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ABSTRACT

The association of vitamin D deficiency with higher prevalence, relapse rate and progression of multiple sclerosis (MS) has stimulated great interest in using vitamin D supplementation as a preventative measure and even a therapy for established MS. However, there is a considerable lack of evidence when it comes to an age/developmental stage-dependent efficacy of vitamin D action and a time-window for the most effective prophylactic treatment remains unclear.

We studied the effect of vitamin D supplementation in myelin oligodendrocyte glycoprotein (MOG)-induced experimental autoimmune encephalomyelitis (EAE), an animal model of MS, at three different developmental stages in rats. Supplementation treatment was initiated: i) prior to gestation and maintained throughout pre- and early postnatal development (gestation and lactation); ii) after weaning, throughout juvenile/adolescence period and iii) in adult age. We observed a marked attenuation of EAE in juvenile/adolescent rats reflected in a less severe CNS inflammation and demyelination, accompanied by a lower amount of IFN-γ producing MOG-specific T cells. Moreover, the cytokine expression pattern in these rats reflected a more anti-inflammatory phenotype of their peripheral immune response. However, the same supplementation regimen failed to improve the disease outcome both in adult rats and in rats treated during pre- and early post-natal development.

Our data demonstrate a developmental stage-dependent efficiency of vitamin D to ameliorate neuroinflammation, suggesting that childhood and adolescence should be the target for the most effective preventive treatment.

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Background

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) associated with demyelination, axonal loss and brain atrophy (Hemmer et al., 2002). MS likely develops in genetically predisposed individuals exposed to certain environmental factors (Fugger et al., 2009; Sawcer et al., 2011). A lack of vitamin D is one of the most frequently reported environmental factors in etiology of MS (Ascherio et al., 2010; Pierrot-Deseilligny and Souberbielle, 2010).

Epidemiological studies indicate that MS prevalence varies with geographical latitude (Pugliatti et al., 2002; Simpson et al., 2011) and it is increased in regions further south or north beyond the 40th parallels (Webb et al., 1988). Furthermore, the correlation between MS incidence

and UVB radiation is 20 times higher than calculated with latitude alone (Sloka et al., 2011). UVB is required for producing active vitamin D in the skin (Holick, 1995) and its production progressively decreases with geographical latitude (Chapuy et al., 1997), indicating an association of vitamin D and MS pathogenesis. Interestingly, MS prevalence is up to 50% lower in children born during autumn compared to those born during spring in Scotland (Willer et al., 2005). This could be related to sunlight/UVB exposure during pregnancy, as the serum levels of vitamin D are the lowest in spring and the highest in autumn. Notably, a recent study reported that MS risk was lower among women whose mothers had increased vitamin D intake during pregnancy (Mirzaei et al., 2011).

Low levels of circulating vitamin D are reported to predict subsequently increased risk for developing MS (Munger et al., 2006) and MS patients themselves are deficient in vitamin D (Nieves et al., 1994). These observations stimulated a great interest in using vitamin D supplementation not only as a preventative measure but also as a therapy for MS and other inflammatory diseases. However, the protective effect of vitamin D supplementation in MS has not yet been unequivocally established (Papeix and Lubetzki, 2013). In development of a successful treatment strategy it should be considered that many lifestyle and environmental factors associated with MS seem to act particularly during childhood and adolescence, such as migration (Gale and Martyn,

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1995), night-shift work (Hedstrom et al., 2011), an increased body mass index (Hedstrom et al., 2012) and even infectious mononucleosis (Handel et al., 2010). It has been reported that vitamin D supplementation during childhood decreases the risk of developing certain autoimmune diseases such as type I diabetes (Forouhi et al., 2008).

Based on these observations we suspected an age-dependent efficacy of vitamin D to prevent or ameliorate neuroinflammation and we aimed to elucidate the most appropriate timing for the treatment. To that end we analyzed the effects of vitamin D supplementation on myelin oligodendrocyte glycoprotein (MOG)-induced experimental autoimmune encephalomyelitis (EAE) in Dark Agouti (DA) rats during different developmental stages. MOG-EAE in DA rats displays striking similarities to MS: a relapsing–remitting disease course, MHC-dependence, histopathological features and pathogenic T- and B-cell responses (Gold et al., 2000; Storch et al., 1998; Weissert et al., 1998). It has been extensively shown that vitamin D can prevent and block progression of EAE in mice (Joshi et al., 2011; Lemire and Archer, 1991; Meehan and DeLuca, 2002; Nashold et al., 2009; Spach et al., 2006). Importantly, the vitamin D receptor (VDR) is not only necessary for the suppressive effect of vitamin D on EAE (Meehan and DeLuca, 2002), but EAE does not develop in animals deficient in vitamin D and VDR (Wang et al., 2012).

Our data demonstrate that only during juvenile/adolescence period, but not during other developmental stages, a five-fold elevated intake of vitamin D was sufficient to significantly ameliorate EAE. The protective effect was reflected in decreased brain inflammation and better myelin and axon preservation accompanied by an anti-inflammatory cytokine expression pattern.

Materials and methods

Ethical statement

All experiments in this study were performed in accordance with the guidelines from the Swedish National Board for Laboratory Animals and the European Community Council Directive (86/609/EEC) under ethical permits approved by the North Stockholm Animal Ethics Committee (Stockholms Norra djurförsöketiska nämnd). Rats were tested according to a health-monitoring program at the National Veterinary Institute (Statens Veterinärmedicinska Anstalt, SVA) in Uppsala, Sweden.

Animals, vitamin D supplementation and EAE induction

Inbred Dark Agouti (DA) rats were housed in the animal facility at Karolinska Hospital (Stockholm, Sweden) in a specific pathogen-free and climate-controlled environment in polystyrene cages containing aspen wood shavings with free access to rodent chow and water with regulated 12-hour light/dark cycles free of UV radiation.

Experimental setting and diet regime based on different contents of vitamin D_3 (cholecalciferol; referred to as vitamin D) is present in detail in Fig. 1. Age-matched inbred female rats were subjected to either: i) supplemented diet containing five-fold increased amount of vitamin D than the regular rat diet (10 International Units/g (IU/g)); ii) regular rat diet containing 2 IU/g of vitamin D; or iii) vitamin D deprived diet (0 IU/g). The supplemented and deprived diet formulations were obtained from TestDiet Limited (London, UK).

