

Immunomodulatory effects of Vitamin D in multiple sclerosis

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Although Vitamin D is best known as a modulator of calcium homeostasis, it also has immune modulating potential. A protective effect of Vitamin D on multiple sclerosis is supported by the reduced risk associated with sun exposure and use of Vitamin D supplements. Moreover, high circulating levels of Vitamin D have been associated with lower risk of multiple sclerosis. In this study, we measured 1,25 (OH)₂ Vitamin D and 25 (OH) Vitamin D levels in multiple sclerosis patients separated into different clinical subgroups according to disease status. In addition, direct effects of 1,25 (OH)₂ Vitamin D on ex vivo CD4+ T cells and myelin-peptide specific T cell lines were investigated to gain more insight into putative regulatory mechanisms in the disease pathogenesis. One hundred and thirty-two Hispanic patients with clinically definite multiple sclerosis were studied, 58 with relapsing remitting multiple sclerosis during remission, 34 during relapse and 40 primary progressive multiple sclerosis cases. Sixty healthy individuals matched with respect to place of residence, race/ethnicity, age and gender served as controls. Levels of 25(OH)D₃ and 1,25(OH)₂D₃, measured by ELISA were significantly lower in relapsing–remitting patients than in controls. In addition, levels in patients suffering relapse were lower than during remissions. In contrast, primary progressive patients showed similar values to controls. Proliferation of both freshly isolated CD4+ T cells and MBP-specific T cells was significantly inhibited by 1,25(OH)₂D₃. Moreover, activated Vitamin D enhanced the development of IL-10 producing cells, and reduced the number of IL-6 and IL-17 secreting cells. Notably, Vitamin D receptor expression was induced by 1,25(OH)₂D₃ in both activated and resting cells. Interestingly, T cells were able to metabolize 25(OH)D₃ into biologically active 1,25(OH)₂D₃, since T cells express α 1-hydroxylase constitutively. Finally, 1,25(OH)₂D₃ also increased the expression and biological activity of indoleamine 2,3-dioxygenase, mediating significant increase in the number of CD4+CD25+ T regulatory cells. Collectively, these data suggest that 1,25(OH)₂D₃ plays an important role in T cell homeostasis during the course of multiple sclerosis, thus making correction of its deficiency may be useful during treatment of the disease.

Keywords: multiple sclerosis; vitamin D; T cells; cytokines; regulatory T cells

Abbreviations: Ag = Antigen; EAE = experimental allergic encephalomyelitis; IDO = indoleamine 2,3-dioxygenase; PBMC = peripheral blood mononuclear cells

Introduction

Several lines of evidence support the hypothesis that autoimmunity plays a major role in multiple sclerosis development

(McFarland and Martin, 2007). It is generally accepted that autoimmune diseases arise from a complex interactions between genetic susceptibility and environmental factors. Geographical variations in the incidence and prevalence of this disease, as well

as studies of populations emigrating from areas of low to areas of high risk, indicate that the environment is also a key factor in its pathogenesis (Ascherio and Munger, 2007; Pugliatti *et al.*, 2008).

Vitamin D is best known as a calcium homeostasis modulator; however both experimental and clinical observations provide evidence that Vitamin D is also one of several important environmental factors that can affect multiple sclerosis prevalence. A protective effect has been supported by reduced risk of the illness associated with sunlight exposure and use of Vitamin D supplements (Munger *et al.*, 2004; van der Mei *et al.*, 2001). A recent longitudinal study conducted among American patients showed decreased risk in white patients with increasing serum levels of 25(OH) Vitamin D (Munger *et al.*, 2006). The evidence obtained from these studies supports the role of Vitamin D as an immunomodulatory molecule.

Vitamin D, ingested orally or formed in the skin following exposure to sunlight is hydroxylized to the major circulating form, 25 (OH) Vitamin D in the liver, levels of which are sensitive to both Vitamin D intake and sun exposure and are markers of Vitamin D availability to tissues, best reflecting Vitamin D status of the patient. However 25(OH) Vitamin D is inert and requires additional hydroxylation, mainly in the kidney, by the 25-hydroxy Vitamin D-1 α hydroxylase (1 α -hydroxylase) to 1,25 (OH)₂ Vitamin D. This is the biologically active metabolite, with most biological effects mediated through binding to the Vitamin D receptor. Expression of Vitamin D receptor and 1- α hydroxylase have been also described in immune cells. For instance, the presence of both molecules has been demonstrated in dendritic cells, macrophages (Overbergh *et al.*, 2000; Veldman *et al.*, 2000), B cells (Chen *et al.*, 2007), and activated T cells (Veldman *et al.*, 2000; Norman, 2006). *In vitro* 1,25(OH)₂ Vitamin D inhibits the differentiation of monocytes into dendritic cells, and suppresses IL-12 secretion as well as surface expression of co-stimulatory molecules (D'Ambrosio *et al.*, 1998; Penna and Adorini, 2000; Adorini, 2002). In addition, evidence supports a model in which 1,25 (OH)₂ Vitamin D mediates a shift of CD4+ T cells to an anti-inflammatory profile (Adorini, 2002; Meehan and DeLuca, 2002; Smolders *et al.*, 2008). Moreover, B cell proliferation, plasma differentiation and immunoglobulin production are inhibited *in vitro* by 1,25 (OH)₂ Vitamin D (Chen *et al.*, 2007). These immunomodulatory effects are consistent with the accelerated onset of experimental allergic encephalomyelitis (EAE), an animal model for multiple sclerosis, in Vitamin D-deficient mice (Cantorna *et al.*, 1996). In contrast, injection of 1,25 (OH)₂ Vitamin D was found to prevent clinical and pathological signs of disease, a phenomenon associated with profound reduction in autoreactive T cells, peptide-specific proliferation and Th1 cell development (Lemire and Archer, 1991).

Tryptophan is an essential amino acid that is catabolized by the rate-limiting enzyme indoleamine 2,3-dioxygenase (IDO) to generate kynurenin. IDO is expressed in different immune cells, such as dendritic cells and activated macrophages (Munn *et al.*, 2002). Data from recent studies indicate that dendritic cells expressing high levels of IDO play an important role in the induction of regulatory T cell responses, and in maintenance of peripheral tolerance (Munn *et al.*, 2002; Meisel *et al.*, 2004). Defects in

IDO-mediated tryptophan metabolism have been associated with tolerance impairment in experimental animal models such as non-obese diabetic mice, in which autoreactive T cells persist during disease progression (Grohmann *et al.*, 2003).

In this study, we measured 1,25 (OH)₂ Vitamin D and 25 (OH) Vitamin D levels in patients with multiple sclerosis, separated into different subgroups according to clinical status. In addition, the direct effects of 1,25 (OH)₂ Vitamin D and IDO on *ex vivo* CD4+ T cells and myelin-peptide specific T cell lines were investigated, to gain more insight into putative regulatory mechanisms of both molecules in multiple sclerosis pathogenesis.

Material and Methods

Patients and control subjects

One hundred and thirty-two Hispanic patients with diagnosis of clinically definite multiple sclerosis according to Poser's and McDonald's criteria were studied. Clinical course of disease was defined following Lublin and Reingold (1996) criteria, and patients were subdivided into three groups: (i) relapsing remitting multiple sclerosis in remission ($n=58$); (ii) relapsing remitting multiple sclerosis during acute exacerbation ($n=34$); and (iii) primary progressive multiple sclerosis ($n=40$). Exacerbations were defined as development of new symptoms or worsening of a pre-existing one, confirmed on neurological examination and lasting at least 48 h, in the absence of fever, and preceded by stability or improvement lasting at least 30 days. Patients with inflammatory, endocrine or major psychiatric disorders were excluded from the study. No patients had received steroids for at least 6 months prior to study entry, nor immunomodulatory, immunosuppressive drugs, or dietary supplements. All patients were living in the city of Buenos Aires (latitude 34°S, longitude 58°W). Sixty healthy individuals, selected to match patients with respect to place of residence, race/ethnicity, age and gender, served as controls. Because relapsing remitting and primary progressive patients differ significantly in age, two control groups were included in the study, one similar to primary progressive patients (control group 1) and another to relapsing remitting ones (control group 2). Thorough clinical and neurological examination, as well as standard chemical and haematological laboratory examinations ruled out presence of underlying disorders in these subjects. Control individuals were not receiving any regular medication, or dietary supplements. Samples from healthy controls were collected during the same months of the year as samples from multiple sclerosis patients. Demographic and clinical characteristics of patients and controls are shown in Table 1.

Determination of serum 25 (OH) Vitamin D and 1,25 (OH)₂ Vitamin D levels

Serum 25 (OH) Vitamin D and 1,25 (OH)₂ Vitamin D levels were measured using commercially available ELISA kits (Immunodiagnostik, Bensheim, Germany) according to manufacturer's instructions. Assay sensitivity levels were: 10 ng/ml and 6.0 pg/ml, respectively. Intra- and interassay variation coefficients were <7.5 and 6.8%, respectively for both assays.

Table 1 Demographic and clinical characteristics of the study population^a

	PPMS	Controls 1	RRRMS	RREMS	Controls 2
<i>n</i>	40	30	58	34	30
Age (years)	49.5 ± 12.7	49.0 ± 5.0	36.8 ± 10	37.5 ± 8.4	33.9 ± 5.9
Female:male	18:22	16:14	41:17	29:5	23:7
EDSS	4.3 ± 1.6	—	1.3 ± 1.1	2 ± 1.4	—
Disease duration (years)	4.1 ± 4.7	—	3.9 ± 4.9	4.3 ± 5.7	—

a Values are expressed as mean ± SD. PPMS=Primary progressive multiple sclerosis; SPMS=Secondary progressive multiple sclerosis; RRRMS=Relapsing remitting multiple sclerosis during remission; RREMS=Relapsing remitting multiple sclerosis during exacerbation; EDSS=Expanded Disability Status Scale.

