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Gender Differences in 1,25 Dihydroxyvitamin D₃ Immunomodulatory Effects in Multiple Sclerosis Patients and Healthy Subjects

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Vitamin D₃ is best known as a calcium homeostasis modulator; however, it also has immune-modulating potential. In this study, we demonstrated that immunomodulatory effects of vitamin D₃ are significantly stronger in females than in males in multiple sclerosis patients, as well as in healthy subjects. Inhibition of self-reactive T cell proliferation and reduction in IFN- γ - and IL-17-secreting cell numbers were considerably greater in females. Furthermore, the increase in IL-10-secreting and CD4⁺CD25⁺FoxP3⁺ regulatory T cell numbers were also greater in females. In parallel with these findings, female subjects had fewer *CYP24A1* transcripts encoding the 1,25-dihydroxyvitamin D₃-inactivating enzyme, as well as greater binding and internalization of vitamin D₃-binding protein, a transporter for vitamin D₃ and its metabolites. These gender-based disparities lead to the accumulation of vitamin D₃ and its metabolites in target cells from female subjects and result in a more potent anti-inflammatory effect. Interestingly, 17- β estradiol reproduced these effects on self-reactive T cells and macrophages from male subjects, suggesting a functional synergy between 1,25-dihydroxyvitamin D₃ and 17- β estradiol, mediated through estrogen receptor α . Collectively, these results demonstrate estrogen-promoted differences in vitamin D₃ metabolism, suggesting a greater protective effect of vitamin D₃-based therapeutic strategies in women. *The Journal of Immunology*, 2010, 185: 4948–4958.

Multiple sclerosis (MS) is an inflammatory demyelinating disease affecting the CNS, in which autoimmune-mediated mechanisms induce myelin injury (1). It is generally accepted that autoimmune diseases, such as MS, arise from a complex interaction between genetic susceptibility and environmental factors (2, 3). The genetic component of MS is thought to result from the action of common allelic variants in several genes (4). However, discordance of MS development between monozygotic twins suggests that additional factors could be involved (5). Geographical differences in disease incidence and prevalence, as well as studies of populations migrating from areas of low risk to areas of high risk, strongly support a role for environmental factors in disease pathogenesis (6, 7).

Vitamin D₃ is best known as a calcium homeostasis modulator; however, experimental and clinical observations provide evidence that vitamin D₃ is also an environmental factor exerting significant influence over immunoregulation, ultimately affecting MS prevalence. A protective effect was proposed after observations in-

dicating reduced risk for disease development associated with sunlight exposure and use of vitamin D₃ supplements (8–15). A recent longitudinal study conducted among American patients showed decreased MS risk in white patients with increased 25-hydroxyvitamin D₃ [25-(OH)D₃] serum levels, and a greater degree of disability has been strongly associated with lower 25-(OH)D₃ levels and reduced sun exposure (16). Moreover, a recent genome-wide association study conducted by the Australian and New Zealand gene consortium identified new MS risk associated with single nucleotide polymorphisms on chromosome 12q13–14 near the gene *CYP27B1*, encoding the enzyme that converts inactive 25-(OH)D₃ into 1,25-dihydroxyvitamin D₃ [1,25-(OH)₂D₃], the biologically active metabolite of vitamin D₃ (17). These findings converge with experimental evidence indicating a protective effect for vitamin D₃ in animal models of MS. For example, experimental autoimmune encephalomyelitis (EAE) can be prevented by administering the active metabolite 1,25-(OH)₂D₃ prior to immunization and ameliorated when given after disease onset (18, 19). Conversely, vitamin D₃ deficiency decreased the time to EAE onset and increased disease severity (19, 20). Interestingly, recent investigations showed that 1,25-(OH)₂D₃ significantly inhibits EAE in female mice, but not in male mice, and that ovariectomy abrogates this protective effect, suggesting a link between female sex hormones and 1,25-(OH)₂D₃ metabolism (21). Synergy between 17- β estradiol (E2) and 1,25-(OH)₂D₃ occurred through 1,25-(OH)₂D₃-mediated enhancement of E2 synthesis, as well as E2-mediated enhancement of CNS vitamin D₃ receptor (VDR) transcription and function (22). Given the evidence indicating that 1,25-(OH)₂D₃ immune response modulation exists in vitro and in animal models, assessing whether this modulation might also be found in MS patients becomes a most interesting question.

Previously, we demonstrated that relapsing-remitting MS patients present reduced serum 25-(OH)D₃ levels and 1,25-(OH)₂D₃ levels compared with healthy subjects, particularly during exacerbations; this suggests that 1,25-(OH)₂D₃-dependent T cell reg-

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Abbreviations used in this paper: DBP, vitamin D-binding protein; E2, 17- β estradiol; EAE, experimental autoimmune encephalomyelitis; EDSS, expanded disability scale score; ER, estrogen receptor; F, females; M, males; MBP, myelin basic protein; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; 25-(OH)D₃, 25-hydroxyvitamin D₃; 1,25-(OH)₂D₃, 1,25-dihydroxyvitamin D₃; RREMS, relapsing-remitting MS during exacerbation; RRRMS, relapsing-remitting MS during remission; TCL, T cell line; VDR, vitamin D receptor.

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ulation may play an important role in maintaining T cell homeostasis in this group of patients (25). In this study, we investigated the immunomodulatory effects of 1,25-(OH)₂D₃ in relation to female hormones. To this end, serum levels of 25-(OH)D₃ and 1,25-(OH)₂D₃ were measured in female and male MS patients. Subsequently, to gain more insight into putative regulatory mechanisms of estrogens and 1,25-(OH)₂D₃ in MS pathogenesis, the immunoregulatory effects of 1,25-(OH)₂D₃ on CD4⁺ T cells and myelin-peptide specific T cells isolated from female and male MS patients were assessed, as well as the mechanism by which E2 might regulate vitamin D₃ metabolism.

Materials and Methods

Patients and control subjects

Ninety-two Hispanic patients with a diagnosis of relapsing-remitting MS, according to Poser's and McDonald's criteria (24, 25), were studied: 34 during exacerbations and 58 in remission. Exacerbations were defined as development of new symptoms or worsening of pre-existing ones, confirmed on neurologic examination and lasting ≥ 48 h, in the absence of fever, and preceded by stability or improvement lasting ≥ 30 d. Patients with inflammatory, endocrine, or major psychiatric disorders were excluded from the study. Patients had not received steroids for ≥ 6 mo prior to study entry nor any other immunomodulatory or immunosuppressive drugs or dietary supplements. All patients lived in the city of Buenos Aires (latitude 34°S, longitude 58°W). No postmenopausal women were included in the study. No differences in clinical disability or disease duration were observed between male and female patients included in this cohort. Thirty healthy individuals, matched for race/ethnicity, age, gender, and place of residence served as controls. Other underlying disorders were ruled out after thorough clinical and neurologic examination and standard chemical and hematological laboratory examinations. Control individuals were not taking any medication or dietary supplements. Samples from healthy controls were collected during the same months of the year as samples from MS patients. Demographic and clinical characteristics of patients and controls are shown in Table I. Blood samples were obtained from relapsing-remitting MS patients during remission for in vitro T cell function analysis in all cases. The study was approved by the Institutional Ethics Committee.

Determination of serum 25-(OH)D₃ and 1,25-(OH)₂D₃ levels

Serum 25-(OH)D₃ and 1,25-(OH)₂D₃ levels were measured using commercially available ELISA kits (Immundiagnostik, Bensheim, Germany), following the 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.