All rats used in this study were weaned at an age of three weeks. Newly weaned pups and three month old, young adult rats were assigned into groups and supplied with one of the three different diets four to five weeks prior to immunization and continuously until the end of the EAE experiment. Each experimental group contained at least six and at most 16 animals (repeated twice, for details please see Supplementary Table 1). In order to analyze the effect of vitamin D during pre- and early postnatal development, DA females were fed with either deprived, regular or supplemented vitamin D diet starting five to six weeks prior to gestation then continuously during gestation, throughout lactation until weaning of the pups. From weaning until
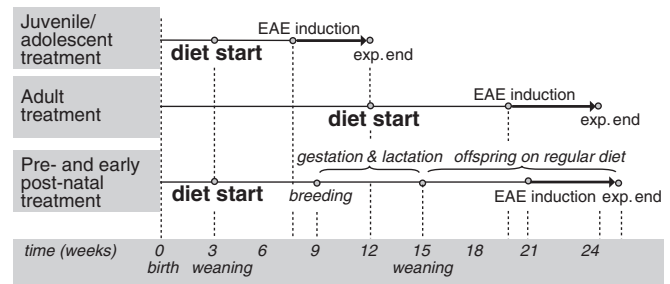

Fig. 1. Diet regimen and experimental outline. For juvenile/adolescent and adult treatments, 3-week-old newly-weaned and 3-month-old, respectively, female rats were assigned into three groups and subjected to either: i) vitamin D deprived diet; ii) diet containing a regular amount of vitamin D or iii) a vitamin D supplemented diet. EAE was induced four to five weeks after initiation of the diet regimen in both juvenile/adolescent and adult rats and lasted for the next 31 days p.i, when the animals were sacrificed for histopathological and IHC analyses. For pre- and early post-natal treatments, 3-week-old newly weaned female rats were subjected to one of the three diets 5 to 6 weeks prior to breeding, throughout gestation and lactation until weaning of the pups. The dams were sacrificed after weaning and their offspring was subsequently subjected to diet containing a regular amount of vitamin D until the end of the experiment. In these pre- and early post-natally treated offspring EAE was induced 6 to 7 weeks after their weaning and lasted 31 days p.i.

the end of the experiment all offspring received regular diet. Six to 11 animals were assigned into each experimental group (repeated twice; Supplementary Table 1). Supplemented, deprived and control littermate offspring were age-matched and immunized to induce EAE at the young adult age of 10–12 weeks.

Myelin oligodendrocyte protein (MOG, amino acids 1–125 from the N terminus) used for EAE induction was expressed in *Escherichia coli* and purified to homogeneity by chelate chromatography (Amor et al., 1994). The purified protein, dissolved in 6 M urea, was dialyzed against phosphate buffered saline (PBS) to obtain a physiological preparation that was stored at −70 °C. To induce EAE, rats were anesthetized with isoflurane (2-chloro-2-(difluoromethoxy)-1,1,1-trifluoro-ethane, Forene, Abbott Laboratories, Chicago, IL, USA) and injected subcutaneously in the tail base with a 200 μL inoculum containing 15 μg MOG in PBS, emulsified 1:1 with incomplete Freund's adjuvant (Sigma-Aldrich, St. Louis, MO, USA). The serum levels of 25-hydroxy vitamin D (25(OH)D) were estimated on day 7 post-immunization (p.i) using a chemiluminescent immunoassay from Diasorin (Diasorin AB, Sundbyberg, Sweden) and a LIAISON® instrument provided by Diasorin AB with equimolar measurement of both 25-OH vitamin D_2 and D_3. ELISA kits for 25(OH)D were purchased from Euroimmun AG (Luebeck, Germany) and used according to the manufacturer's instructions to confirm the measurements of 25(OH) D serum levels. This ELISA detects 25-OH vitamin D_2 and D_3 specifically and cross-reactions with other metabolites such as vitamin D_2, D_3 and cholesterol were less than 0.02% and 0.04%, respectively.

EAE phenotyping

Rats were monitored daily for clinical signs of EAE from day 7 p.i until sacrificed on day 30 or 31 p.i. The scoring of clinical symptoms was performed as follows: 0 = no detectable clinical signs; 1 = tail weakness or paralysis; 2 = hind limb hemiparesis or paraparesis; 3 = hind limb paralysis and 4 = tetraplegy or moribund. The following disease parameters were assessed for each animal: onset of EAE (the first day with clinical disease manifestation), maximum EAE score (the highest clinical score observed during EAE), cumulative EAE score (the sum of daily clinical scores) and duration of EAE (the number of days with manifested disease).

Quantitative real-time PCR

Five to seven animals from each diet regimen (experiment repeated twice) were sacrificed on day 7 p.i using CO_2 and draining inguinal lymph nodes were collected in DMEM (Gibco-BRL, Grand Island, NY,

USA) before being mechanically separated and passed through a mesh screen to obtain single cell suspension (SCS). Total RNA was extracted from the SCS using the RNeasy kit (Qiagen, Hilden, Germany) and the QIAcube (Qiagen) including on column DNA-digestion for fully automated sample preparation. RNA concentration and purity was determined through measurement of A260/A280 ratio with a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Confirmation of RNA quality was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). cDNA was prepared using the iScript kit (Bio-Rad, Hercules, CA, USA). Quantitative real-time PCR (qPCR) was performed using a CFX384 touch real time PCR detection system (Bio-Rad) with a two-step PCR protocol (95 °C for 10 min followed by 40 cycles at 95 °C for 10 s and 60 °C for 30 s and 45 cycles of melt curve analysis), using SYBR Green (Bio-Rad) as the fluorophore. Relative expression levels, corrected for amplification efficiency, were analyzed using the CFX manager software (Bio-Rad). Relative expression was calculated as the ratio between the target and *Gapdh*. Serial 10-fold dilutions from a pool of undiluted samples within the study were used as standard. The following primers were used for SYBR Green: *Gapdh*_fw TCA ACT ACA TGG TCT ACA TGT TCC AG, *Gapdh*_rev TCC CAT TCT CAG CCT TGA CTG; *Il2*_fw CGA TAA TT A CAA GAA TCT GAA ACT CC, *Il2*_rev TTT CCA GCG TCT TCC AAG TG; *Il4*_fw CTT ACG GCA ACA AGG AAC ACC, *Il4*_rev CTT TCA GTG TTG TGA GCG TGG; *Icam*_fw GCA GAT GGT GTC CCG CTG CC, *Icam*_rev CCA CGA TCA CGA AGC CCG CA; *Ifnγ*_fw AAA GAC AAC CAG GCC ATC AGC, *Ifnγ*_rev TGG CGA TGC TCA TGA ATG C; *Cd80*_fw TGA CTT CCC TAC CCC CAA CAT, *Cd80*_rev CGG AAG CAA AGC AGG TAA TCC; *Foxp3*_fw TGC CAC CTG GAA TCA ATG T, *Foxp3*_ rev CGT GGG AAG GTG CAG AGT AGA; *il17a*_fw CTC AGA CTA CCT CAA CCG TTC C, *il17a*_ rev GTG CCT CCC AGA TCA CAG AAG; *Rorc*_fw TTT GAA GGC AAA TAC GGT GGT G, *Rorc*_rev TGT CGA TGA GTC TTG CAG AGA TG.