Antigen preparation

MBP_{83–102}, MBP_{143–168} and MOG_{63–87} peptides were synthesized using an automated peptide synthesizer, and purity was assessed using high-pressure liquid chromatography analysis.

CD4+ cell enrichment and generation of MBP- and MOG-peptide reactive T cell lines

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque density gradient centrifugation (Pharmacia LKB Biotechnology, Piscataway, NJ), and CD4+ T cells were positively selected using Dynabeads M-450 CD4 beads (DynaL Biotech ASA, Oslo, Norway). Separation was monitored using flow cytometry analysis, demonstrating over 97% purity. MBP- and MOG-peptide specific T cell lines were expanded from peripheral blood as previously described (Correale et al., 1995). Briefly, 5×10^6 PBMC were stimulated with optimal concentrations of MBP or MOG peptides (10–20 µg/ml). After 5–7 days, cells were re-cultured in fresh medium containing 50 U/ml of recombinant human IL-2 (rIL-2; R&D Systems, Minneapolis) for an additional week. Re-stimulation cycles with autologous irradiated PBMCs (3000 Rads) as antigen presenting cells plus peptide, followed by expansion with rIL-2, were repeated weekly. After four cycles of restimulation and expansion, T-cell lines were evaluated using standard proliferation assays. Cutoff value for a positive response was set at stimulation index >3. All MBP- and MOG-reactive T-cell lines were >93% CD4+.

T helper cell polarization

Human CD4+ T cells were purified from PBMCs of relapsing remitting patients and healthy controls as described above, and cultured for 5 days with PHA (1 µg/ml; Sigma-Aldrich), and rIL-2 (50 U/ml; R&D Systems) in neutral or polarizing conditions as described (Hannier et al., 2002): Th1, IL-12 (2 ng/ml; BD Biosciences, San Diego, CA), plus anti-IL-4 mAb (100 ng/ml; BD Biosciences) and Th2, IL-4 (5 ng/ml; Sigma) plus anti-IL-12 (2 µg/ml; BD Biosciences). For Th-17 differentiation, naïve CD4+ T cells were stimulated for 5 days (Bettelli et al., 2006) with plate bound anti-CD3 (5 µg/ml; ATCC) and soluble anti-CD28 (1 µg/ml; BD Biosciences) in the presence of TGF-β (3 ng/ml), IL-6 (20 ng/ml) and IL-23 (20 ng/ml, all from R&D Systems), and neutralizing antibodies anti IL-4 (10 µg/ml), and anti-IFN-γ (10 µg/ml), both from BD Biosciences. Cultures were supplemented with rIL-2 (50 U/ml; R&D Systems) on Days 2 and 4.

Generation of dendritic cells

Dendritic cells were generated as previously described (Correale and Farez, 2007). Briefly, monocytes were positively selected from PBMCs isolated from relapsing remitting patients and healthy controls using anti-CD14 coated magnetic beads (Miltenyi Biotec, Bergisch

Glandbach, Germany). Cells were cultured in the presence of 100 ng/ml of GM-CSF (R&D Systems), and 50 ng/ml of rhIL-4 (R&D Systems). On Days 2 and 4, half the culture medium was replaced maintaining the same GM-CSF and IL-4 concentrations. After 5 days, 100 ng/ml of LPS were added to stimulate dendritic cell maturation. Contamination with CD3+ T cells was verified by flow cytometry and found to be <0.4%.

Stimulation of CD4+ T cells and peptide-specific T-cell lines, and proliferation assays

For peptide Antigen (Ag)-specific stimulation, T-cell lines were cultured at a density of 5×10^4 cells/well in the presence of 5×10^3 adherent irradiated autologous PBMC as the source of antigen presenting cells, and 10 µg/ml of appropriated peptide, in the presence or absence of 1,25 (OH)₂ Vitamin D, or the analogues 24,25 (OH)₂ Vitamin D and 25,26 (OH)₂ Vitamin D (all from Sigma-Aldrich, St Louis; MO). For non-specific stimulation, peptide-specific T-cell lines (5×10^4 cells/well) were cultured in the presence of 1 µg/ml of immobilized anti-CD3 mAb (OKT3; American Type Culture Collection, Manassas, VA), in the presence or in the absence of 1,25 (OH)₂ Vitamin D. Irradiated PBMC were not included under these conditions. Meanwhile, purified CD4+ T cells were plated at a density of 5×10^4 cells/well and stimulated with 1 µg/ml of PHA (Sigma-Aldrich). After stimulation, T cell proliferation was examined in a standard 60-h assay measuring [³H]thymidine (ICN Biomedicals, Irvine, CA) incorporation, as previously described (Correale et al., 1995).

1,25 (OH)₂ Vitamin D and its analogues were dissolved in absolute ethanol at a stock concentration of 0.01 M, and further diluted in RPMI 1640 medium to the tested concentrations. Additional controls included viability studies using trypan blue dye exclusion, and the addition of ethanol diluted to concentrations equivalent to those used for 1,25 (OH)₂ Vitamin D or its analogues dilutions. In all experiments, 1,25 (OH)₂ Vitamin D was used at the pharmacological dose of 10 nM. This dose is ~82- to 550-fold higher than circulating levels in healthy individuals.

Real-time quantitative RT-PCR analysis

For quantitative assessment of relative mRNA levels, total RNA was prepared using TRIzol LS reagent (Invitrogen, Carlsbad, CA) following manufacturer instructions. RNA was reverse transcribed using a M-MLV RT reverse transcription kit with random hexamer primers (Invitrogen). Relative levels of VDR, 1-α hydroxylase, and IDO mRNAs were determined by real-time PCR (ABI 7000 sequence detection system; Applied Biosystems, Foster City, CA), using a SYBR green PCR mix. Values obtained were normalized to the amount of GAPDH. Primer sequences used were as follows: GAPDH: forward 5'-GAAG GTGAAGTCGGAGTC-3', reverse 5'-GAAGATGGTGATGGGATTTC-3'; VDR: forward 5'-CTTCAGGCGAAGCATGAAGC, reverse 5'-CCTTCA TCATGCCGATGTCC-3'; 1-α hydroxylase: forward 5'-TTGGCAAGCC

CAGCTGTAT-3', reverse 5'-TGTGTTAGGATCTGGGCCAAA-3'; IDO: forward 5'-ACTGGAGGCACTGATTA-3', reverse 5'-ATTAGTTTGTG GCTCTGTTA-3'. Each specific primer was used at a final concentration of 250 nM.

Quantification of secreted cytokines

The number of CD4+ T cells or MBP- and MOG-peptide specific T cells secreting IL-4, IL-6, IL-10, IL-17 and IFN- γ was evaluated using commercially available kits for single-cell resolution enzyme-linked immunospot (ELISPOT) assays following manufacturer instructions (R&D Systems, Minneapolis, MN, USA), in the presence and in the absence of 1,25 (OH) $_2$ Vitamin D. The number of cytokine secreting cells was calculated by subtracting the numbers of spots obtained in control cultures without Ag stimulation, from the number of spots obtained in cultures exposed to stimulating Ag. Results are reported as number of spots per 10 5 PBMC. To assess the role of IL-10 as an autocrine factor, a neutralizing anti IL-10 receptor mAb (10 μ g/ml; Pharmingen, San Diego, CA) was added to culture in some experiments.

Polarized T helper cells were stimulated with 1 μ g/ml of plate-bound anti-CD3 monoclonal antibody (ATCC). Supernatants were removed at 36 (IL-4), or 72 h (IFN- γ , and IL-17), and cytokines measured using commercially available ELISA kits, purchased from R&D Systems.

Measurement of IDO activity

CD4+ T cells, as well as MBP and MOG peptide-specific T-cell lines were cultured in the presence or in the absence of 1,25 (OH) $_2$ Vitamin D. Seventy-two hours after culture, supernatants were collected and kynurenine concentrations were measured with HPLC using a reverse-phase column as previously described (Widner *et al.*, 1997). Kynurenine was detected using UV detection at 360 nm wavelength, and values referred to a calibration curve previously constructed with defined kynurenine concentrations.

Evaluation of CD4+CD25+FoxP3+ regulatory T cells

To evaluate the role of 1,25 (OH) $_2$ Vitamin D on regulatory T cell induction, 5 \times 10 4 PBMC were stimulated with soluble anti-CD3, and soluble anti-CD28 (BD Biosciences) antibodies, both at 5 μ g/ml concentration, in the presence or the absence of 1,25 (OH) $_2$ Vitamin D. After 7 days, the number of CD4+CD25+FoxP3+ T cells was evaluated by flow cytometry, using commercially available regulatory T cell staining kits following the manufacturer instructions (eBioscience, San Diego, CA). To evaluate the role of IDO in regulatory T cell induction, the IDO inhibitor 1-methyl-D-tryptophan (1-MT; Sigma-Aldrich) was added to the cultures in some experiments at a concentration of 1000 μ M.

To evaluate 1,25 (OH) $_2$ Vitamin D activity induced by regulatory T cells, CD4+ CD25- and CD4+CD25 high populations were isolated by FACS sorting. Sort gates were restricted to the population of lymphocytes by forward and side scatter properties. Large, activated T cells were excluded. To ensure purity of isolated populations, a portion of each sample was re-analysed, and purity was determined to be >95%. Variable numbers of CD4+ CD25+ cells were added to a constant number of CD4+CD25- indicator cells (3 \times 10 3 cells/well), in order to achieve CD4+CD25+:CD4+CD25- ratios of 10:1, 5:1, 2.5:1 and 1:1, respectively. Stimulation was provided by the addition of soluble anti-CD3, and soluble anti-CD28 (BD Biosciences), both at 5 μ g/ml concentrations in the presence of 3 \times 10 4 /well irradiated PBMC, depleted of CD3+ T cells as a source of antigen presenting cells. Proliferation was determined on Day 6, with [3 H] thymidine added

during the last 18 h of culture. Mean counts per minute \pm SEM were calculated for triplicate measurements. To measure IFN- γ production, supernatants were removed from each well before [3 H] thymidine addition, and analysed using commercially available ELISA kits (R&D systems).

