IL-10 Signaling Is Essential for 1,25-Dihydroxyvitamin D_3-Mediated Inhibition of Experimental Autoimmune Encephalomyelitis[1]

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Multiple sclerosis (MS) results from an aberrant, neuroantigen-specific, T cell-mediated autoimmune response. Because MS prevalence and severity decrease sharply with increasing sunlight exposure, and sunlight supports vitamin D_3 synthesis, we proposed that vitamin D_3 and 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) may protect against MS. In support of this hypothesis, 1,25-$(OH)_2D_3$ strongly inhibited experimental autoimmune encephalomyelitis (EAE). This inhibition required lymphocytes other than the encephalitogenic T cells. In this study, we tested the hypothesis that 1,25-$(OH)_2D_3$ might inhibit EAE through the action of IL-10-producing regulatory lymphocytes. We report that vitamin D_3 and 1,25-$(OH)_2D_3$ strongly inhibited myelin oligodendrocyte peptide ($MOG_{35–55}$)-induced EAE in C57BL/6 mice, but completely failed to inhibit EAE in mice with a disrupted *IL-10* or *IL-10R* gene. Thus, a functional IL-10-IL-10R pathway was essential for 1,25-$(OH)_2D_3$ to inhibit EAE. The 1,25-$(OH)_2D_3$ also failed to inhibit EAE in reciprocal, mixed bone marrow chimeras constructed by transferring *IL-10*-deficient bone marrow into irradiated wild-type mice and vice versa. Thus, 1,25-$(OH)_2D_3$ may be enhancing an anti-inflammatory loop involving hemopoietic cell-produced IL-10 acting on brain parenchymal cells and vice versa. If this interpretation is correct, and humans have a similar bidirectional IL-10-dependent loop, then an IL-10-IL-10R pathway defect could abrogate the anti-inflammatory and neuroprotective functions of sunlight and vitamin D_3. In this way, a genetic IL-10-IL-10R pathway defect could interact with an environmental risk factor, vitamin D_3 insufficiency, to increase MS risk and severity. ***The Journal of Immunology,*** **2006, 177: 6030–6037.**

Multiple sclerosis (MS)[3] shows a striking gradient of increasing prevalence with increasing latitude (1). Worldwide studies have attributed this gradient to a strong link between low sunlight exposure and high MS risk (2–5). Because sunlight exposure produces vitamin D_3 (6), we hypothesized that the high MS risk with low sunlight exposure might reflect insufficient sunlight-catalyzed vitamin D_3 synthesis to support the immunoregulatory functions of the vitamin D_3 hormone, 1,25-dihydroxyvitamin D_3 (7).

Consistent with this hypothesis, 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) strongly inhibited experimental autoimmune encephalomyelitis (EAE), an MS model (8, 9). The 1,25-$(OH)_2D_3$-mediated inhibition of EAE required *Rag-1*-dependent lymphocytes other than the encephalitogenic $CD4^+$ T cells (10). To explain this result, we hypothesized that 1,25-$(OH)_2D_3$ might strengthen the function of lymphocytes that regulate potentially autoreactive T cell responses (10). The $CD4^+CD25^+$ lymphocytes that suppress EAE do so via an IL-10-dependent mechanism (11–14). IL-10 is a potent inhibitor of APC function, inflammatory T cell activation, cytokine synthesis, and chemokine synthesis (15). Increases in spinal cord IL-10 correlated with EAE remissions (16). Mice with transgenic *IL-10* resisted EAE (17), whereas mice with a disrupted *IL-10* gene were highly EAE susceptible (18, 19). These data indicate that the IL-10-IL-10R pathway has very important suppressor functions in EAE.

The present experiments evaluated vitamin D_3- and 1,25-$(OH)_2D_3$-mediated protection from myelin oligodendrocyte peptide ($MOG_{35–55}$)-induced EAE in C57BL/6 (B6) mice with targeted disruptions of the *IL-10* or *IL-10R* genes to test the hypothesis that the inhibition mechanism might be IL-10 dependent. Having established that the IL-10-IL-10R pathway was essential, we also evaluated irradiation bone marrow (BM) chimeras to pinpoint the source of the essential IL-10. Furthermore, we investigated a possible direct effect of the hormone on IL-10 or IL-10R expression. We present a model for a bidirectional, IL-10-dependent, anti-inflammatory loop to explain how vitamin D may implement sunlight's protective biological effects in MS, and discuss the implications of this model for vitamin D-based preventive and therapeutic strategies in MS.