FACS analysis

Six animals from each experimental group/diet regimen (experiment repeated twice) were sacrificed 7 days p.i. Obtained SCS from the draining inguinal lymph nodes was subsequently washed and cells were resuspended in PBS. A triple staining procedure was performed with the following targets: CD3-FITC, CD4-APC and CD45RA-PE (incubation with directly labeled primary antibodies purchased from BD Biosciences, San Jose, CA, USA, 1:100, for 30 min at 4 °C). Target staining and control staining were visualized using a FACS Calibur (BD, Franklin Lakes, NJ, USA) with CellQuest software (version 3.2.1f1, BD) and analyzed using Kaluza software (Beckman Coulter, CA, USA).

Elispot analysis

Four spleens were harvested from each vitamin D supplemented and deprived group (repeated twice) on day 10 p.i and placed in DMEM before being mechanically separated and passed through a mesh screen. Splenocytes of the rats treated with vitamin D supplemented or deprived diets were used for IFN-γ Elispot analysis (R&D systems) in 96 well plates. Concanavalin A (ConA, 0.125 ng/μL), myelin oligodendrocyte protein (MOG, 0.5 ng/μL) and myelin basic protein (MBP, 0.5 ng/μL) were used to stimulate 50,000 splenocytes/well. After 48 h incubation, IFN-γ production was visualized by antibody staining according to the manufacturer's protocol and the colony forming units (CFUs) were visualized using an AID Elispot reader (AID, Germany).

Histopathological and immunohistochemical analyses

On day 31 p.i, seven to 12 animals from each diet regimen were euthanized using CO_2 and perfused via the left heart ventricle with PBS followed by 4% paraformaldehyde. Paraformaldehyde-fixed 3–5 mm thick paraffin embedded sections of the brain and spinal cord were dewaxed in xylol, rehydrated and then stained with hematoxylin &

eosin (HE) and Luxol Fast Blue (Kluever, KL) to assess tissue inflammation and demyelination, respectively. The inflammatory index (I.I.) and demyelination score (DM) were determined from the number and size of demyelinated lesions in each animal on an average of fifteen complete spinal cord cross-sections as previously described (Storch et al., 1998).

For immunohistochemistry (IHC), paraffin sections of the spinal cord were treated as previously described (Bradl et al., 2005). After performing antigen retrieval as previously described (Adzemovic et al., 2012), sections were incubated with 10% fetal calf serum (FCS) in 0.1 mol/L PBS prior to incubation with primary antibody at 4 °C, overnight. After washing in PBS, sections were incubated with biotinylated secondary antibody in 10% FCS in PBS for 1 h at room temperature (anti-mouse or -rabbit, Amersham Pharmacia Biotech, 1:200). Finally, avidin peroxidase (1:100, Sigma, Vienna, Austria) was applied for 1 h at room temperature and subsequently visualized with 3,3′diaminobenzidine-tetrahydrochloride (DAB, Sigma, Austria). Evaluation was performed on at least fifteen whole spinal cord cross-sections. Imaging was performed using a Leica Polyvar 2 microscope. In adjacent serial sections IHC was performed by using antibodies against the following targets: lysosomes of rat activated macrophages and microglia cells (ED1, mouse anti-rat monoclonal antibody, AbD Serotec, Germany; 1:1000), rat T lymphocytes (W3/13, mouse anti-rat, Abcam, UK, 1:100) and anti-amyloid precursor protein (APP, mouse anti-rat, Millipore Chemikon, 1:500), complement components (mouse monoclonal anti-C1q antibody (Abcam UK, 1:100), mouse monoclonal complement C3 antibody (Novus Biologicals, UK, 1:400), rabbit polyclonal anti-C5b-9 antibody (Bioss, USA, 1:500)) and immunoglobulins (goat anti-rat IgG (biotinylated, GE Healthcare, 1:200) and goat anti-rat IgM antibody (biotinylated, Novus Biologicals, UK, 1:1000)). Control sections were incubated in the absence of primary antibody with non-immune FCS.

Statistical analysis

Statistical analyses of clinical EAE parameters, histopathology and qPCR data were performed using non-parametric Kruskal–Wallis test followed by Dunn's multiple comparison test for all pairs of analyzed groups. Statistical analyses of FACS data were performed with ANOVA followed by Bonferroni's multiple comparison for all pairs of analyzed groups. Since the regular diet (2 IU/g vitamin D) was primarily used as a control, statistical significances indicated in the figures refer to comparison between vitamin D deprived and vitamin D supplemented groups. Elispot data comparisons between the two diet regimens, for each stimulation, were analyzed with t-test.

Results

A vitamin D supplementation regimen is sufficient to ameliorate EAE only in juvenile/adolescent rats, but not in the other developmental stages