Statistical analysis

Differences in the levels of 25 (OH) Vitamin D and 1,25 (OH) $_2$ Vitamin D between distinct populations were analysed with a two-tailed Student's *t*-test. Differences in immunological variables were tested for significance using Mann-Whitney U-test. In all cases, *P*-values below 0.05 were considered statistically significant.

Results

Relapsing remitting multiple sclerosis patients have significantly reduced levels of 25 (OH) Vitamin D and 1,25 (OH) $_2$ Vitamin D

25 (OH) Vitamin D serum levels were significantly lower in relapsing remitting patients (47.3 \pm 9.0 ng/ml during remission and 38.5 \pm 8.7 ng/ml during exacerbations) than in healthy controls (61.2 \pm 5.6 ng/ml; Fig. 1A). Additional analysis demonstrated significantly lower levels of 25 (OH) Vitamin D in patients during exacerbations compared with patients in remission (Fig. 1A). In contrast, 25 (OH) Vitamin D levels in primary progressive patients (52.2 \pm 6.5 ng/ml) were not statistically different from those observed in healthy controls (52.7 \pm 8.2 ng/ml; Fig. 1B). Likewise, 1,25 (OH) $_2$ Vitamin D serum level were also significantly lower in relapsing remitting patients (29.2 \pm 5.6 pg/ml during remission, and 23.0 \pm 5.6 pg/ml during exacerbations) than in healthy controls (35.1 \pm 6.6 pg/ml; Fig. 1C). Once again, 1,25 (OH) $_2$ Vitamin D levels were significantly lower in patients with relapsing remitting symptoms during exacerbations compared with patients in remission (Fig. 1C). In contrast, 1,25 (OH) $_2$ Vitamin D serum levels in patients with primary progressive multiple sclerosis (32.5 \pm 5.6 pg/ml) were not statistically different from levels in normal controls (31.3 \pm 6.1 pg/ml; Fig. 1D). Overall, these results indicated that relapsing remitting patients, had decreased levels of 25 (OH) Vitamin D and 1,25 (OH) $_2$ Vitamin D, particularly during exacerbations. In subsequent experiments impact of Vitamin D on T cell function was examined.

1,25 (OH) $_2$ Vitamin D inhibits proliferation of T cells

1,25 (OH) $_2$ Vitamin D capacity to inhibit T cell proliferation was assessed in comparison with the analogues 24,25 (OH) $_2$ Vitamin D and 25,26 (OH) $_2$ Vitamin D used as controls. None of the molecules affected CD4+ T cell survival, as measured by trypan blue dye exclusion. All three compounds were tested in a PHA-induced proliferation assay using *ex vivo* CD4+ T cells and in CD4+ MBP-peptide specific T cell lines stimulated with the cognate Ag, isolated from both relapsing remitting patients during remission

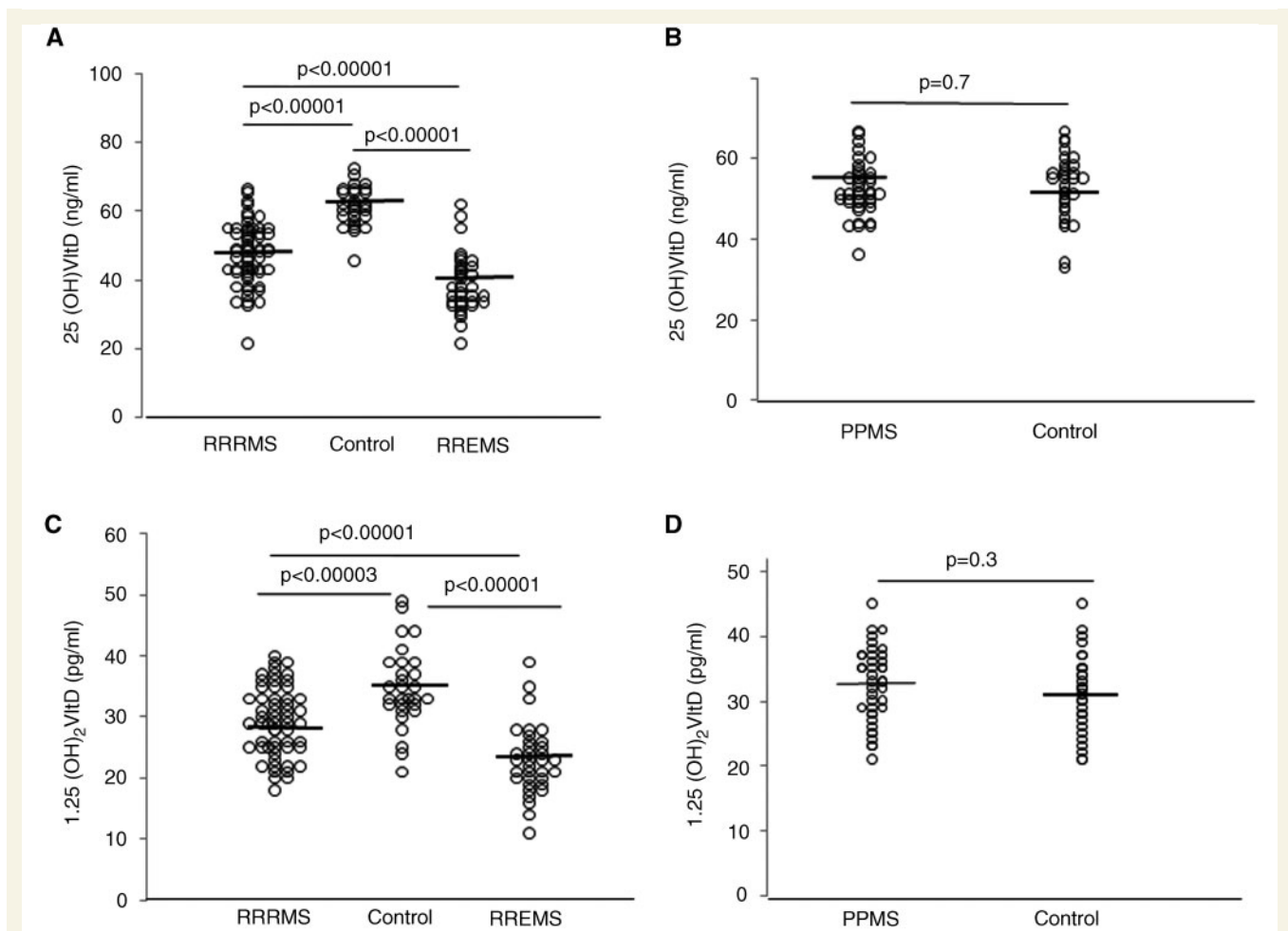


Figure 1 Patients with relapsing remitting multiple sclerosis have decreased 25(OH) Vitamin D and 1,25 (OH)₂ Vitamin D levels. Serum levels of 25(OH) Vitamin D and 1,25 (OH)₂ Vitamin D were measured by ELISA. (A) 25(OH) Vitamin D levels in relapsing remitting patients during remission ($n=58$) and exacerbations ($n=34$) compared with normal controls ($n=30$). (B) 25(OH) Vitamin D levels in patients with primary progressive multiple sclerosis ($n=40$) compared with normal controls ($n=30$). (C) 1,25 (OH)₂ Vitamin D levels in relapsing remitting patients during remission ($n=58$) and exacerbations ($n=34$) compared with normal controls ($n=30$). (D) 1,25 (OH)₂ Vitamin D levels in primary progressive patients ($n=40$) compared with normal controls ($n=30$). Each circle represents average Vitamin D levels from a single individual. Horizontal lines indicate mean group values.

and from healthy controls. As illustrated in Fig. 2A and B, both *ex vivo* CD4⁺ T cells and MBP-peptide specific T cell proliferation were inhibited by 1,25 (OH)₂ Vitamin D in concentration-dependent manner. In contrast, the analogues 24,25 (OH)₂ Vitamin D and 25,26 (OH)₂ Vitamin D did not inhibit CD4⁺ T cell proliferation (Fig. 2C and D), even after testing at concentrations 100-fold higher than those used for 1,25 (OH)₂ Vitamin D. Percentages of inhibition mediated by 1,25 (OH)₂ Vitamin D did not differ between relapsing remitting patients and healthy controls.

VDR is induced by T cell activation and 1,25 (OH)₂ Vitamin D

VDR expression has been described in monocytes and activated lymphocytes (Veldman *et al.*, 2000; Norman, 2006). We therefore chose to examine the expression of VDR on CD4⁺ T cells, isolated

from relapsing remitting patients and healthy controls, using quantitative PCR. Both freshly isolated CD4⁺ T cells, and resting MBP-peptide specific T cells, expressed very low levels of VDR mRNA (Fig. 3). After activation, a 5- to 10-fold increase in CD4⁺ T cells and MBP peptide specific T-cell lines VDR expression was observed ($P < 0.0001$). Moreover, 1,25 (OH)₂ Vitamin D caused a significant increase in VDR expression (3- to 7-fold; $P < 0.0001$) on both un-stimulated and activated CD4⁺ T cells, as well as on un-stimulated and activated MBP peptide-specific T-cell lines (Fig. 3A and B). 1,25 (OH)₂ Vitamin D increased VDR expression in both relapsing remitting patients and healthy controls.