Ag preparation

Myelin basic protein (MBP)_{83–102} (ENPVVHFFKNIVFPRTPPPS), MBP_{143–168} (GVDAQGTLSKIFKLGGRDSRSGSPMA), and myelin oligodendrocyte glycoprotein (MOG)_{63–87} (PEYRGRTELLKDAIGEGKVTLRIRN) peptides were synthesized with an automated peptide synthesizer using FAST-MOC chemistry (26). Peptides were cleaved from resin using trifluoroacetic acid, chromatographed on Sephadex G-10 with 30% acetic acid, and lyophilized. Each peptide was analyzed by HPLC and was found to have the amino acid composition expected.

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

PBMCs were isolated by Ficoll-Hypaque density-gradient centrifugation (Pharmacia, Peapack, NJ), and CD4⁺ T cells were positively selected using Dynabeads M-450 CD4 beads (DynaL Biotech, Oslo, Norway). Separation was monitored using flow-cytometry analysis, demonstrating >97% purity. MBP- and MOG-peptide specific T cell lines (TCLs) were expanded from peripheral blood, as previously described (27). Briefly, 5 × 10⁶ PBMCs were stimulated with optimal concentrations of MBP or MOG peptides (10–20 μg/ml). After 5–7 d, cells were recultured in fresh medium containing 50 U/ml human rIL-2 (R&D Systems, Minneapolis, MN) for an additional week. Restimulation cycles with autologous irradiated PBMCs (3000 rad) as APCs plus peptide, followed by expansion with rIL-2, were repeated weekly. After four cycles of restimulation and expansion, TCLs were evaluated using standard proliferation assays. Cut-off values for a positive response were set at stimulation index >3. All MBP- and MOG-reactive TCLs were >93% CD4⁺.

For macrophages, isolation monocytes were positively selected from PBMCs using anti-CD14-coated magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany), following the manufacturer's instructions. After separation, monocytes were resuspended in RPMI 1640 medium (JRH Biosciences, Lenexa, KS) containing 5% heat-inactivated human AB⁺ serum (Sigma-Aldrich, St Louis, MO) and 2 mM L-glutamine (JRH Biosciences), plated at a density of 4 × 10⁵ cells/cm² on 24-well Falcon Primaria plates (BD Biosciences, San Jose, CA), and allowed to differentiate for 5–7 d at 37°C and 5% CO₂. Viability was assessed by trypan blue dye exclusion, and monocyte enrichment was established with an α-naphthyl acetate kit (nonspecific esterase; Sigma-Aldrich), following the manufacturer's instructions. Cells obtained with this protocol showed >98% viability and >95% esterase positivity.

Stimulation of CD4⁺ T cells and peptide-specific TCLs and proliferation assays

For peptide Ag-specific stimulation, TCLs were cultured at a density of 5 × 10⁴ cells/well in the presence of 5 × 10³ adherent irradiated autologous PBMCs, as the source of APCs, and 10 μg/ml the appropriate peptide, with and without 1,25-(OH)₂D₃ (Sigma-Aldrich). For nonspecific stimulation, peptide-specific TCLs (5 × 10⁴ cells/well) were cultured in the presence of 1 μg/ml immobilized anti-CD3 mAb (OKT3; American Type Culture Collection, Manassas, VA) with and without 1,25-(OH)₂D₃. Irradiated PBMCs were not included under these conditions. Meanwhile, purified CD4⁺ T cells were plated at a density of 5 × 10⁴ cells/well and stimulated with 1 μg/ml 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 (27). Thymidine incorporation (cpm) was calculated as the mean value of triplicate cultures. 1,25-(OH)₂D₃ was dissolved in absolute ethanol at a stock concentration of 0.01 M and further diluted in RPMI 1640 medium to the concentrations tested. Additional controls included viability studies using trypan blue dye exclusion, the addition of ethanol diluted to concentrations equivalent to those used for 1,25-(OH)₂D₃ dilutions, and serum-free medium without phenol red. This last control was included to rule out possible estrogen-like activity of phenol red (28). No autologous serum was used in any of the culture conditions.

Quantification of cytokines secreted

The number of CD4⁺ T cells or MBP- and MOG-peptide specific T cells secreting IL-10, IL-17, and IFN-γ was evaluated using commercially available kits (R&D Systems) for single-cell resolution ELISPOT assays, following the manufacturer instructions, with and without 1,25-(OH)₂D₃. Cytokine-secreting cell numbers were calculated by subtracting the number 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 the number of spots per 10⁵ PBMCs. Optimal doses of 1,25-(OH)₂D₃ were established in preliminary experiments. As for proliferation assays, serum-free medium without phenol red was included in parallel experiments as control.

Evaluation of CD4⁺CD25⁺FoxP3⁺ regulatory T cells

To evaluate the role of 1,25-(OH)₂D₃ on regulatory T cell induction, 5 × 10⁴ PBMCs from male and female MS patients were stimulated with soluble anti-CD3 and soluble anti-CD28 (BD Biosciences) Abs, both at 5 μg/ml, with and without 1,25-(OH)₂D₃. After 7 d, the number of CD4⁺CD25⁺FoxP3⁺ T cells was evaluated by flow cytometry, using commercially available regulatory T cell-staining kits (eBioscience, San Diego, CA), following the manufacturer's instructions. Optimal doses of 1,25-(OH)₂D₃ were established in preliminary experiments. Serum-free medium without phenol red was included in parallel experiments as control.

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 the manufacturer's instructions. RNA was reverse transcribed using an M-MLV RT reverse-transcription kit with random hexamer primers (Invitrogen). Relative levels of *VDR*, *CYP27B1*, and *CYP24A1* transcripts 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 GAPDH transcripts as an internal control. These values were then standardized such that a minimum value of 1.0 was assigned to the group with the lowest gene expression. The following primer sequences were used: GAPDH: forward 5'-GAAGGTGAAGTCG-GAGTC-3', reverse 5'-GAAGATGGTATGGGATTTC-3'; *VDR*: forward 5'-CTTCAGGCGAAGCATGAAGC-3', reverse 5'-CCTTCATCATGCCG-

ATGTCC-3'; *CYP27B1*: forward 5'-TTGGCAAGCGCAGCTGTAT-3', reverse 5'-TGTGTTAGGATCTGGGCCAAA-3'; and *CYP24A1*: forward 5'-CAAACCGTGGAGGCCTATC-3', reverse 5'-AGTCTTCCCCTCC-AGGATCA-3'.

Measurement of circulating vitamin D-binding protein and vitamin D-binding protein uptake

Free circulating vitamin D-binding protein (DBP) levels were measured in serum from male and female MS patients using a commercially available kit for ELISA (Immundiagnostik), following the manufacturer's instructions. Assay sensitivity level was 1.23 ng/ml. Intra- and interassay variation coefficients were <5% and <11%, respectively.

Quantitation of DBP uptake was assessed, as described previously, but with some modifications (29, 30). Briefly, DBP (Calbiochem, Darmstadt, Germany) was conjugated with FITC using a commercial kit (Sigma-Aldrich). The final FITC/protein molar ratio was 1:1. Prior to all experiments, cells were incubated for 30 min at 37°C in RPMI 1640 serum-free medium (JRH Biosciences) to deplete them of endogenous proteins and proteins internalized from the culture medium. Cells (10^5 /tube) harvested at different times were incubated at 4°C (to inhibit uptake) or 37°C (optimal uptake temperature) in OVA/RPMI 1640 medium containing 2 μ M FITC-DBP. Tubes were previously coated for 2 h at 37°C with a solution of 1.5% OVA in PBS. After incubation, cells were washed in ice-cold PBS containing 5% FCS and 0.1% NaN_3 to stop internalization. Cells were then fixed with 4% paraformaldehyde in PBS, and samples were analyzed by FACScan using CellQuest software (BD Biosciences). Relative fluorescence intensity of ≥ 5000 cells per sample was recorded as single-parameter histograms, and the mean fluorescence intensity was calculated for each histogram as an indicator of the number of DBP molecules incorporated per cell at the different times and culture conditions specified.