We analyzed the effect of the same vitamin D supplementation regimen on EAE at different developmental stages (Fig. 1). The supplementation was associated with a significantly milder EAE course in juvenile/adolescent rats, in contrast to the groups receiving regular or vitamin D deprived diet (Fig. 2A). In addition, both maximal and cumulative EAE scores and EAE duration were significantly reduced in juvenile/adolescent rats fed with the supplemented diet (Fig. 2B). The EAE incidence was 100% in the rats subjected to vitamin D deprived or regular diets and 89% in the rats receiving the supplementation. In contrast to juveniles/adolescents, this supplementation was insufficient to affect the severity of the clinical disease either in adult (Figs. 2C and D) or in the pre- and early post-natally treated rats (Figs. 2E and F). Notably, the five-fold enhanced vitamin D supplemented diet increased serum levels of 25(OH)D comparably in juvenile/adolescent and adult rats in contrast to rats receiving the vitamin D deprived diet: 385.4 ± 83.5 nmol/L versus (vs.) 11.3 ± 2.6 nmol/L (MV ± SD) in juvenile/adolescent and 389.0 ± 32.5 nmol/L vs. 93.9 ± 12.8 nmol/L (MV ±
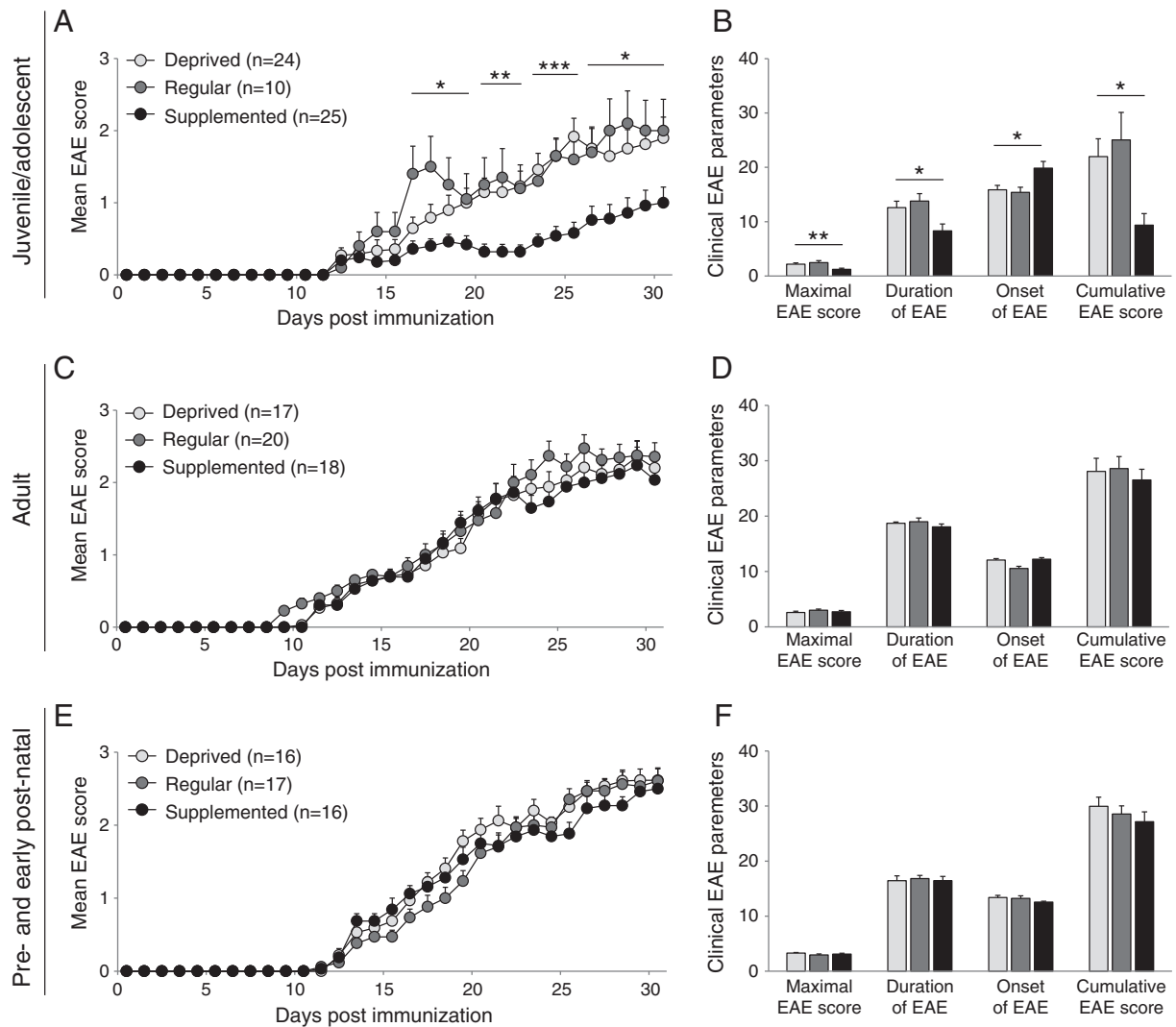
Fig. 2. The vitamin D supplementation regimen ameliorates clinical symptoms in juvenile/adolescent, but not in adult rats or in rats treated during pre- and early post-natal development. (A) Juvenile/adolescent rats subjected to the vitamin D supplemented diet developed less severe EAE than the groups receiving either vitamin D deprived or the regular diet. Such effect of the five-fold vitamin D enriched diet was not observed either in (C) adult or in (E) pre- and early post-natally treated rats. Graphical presentations of the maximal and cumulative EAE scores, duration and onset of EAE in juvenile/adolescent (B), adult and (D) pre- and early post-natally treated rats (F); data obtained from the experiments shown in A, C and E, respectively. Graphs represent data from two pooled independent experiments with similar outcome (details are given in Supplementary Table 1). All disease parameters presented were significantly downregulated due to the supplementation regimen in juveniles/adolescents, but not in adult or in rats treated during pre- and post-natal development. Error bars represent SEM. Statistics were calculated using Kruskal–Wallis test with Dunn's correction for multiple testing ($p < 0.05 = *$, $p < 0.01 = **$, $p < 0.001 = ***$). Statistical significances refer to comparison between vitamin D deprived and vitamin D supplemented groups.

SD) in adult rats. Serum levels of 25(OH)D of 193.0 ± 18.5 nmol/L ($MV \pm SD$) were measured in juveniles/adolescents fed with the diet containing a regular amount of vitamin D.

According to histopathological and IHC analyses, juveniles/adolescents, but not adult rats subjected to the supplemented diet displayed significantly less severe neuroinflammation and myelin loss than the age-matched groups receiving either vitamin D deprived or regular diets (Fig. 3). Milder clinical disease course in juvenile/adolescent rats subjected to the supplemented diet was mirrored in less numerous, lower size demyelinated lesions, overall lower recruitment of ED1^{+} macrophages and less abundant axonal damage. The amount of infiltrating W3/13[+] lymphocytes did not, however, significantly differ

between the diet regimens in both age groups. The pathology of juvenile/adolescent spinal cord affected with demyelinated lesions was further analyzed for antibody extravasation and complement activation. Staining with isotype-specific antibodies against IgG and IgM revealed the highest immunoreactivity in the groups that were receiving either regular or vitamin D deprived diet (Fig. 4). Similar to C3 (Fig. 4), deposition of C1q complement component was generally reduced upon vitamin D supplementation (data not shown), corresponding to overall lower extent of demyelination observed in this group. Notably, in contrast to rats that received vitamin D deprived diet or regular amounts of vitamin D, the presence of C5b-9 was almost completely abolished in the vitamin D supplemented group (Fig. 4).