T cells metabolize 25 (OH) Vitamin D to 1,25 (OH)₂ Vitamin D

Synthesis of 1,25 (OH)₂ Vitamin D from the major circulating form of Vitamin D, 25 (OH) Vitamin D, is catalyzed by 1 α hydroxylase,

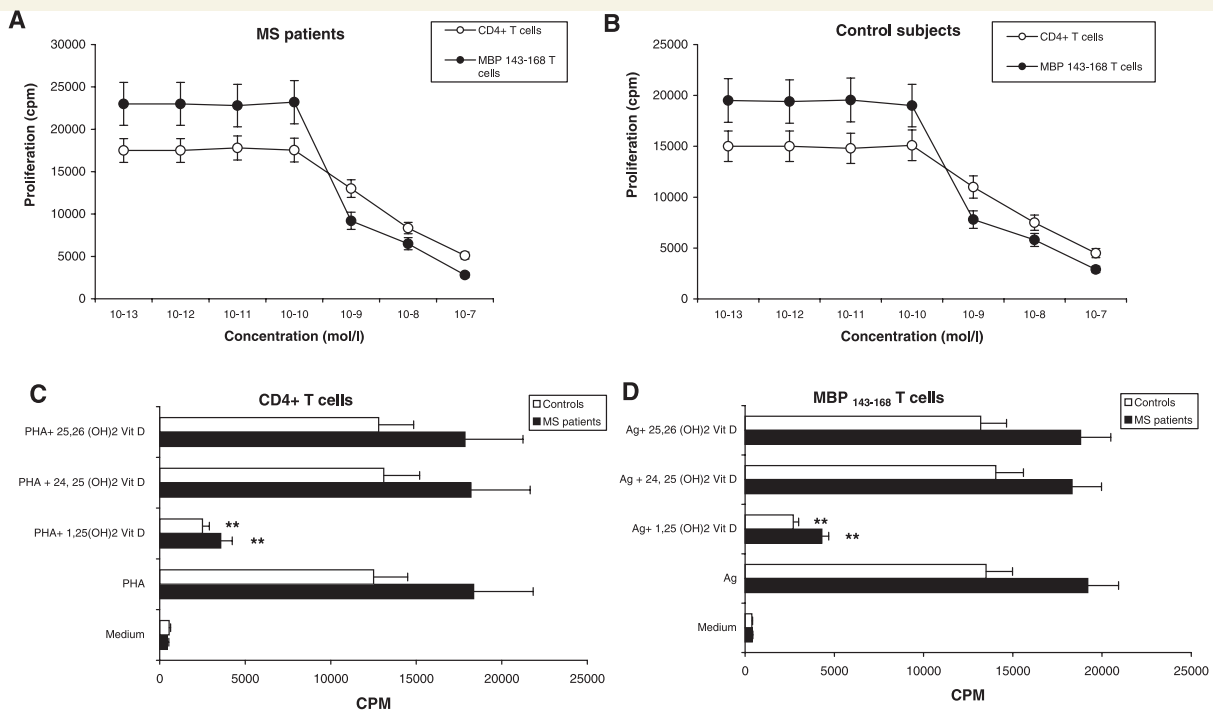


Figure 2 Vitamin D inhibits the proliferation of human CD4+ T cells and MBP peptide-specific T-cell lines. (A–B) Inhibition of CD4+ T cells (open circles) and MBP peptide-specific T-cell lines (full circles) is dose dependent. Results are presented as mean ± SEM values from 20 relapsing remitting multiple sclerosis patients and 20 control subjects. Purified CD4+ T cells (C), and MBP peptide-specific T-cell lines (D) were cultured in the presence or in the absence of 1,25 (OH)₂ Vitamin D (10 nM), or the Vitamin D analogues 25,26 (OH)₂ Vitamin D and 24,25 (OH)₂ Vitamin D. After stimulation, T cell proliferation was examined in a standard 60-h assay measuring [³H]thymidine incorporation. Data represent mean ± SEM values from 20 relapsing remitting multiple sclerosis patients and 20 control subjects. ***P* < 0.0001 compared with untreated cultures. Percentages of inhibition mediated by 1,25 (OH)₂ Vitamin D were similar in both relapsing remitting patients and healthy controls.

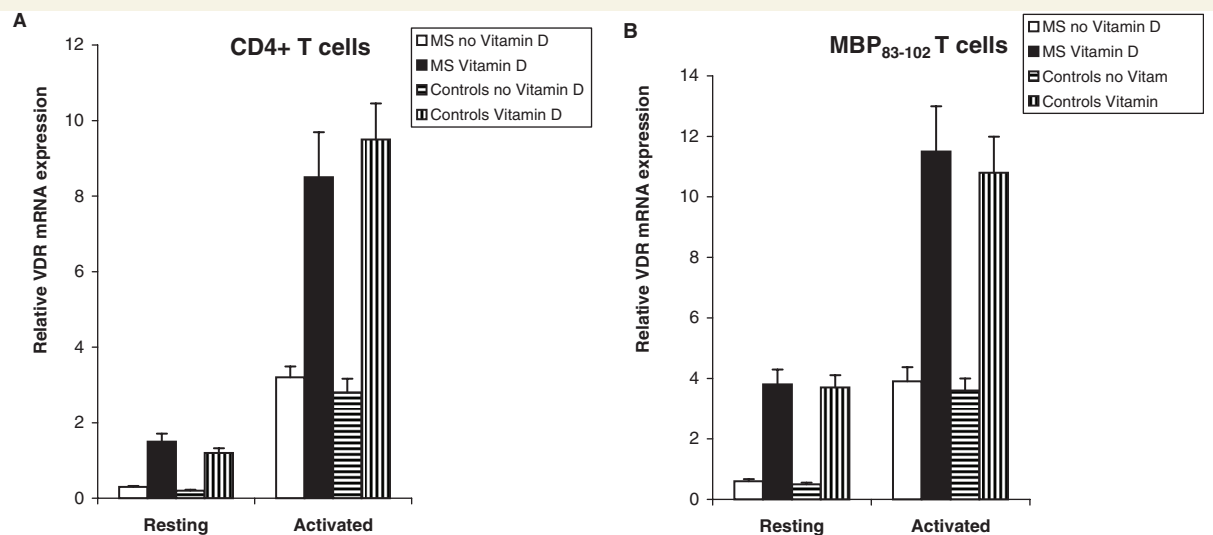


Figure 3 VDR is upregulated on activated T cells, and after exposure to 1,25 (OH)₂ Vitamin D. Purified CD4+ T cells and MBP peptide-specific T-cell lines were cultured in resting state and after activation in the presence or in the absence of 1,25 (OH)₂ Vitamin D (10 nM). Total RNA was extracted after 3 days in culture, and gene expression was detected by quantitative RT-PCR. Data are expressed as VDR mRNA relative to GAPDH, mean values ± SEM of mRNA expression in *ex vivo* CD4+ T cells isolated from 25 relapsing remitting multiple sclerosis patients, and 20 healthy controls (A), as well as from 35 myelin peptide-specific T-cell lines isolated from 20 relapsing remitting patients, and 30 myelin peptide-specific T-cell lines isolated from 20 healthy subjects (B). No significant differences were observed between relapsing remitting patients and healthy controls.