Statistical analysis

Differences in 25-(OH) D_3 and 1,25-(OH) D_3 levels between males and females were analyzed using the two-tailed Student *t* test. Differences in immunological variables were tested for significance using the Mann-Whitney *U* test. In all cases, *p* values <0.05 were considered statistically significant.

Results

Female and male subjects showed similar serum levels of 25-(OH) D_3 and 1,25-(OH) D_3

In a first approach to investigate whether gender differences existed in relation to immunological effects of vitamin D_3 , serum levels of 25-(OH) D_3 and 1,25-(OH) D_3 were quantified in male and female MS patients, as well as in healthy controls (Table I). As illustrated in Fig. 1, serum levels of 25-(OH) D_3 were similar in females (119.75 \pm 22.5 nM in remission, 95.75 \pm 21.25 nM during exacerbations, and 153.75 \pm 15 nM in healthy controls) and in males (114.75 \pm 23 nM in remission, 98 \pm 26.75 nM during exacerbations, and 150.25 \pm 10.75 nM in healthy controls). This was also the case for 1,25-(OH) D_3 serum levels in females (72 \pm 13.2 pM during remission, 55.4 \pm 13.92 pM during exacerbations, and 84 \pm 16.56 pM in healthy controls) and males (65.28 \pm 13.2 pM during remission, 54.24 \pm 13.2 pM during exacerbations, and 84.96 \pm 15.36 pM in healthy controls). Additional analyses demonstrated significantly lower serum levels of

Table I. Demographic and clinical characteristics of the study population

Characteristics	RRRMS	RREMS	Controls
No. of subjects	58	34	30
Age (y; mean \pm SD)	36.8 \pm 10	37.5 \pm 8.4	33.9 \pm 5.9
Females/males (<i>n</i>)	41:17	29:5	23:7
EDSS (mean \pm SD)	1.3 \pm 1.1	2.0 \pm 1.4	
Disease duration (y; mean \pm SD)	3.9 \pm 4.9	4.3 \pm 5.7	

EDSS, expanded disability scale score; RREMS, relapsing-remitting MS during exacerbation; RRRMS, relapsing-remitting MS during remission.

25-(OH) D_3 , and 1,25-(OH) D_3 in female patients during exacerbations compared with those observed during remissions (*p* < 0.0001). Moreover, 25(OH) D_3 and 1,25(OH) D_3 serum levels were also significantly lower in female MS patients during remission and exacerbation compared with values observed in control subjects (*p* < 0.0001 and *p* = 0.0006, respectively). In contrast, in male MS patients, 25-(OH) D_3 and 1,25-(OH) D_3 serum levels were similar during exacerbations and remissions (*p* = 0.18 and *p* = 0.14, respectively). These results are probably influenced by the reduced number of male patients included in this investigation, particularly those experiencing exacerbations (*n* = 5). Nevertheless, additional analysis demonstrated significantly lower serum levels of 25-(OH) D_3 in male MS patients during remissions and exacerbations compared with healthy controls (*p* = 0.0003 and *p* = 0.01, respectively), as well as significantly lower levels of 1,25-(OH) D_3 under both clinical conditions in comparison with control subjects (*p* = 0.003 and *p* = 0.002). Overall, these results demonstrated no gender differences in the levels of vitamin D_3 metabolites in MS patients during the same clinical course of the disease or in healthy controls. Therefore, the impact of 1,25-(OH) D_3 on T cell function in males and females was examined in subsequent experiments.

Inhibition of T cell proliferation by 1,25-(OH) D_3 is significantly stronger in females compared with males with MS

1,25-(OH) D_3 T cell proliferation inhibition was assessed in PHA-induced proliferation assays, using ex vivo CD4⁺ T cells and in MBP peptide-specific TCLs stimulated with the cognate Ag, isolated from male and female MS patients. 1,25-(OH) D_3 did not affect CD4⁺ T cell survival measured by trypan blue dye exclusion. Ex vivo CD4⁺ T cell and MBP peptide-specific T cell proliferation were inhibited by 1,25-(OH) D_3 in a concentration-dependent manner. As shown in Fig. 2, concentrations required to inhibit 50% of CD4⁺ T cells and MBP peptide-specific T cell proliferation were significantly lower in cells isolated from females (10^{-11} – 10^{-10} mol/l) than from males (10^{-8} – 10^{-7} mol/l; *p* < 0.0001), indicating that T cell proliferation inhibition was significantly stronger in females with MS. 1,25-(OH) D_3 -mediated inhibition did not differ between MS patients and healthy controls.

Cytokine responses reveal significant gender differences in 1,25-(OH) D_3 effects

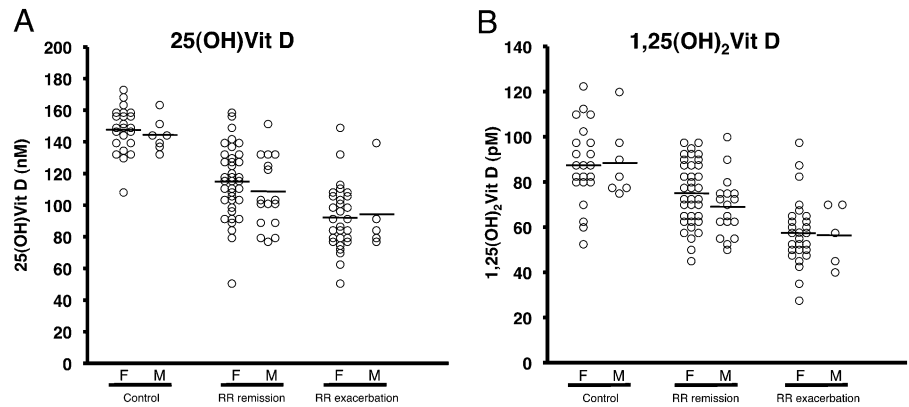
The impact of 1,25-(OH) D_3 on T cell cytokine production of IFN- γ , IL-17, and IL-10 was measured in males and females, using ELISPOT assays, in purified CD4⁺ T cells and in myelin peptide-specific TCLs stimulated with immobilized anti-CD3 mAb, in the presence and absence of 1,25-(OH) D_3 . Fig. 3 demonstrates the significant differences observed between the genders in the secretion patterns of these three cytokines.

1,25-(OH) D_3 reduced the number of IFN- γ - and IL-17-producing cells significantly more in females than in males, for CD4⁺ T cells, as well as in MBP- and MOG-specific T cells. Likewise, the increase in IL-10-secreting cell numbers after exposure to 1,25-(OH) D_3 was significantly greater in female MS patients for both cell populations (*p* < 0.0001). These findings were similar in both cultures from MS patients and healthy controls.

Increase in number of CD4⁺CD25⁺FoxP3⁺ regulatory T cells induced by 1,25-(OH) D_3 is greater in female MS patients

To assess the influence of gender on CD4⁺CD25⁺FoxP3⁺ induction by 1,25-(OH) D_3 , PBMCs were stimulated with soluble anti-CD3 and soluble anti-CD28 in the presence and absence of 1,25-(OH) D_3 , and subsequent CD4⁺CD25⁺FoxP3⁺ regulatory T cell

FIGURE 1. Levels of 25-(OH)D₃ and 1,25-(OH)₂D₃ by gender in MS patients and healthy controls. Serum levels of 25-(OH)D₃ and 1,25-(OH)₂D₃ in MS patients in remission (*n* = 58) and during acute exacerbations (*n* = 34), as well as in healthy controls (*n* = 30), were measured by ELISA. Each circle represents average vitamin D₃ metabolite levels for a single individual. Horizontal lines indicate mean group values. F, females, M, males.



numbers were measured using flow cytometry. As shown in Fig. 4, CD4⁺CD25⁺FoxP3⁺ regulatory T cell number was not influenced by gender in the absence of 1,25-(OH)₂D₃ (6 ± 0.9% in males versus 5 ± 0.65% in females). Cultures developed in the presence of 1,25-(OH)₂D₃ led to a significant increase in CD4⁺CD25⁺FoxP3⁺ T cell number in male and female MS patients; however, total amounts generated in females were significantly greater than in males (33 ± 4.6% versus 15 ± 1.8%; *p* < 0.001). In all cases, isolated CD4⁺CD25⁺FoxP3⁺ T cells showed low or undetectable expression of CD127. No significant differences were observed between MS patients and controls.