Fig. 3. The vitamin D supplementation regimen ameliorates CNS inflammation and demyelination in juvenile/adolescent, but not in adult rats. Group representative images of the spinal cord cross-sections of rats subjected to different levels of vitamin D in their diet. Histopathological and IHC analyses performed on 7–12 paraffin embedded spinal cords harvested on day 31 p.i of each experimental group/diet regime, for both juvenile/adolescent and adult rats. Tissue cross-sections were stained with Kluever (KL; AI–CI and JI–LI), Hämalaun & eosin (HE; AII–CII and JII–LII) and also against ED1 (AIII–CIII and JIII–LIII), W3/13 (AIV–CIV and JIV–LIV) and APP (AV–CV and JV–LV) in order to reveal demyelination score (DM; D, M) and inflammatory index (I.I.; E, N) in juvenile/adolescent and adult rats, respectively and also recruitment of ED1^{+} macrophages (F O), W3/13^{+} T cells (G, P) and axonal damage (APP; H, Q). Error bars represent SEM. Statistics were calculated using Kruskal–Wallis test with Dunn's correction for multiple testing ($p < 0.05 = *$, $p < 0.01 = **$). Statistical significances refer to comparison between vitamin D deprived and vitamin D supplemented groups.
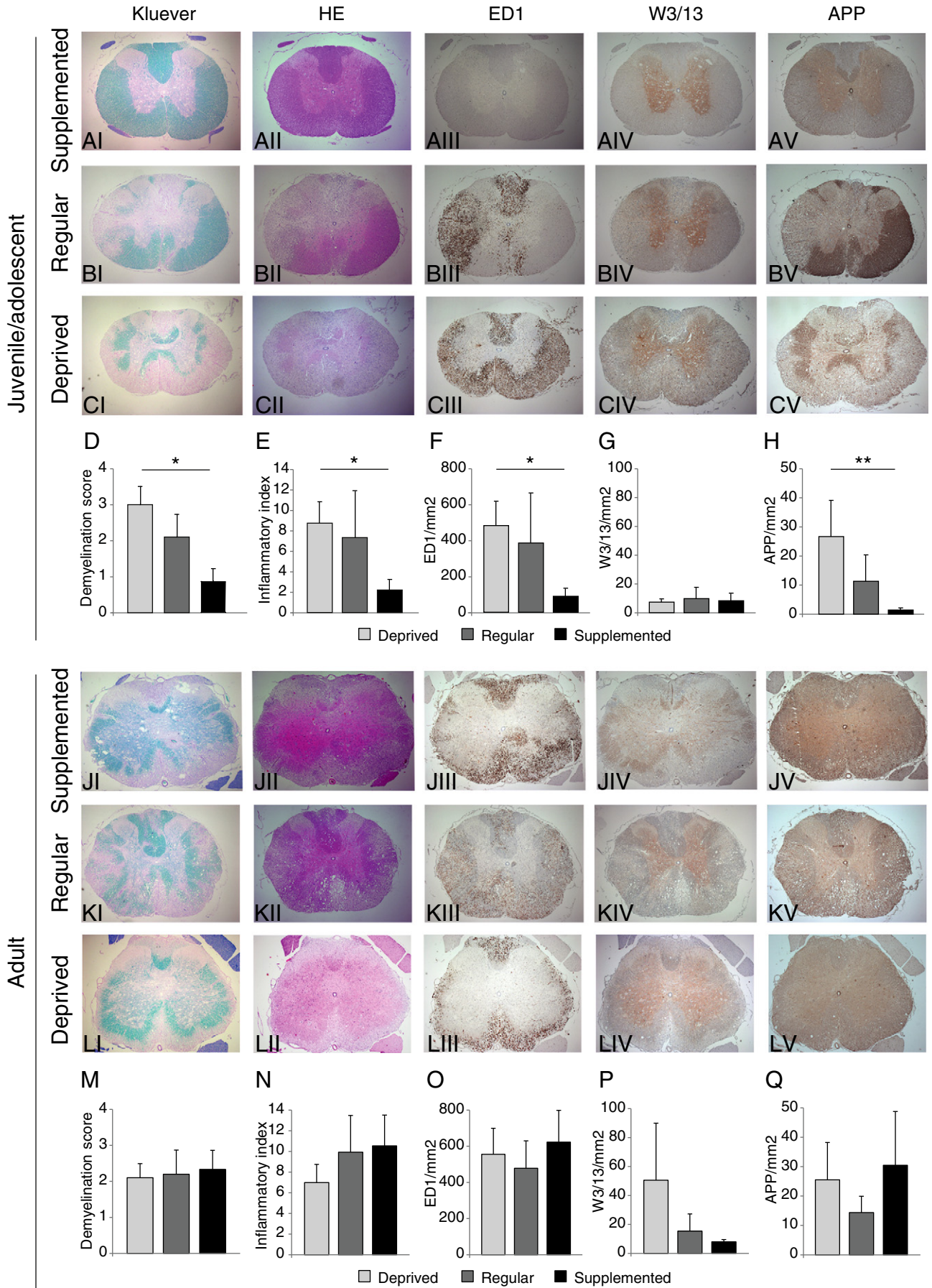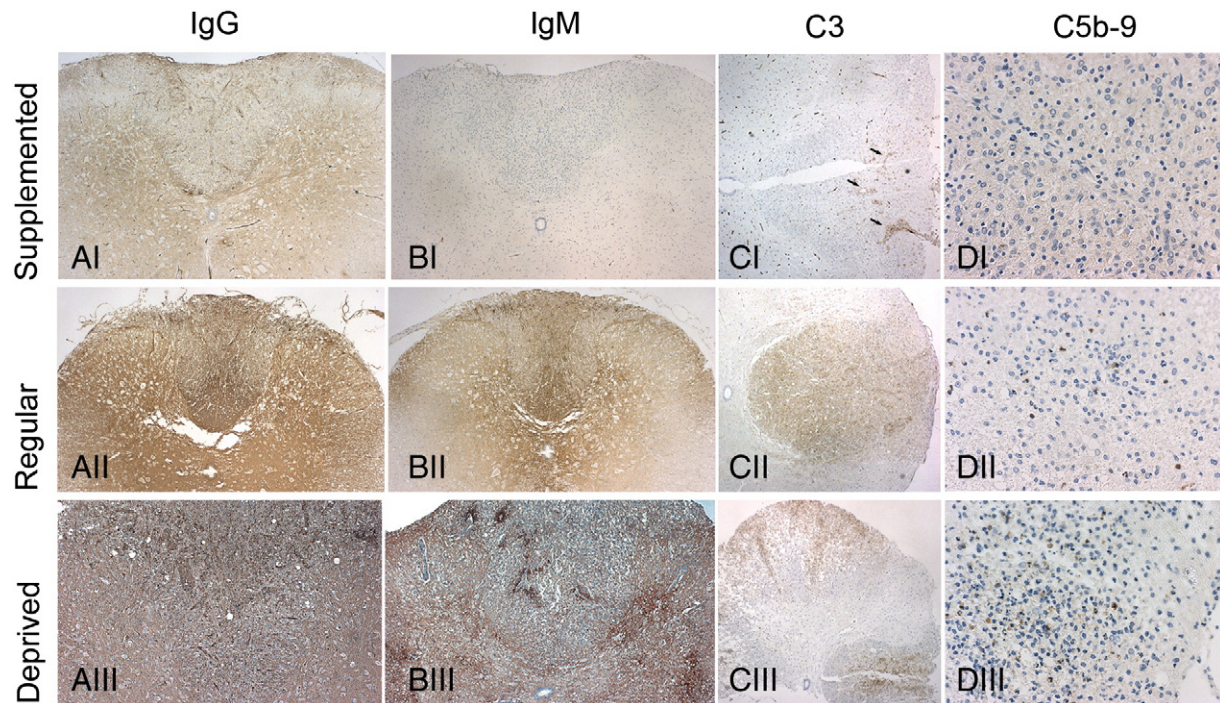
Fig. 4. Antibody extravasation and complement activation in the spinal cord of juvenile/adolescent rats affected by inflammation/demyelination evidenced the humoral immunity. Group-representative neuropathology of spinal cord in juvenile/adolescent rats with EAE fed either with vitamin D supplemented (AI–DI), regular (AII–DII) or deprived (AIII–DIII) diet (IHC was performed on the same tissue as histopathology and IHC presented in Fig. 3). Analysis of the Ig isotypes IgG (AI–AIII) and IgM (BI–BIII) revealed the lowest immunoglobulin reactivity in the vitamin D supplemented group (AI, BI respectively) compared to the other two diet regimens (AII, BII and AIII, BIII, respectively; magnification ×100). Staining for C3 (CI–CIII; magnification ×100) revealed massive depositions upon regular diet (CII) and vitamin D deprived diet (CIII) matching the demyelinated areas. C3 deposition in the treated animal (CI) is much reduced and mainly restricted to the perivascular area (arrows). C5b-9 was detected in the lesions of rats that received regular diet (DII) and vitamin D deprived diet (DIII). Virtually no C5b-9 depositions in the lesions of the vitamin D supplemented rat (DI). Staining revealed also the presence of granulocytes in the lesions of rats fed with vitamin D deprived (DIII) and the regular diet (DII), whereas the infiltrate in the vitamin D supplemented animals is mainly composed of mononuclear cells (DI).

