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

Jorge Correale, María C. Ysraeleit, and María I. Gaitán

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.


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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; RRREMS, relapsing-remitting MS during remission; TCL, T cell line; VDR, vitamin D receptor.

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 indicating 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-
ulation may play an important role in maintaining T cell homeostasis in this group of patients (25). In this study, we inves-
tigated the immunomodulatory effects of 1,25-(OH)\(_2\)D\(_3\) in relation to female hormones. To this end, serum levels of 25-(OH)D\(_3\) and 1,25-(OH)\(_2\)D\(_3\) were measured in female and male MS patients. Subsequently, to gain more insight into putative regulatory mech-
anisms of estrogens and 1,25-(OH)\(_2\)D\(_3\) in MS pathogenesis, the immunoregulatory effects of 1,25-(OH)\(_2\)D\(_3\) 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 D\(_3\) 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, con-
ﬁrmed 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 ex-
cluded 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 II. Other controls were obtained from patients undergoing 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)D\(_3\) and 1,25-(OH)\(_2\)D\(_3\) levels

Serum 25-(OH)D\(_3\) and 1,25-(OH)\(_2\)D\(_3\) 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 coef-
ficients were <7.5% and <6.8%, respectively.

Ag preparation

Myelin basic protein (MBP)\(_{83–102}\) (ENPVVHFFKNIVFPRTPPPPS), MBP\(_{83–102}\) (GVDAGQTL5KJ5FLGDRSD5PSMA), and myelin oligo-
dendrocyte glycoprotein (MOG)\(_{83–97}\) (PEYGR5EKL5DAIGEGKVTL5-
RIRN) 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 \(\times\) 10\(^5\) PBMCs were stimulated with optimal concentrations of MBP or MOG peptides (10–20 µg/ml). After 5–7 d, cells were recultured in fresh me-
derium 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 after four cycles of restimulation and 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 \(\times\) 10\(^6\) cells/cm\(^2\) on 24-well Falcon Primaria plates (BD Biosciences, San Jose, CA), and allowed to differ-
centrate for 5–7 d at 37˚C and 5% CO\(_2\). Viability was assayed by try-
pan blue dye exclusion, and monocyte enricement 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 \(\times\) 10\(^5\) cells/well in the presence of 5 \(\times\) 10\(^{-5}\) M adherent irradiated PBMCs, as the source of APCs, and 10 µg/ml the appropriate peptide, with and without 1,25-(OH)\(_2\)D\(_3\), as described previously (25). In these conditions, cells were not included control CD4\(^+\) T cells were plated at a density of 5 \(\times\) 10\(^5\) 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 [\(^3\)H]thymidine incorporation. The proliferative response was measured as incorporation of radioactivity into thymidine by CD4\(^+\) T cells. The incorporation of radioactivity was determined using a scintillation counter (Wallac 1450, Wallac, Vantaa, Finland).

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 measured using commercially available kits (R&D Systems) for single-cell resolution ELISPot assays, following the manufacturer’s instructions, with and without 1,25-(OH)\(_2\)D\(_3\). 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 10\(^5\) PBMCs. Optimal doses of 1,25-(OH)\(_2\)D\(_3\) were established in preliminary experiments. For environ-
mental conditions, 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)\(_2\)D\(_3\) on regulatory T cell induction, 5 \(\times\) 10\(^5\) 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)\(_2\)D\(_3\). After 7 d, the number of CD4\(^+\)CD25\(^+\)FoxP3\(^+\) T cells was measured by flow cytometry, using commercially available regulatory T cell–staining kits (eBioscence, San Diego, CA), following the manufacturer’s instructions. Optimal doses of 1,25-(OH)\(_2\)D\(_3\) 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 pre-
pared using TRIzol LS reagent (Invitrogen, Carlsbad, CA), following the manufacturer’s instructions. RNA was reverse transcribed using M-MLV RT–reverse-transcription kit with random hexamer primers (Invitrogen). Relative levels of VDR, CYP27B1, and CYP24A1 transcripts were de-
termined by real-time PCR (ABI 7000 sequence detection system; Ap-
plied 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′-GAAGTGTAGACATTGCG-
GAGTC-3′, reverse 5′-AAAGATGTTGATGGATATTC-3′; VDR: forward 5′-CTTCAGGCGAAGCATTGAAGC-3′, reverse 5′-CTTCATTGATGCG-

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ATGTC3; CYP27B1: forward 5'-TTGGCAAGCAGGCTGTAT3', reverse 5'-TGTGTAAGATGCTGCCAA3'; and CYP24A1: forward 5'-CCAACTGGAGGAAGCTACT-3', reverse 5'-AGTCTTCCCTTCC-AAGATCA3'.