Gender influence on vitamin D₃ metabolism

To further investigate the sex-based disparity in immunological effects, vitamin D₃ metabolism was analyzed in males and females. VDR expression has been described in monocytes and activated lymphocytes (31, 32). Therefore, we chose to examine VDR expression, using quantitative PCR, on MBP- and MOG-specific TCLs isolated from male and female MS patients. As reported previously, resting MBP and MOG peptide-specific TCLs expressed low levels of VDR mRNA, which increased 5–10-fold after activation (*p* < 0.0001; Fig. 5A). Moreover, 1,25-(OH)₂D₃ treatment caused a significant increase in VDR expression (3–7-fold; *p* < 0.001) in resting and activated MBP and MOG peptide-specific TCLs (Fig. 5A). Under all conditions studied, male and female patients expressed similar levels of VDR mRNA.

Tissue availability of active vitamin D₃ metabolite 1,25-(OH)₂D₃ is dependent on the expression of the activating enzyme 1-(OH)ase, and its catabolic counterpart 24-(OH)ase (33, 34). These enzymes were recently found in different tissues (22, 33). Therefore, we studied the expression and regulation of *CYP27B1* and *CYP24A1* transcripts in MBP and MOG peptide-specific T cells. The *CYP27B1* gene encodes the enzyme 1-(OH)ase, whereas the *CYP24A1* gene encodes the enzyme 24-(OH)ase. As

shown in Fig. 5B, resting autoreactive T cells constitutively expressed *CYP27B1* mRNA. Levels of expression were significantly upregulated following activation but were not induced further by 1,25-(OH)₂D₃ nor did transcript expression vary significantly with gender. Male and female patients constitutively expressed similar amounts of T cell *CYP27B1* transcripts. In contrast, exposure to 1,25-(OH)₂D₃ significantly increased *CYP24A1* transcripts in self-reactive T cells from males but not females (Fig. 5C). Interestingly, activation of self-reactive T cells from females inhibited *CYP24A1* gene induction (Fig. 5C). Overall, these data suggest that T cells from males and females synthesize 1,25-(OH)₂D₃ at similar rates, but females inactivate it more slowly, favoring accumulation in self-reactive T cells. Similar results were obtained using CD4⁺ T cells specific for tetanus toxoid, streptolysin O, influenza hemagglutinin_{307–319} peptide, and polyclonally stimulated T cells. No significant differences were observed between MS patients and healthy controls.

DBP is a serum glycoprotein, synthesized predominantly in the liver, which reversibly binds and transports vitamin D₃ and its metabolites to target cells. Numerous studies detected DBP bound to the surface of several cell types, including human macrophages and lymphocytes, supporting the concept that direct substrate trafficking is pivotal in vitamin D₃ regulation (35–37). Therefore, we studied DBP uptake in CD4⁺ T cells and macrophages isolated from male and female MS patients. As illustrated in Fig. 6A, females and males showed similar circulating DBP levels (32 ± 5.04 mg/dl versus 36 ± 5.3 mg/dl). In the next experiments, we examined whether DBP uptake differed in males and females. DBP binding and internalization to ex vivo CD4⁺ T cells, MBP peptide-reactive T cells, and macrophages was a time-dependent function requiring cell activation. Amounts of DBP bound and internalized by resting cells were low; they increased progressively after stimulation, attained peak values at 48–72 h, and decreased thereafter (Fig. 6B–D). Cells isolated from male and

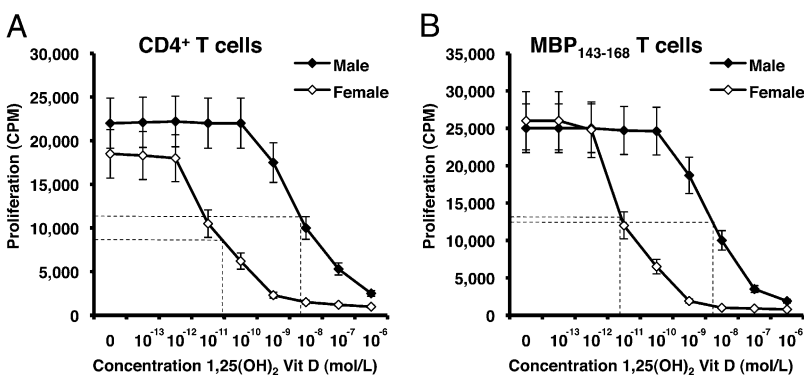
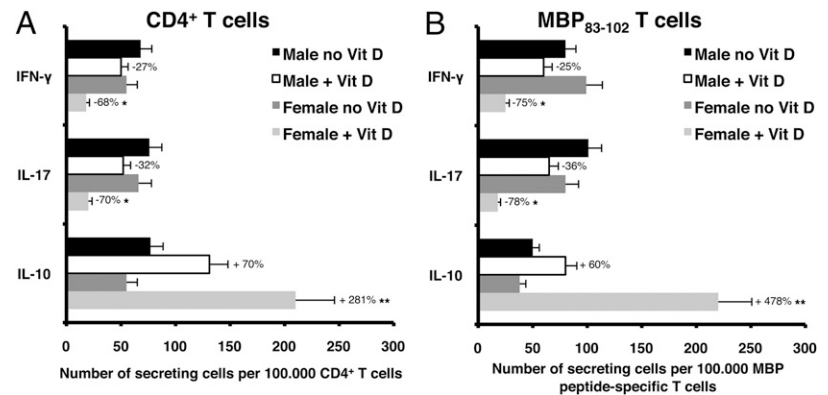


FIGURE 2. 1,25-(OH)₂D₃ inhibition of T cell proliferation is stronger in females. Purified CD4⁺ T cells (A) and MBP peptide-specific T cells (B) from males and females were cultured in the presence of different concentrations of 1,25-(OH)₂D₃. After stimulation, T cell proliferation was examined in a standard 60-h assay measuring [³H]thymidine incorporation. Results are presented as mean ± SEM from 25 female and 17 male MS patients.

FIGURE 3. Gender influence on cytokine secretion. Purified CD4⁺ T cells (A) and MBP-specific TCLs (B) isolated from female and male MS patients were stimulated with plate-bound anti-CD3 mAb in the presence and absence of 1,25-(OH)₂D₃ (10 nM). After 72 h in culture, the number of cytokine-secreting cells was evaluated using ELISPOT assays. Percentages indicate changes in the number of cytokine-secreting cells in the presence of 1,25-(OH)₂D₃ compared with those in the absence of 1,25-(OH)₂D₃ in females and males. Data represent the mean ± SEM of nine independent experiments conducted in triplicate from different donors. **p* < 0.01; ***p* < 0.0001 versus male 1,25-(OH)₂D₃-treated T cells.



female MS patients followed similar time-dependent profiles. However, the amount of DBP associated with activated cells from female MS patients was 2–5-fold greater than from males with MS (Fig. 6B–D; *p* < 0.0001). This effect was similar in cultures from MS patients and healthy controls.