The supplementation regimen favors an anti-inflammatory phenotype predominantly in juvenile/adolescent rats compared to the other developmental stages

In order to investigate whether the five-fold increased regular vitamin D intake is associated with differential immune activation, we analyzed mRNA expression levels of cytokines in the inguinal lymph nodes harvested 7 days p.i. At this stage susceptible DA rats mount a strong immune response against MOG, which subsequently leads to infiltration of cells into CNS and signs of clinical disease (Thessen Hedreul et al., 2009). Expression level of *Il4*, a key effector cytokine of the anti-inflammatory Th2-like immune response, was significantly higher in juvenile/adolescent rats subjected to the supplemented diet in contrast to those fed with vitamin D deprived food (Fig. 5A). In addition, we observed decreased *Ifnγ* and *Il2* mRNA expression levels in juvenile/adolescent rats subjected to the supplemented diet, both of these cytokines being associated with a pro-inflammatory Th1-like immune response. *Rorc*, which encodes RORgt, a transcription factor promoting T cell differentiation into Th17 cells was decreased with vitamin D supplementation regimen. The differences in *Il17* expression were, however, not significantly different between experimental groups of all three ages/developmental stages (data not shown). Further, expression levels of the co-stimulatory molecule *Cd80* and the adhesion molecule *Icam* were significantly decreased in juvenile/adolescent rats fed with the supplemented diet (Fig. 5A).

None of these changes were observed in adult rats (Fig. 5B) except for differential *Foxp3* expression. Interestingly, upregulation of the regulatory T cell transcription factor observed in both juvenile/adolescent and adult rats directly exposed to vitamin D supplementation in the diet was not detected in the indirectly supplemented offspring. Notably, this offspring that received supplementation during pre- and early

postnatal development via mothers expressed a tendency to downregulate *Ifnγ* (Fig. 5C).

Influence of the five-fold enhanced vitamin D intake on the main immune cell populations prior to clinical disease onset

To investigate the impact of the same vitamin D supplementation regimen at different developmental stages on the main immune cell types during the initial phase of EAE we performed FACS analysis on ex vivo inguinal lymph node cells harvested on day 7 p.i. The CD4$^+$ cell subset was generally more abundant in young than in adult experimental animals subjected to the supplemented diet, which was accompanied by significantly lower amounts of CD45RA$^+$ B cells. We did not observe such a trend in CD45RA$^+$/CD4$^+$ cell ratio in juvenile/adolescent and in the pre- and early post-natally treated rats subjected to the vitamin D deprived diet (Figs. 6A, C). In contrast to rats treated in the earlier developmental stages, the same supplementation regimen made no significant impact on the main immune cell populations in adult age (Fig. 6B). We observed no age or diet regimen-dependent difference in amounts of CD8$^+$ T within all experimental groups.

Suppressed T cell responses towards MOG predominantly in juvenile/adolescent rats receiving vitamin D supplementation