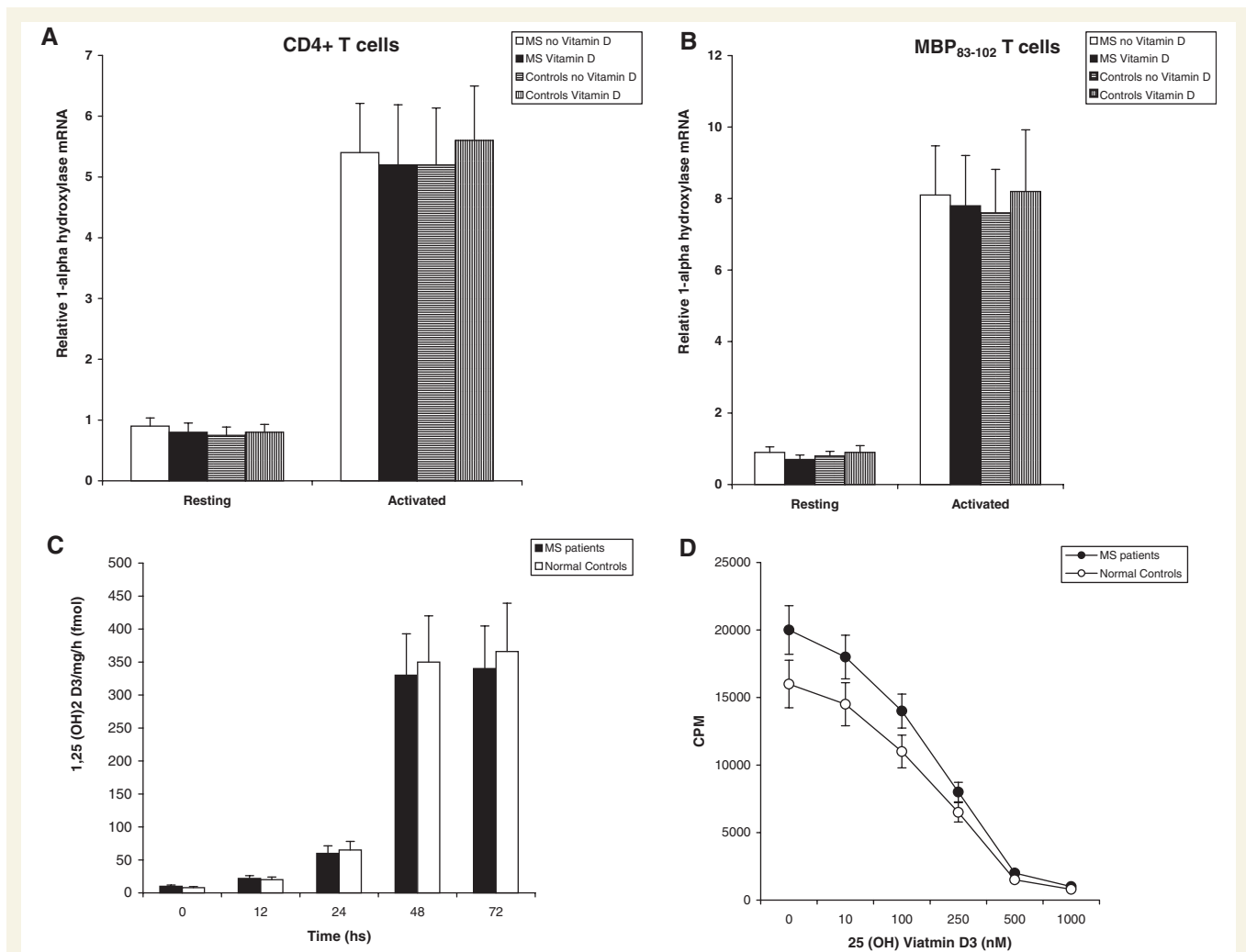


Figure 4 CD4+ and autoreactive T cells can metabolize 25(OH) Vitamin D into 1,25(OH)₂ Vitamin D. *Ex vivo* CD4+ T cells (A) and MBP-peptide specific T cells (B) constitutively expressed 1 α -hydroxylase mRNA, upregulated by activation, but not further induced by 1,25(OH)₂ Vitamin D. Cells were stimulated with PHA or the cognate peptide after 5 days in culture, 1 α -hydroxylase mRNA expression was determined by quantitative RT-PCR and expressed relative to GAPDH. The mean \pm SEM of eight independent experiments is shown. (C) Regulation of 1 α -hydroxylase. Cells were stimulated with or without 1,25(OH)₂ Vitamin D (10 nM) for 12, 24, 48 and 72 h. [³H]-25(OH) Vitamin D was included for the final 4 h. Data shown correspond to 1,25(OH)₂ Vitamin D production (1 α -hydroxylase activity). Results are expressed as mean \pm SEM of triplicate cultures, represent seven independent experiments. (D) *Ex vivo* CD4+ T cells from relapsing remitting multiple sclerosis patients and healthy controls were stimulated with PHA (1 μ g/ml) in the presence of different concentrations of 25(OH) Vitamin D ranging from 0 to 1000 nM. T cell proliferation was examined in a 60-h assay measuring [³H]-thymidine uptake. Data represent mean \pm SEM values from 10 relapsing remitting patients and 10 healthy control subjects. Percentages of inhibition were similar in both relapsing remitting patients and healthy subjects.

an enzyme recently found to be present in different tissues (Hewison *et al.*, 2007). Thus, we study expression and regulation of 1 α -hydroxylase mRNA by CD4+ T cells. As shown in Fig. 4A and B, both resting *ex vivo* CD4+ T cells and MBP-peptide specific T cells constitutively expressed 1 α -hydroxylase mRNA. Levels of expression were significantly up-regulated following activation, but not further induced by 1,25(OH)₂ Vitamin D. To determine whether this up-regulation had functional consequences, two different sets of experiments were performed. First, 1 α -hydroxylase activity in purified CD4+ T cells was measured. In this assay, cells were stimulated with PHA for different time periods, [³H]-25(OH) Vitamin D was included for a further 4 h, and conversion to

[³H]-1,25(OH)₂ Vitamin D measured using thin-layer chromatography. As shown in Fig. 4C, activity of 1 α -hydroxylase was induced 8- to 10-fold after 24 h of incubation, increasing 25- to 33-fold after 48 h. In a second group of experiments, different concentrations of the precursor 25(OH) Vitamin D were added to purified CD4+ T cells stimulated with PHA, and the proliferation of CD4+ T cells examined as read out of the assay. As illustrated in Fig. 4D, 25(OH) Vitamin D decreased the proliferation of CD4+ T cells in a concentration-dependent manner. Collectively, these results indicate that CD4+ T cells are capable of metabolizing 25(OH) Vitamin D to 1,25(OH)₂ Vitamin D, which in turn inhibits T cell function.

Effects of 1,25 (OH)₂ Vitamin D on cytokine secretion by CD4+ T cells

The impact of 1,25 (OH)₂ Vitamin D on cytokines production by T cells was assessed using purified CD4+ T cells as well as MBP-peptide specific T cell lines, stimulated with immobilized anti-CD3 mAb, in the presence and in the absence of 1,25 (OH)₂ Vitamin D. T cells were assessed under these conditions for their ability to secrete IFN- γ , IL-4, IL-6, IL-10, and IL-17. Data on Fig. 5A and B show changes in cytokine-secreting cell numbers following culture with 1,25 (OH)₂ Vitamin D relative to vehicle-treated cells in relapsing remitting patients and healthy controls. 1,25 (OH)₂ Vitamin D led to increased number of IL-10 producing T cells and decreased numbers of IL-6 and IL-17 producing cells. No changes were observed in numbers of T cells producing IL-4 or IFN- γ . Similar results were achieved when T cells were stimulated either with PHA or the cognate peptide in the presence of antigen presenting cells (data not shown). These findings were similar in both relapsing remitting patients and control subjects.

Because IL-10 is a positive autocrine factor enhancing development of IL-10 producing T cells, we assessed the role of IL-10 in this process. Using an antigen presenting cell free system, where T cells were stimulated with anti-CD3 mAb we found that the combination of 1,25 (OH)₂ Vitamin D and IL-10 led to enhanced numbers of human IL-10 producing T cells, compared with the effects of 1,25 (OH)₂ Vitamin D alone (Fig. 5C and D). In addition, the number of IL-10 producing T cells was significantly abrogated when a neutralizing anti-IL-10 receptor mAb was simultaneously added to cultures. Isotype control mAb had no effect (Fig. 5C and D). These results confirm that IL-10 is a positive autocrine factor that acts directly on T cells in the absence of antigen presenting cells, enhancing the action of 1,25 (OH)₂ Vitamin D. Similar results were observed in both relapsing remitting patients and healthy controls.

To evaluate 1,25 (OH)₂ Vitamin D effects on significant number of cytokine producing cells, experiments using polarized Th1, Th2, and Th17 effector cells generated in short-term cultures of purified CD4+ T cells from both relapsing remitting patients and healthy controls were performed. Polarized phenotypes were confirmed by analysing IFN- γ , IL-4 and IL-17 producing CD4+ T cell percentage as well as IFN- γ , IL-4 and IL-17 secretion levels. In addition, transcription factor T-bet (for Th1 cells), GATA-3 (for Th2 cells) and ROR γ t (for Th17 cells) were also measured (data not shown). As shown on Fig. 6, 1,25 (OH)₂ Vitamin D significantly inhibited IL-17 production in both relapsing remitting patients and healthy controls compared with vehicle treated polarized Th17 cells, compared with 1,25 (OH)₂ Vitamin D, which had no effect on IL-4 production by Th2 polarized cells. VDR agonists have been found to directly inhibit Th1 cytokines such as IL-2 and IFN- γ (Adorini and Penna, 2008). To assess the relationship between 1,25 (OH)₂ Vitamin D and IFN- γ secretion, we used Th1 polarized cells. Although difference observed were not statistically significant, 1,25 (OH)₂ Vitamin D treatment resulted in a trend of reduced IFN- γ production, both in relapsing remitting patients and healthy controls ($P=0.055$; Fig. 6)

1,25 (OH)₂ Vitamin D induces CD4+CD25+ FoxP3+ T cells through an IDO-mediated pathway

IDO is a key enzyme in tryptophan metabolism, catalysing its degradation to generate kynurenine. IDO induced tryptophan catabolism suppresses in turn T cell responses and promotes immune tolerance in autoimmunity, mammalian pregnancy and allergic inflammation. RT-PCR analysis indicated IDO mRNA presence in dendritic cells derived from both relapsing remitting patients and healthy controls. Likewise, both resting *ex vivo* CD4+ T cells, and MBP-peptide specific T cells from both populations constitutively expressed IDO mRNA, although at significantly lower levels (Fig. 7A; $P<0.0001$). Expression levels were significantly increased after 1,25 (OH)₂ Vitamin D exposure (Fig. 7B; $P<0.0001$). Culture of MBP-peptide specific T cells in complete medium containing tryptophan in the presence of 1,25 (OH)₂ Vitamin D, induced production of significantly higher levels of kynurenine, compared with those observed in the absence of 1,25 (OH)₂ Vitamin D. Interestingly, the addition of IDO inhibitor 1-MT significantly abrogated kynurenine production (Fig. 7C). These data suggest that IDO expressed by CD4+ T cells is functionally active. IDO has a role in regulatory T cell generation under both physiological and pathological conditions. Likewise, 1,25 (OH)₂ Vitamin D and its analogues enhance CD4+CD25+ regulatory T cell numbers, inhibiting autoimmune diseases and graft rejection in different experimental models. On the basis of this information, we studied IDO and 1,25 (OH)₂ Vitamin D regulatory T cell generating capacity, for which PBMC were cultured in the presence and in the absence of 1,25 (OH)₂ Vitamin D and the IDO inhibitor 1-MT. As shown in Fig. 7D culture of PBMC in the presence of 1,25 (OH)₂ Vitamin D significantly increased CD4+CD25+FoxP3+ T cell percentage. The addition of 1-MT to cultures restored the of CD4+CD25+FoxP3+ T cell percentage to levels observed prior to adding 1,25 (OH)₂ Vitamin D (Fig. 7D), suggesting that Vitamin D induces CD4+CD25+FOxP3+ regulatory T cells through an IDO dependent pathway. Cell viability when cultured in presence of 1-MT did not differ from that of cells cultured in medium alone. We also analysed IDO mRNA expression on CD4+CD25-, and CD4+CD25+ T cells, in both relapsing remitting patients and healthy controls, and found no differences between either cell population (Fig. 8A).