Materials and Methods

Mice

We purchased the B6, B6.SJL-*Ptprc*^{a} *Pep3*^{b}/BoyJ (B6.CD45.1), and C57BL/6-*Il10*^{tm1Cgn} mice (B6.IL-$10^{-/-}$) from The Jackson Laboratory. Genentech donated the CFR2.4 (B6.IL-10R$\beta^{-/-}$) breeder mice; they were genotyped for the loss of *IL-10R*β and the acquisition of *neo* as described (20). Mice were housed in our pathogen-free mouse colony at 23°C with 40–60% humidity, 12-h light-dark cycles, and ad libitum access to water.

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[3] Abbreviations used in this paper: MS, multiple sclerosis, 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; BM, bone marrow; EAE, experimental autoimmune encephalomyelitis; MBP, myelin basic protein; $MOG_{35–55}$, myelin oligodendrocytic glycoprotein peptide; VDR, vitamin D receptor.

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Before experiments, they were fed Lab Diet no. 5008 (PMI Nutrition International) containing 0.33 μg/day vitamin D_3 and 1% calcium. Experiments used male and female mice aged 6–8 wk (age- and sex-matched within experiments). The Institutional Animal Care and Use Committee approved all of the experimental protocols.

Antigens

The $MOG_{35–55}$ was purchased from BioSynthesis and had the amino acid sequence MEVGWYRSPFSRVVHLYRNGK.

Vitamin D_3 or 1,25-$(OH)_2D_3$ treatment and EAE induction

A nutritionally complete synthetic diet was formulated as we described (21), except that vitamin D was omitted. The vitamin D_3 (Sigma-Aldrich) was added to this diet in an amount to provide 1 μg/day. Alternatively, the 1,25-$(OH)_2D_3$ (Sigma-Aldrich) was added in an amount to provide 50 ng/day to females and 100 ng/day to males. These doses significantly inhibited EAE in previous studies (9, 10, 22–26). Fresh diet was provided three times per week. For prevention studies, mice were fed the synthetic diets before EAE induction (10). For treatment studies, EAE was induced in chow-fed mice, and at the peak of disease, 200 ng of 1,25-$(OH)_2D_3$ in oil or oil only as the placebo was injected i.p (27). EAE was induced and clinical EAE severity was assessed daily exactly as described (10). Blood samples were obtained before EAE induction and at the end of the study.

Histopathology

For histopathological evaluation, mice were euthanized and perfused with saline. The spinal cords were removed, divided into six equal segments, frozen in OCT compound (Sakura Finetek). And sectioned transversely (10 μm). The cryosections were fixed in 4% paraformaldehyde, stained with Gill's no. 3 H&E Y (Sigma Diagnostics), and examined using a Zeiss Axioskop microscope equipped with a Plan-Neofluar ×20/0.5 objective. Bright field images were acquired with AxioVision 3.0 software controlling an Axiocam digital camera. For the histopathology analysis, each of six sections per mouse was divided into quadrants, and each quadrant was scored in a blinded fashion as 0 or 1, based on the absence or presence, respectively, of infiltrating inflammatory cells. The histopathology score was recorded as the percentage of spinal cord quadrants that showed a readily identifiable inflammatory cell infiltrate.

Serum calcium analysis

Blood was collected, clotted, and centrifuged (2000 × *g* for 10 min) at 6°C. The serum was decanted and stored at −20°C. The samples, standards, and buffer blanks (2 μl each) were aliquoted into duplicate wells of a 96-well plate. The calcium detection reagent was prepared according to the manufacturer's directions (Sigma Diagnostics), and 0.25 ml was added to each well. The absorbance at 570 nm less the blank was measured 10 to 30 min later. The Ca^{2+} mM/L serum was determined from a standard curve.