Estrogens modulate vitamin D₃ metabolism

We next investigated possible E2-mediated inhibition of *CYP24A1* transcripts and enhancement of DBP uptake. Resting and activated MBP-specific T cells and macrophages from male MS patients were cultured for 72 h, with and without E2, before assessing *CYP24A1* transcripts and DBP uptake. Treatment with E2 significantly reduced *CYP24A1* transcripts in resting and activated T cells from males (Fig. 7A; *p* < 0.001), and it significantly enhanced DBP uptake in CD4⁺ T cells and macrophages (Fig. 7B, 7C; *p* < 0.001). Progesterone had no effect on *CYP24A1* transcript suppression or DBP uptake enhancement, and the combination of progesterone plus E2 showed similar effects to E2 alone (Fig. 7A–C). Of note, the estrogen receptor (ER) antagonist ICI 182780 reversed E2 effects on *CYP24A1* transcripts and DBP uptake, demonstrating that these effects were ER-signaling dependent (Fig. 7A–C). To determine the relative contribution of ERα and ERβ to E2 effects, male MBP-specific T cells and macrophages were treated with ER-selective ligands. Treatment with propyl-

pyrazole triol, an ERα agonist, reproduced effects observed with E2. In sharp contrast, the ERβ agonist diarylpropionitrile had no effect on 1,25-(OH)₂D₃ metabolism (Fig. 7A–C).

To establish whether changes in vitamin D₃ metabolism induced by E2 translated into functional changes in autoreactive T cells, we stimulated MBP peptide-specific TCLs from male MS patients with the cognate peptide, in the presence of 1,25-(OH)₂D₃ plus E2, and examined T cell proliferation, IL-10 secretion, and regulatory T cell induction, as markers of immunosuppressant induction. The addition of E2 to MBP peptide-specific T cells from male MS patients cultured in the presence of 1,25-(OH)₂D₃ caused a significant increase in the suppression of T cell proliferation (Fig. 7D; *p* < 0.001). Thus, 1,25-(OH)₂D₃ concentrations required to inhibit 50% of male MBP peptide-specific T cell proliferation in the presence of E2 were similar to those observed in cells isolated from females (Fig. 7D). Likewise, addition of E2 to male MBP peptide-specific T cells cultured in the presence of 1,25-(OH)₂D₃ significantly increased the numbers of IL-10-secreting T cells, as well as the induction of CD4⁺CD25⁺FoxP3⁺ regulatory T cells (Fig. 7E, 7F; *p* < 0.001). Notably, all of these effects were reversed by the ER antagonist ICI 182780. These results are consistent with the concept that E2 modifies vitamin D₃ metabolism, inducing greater immunosuppressive effects. Results were similar in MS patients and healthy subjects.

Because phenol red was reported to exhibit estrogenic effects (28), it was important to exclude the possibility that it had contributed to or was responsible for E2 effects observed in different immunological responses. A second concern was that culturing of female T cells in vitro depleted their E2 content. Thus, phenol red-free medium was used in parallel cultures with phenol red-containing medium. Twelve MBP₈₃₋₁₀₂ TCLs derived from eight MS patients (four females and four males) were selected for these control experiments. Moreover, ex vivo CD4⁺ T cells isolated from the same group of subjects were also tested in parallel experiments. T cells were evaluated for proliferation, FoxP3 expression, and IL-10 and IFN-γ secretion in the presence of increasing concentrations of E2. The data presented as supplemental material indicate that E2 effects were the same whether phenol red was present in the medium. Baseline proliferation, FoxP3 expression, and IL-10 and IFN-γ secretion also were not affected by the presence of phenol red. Finally, the addition of ethanol, diluted in the absence of E2 to duplicate ethanol concentrations present at the highest tested dose of E2, had no effect on proliferation, FoxP3 expression, or cytokine secretion (data not shown). Results were similar for MBP₈₃₋₁₀₂ TCLs and ex vivo-isolated CD4⁺ T cells. Overall, the effects observed on proliferation, cytokine secretion, and FoxP3 expression could only be explained by the presence of E2 and not by any other experimental parameter.

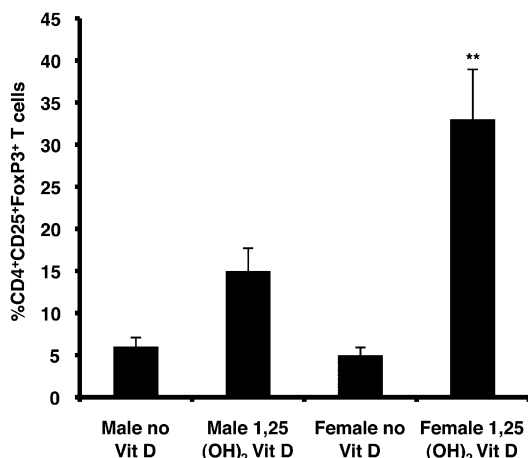


FIGURE 4. Induction of CD4⁺CD25⁺FoxP3⁺ regulatory T cells by 1,25-(OH)₂D₃ is greater in females than in males. PBMCs isolated from females and males were stimulated as described in *Materials and Methods* in the presence and absence of 1,25-(OH)₂D₃ (10 nM), and the percentage of CD4⁺CD25⁺FoxP3⁺ regulatory T cells was measured by flow cytometry. Data represent mean ± SEM from 25 female and 17 male MS patients. ***p* < 0.0001 versus male 1,25-(OH)₂D₃-treated T cells.