Regulatory properties of CD4+CD25+ FoxP3+ T cells isolated after 1,25 (OH)₂ Vitamin D exposure were further investigated by testing ability of these cells to suppress proliferative responses and IFN- γ secretion by CD4+CD25- cells. To do this, CD4+CD25- T cells were stimulated with anti-CD3 and anti-CD28 mAb, while increasing numbers of autologous CD4+CD25+ cells were added. As illustrated in Fig. 8B CD4+CD25+FoxP3+ cells generated by exposure to 1,25 (OH)₂ Vitamin D were able to suppress the proliferation of indicator CD4+CD25- T cells, titrating from high to low ratios of CD4+CD25+:CD4+CD25-. Furthermore, CD4+D25+FoxP3+ cells suppressed the proliferation of CD4+CD25- T cells in response to PHA ($66\pm 18\%$, at ratio 10:1), and to plate-bound anti-CD3 stimulation ($75\pm 20\%$, at ratio 10:1), suggesting that the suppressive effect of CD4+D25+FoxP3+

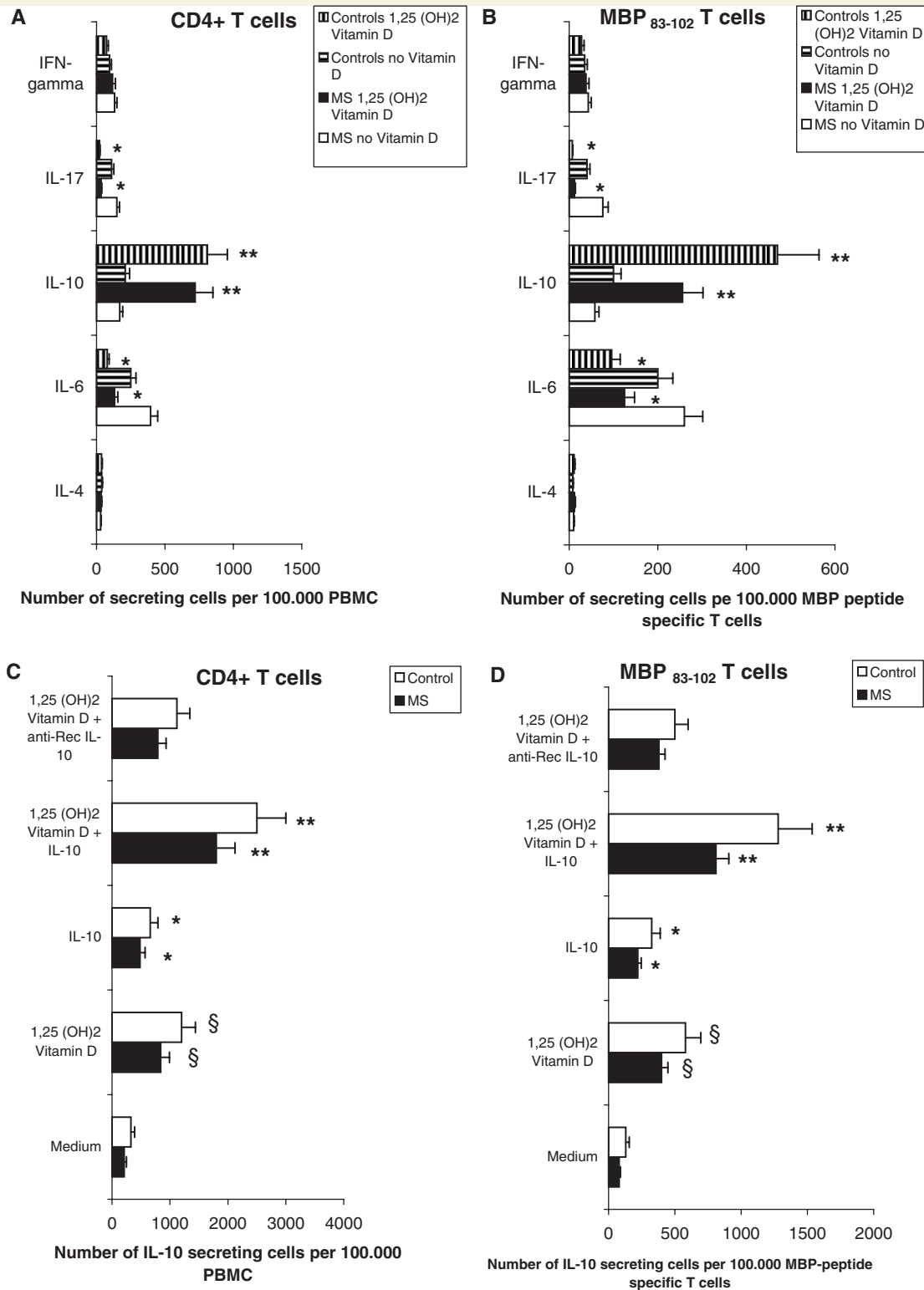


Figure 5 1,25 (OH)₂ Vitamin D increases the development of IL-10 secreting cells and down-regulates the number of IL-6 and L-17 secreting cells. Purified CD4+ T cells (A) and MBP peptide-specific T-cell lines (B) isolated from both relapsing remitting multiple sclerosis patients and healthy controls, were stimulated with 1 µg/ml of plate-bound anti-CD3 mAb in the presence or in the absence of 1,25 (OH)₂ Vitamin D (10 nM). After 72 h in culture the number of cytokine secreting cells was evaluated using ELISPOT assays. In some experiments CD4+ T cells (C) or MBP peptide-specific T-cell lines (D) were incubated with IL-10 (ng/ml) alone or in combination with 1,25 (OH)₂ Vitamin D. In other groups of experiments neutralizing anti IL-10 receptor mAb (10 µg/ml) was simultaneously added to culture, and the number of IL-10 secreting cells evaluated using ELISPOT assays. Results of 16 experiments are shown. *P=0.01, ^SP=0.001, **P<0.0001, compared with untreated cells.

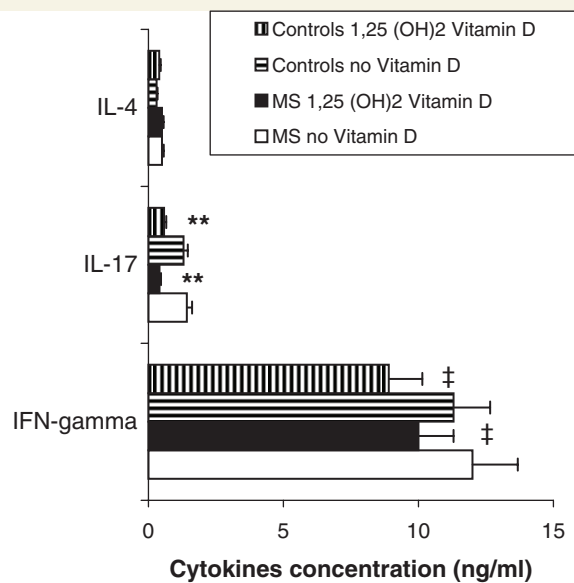


Figure 6 1,25 (OH)₂ Vitamin D downregulated IL-17 production in Th17 polarized cells. Purified CD4⁺ T cells isolated from relapsing remitting multiple sclerosis patients and healthy controls were polarized as described in Materials and Methods to obtain Th1, Th2 and Th17 cells. Polarized T helper cells were stimulated with plate bound anti-CD3 mAb, and with supernatant collected at 36 (IL-4), and 72 h (IFN- γ and IL-17). Cytokine secretion was measured using ELISA. Data represent mean \pm SEM values from 20 relapsing remitting patients and 20 control subjects. ** $P < 0.0001$, ‡ $P = 0.055$, compared with untreated cells.

is independent of antigen presenting cells. Moreover, prevention of cell contact abolished the regulatory function that CD4⁺CD25⁺ Treg cells exert on CD4⁺CD25⁻ T cells (data not shown). In addition, CD4⁺CD25⁺FoxP3⁺ T cells generated after 1,25 (OH)₂ Vitamin D exposure were also capable of suppressing the production of IFN- γ by CD4⁺CD25⁻ T cells activated by anti CD3/CD28 mAbs (Fig. 8C). Overall, these data suggest that 1,25 (OH)₂ Vitamin D induced CD4⁺CD25⁺FoxP3⁺ T cell development through an IDO-mediated pathway while retaining immunosuppressive activity and may thus be considered bona fide regulatory T cells. Addition of 1MT to culture media did not affect the suppressive action of CD4⁺CD25⁺ Treg cells on CD4⁺ T cell proliferation (Fig. 8D), or IFN- γ secretion (Fig. 8E); suggesting that IDO has impact on Treg cell development induced by Vitamin D, but does not affect regulatory properties of these same cells. In contrast, addition of IDO inhibitors to either CD4⁺ T cells or MBP-peptide specific T cell cultures abrogated 1,25 (OH)₂ Vitamin D mediated inhibitory effects on T cells proliferation (Fig. 8F). This effect was significantly higher in cultures from healthy subjects, suggesting differences in IDO-mediated tryptophan deprivation.

Discussion

In this study, we show that relapsing remitting patients, particularly during exacerbations, have reduced serum levels of 25 (OH)

Vitamin D, and 1,25 (OH)₂ Vitamin D compared with healthy subjects, suggesting that in this group of patients Vitamin D-dependent T cell regulation may play an important role in maintaining T cell homeostasis. Recently, high circulating levels of 25 (OH) Vitamin D have been associated with a lower risk of MS (Munger *et al.*, 2006). The results of this study showed serum levels of 25 (OH) Vitamin D, and 1,25 (OH)₂ Vitamin D in primary progressive patients were similar to those observed in healthy individuals. Consistent with these observations, a growing body of evidence indicates that distinct pathogenic processes mediate brain damage in different groups of multiple sclerosis patients. Active focal inflammatory demyelinating lesions in the white matter are mainly present in patients with relapsing remitting multiple sclerosis for example, while diffuse injury of normal-appearing white matter, cortical demyelination and signs of oligodendrocyte dystrophy are mainly hallmarks of the primary progressive disease (Lucchinetti *et al.*, 2000; Kutzelnigg *et al.*, 2005). Moreover, while immunomodulatory and immunosuppressive treatments are beneficial during acute and relapsing stages of the disease, they have no effect in primary progressive patients (Noseworthy *et al.*, 2000; Goodin *et al.*, 2002). Overall, these findings would appear to indicate that pathological features additional to classic inflammatory demyelinating lesions might be important in primary progressive pathogenesis.