To detect potential vitamin D level-dependent differences in response to the specific CNS antigen MOG at different developmental stages, we performed IFN-γ Elispot analyses on day 10 p.i with ex vivo splenocytes. The specific T cell response towards MOG antigen was reduced by 80% in the juvenile/adolescent rats receiving the supplemented diet compared to those fed with the vitamin D deprived diet (Fig. 6D). A significant modulation of the MOG-specific autoimmune response was also
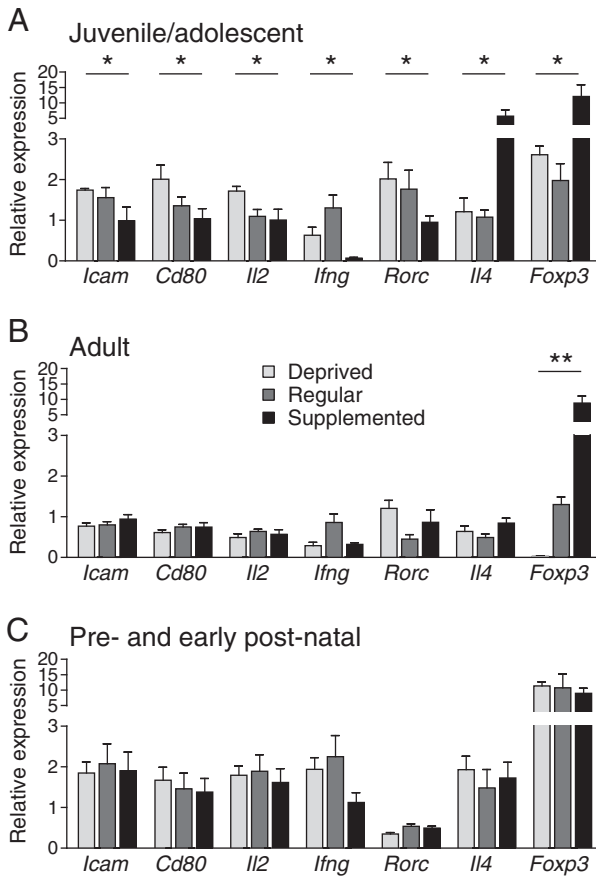
Fig. 5. The vitamin D supplementation regimen favors an anti-inflammatory response predominantly in juvenile/adolescent rats. (A–C) Graphs presenting mRNA levels of indicated targets in the inguinal lymph nodes harvested on day 7 p.i from juvenile/adolescent, adult and pre- and early post-natally treated rats, respectively, subjected to different amounts of vitamin D through the diets; analysis performed by qPCR (n = 5–7 animals per condition, experiments repeated twice). (A) Juvenile/adolescent rats subjected to vitamin D supplemented diet displayed upregulated mRNA levels of Il4, while the expression of the adhesion molecule Icam, co-stimulator Cd80, Cd4 and the interleukins Il2 and Ifnγ was significantly downregulated compared to vitamin D deprived rats. (B) Adult rats did not show vitamin D regimen-dependent differences in mRNA target levels. (C) Rats treated during pre- and early post-natal development tended to differentially express Ifnγ. Error bars represent SEM. Statistics were calculated using Kruskal–Wallis test with Dunn's correction for multiple testing (p < 0.05 = *, p < 0.01 = **). Statistical significances refer to comparison between vitamin D deprived and vitamin D supplemented groups.

Fig. 6. The five-fold enhanced vitamin D intake has differential impact on the main immune cell populations and antigen specific immune responses at different developmental stages. Graphs presenting cell counts of $CD4^+$ and $CD8^+$ T cells and $CD45RA^+$ B cells in lymph nodes harvested on 7 day p.i from (A) juvenile/adolescent, (B) adult and (C) the rats treated during pre- and early post-natal development subjected to different amounts of vitamin D in their diet as analyzed by flow cytometry (n = 6 for each experimental group/diet regime, experiments repeated twice). (D) Graph presenting the number of MOG-specific T cells in the splenocytes harvested on day 10 p.i from juveniles/adolescents, adult and pre- and early post-natally treated rats, respectively, subjected to either supplemented or vitamin D deprived diet (ConA was used as a positive control, MBP as a non-specific antigen; 4 animals per diet regimen, experiment repeated twice in both juvenile/adolescent and adult rats). Juvenile/adolescent and rats treated during pre- and early post-natal development, but not adult rats subjected to a vitamin D supplemented diet had significantly lower numbers of MOG-specific T cells comparing to rats fed with vitamin D deprived diet. Error bars represent SEM. Statistics for FACS data were calculated using ANOVA with Bonferroni correction for multiple testing and Elispot differences were analyzed using t-test (p < 0.05 = *, p < 0.01 = **). Statistical significances refer to comparison between vitamin D deprived and vitamin D supplemented groups.

observed in the pre- and early post-natally supplemented rats. Nevertheless, such vitamin D supplementation-related effect was not observed in adult rats. Importantly, the responses to ConA (positive control) and myelin basic protein (MBP, non-specific CNS antigen) were similar between the groups.

Discussion

Numerous studies provide evidence for strong associations of vitamin D with development of MS and EAE, indicating prospects for treatment of the disease based on vitamin D supplementation. However, a conclusive demonstration of the efficacy of vitamin D in MS is still missing (Papeix and Lubetzki, 2013), especially in terms of the most sensitive age/developmental stage. We aimed to address this question by investigating the effect of the same vitamin D supplementation regimen during different developmental stages in a setting of rat neuroinflammation. Since confounding factors such as body mass index, season and lifestyle can additionally affect susceptibility to MS (Handel et al., 2011), our study was performed in age-matched inbred female rats housed under equal environmental conditions, which
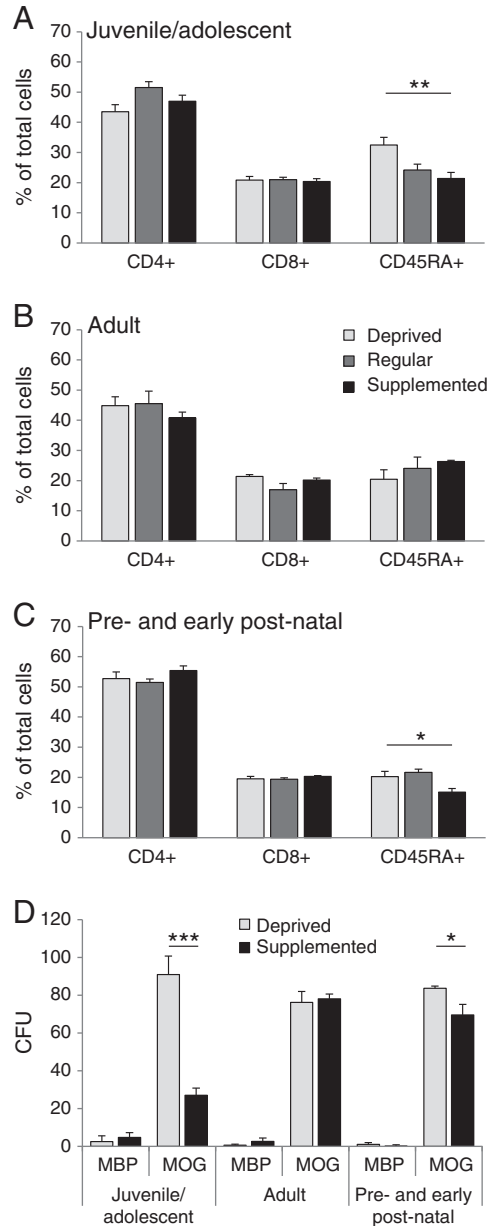
ruled out the effect of age, gender and genetic background. While it significantly ameliorated EAE in juvenile/adolescent rats, the same supplementation regimen was insufficient to affect clinical disease in both adult rats and rats treated during pre- and early post-natal

development. Thus, our observations indicate that the juvenile/ adolescence period has the highest sensitivity to vitamin D. Our data conform with many epidemiological studies that associate sun exposure during childhood and adolescence with the lower risk for MS (Dalmay et al., 2010; Islam et al., 2007; Kampman et al., 2007; McDowell et al., 2010; van der Mei et al., 2003). Moreover, women who received higher levels of vitamin D from supplements during adolescence have a reduced risk for developing MS (Munger et al., 2011). Finally, a retrospective study in pediatric MS reported that every 10 ng/mL increase in serum vitamin D3 level was associated with a 34% decrease in the rate of subsequent disease relapses (Mowry et al., 2010).