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 (10^6/tube) harvested at different times were incubated at 4°C (to inhibit uptake) or 37°C (optimal uptake temperature) in OVA/RPMI 1640 containing 2 μM 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% NaN3 to stop internalization. Cells were then fixed with 4% paraformaldehyde in PBS, and samples were analyzed by FACSscan 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)D3 and 1,25-(OH)2D3 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)D3 and 1,25-(OH)2D3

In a first approach to investigate whether gender differences existed in relation to immunological effects of vitamin D3, serum levels of 25-(OH)D3 and 1,25-(OH)2D3 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)D3 were similar in females (119.75 ± 22.5 nM in remission, 95.75 ± 21.25 nM during exacerbations, and 153.75 ± 15 nM in healthy controls) and in males (114.75 ± 23 nM in remission, 98 ± 26.75 nM during exacerbations, and 150.25 ± 10.75 nM in healthy controls). This was also the case for 1,25-(OH)2D3 serum levels in females (72 ± 13.2 pM during remission, 55.4 ± 13.92 pM during exacerbations, and 84 ± 16.56 pM in healthy controls) and in males (65.28 ± 13.2 pM during remission, 54.24 ± 13.2 pM during exacerbations, and 84.96 ± 15.36 pM in healthy controls). Additional analyses demonstrated significantly lower serum levels of 25-(OH)D3, and 1,25-(OH)2D3 in female patients during exacerbations compared with those observed during remissions (p < 0.0001). Moreover, 25(OH)D3 and 1,25(OH)2D3 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)D3 and 1,25(OH)2D3 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)D3 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)2D3 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 D3 metabolites in MS patients during the same clinical course of the disease or in healthy controls. Therefore, the impact of 1,25-(OH)2D3 on T cell function in males and females was examined in subsequent experiments.

Inhibition of T cell proliferation by 1,25-(OH)2D3 is significantly stronger in females compared with males with MS

1,25-(OH)2D3 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)2D3 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)2D3 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)2D3–mediated inhibition did not differ between MS patients and healthy controls.

Cytokine responses reveal significant gender differences in 1,25-(OH)2D3 effects

The impact of 1,25-(OH)2D3 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)2D3. Fig. 3 demonstrates the significant differences observed between the genders in the secretion patterns of these three cytokines.

1,25-(OH)2D3 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)2D3 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)2D3 is greater in female MS patients

To assess the influence of gender on CD4+CD25+FoxP3+ induction by 1,25-(OH)2D3, PBMCs were stimulated with soluble anti-CD3 and soluble anti-CD28 in the presence and absence of 1,25-(OH)2D3, and subsequent CD4+CD25+FoxP3+ regulatory T cell
isolated CD4+CD25+FoxP3+ T cells showed low or undetectable effects, vitamin D3 metabolism was analyzed in males and To further investigate the sex-based disparity in immunological expression of CD127. No significant differences were observed in males (33.4.6% versus 15.6.8%; 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 hemagglutinin307–319 peptide, and polyclonally stimulated T cells. No significant differences were observed between MS patients and healthy controls.

**DBP**

DBP is a serum glycoprotein, synthesized predominantly in the liver, which reversibly binds to 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...
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 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 propylpyrazole 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 modulates 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.
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
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 estradiol 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...
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 1-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 (ERα and ERβ), 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 ERα-selective agonist reproduced effects observed with E2. In sharp contrast, the ERβ agonist showed no effect on 1,25-(OH)2D3 metabolism. This suggests that E2’s effects on vitamin D metabolism are mediated primarily through ERα and is consistent with observations indicating that immunomodulatory effects of E2 are dependent on ERα 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 ERα (74). These results are consistent with observations showing treatment of EAE with an ERα-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 ERβ-selective ligand, although treatment with ERα or ERβ 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 cubulin, 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 hsp70–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/cubulin 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 ERα. 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 subgroup of MS patients.

Studies relating to these observations should be extended to the mechanistic evaluation of other sexually dimorphic hormones, such as prolan, and growth hormone. Correction of vitamin D 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
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References


Supplemental Figure. The effects of E2 on proliferation, FoxP3 expression and cytokine secretion was not affected by culture conditions. MBP\textsubscript{83-102} T cell lines (panels A to C), and ex vivo CD4+ T cells (panels D to G) isolated from 4 female and 4 male MS patients were cultured using phenol red containing medium, or phenol red free-medium, stimulated with the cognate peptide (10 μg/ml) or PHA (10 μg/ml) respectively, in the presence of either 0, 1, 5 or 10 nM of E2, and in the absence of 1,25-(OH)\textsubscript{2}D\textsubscript{3}. Proliferation assays, number of cytokine secreting cells and percentage of CD4+CD25+FoxP3+ regulatory T cells were evaluated as described in Material and Methods. PR= Phenol red.