Serum concentration of 25 (OH) Vitamin D is a reflection of the balance between Vitamin D intake in food, its synthesis from pro-vitamins in the skin under the influence of UV light, and its catabolism by 24-hydroxylase. Thus, either a decrease in synthesis or an increase in 25 (OH) Vitamin D degradation could explain low serum levels observed in multiple sclerosis patients. Some studies associating higher dietary intake of Vitamin D with protection against autoimmune diseases have been published (Hyppönen *et al.*, 2001; Merino *et al.*, 2004), and inverse correlation between multiple sclerosis prevalence and sunlight exposure has been observed both in early ecological studies, as well as in recent case-control ones (Acheson *et al.*, 1960; van der Mei *et al.*, 2003; Islam *et al.*, 2007). Likewise, seasonal changes in Vitamin D levels have also been reported as low during winter months and elevated in summer ones. Furthermore, correlation of seasonality has also been found between low Vitamin D levels and multiple sclerosis flares (Bamford *et al.*, 1983). Despite the fact that both patients and control subjects included in this investigation lived in the same area, and that samples from both groups were collected during the same months of the year, it is important to consider that patients frequently have difficulty exercising or moving about and therefore spend less time outdoors and are less exposed to sunlight. Measurement of sunlight exposure in patients compared with control subjects using specific questionnaires is currently under investigation. Likewise, influence of 24-hydroxylase on 25 (OH) Vitamin D levels, not assessed in this study, is now under examination in ongoing studies at our laboratory.

1,25 (OH)₂ Vitamin D effects are mediated primarily through interaction with intracellular VDR (Adorini and Penna, 2008), although recent reports have highlighted rapid non-genomic effects of 1,25 (OH)₂ Vitamin D (Norman, 1998). VDR is present in different tissues including cells of the immune system such as monocytes, dendritic cells and B cells (Veldman *et al.*, 2000;

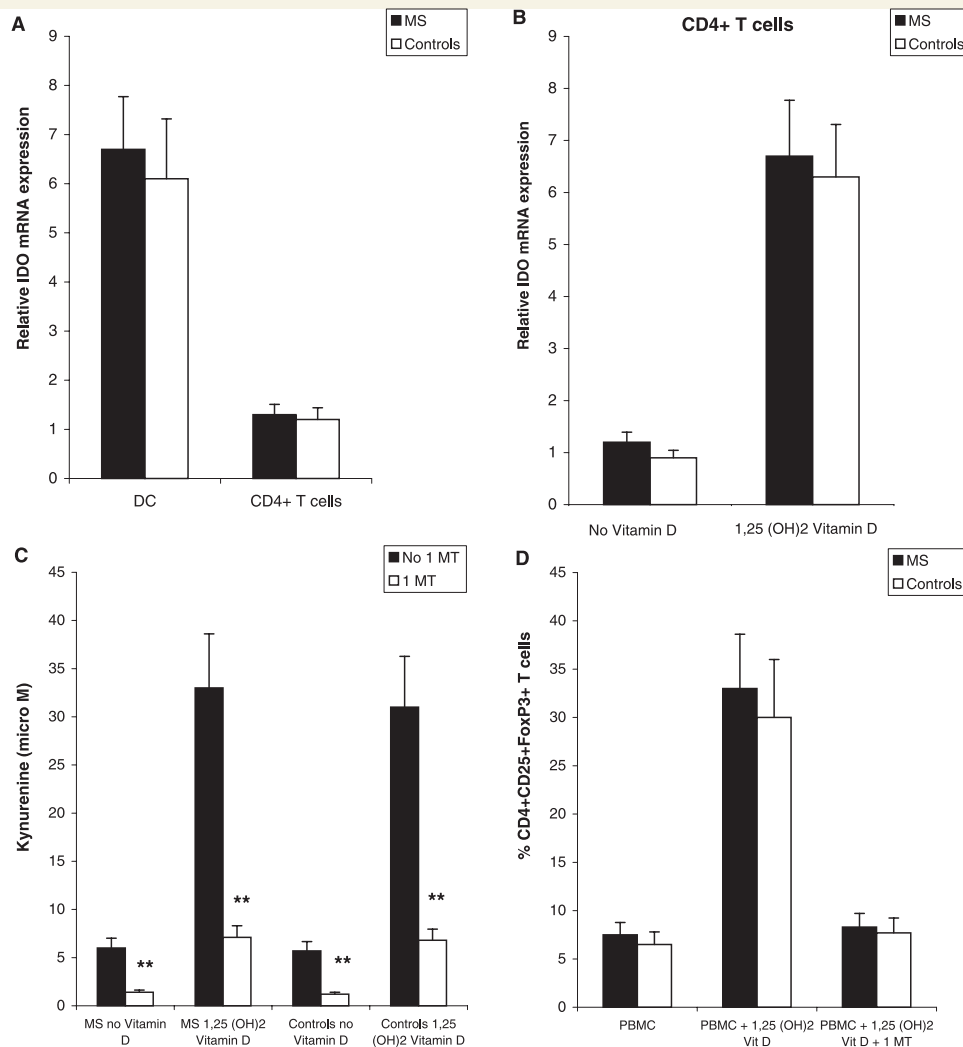


Figure 7 (A) Dendritic cells, and CD4+ T cells from relapsing remitting multiple sclerosis patients, and healthy controls constitutively expressed IDO. (B) IDO expression was up-regulated by 1,25 (OH)₂ Vitamin D. Cells were cultured for 3 days in the presence and in the absence of 1,25 (OH)₂ Vitamin D, IDO mRNA expression was determined by quantitative RT-PCR and expressed relative to GADPH. (C) *Ex vivo* CD4+ derived from relapsing remitting patients and healthy controls, were cultured in medium supplemented with additional tryptophan with or without 1,25 (OH)₂ Vitamin D for 48 h. Kynurenine in the culture supernatant was measured by HPLC. The addition of the IDO inhibitor 1-MT abrogated the 1,25 (OH)₂ Vitamin D effect. (D) Culture of PBMC in the presence of 1,25 (OH)₂ Vitamin D significantly increased the percentage of CD4+CD25+FoxP3+ regulatory T cells as measured by flow cytometry. Once again the addition of the IDO inhibitor 1-MT abrogated 1,25 (OH)₂ Vitamin D effect. Data represent mean \pm SEM values from 20 relapsing remitting patients and 18 control subjects. ** $P < 0.0001$.

Norman, 2006; Chen *et al.*, 2007). We demonstrated that VDR mRNA is constitutively expressed in CD4+ T cells, and is upregulated following activation and 1,25 (OH)₂ Vitamin D exposure. Likewise, 1- α hydroxylase, mainly found in renal tubule cells, was recently described in a wide variety of tissues (Hewison *et al.*, 2007), and cloning studies have revealed the same mRNA for 1- α hydroxylase in renal and extra-renal tissues (Monkawa *et al.*, 1997). However, although the enzyme present in immune cells is identical to the renal form, its regulation seems to be under a different control system, mediated mainly by immune signals, such as antigenic stimulators or inflammatory mediators like IFN- γ (Hewison *et al.*, 2003; Overbergh *et al.*, 2006). Interestingly, we found that 1- α hydroxylase mRNA was also expressed in resting

CD4+ T cells at low levels, and increased its expression significantly after stimulation, but not following 1,25 (OH)₂ Vitamin D exposure. Moreover, we found that 25 (OH) Vitamin D could be metabolized to 1,25 (OH)₂ Vitamin D by CD4+ T cells, representing local production of active Vitamin D, as was previously demonstrated for macrophages, dendritic cells and thyroid tissue.

Activation of the VDR is known to alter transcription, proliferation and differentiation of immune cells (Dong *et al.*, 2005; Muthian *et al.*, 2006; Adorini and Penna, 2008). In this study, we demonstrated that activated Vitamin D has different immunoregulatory effects on CD4+ T cells, namely it (i) inhibits CD4+ T cell proliferation; (ii) enhances IL-10 secreting cell development, and inhibits IL-6 and IL-17 producing cell development;