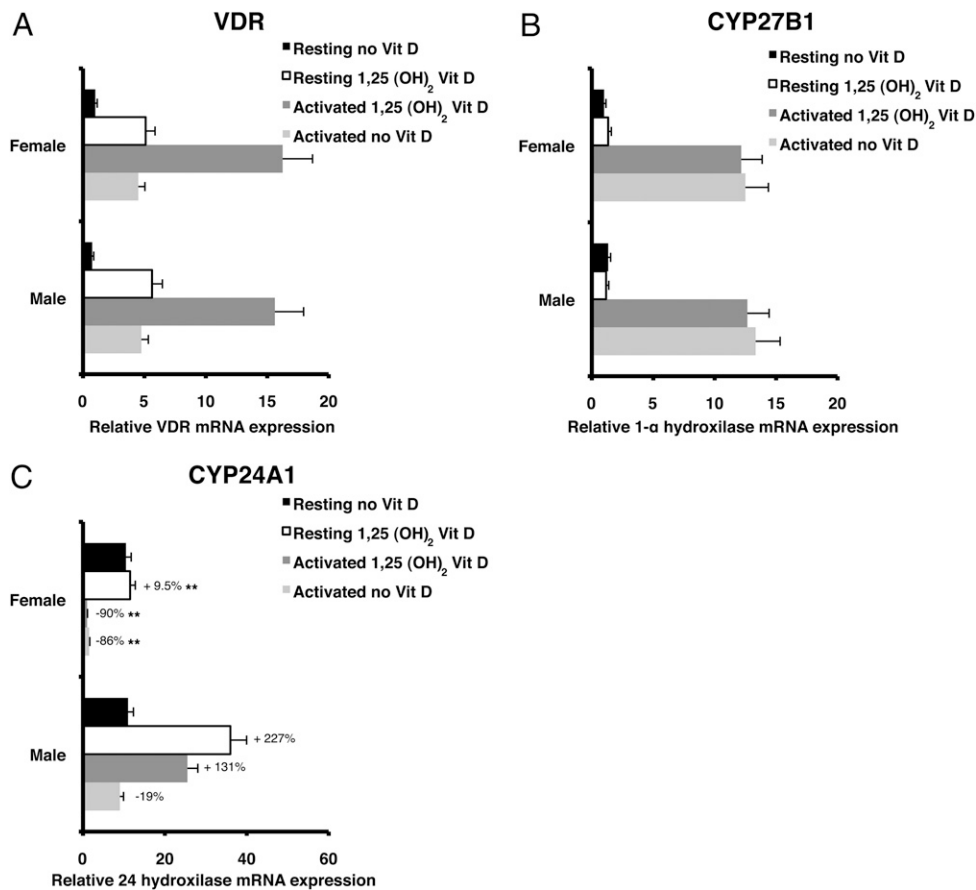


FIGURE 5. *CYP24A1* transcript induction is lower in self-reactive T cells from females. Resting and activated MBP and MOG peptide-specific T cells were cultured in the presence and absence of 1,25-(OH)₂D₃ (10 nM). After 3 d of culture, mRNA was extracted and *VDR* (A), *CYP27B1* (B), and *CYP24A1* (C) gene expression was quantified by RT-PCR. Values obtained were normalized to the housekeeping gene GAPDH. Values are expressed relative to mRNA, with a minimum of 1.0 assigned to the group with the lowest gene expression. In A and B, the level of mRNA in female resting cells not treated with vitamin D₃ is set at 1. In C, the level of mRNA in female activated vitamin D₃-treated cells is set at 1. Values are reported as mean ± SEM from 30 MBP and 15 MOG peptide-specific TCLs isolated from 17 female and 15 male MS patients. In C, percentages indicate changes in *CYP24A1* transcript expression under different conditions compared with resting cells in the absence of 1,25-(OH)₂D₃. ***p* < 0.0001 versus male 1,25-(OH)₂D₃-treated cells.

Discussion

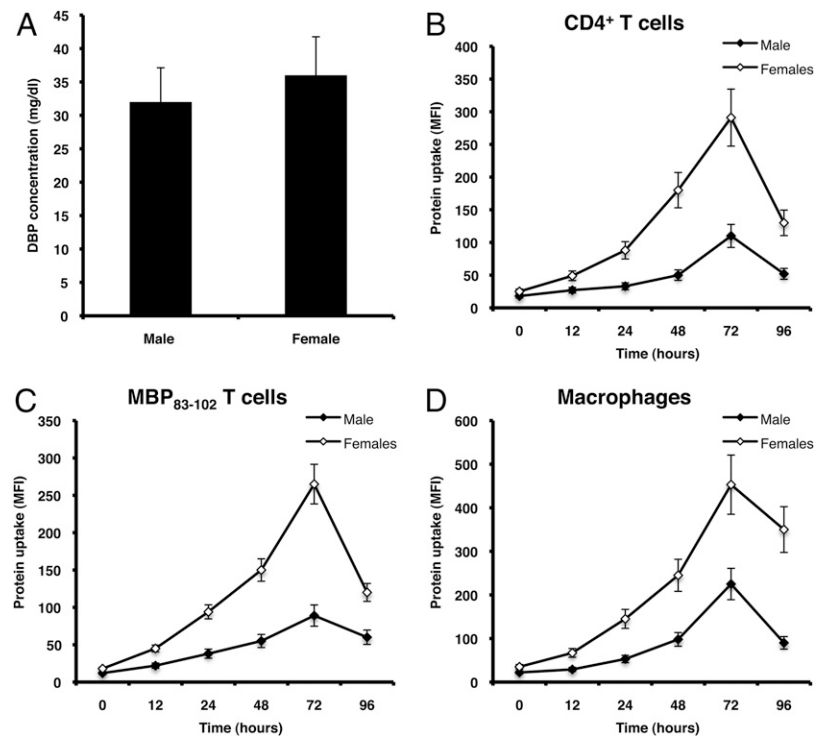
We presented evidence indicating that immunomodulatory effects of 1,25-(OH)₂D₃ are significantly stronger in female MS patients than in male MS patients. Serum levels of 25-(OH)D₃ and 1,25-(OH)₂D₃ were similar in men and women with MS. However, activated 1,25-(OH)₂D₃ showed a striking, gender-based disparity with respect to immunoregulatory effects on CD4⁺ T cells. Inhibition of T cell proliferation was significantly stronger in females, 1,25-(OH)₂D₃ caused a greater reduction in IFN-γ- and IL-17-producing cell numbers and a greater increase in IL-10-secreting cells in females compared with males, and the increase in the number of CD4⁺CD25⁺FoxP3⁺ regulatory T cells induced by 1,25-(OH)₂D₃ was also greater in female MS patients. In correlation with these immunomodulatory changes, female MS patients had fewer *CYP24A1* transcripts encoding the 1,25-(OH)₂D₃-inactivating enzyme and presented greater binding and internalization of DBP to ex vivo CD4⁺ T cells, MBP peptide-reactive T cells, and macrophages. Overall, these findings suggest that females inactivate 1,25-(OH)₂D₃ more slowly, allowing it to accumulate and, thus, triggering more potent anti-inflammatory effects.

Although the incidence rates of MS are 2–3-fold greater in women than in men, gender differences in vitamin D₃ metabolism were not evident in the circulation. However, it is still possible that differences in the active metabolite may be evident at local sites of

inflammation, as reported in the EAE model, in which vitamin D₃ inhibited severe EAE only in female mice, associated with increased concentrations in the CNS (21). Indeed, activated myeloid and lymphoid lineage cells upregulate the *CYP27B1* gene and local 1,25-(OH)₂D₃ production for paracrine and intracrine use (33, 38). Interestingly, ovariectomy abrogates this protective effect (21), and diestrus-level E2 implants restore vitamin D₃-mediated EAE resistance in ovariectomized female mice (22). In this model, the sex-based disparity in EAE susceptibility was associated with *CYP24A1* transcript suppression at the spinal cord level, leading to 1,25-(OH)₂D₃ accumulation, as well as enhancement of *VDR* gene expression in the CNS of female mice (21). In our study, just as was observed in the EAE model, activation of self-reactive T cells from females, but not from males, inhibited *CYP24A1* gene induction. However, although *VDR* mRNA was upregulated in self-reactive CD4⁺ T cells following activation and 1,25-(OH)₂D₃ exposure, transcript expression did not vary significantly with gender. Discrepancies may be due to differences in the tissues investigated or, alternatively, to differences in vitamin D₃ metabolism between humans and mice.

It is important to note that in these experiments 1,25-(OH)₂D₃ was used at supraphysiological doses, ~120-fold greater than circulating levels found in healthy individuals. It seems likely that increasing 1,25-(OH)₂D₃ levels could reduce the risk for MS. If so, it is important to establish optimal vitamin D₃ levels and verify

FIGURE 6. DBP uptake is greater in CD4⁺ T cells and macrophages from female MS patients than from male MS patients. *A*, Circulating DBP levels were evaluated in female and male MS patients using an ELISA kit. Data represent results from 25 female and 15 male MS patients. *B–D*, One hundred thousand ex vivo CD4⁺ T cells, MBP-specific T cells, or macrophages from female and male MS patients were harvested at different times after stimulation with PHA (10 μg/ml), cognate MBP peptide (10 μg/ml), or LPS (5 ng/ml), respectively, and incubated at 4°C or 37°C for 60 min in OVA/RPMI 1640 medium containing 2 μM of FITC-DBP. DBP uptake was assessed using flow cytometry. Data represent mean ± SEM of seven independent experiments conducted in triplicate from samples from different donors.



whether vitamin D₃ supplements administered at these levels contribute to prevention without producing side effects. It was suggested that vitamin D₃ should be given at a dose equivalent to 1000–3000 IU daily, to maintain levels at 100 nM/l and, thus, to keep the risk for toxic side effects low (39). However, confirmation of safety and efficacy in a large randomized trial is needed before general recommendations can be made. Optimal vitamin D₃ supplementation may well be a very individual matter, because there is significant interpersonal variation in response to supplementation. Data from our laboratory obtained in Hispanic individuals suggest that 25-(OH)D₃ levels associated with remission range between 115 and 120 nM, whereas levels associated with relapse are closer to 96–98 nM. These values are distinct from results reported for Finnish individuals: 60 nM during remissions and 44–47 nM during relapses, with no relapses observed for values >85 nM (14). The discrepancies observed between the Finnish and Hispanic individuals merit further investigation. Moreover, it is important to note that international standards for serum 25-(OH)D₃ have been established from studies of calcium metabolism and bone health and that greater 25-(OH)D₃ concentrations may be required for optimal immune function (40).