Real-time PCR

Real-time PCR was performed as described (28) with minor modifications (29). In brief, total RNA was extracted using TRI Reagent (Molecular Research Center) and reverse transcribed from an oligo(dT) primer using the Reverse Transcription System (Promega). Real-time PCR was performed with SYBRGreen PCR Master Mix (Applied Biosystems) in a GeneAmp 5700 Sequence Detection System. Purified and quantified control cDNA was serially diluted, and amplified in each run; the control cDNA vs the threshold cycle formed a standard curve. The transcript abundance, determined with the aid of the standard curve, is reported relative to GAPDH. Published primers for the GAPDH and IFN-γ (30), and for Foxp3 (31) were used. The IL-10 primers were 5′-CCC TTT GCT ATG GTG TCC TT-3′ and 5′-TGG TTT CTC TTC CCA AGA CC-3′; they were designed with Primer Express (Applied Biosystems).

Flow cytometry

For flow cytometric studies, PBS perfusion was done and single-cell suspensions of spinal cord cells or splenocytes were prepared as described (10, 32). In brief, dissociated spleen cells in cold staining buffer (5% heat-inactivated FBS and 0.1% sodium azide in PBS, pH 7.3) were depleted of RBC, and stained (10^6 cells/sample) for 30–40 min on ice with predetermined optimal amounts of FITC-, PE-, or allophycocyanin-conjugated mAb. Spinal cord cells were centrifuged through a Percoll gradient, washed, and stained as previously described (10). Reference samples were stained with fluorochrome-coupled isotype control mAb, or single-color specific mAb stains for compensation. Stained samples were analyzed on a FACSCalibur (BD Biosciences) using CellQuest software. The fluorescent mAbs to IFN-γ, IL-10, IL-10R, CD4, CD11b, CD25, CD45.2, and CD45.1 were purchased from Southern Biotechnology Associates or BD Biosciences.

Intracellular staining for cytokines

To enumerate cytokine-producing cells, EAE was induced, and when the placebo-treated mice reached stage 2 EAE (20 days post MOG immunization), all mice were euthanized, and the spinal cord and cervical lymph node cells were collected and analyzed (33). The cells were stimulated overnight with immobilized Abs to CD3, cultured 4 h with monensin, washed, and stained with FITC-coupled Abs to CD4 or isotype control Abs. The stained cells were paraformaldehyde-fixed, permeabilized in buffer with 0.01% Triton X-100, and stained with PE-coupled Abs to IFN-γ and allophycocyanin-coupled Abs to IL-10 or isotype control Abs (BD Pharmingen). Flow cytometric data were acquired on a FACSCalibur using CellQuest software (BD Biosciences).

Irradiation BM chimeras

Irradiation BM chimeras were constructed by a standard protocol (34, 35). In brief, recipient mice (6 wk of age) were given antibiotics in the drinking water for 3 days, irradiated with a total of 1200 rad (2 doses 4 h apart), injected i.v. with 2–5 × 10^6 BM cells, and maintained in a sterile environment with antibiotics for 2 wk. Thereafter, they were maintained under standard conditions without antibiotics. At 6–7 wk posttransplantation, blood lymphocytes were stained and analyzed for CD45.1 and CD45.2 to quantify donor BM engraftment. At 7 wk posttransplantation, the mice were placed on the 1,25-$(OH)_2D_3$-supplemented or placebo control diet. At 8 wk posttransplantation, EAE was induced and evaluated as above.

Statistical analysis

Individual mice were analyzed and the mean and SD were calculated for each group of mice. Experiments were repeated at least once. The group sizes are given in the table and figure legends. The significance of differences between the group means was determined using the Mann-Whitney *U* test ($n \leq 16$), Student's *t* test ($n > 16$), or χ^2 test (binomial data) as indicated (36); $p < 0.05$ was considered significant.