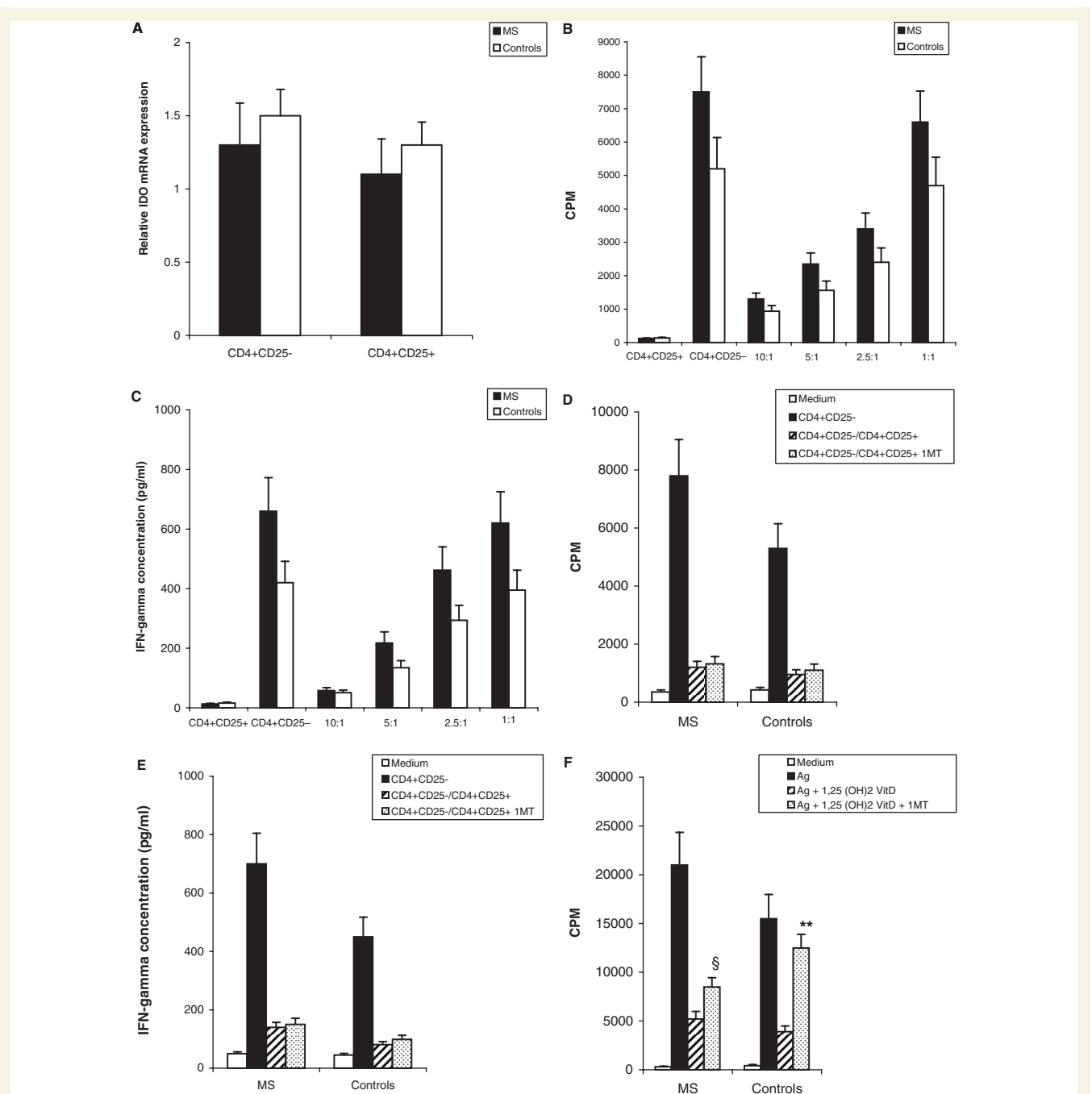


Figure 8 (A) Both CD4+CD25– and CD4+ CD25+ T cells constitutively expressed similar amounts of IDO mRNA. (B) CD4+CD25+ FoxP3+ regulatory T cells induced by 1,25 (OH)₂ Vitamin D mediated suppression of proliferation induced by anti-CD3/anti CD-28 in CD4+CD25– target T cells, titrating from high to low ratios of CD4+CD25+: CD4+CD25–. Data represent mean values ± SEM of 16 experiments. (C) CD4+CD25+FoxP3+ regulatory T cells induced by 1,25 (OH)₂ Vitamin D were potent suppressors of IFN-γ secretion induced by anti-CD3/anti-CD28 in CD4+CD25– target cells. Data represent mean values ± SEM of 16 experiments. (D) CD4+CD25+Foxp3+ T cells induced by 1,25 (OH)₂ Vitamin D were co-cultured with CD4+CD25– T cells at a ratio 10:1, and stimulated with anti-CD3 and soluble anti-CD28 mAbs, both at 5 μg/ml concentrations in the presence of irradiated PBMC, depleted of CD3+ T cells as a source of antigen presenting cells; 1 MT was added at a concentration of 1000 μM. Data represent mean ± SEM values from 18 relapsing remitting multiple sclerosis patients and 16 healthy controls. (E) Culture conditions were similar to those described for panel (D). To measure IFN-γ, supernatant was removed from each well and analyzed using commercially available ELISA kits. Data represent mean ± SEM values from 18 relapsing remitting patients and 16 healthy controls. (F) MBP- and MOG-peptide-specific T cell lines from relapsing remitting patients and control subjects were cultured as described in Materials and methods section, in the presence and in the absence of 1,25 (OH)₂ Vitamin D, and 1 MT at 1000 μM concentration. Data represent mean ± values from 18 relapsing remitting patients and 18 healthy controls. Similar results were observed using *ex vivo* CD4+ T cells. ****P* < 0.0001 and [§]*P* = 0.001 compared with 1,25 (OH)₂ Vitamin D treated cultures.

and (iii) induces CD4+CD25+ FoxP3+ regulatory T cells through an IDO-mediated pathway.

1,25 (OH)₂ Vitamin D inhibition of cell proliferation has been reported in lymphocytes and different cancer cell lines (Munker *et al.*, 1996; Li *et al.*, 2004; Chen *et al.*, 2007; Smolders *et al.*, 2008). Proliferation inhibition has been linked to different mechanisms, such as inducing gene transcription of CDK inhibitors p21 and p27, which inhibit cell cycle progression (Liu *et al.*, 2002; Li *et al.*, 2004; Chen *et al.*, 2007). Alternatively, 1,25 (OH)₂ Vitamin D may help to limit uncontrolled proliferation through apoptosis induction, since dendritic cells stimulated with 1,25 (OH)₂ Vitamin D or Vitamin D analogues induce T cell apoptosis (van Halteren *et al.*, 2004). Interestingly, in a recent study apoptosis induction of activated CD4+ T cells was observed in the CNS and spleen of EAE animals (Pedersen *et al.*, 2007).

Active Vitamin D is known to inhibit the activation and action of different transcription factors involved in cytokine gene regulation (Alroy *et al.*, 1995; D'Ambrosio *et al.*, 1998; Takeuchi *et al.*, 1998). A combination of 1,25 (OH)₂ Vitamin D and dexamethasone has been shown to induce human and mouse naïve CD4+ T cells to differentiate *in vitro* into IL-10 secreting cells (Barrat *et al.*, 2002). In the present study, upregulation of IL-10 by 1,25 (OH)₂ Vitamin D stimulation was also present in CD4+ T cells from MS patients in the absence of dexamethasone. Similar results have been previously shown in patients with Crohn's disease (Bartels *et al.*, 2007). Our data also showed that IL-10 itself had synergistic action with 1,25 (OH)₂ Vitamin D. These observations are supported by experiments in the EAE model, where 1,25 (OH)₂ Vitamin D significantly inhibits the disease in wild-type animals, but not in IL-10 or IL-10 receptor knockout strains, indicating that the IL-10/IL-10 receptor pathway is essential for 1,25 (OH)₂ Vitamin D-mediated EAE inhibition (Spach *et al.*, 2006). Aside from up-regulated IL-10 production, we observed significant down-regulation of IL-6, and IL-17 secreting T cells. Investigators have found TGF-β, and IL-6 are critical factors for Th17 development (Weaver *et al.*, 2007). These data are in agreement with the known inhibition of IL-17 production by VDR agonists during the course of experimental autoimmune prostatitis (Penna *et al.*, 2006). In contrast to other groups, we observed no significant effect on IFN-γ production. Even when CD4+ T cells were polarized to a Th1 phenotype, 1,25 (OH)₂ Vitamin D treatment resulted in a trend of reduced IFN-γ production, although the level of reduction was not statistically significant. 1,25 (OH)₂ Vitamin D capacity to inhibit T cell IFN-γ production has been previously reported, but could not be confirmed by other studies (Cantorna *et al.*, 1998; Nashold *et al.*, 2001; Mahon *et al.*, 2003; Muthian *et al.*, 2006). Overall, these data support the notion that 1,25 (OH)₂ Vitamin D plays an important role in shaping the development of T cell responses, inducing T cells with immunosuppressive properties. It is important to note that in these experiments 1,25 (OH)₂ Vitamin D was used at pharmacological doses ~82- to 525-fold higher than circulating levels found in healthy individuals. It appears likely that increasing Vitamin D levels could reduce the risk of multiple sclerosis. If so, it is important to establish optimal Vitamin D levels, and to verify whether Vitamin D supplements administered at these levels contribute to prevention without producing side effects.

An important effect of 1,25 (OH)₂ Vitamin D is the induction of T cells with regulatory properties (Adorini and Penna, 2008). Regulatory T cells are induced by the modulation of antigen presenting cells, particularly dendritic cells. Notably, *in vitro* treatment of dendritic cells with 1,25 (OH)₂ Vitamin D leads to down-regulated expression of co-stimulatory molecules and reduced IL-12 production, with acquisition of a tolerogenic phenotype (Adorini, 2003). These dendritic cells could favour peripheral tolerance not only by inhibiting effector T cell activation, but also by inducing the differentiation of CD4+CD25+FoxP3+ regulatory T cells able to arrest the development of autoimmune responses. In our study the percentage of CD4+CD25+FoxP3+ was significantly increased when PBMC were cultured in the presence of 1,25 (OH)₂ Vitamin D. In the present study we show that this process is mediated by an IDO-dependent pathway.

IDO has been widely investigated for the induction of immunological tolerance, and its expression associated with an increased number of circulating CD4+CD25+FoxP3+ T cells in physiological and pathological conditions (Munn *et al.*, 1998; Uyttenhove *et al.*, 2003). This effect could be explained through different mechanisms: (i) expansion of CD4+CD25+FoxP3+; (ii) increased apoptosis of CD4+CD25- over CD4+CD25+ T cells; and (iii) conversion of CD4+CD25- into CD4+CD25+ T cells (Curti *et al.*, 2007). Different models support the notion that microenvironments containing reduced tryptophan concentration and high kynurenine concentration favor the emergence of CD4+CD25+FoxP3+ T cells by conversion from CD4+CD25- T cells (Fallarino *et al.*, 2006; Curti *et al.*, 2007). Thus, the role of IDO has shifted from that of a metabolic regulator of tryptophan availability to one that is central to immune homeostasis. Indeed, it has been recently proposed that IDO acts as a bridge between dendritic cells and CD4+ regulatory T cells (Puccetti and Grohmann, 2007).

Overall, 1,25 (OH)₂ Vitamin D affects the immune system at different levels through different mechanisms, conferring on the whole an immunosuppressive effect. Correction of 1,25 (OH)₂ Vitamin D deficiency may be useful to suppress autoimmune disorders such as MS. Nevertheless, before conclusions can be drawn, further investigations are needed to determine optimal dose. Similarly, studies are warranted to compare efficacy and safety of different Vitamin D analogues with less hypercalcemic effects.

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