Gender differences in MS incidence are not fully understood but might include sex-related differences in immune responsiveness, sex hormone influence, or sex-linked genetic factors (41). Female mice produce more Abs and show increased cell-mediated immunity after immunization than male mice (42). Also noteworthy is the fact that women have greater absolute CD4⁺ lymphocyte counts relative to males, likely contributing to increased immune response (43). In animal models, transfer of MBP-sensitized lymph node cells into female mice results in severe EAE, whereas transfer into males attenuates disease symptoms (44). Much attention has been directed toward the actions of sex hormones, such as estrogens, progesterone, and testosterone, during the course of autoimmune diseases, and many findings in humans have been borne out in animal models. For example, administration of E2 confers protection against EAE, collagen-induced arthritis, and adjuvant arthritis in mice and rats (45–47). In addition,

castration of female mice shortens time to EAE onset (45). Perhaps the most striking evidence of the modulatory effects of sex hormones on immune function in MS comes from pregnancy. Clinical disease is ameliorated, particularly during the third trimester, when estrogen and progesterone levels are highest, and worsens during the postpartum period, when sex hormone concentrations decrease (48). Also, following the natural fluctuations in sex hormone levels during the menstrual cycle, MS and rheumatoid arthritis symptoms worsen prior to the onset of menses (49). However, studies have not been performed to determine how autoimmune responses change longitudinally through the menstrual cycle, as well as during oral contraceptive use or estrogen-replacement therapy. The striking protective effects of E2 on EAE raise the possibility that estrogens might be beneficial in MS. A pilot study reported symptom improvement in postmenopausal MS patients receiving hormone-replacement therapy (50). Furthermore, in a phase I clinical trial, female MS patients treated with oral estriol showed decreased delayed-type hypersensitivity reactions to tetanus-recall Ags, decreased PBMC IFN-γ production, as well as reduced numbers and volumes of gadolinium-enhancing lesions in the brain (51). The greater incidence of MS in females seems to be contradictory to modulatory effects of estrogens suppressing autoimmunity. However, these discrepancies might be partly reconciled by the fact that estrogens possess biphasic dose effects: lower levels facilitate immune responses, whereas higher ones similar to those present in pregnancy suppress such responses (52).

Estrogens exert different immunoregulatory effects on the immune system (53). They inhibit total cell, as well as macrophage and T cell, infiltration into the CNS. This marked reduction in inflammatory cells is reflected in a strong reduction in chemokine and chemokine receptor expression in CNS and lymph node cells (54, 55). Estrogens also alter macrophage and dendritic cell APC function, blocking Th1 cell activation involved in EAE induction and increased IL-10 secretion (52, 56). Estrogens increase FoxP3 expression *in vivo* and *in vitro*, expanding the regulatory T cell compartment (57), and they reduce IL-12, TNF-α, and IFN-γ