There is a considerable variation in the supplementation strategies and doses proven to successfully ameliorate EAE in rodents (Joshi et al., 2011; Lemire and Archer, 1991; Nashold et al., 2009; Nataf et al., 1996; Spach et al., 2006). We demonstrated that a five-fold higher than regular amount of vitamin D, when administered in a continuous, physiological way via diet, is sufficient to ameliorate neuroimmunological disease in juvenile/adolescent rats, but not in the other developmental stages. Nevertheless, this supplementation significantly elevated 25(OH)D serum levels in both juvenile/adolescent and adult rats.

Vitamin D exhibits a variety of immunomodulatory functions (Mora et al., 2008). Human in vitro studies demonstrated an anti-proliferative effect of vitamin D on T and B cells (Chen et al., 2007; Iho et al., 1986; Lemire et al., 1984; Rigby et al., 1984) and an increased number of regulatory T cells (T regs) upon stimulation with vitamin D (Barrat et al., 2002; Jeffery et al., 2009). Indeed, both juvenile/adolescent and adult rats directly exposed to higher than regular levels of vitamin D in the diet increased *Foxp3* expression. In concord with previously in vitro demonstrated suppressive effect of vitamin D on Th17 cells (Chang et al., 2010), we observed vitamin D supplementation-conditioned downregulation of *Rorc*, a master regulator of this T cell-type. Our data confirmed that the effect on T cells is at least partially mediated by vitamin D-induced inhibition of IL-2 (Bhalla et al., 1986; Rigby et al., 1984) through a downregulation of *Il2* in the supplemented group. Together with a decrease in *Ifnγ* and an increase in *Il4*, which has previously been reported in mice (Boonstra et al., 2001), our data support the protective immunomodulatory effect of vitamin D, which resulted in reduced number of IFNγ producing MOG-specific T cells, and ameliorated autoimmune response in the juvenile/adolescent rats subjected to vitamin D supplementation.

Vitamin D supplementation in juvenile/adolescent rats led to a milder clinical disease reflected in less severe CNS inflammation and demyelination, and even to a decreased CD45RA$^+$/CD4$^+$ cell ratio in the periphery. Besides CD4$^+$ T cells, B-cells and antibodies may also contribute to MS-lesion pathogenesis (Archelos et al., 2000). Similar to the previous observation in marmoset monkeys (Merkler et al., 2006), MOG-induced EAE in our study reflected MS pattern II lesions (Lucchinetti et al., 2000) with the lowest level of antibody/complement-mediated tissue damage in juvenile/adolescent rats fed with vitamin D supplemented diet.

Post-natal supplementation resulted in a less severe EAE in mice (Fernandes de Abreu et al., 2011). The five-fold enhanced vitamin D supplemented diet was still not sufficient to reduce the clinical disease symptoms in pre- and early post-natally treated rats. The supplemented offspring displayed, nevertheless, a tendency towards protective immunomodulation through a decreased number of CD45RA$^+$ B cells, a suppressed T cell response towards MOG, as well as a tendency to downregulate *Ifnγ*. Fernandes de Abreu et al. observed that prenatal vitamin D deprivation protected offspring mice against EAE later in adulthood (Fernandes de Abreu et al., 2010) and even speculated about the outcome of a prolonged gestational deprivation sustained until weaning (Fernandes de Abreu et al., 2011). In our hands, vitamin D deprivation sustained throughout pre- and early post-natal rat development caused no changes in susceptibility to EAE in adulthood. This additionally indicates the importance of the early post-natal period

(lactation), which has been shown to have a high impact on the rat development (Feron et al., 2005; Lester et al., 1982). It is likely that such offspring could not benefit from the protective effect gained during pre-natal hypovitaminosis (Fernandes de Abreu et al., 2010), due to the vitamin D deprivation continuously being sustained until weaning. Nevertheless, it seems that the exposure to vitamin D during juvenile/ adolescent development successfully compensated for the early post-natal deprivation. These observations additionally emphasize the sensitivity of the juvenile/adolescent age to vitamin D treatment.

Apart from increasing the levels of *Foxp3*, the supplementation made no significant impact on any other disease aspects in adult rats. Accordingly, one may assume that vitamin D treatment in adult patients undergoing MS may not have the desired therapeutic effect, despite increased serum levels of 25(OH)D. To this end, randomized phase 2 studies performed in adult MS patients deemed the influence of vitamin D supplementation as either ineffective (Kampman et al., 2012) or insignificant (Burton et al., 2010). Another small placebo-controlled trial with vitamin D as an add-on treatment to interferon-β in MS patients revealed significantly lower numbers of T1 enhancing lesions, but no significant effect on MRI parameters or clinical disease variables (Soilu-Hanninen et al., 2012). However, despite the lack of impact on all disease aspects, fairly high supplementation doses still exhibit a considerable effect on adult MS pathologies (Burton et al., 2010; Kimball et al., 2011).

Conclusion

Our data demonstrate that vitamin D has an age-dependent efficacy to ameliorate MS-like neuroinflammation. Equivalent vitamin D supplementation regimen carried throughout juvenile/adolescent developmental stage successfully ameliorated EAE, while it was insufficient to alter the disease severity in adult rats, despite comparably increasing the levels of 25(OH)D in serum. Moreover, the same supplementation was not sufficient to directly affect the clinical disease course in rats treated during pre- and early post-natal development. Nevertheless, it induced a certain degree of immunomodulation, similar to that observed in the juvenile/adolescent rats. These results suggest that in adulthood and for prenatal prevention purposes probably much higher doses of supplementation would be required for achieving the protective effect observed in juvenile/adolescent rats. Thus vitamin D supplementation is most efficient in ameliorating neuroinflammation when administered at juvenile/adolescent age.

Supplementary data to this article can be found online at http://dx. doi.org/10.1016/j.expneurol.2013.08.002.

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