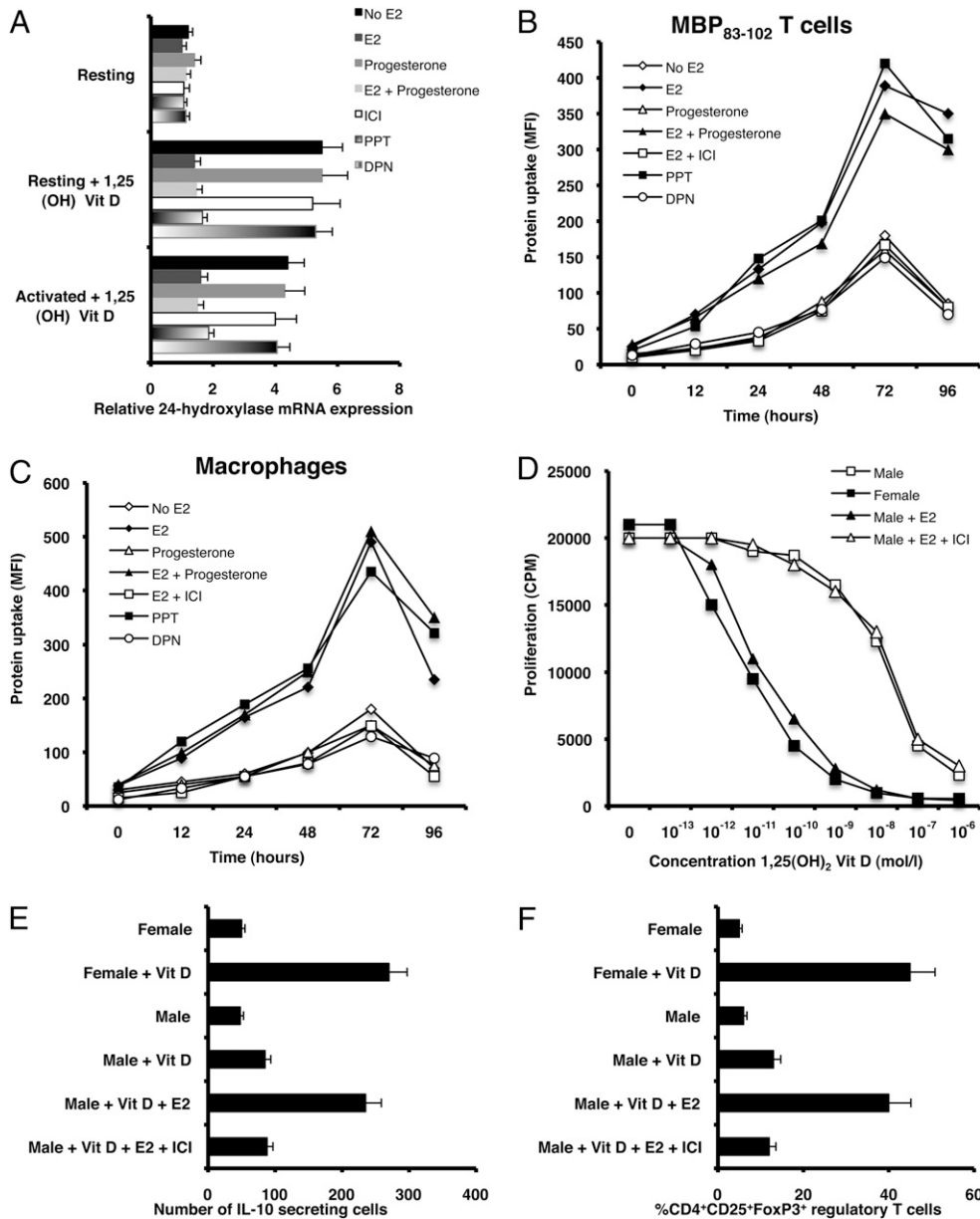


FIGURE 7. Influence of estrogens on 1,25-(OH)₂D₃ metabolism. *A*, Resting and activated MBP- and MOG-specific TCLs from male MS patients were cultured under different conditions in the presence or absence of 1,25-(OH)₂D₃, as indicated. Additional treatments included E2 (10 nM), propyl-pyrazole triol (100 nM), diarylpropionitrile (1 nM), or ICI 182780 (1 μM). Total RNA was extracted from male cells, and *CYP24A1* gene expression was measured using quantitative RT-PCR, as described in Fig. 5. The level of E2-treated resting cells was set at 1. *B* and *C*, MBP peptide-specific T cells and macrophages from male MS patients were cultured under different conditions, as indicated in *A*, and DBP uptake was assessed using flow cytometry. *D*, MBP peptide-specific T cells from males and females were cultured in the presence of different concentrations of 1,25-(OH)₂D₃, with and without E2 (10 nM) and ICI 182780 (1 μM). After stimulation, T cell proliferation was examined in a standard 60-h assay measuring [³H]thymidine incorporation. *E*, MBP-specific TCLs isolated from female and male MS patients were cultured under different conditions in the presence or absence of 1,25-(OH)₂D₃, as indicated, and stimulated with the cognate peptide (10–15 μg/ml). After 72 h, the number of cytokine-secreting cells was evaluated using ELISPOT assay. Similar results were observed when cells were stimulated with plate-bound anti-CD3 mAb. *F*, PBMCs isolated from females and males were cultured in different conditions in the presence and absence of 1,25-(OH)₂D₃ (10 nM) as indicated; percentages of CD4⁺CD25⁺FoxP3⁺ regulatory T cells were measured by flow cytometry. All experiments were conducted in serum-free media. Reagents were dissolved in absolute ethanol at a stock concentration of 0.01 M and further diluted in serum-free medium. The addition of ethanol in concentrations similar to those used for the dilutions of the different reagents did not affect *CYP24A1* transcript amounts or DBP uptake. For *A*, *B*, *C*, and *E*, data represent mean ± SEM of triplicate cultures from nine independent experiments. For *D* and *F*, data represent mean ± SEM from 25 female and 17 male MS patients.

secretion and strongly enhance IL-10 and TGF-β3 secretion (52, 54, 55, 58).

In addition to their role in the immune system, estrogens and 1,25-(OH)₂D₃ have important neuroprotective effects on the CNS. E2 promotes neuron growth and differentiation, acting in concert with different neurotrophins, including brain-derived neurotrophic factor, nerve growth factor, and neurotrophins 3 and 4/5 (59).

These actions promote axon and myelin survival. Likewise, VDR-immunoreactive neurons were described in the hypothalamus and the hippocampus, where 1,25-(OH)₂D₃ seems to support cell survival (60, 61). Also, in culture systems, VDR transcripts were detected in astrocytes and oligodendrocytes (62, 63). These cells respond to 1,25-(OH)₂D₃-enhancing transcripts encoding NGF and its low-affinity receptor the p75 protein (63, 64). Furthermore,

1,25-(OH)₂D₃ enhances calcium-binding protein expression and downregulates synthesis of L-type calcium channels, decreasing neuronal vulnerability to excitotoxic insults (65, 66). Overall, these 1,25-(OH)₂D₃-mediated changes suggest that active vitamin D₃ may mitigate different processes linked to CNS damage.

These observations indicate that synergy may exist between E2 and 1,25-(OH)₂D₃ in the control of EAE and MS. Indeed, estrogen stimulates 1,25-(OH)₂D₃ accumulation during ovulation and pregnancy, when its levels are high, and active vitamin D₃ levels increase in postmenopausal women receiving estrogen therapy (67–69). Different mechanisms have been proposed to explain this synergy: E2 is able to suppress *CYP24A1* transcripts, leading to 1,25-(OH)₂D₃ accumulation (21); in rodents, E2 enhances VDR biosynthesis in different tissues (21, 70); and E2 induces greater binding and internalization of DBP to self-reactive T cells and macrophages, allowing 1,25-(OH)₂D₃ to accumulate in immune cells.

Estrogen action is largely mediated through nuclear hormone receptors. Two ER isoforms have been identified (ER α and ER β), with a selective agonist existing for each (71, 72). The effects of E2 on 1,25-(OH)₂D₃ metabolism are reversed by the ER antagonist ICI 182780, suggesting that E2 effects are mediated by ER. By comparing the effects of ER-selective ligands, we found that an ER α -selective agonist reproduced effects observed with E2. In sharp contrast, the ER β agonist showed no effect on 1,25-(OH)₂D₃ metabolism. This suggests that E2's effects on vitamin D metabolism are mediated primarily through ER α and is consistent with observations indicating that immunomodulatory effects of E2 are dependent on ER α signaling (53, 73). Expression of both ERs was reported on lymphocytes, and it was recently shown that E2-mediated suppression of certain genes, such as IFN- γ and RANTES in the EAE model, is dependent on the presence of ER α (74). These results are consistent with observations showing treatment of EAE with an ER α -selective ligand reduces CNS inflammation, resulting in delayed onset, as well as decreased incidence and severity of disease. In contrast, little or no effect was observed with ER β -selective ligand, although treatment with ER α or ER β ligands was neuroprotective, as evidenced by the preservation of axons and decreased neuronal abnormalities in gray matter (71, 73).

Tissue availability of 1,25-(OH)₂D₃ is dependent on the expression of the activating enzyme 1-(OH)ase and its catabolic counterpart 24-(OH)ase (34). The activity of these two enzymes is controlled, at least in part, by DBP's affinity for the precursor 25-(OH)D₃. DBP is a serum glycoprotein that binds and transports vitamin D₃ and its metabolites to target cells (75). Human DBP has been linked to several membrane components of peripheral blood monocytes, lymphocytes, and neutrophils, supporting the concept of a cell surface-binding site for DBP (35–37). In addition, studies demonstrated that *in vivo* endocytic uptake of the vitamin D₃-DBP complex is mediated by megalin and cubilin, two members of the low-density lipoprotein-like receptor protein family (76, 77). However, the mechanism by which vitamin D₃ metabolites dissociate from the internalized complex and travel to specific intracellular destinations is not known. It has been proposed that the hsp-70-related family of intracellular DBPs plays a central role in this transport process (78). In this study, we demonstrated that amounts of DBP associated with activated cells from female MS patients are 2–5-fold greater than those from males. Furthermore, treatment of male cells with E2 significantly enhanced DBP uptake in CD4⁺ T cells and macrophages. Interestingly, megalin expression in murine mammary tissue is highest in glands removed during pregnancy and lactation (77), and supplementation of mice with estrogens and progesterone

to stimulate pregnancy enhances megalin expression in ductal mammary epithelial cells (77). Moreover, studies with megalin knockout mice demonstrated megalin-mediated endocytosis plays a critical role in several tissues, including the thyroid gland and male and female reproductive organs (79, 80). The impact of E2 on the megalin/cubilin complex and intracellular DBPs, as well as its association with DBP uptake and vitamin D₃ metabolism in self-reactive T cells and macrophages is under investigation in our laboratory.

In summary, the results presented demonstrate a functional synergy between the immunomodulatory effects of 1,25-(OH)₂D₃ and E2 in MS patients that is mediated by ER α . Gender differences in vitamin D₃ metabolism promoted by estrogens suggest greater protective effects for vitamin D₃-based therapeutic strategies in women. Conversely, combined deprivation of vitamin D₃ and estrogens, present in menopausal women, could induce significant disease progression in this subgroups of MS patients. Studies relating to these observations should be extended to the mechanistic evaluation of other sexually dimorphic hormones, such as prolactin, and growth hormone. Correction of vitamin D₃ deficiency, in combination with E2, may be useful to suppress autoimmune diseases, such as MS. However, before any conclusions can be drawn, further investigations are needed to determine the optimal doses of both hormones to avoid adverse effects. Likewise, studies are warranted to compare the efficacy and safety of different vitamin D₃ analogs with less hypercalcemic activity, as well as selective ER modulators, such as raloxifene, which has the beneficial properties of estrogens but with a reduced adverse effect profile.

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Disclosures

The authors have no financial conflicts of interest.

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