Results

Vitamin D_3 inhibited EAE in wild-type (Wt) but not IL-$10^{-/-}$ mice

The experiments reported here used $MOG_{35–55}$-induced EAE in B6 mice to investigate why *Rag-1*-dependent lymphocytes other than the neuroantigen-reactive CD4 T cells were needed for 1,25-$(OH)_2D_3$ to inhibit EAE (10). To test the hypothesis that the vitamin D_3 system might be enhancing the function of IL-10-producing regulatory lymphocytes, we studied vitamin D_3-mediated inhibition of $MOG_{35–55}$-induced EAE in mice with a Wt or disrupted *IL-10* gene. Mice were fed synthetic diets that provided 0 (−D diet) or 1 μg/day (+D diet) of vitamin D_3 beginning at age 4 wk exactly as described (26). At age 8 wk, the +D mice had 56 ± 3 nM/L serum 25-hydroxyvitamin D_3, whereas the −D mice had 8 ± 3 nM/L. All mice were then primed with $MOG_{35–55}$ and evaluated for EAE disease. The +D diet significantly reduced the incidence, peak clinical score, and cumulative disease index for $MOG_{35–55}$-induced EAE in female but not male B6 mice (Table I). These results confirm the female-specific protective effect of dietary vitamin D_3 that we reported previously for MBP-induced EAE in B10.PL mice (26). However, the +D diet did not inhibit $MOG_{35–55}$-induced EAE in B6.IL-$10^{-/-}$ mice (Table I). We conclude that vitamin D_3-mediated inhibition of EAE requires *IL-10* gene expression.

1,25-$(OH)_2D_3$ inhibited EAE in Wt but not IL-$10^{-/-}$ mice

One explanation for the failure of vitamin D_3 to inhibit EAE in B6.IL-$10^{-/-}$ mice could be an unanticipated requirement for IL-10 to stimulate the conversion of the biologically inactive vitamin D_3 and 25-hydroxyvitamin D_3 compounds into the biologically active hormone, 1,25-$(OH)_2D_3$. To bypass a possible vitamin D_3 metabolism problem in B6.IL-$10^{-/-}$ mice, we repeated the

Table I. *The vitamin D_3 reduced the incidence and severity of EAE in female B6 mice, but not in male B6 mice or B6.IL-10$^{-/-}$ mice of either gender*[a]

Strain	Sex	Treatment	Incidence[b] (%)	Onset (day)	Peak Clinical Score	Cumulative Disease Index[c]
C57BL/6	F	Placebo	100	13.0 ± 1.0	2.5 ± 0.5	17.4 ± 6.3
C57BL/6	F	Vitamin D_3	60*	13.3 ± 1.5	0.9 ± 1.2*	5.9 ± 7.4*
C57BL/6	M	Placebo	100	13.0 ± 1.0	2.0 ± 1.0	16.4 ± 8.3
C57BL/6	M	Vitamin D_3	100	12.8 ± 1.1	2.3 ± 0.8	19.0 ± 8.9
B6.IL-10$^{-/-}$	F	Placebo	100	12.4 ± 0.9	1.9 ± 0.9	15.5 ± 6.4
B6.IL-10$^{-/-}$	F	Vitamin D_3	100	12.2 ± 0.4	2.9 ± 0.5	20.8 ± 6.5
B6.IL-10$^{-/-}$	M	Placebo	100	12.3 ± 0.5	2.3 ± 0.6	16.9 ± 5.5
B6.IL-10$^{-/-}$	M	Vitamin D_3	100	12.2 ± 0.4	2.8 ± 0.3	21.7 ± 6.1

[a] Adult mice were fed synthetic diets formulated to provide 0 or 1 μg/day of vitamin D_3 for 1 mo. EAE was induced with MOG_{35-55} peptide, synthetic diet feeding was continued, and disease severity was scored daily. The clinical data shown are the composite mean ± SD of (five mice per group). Within gender comparisons were performed using the Mann-Whitney test; *, $p < 0.03$.

[b] Mice with a clinical score ≥1 for 2 consecutive days were considered to have EAE.

[c] A cumulative disease index for each mouse was calculated as the sum of the mouse's daily clinical scores for the first 21 days postimmunization; the data shown are the cumulative disease indices for each group (mean ± SD).

experiment except that biologically active 1,25-(OH)$_2$D$_3$ was used in place of vitamin D$_3$. The 1,25-(OH)$_2$D$_3$-fed male and female B6 had a lower EAE incidence ($p < 0.001$), decreased mortality ($p < 0.05$), and lower peak severity and cumulative disease index ($p < 0.0001$) than the placebo-fed controls (Fig. 1*A* and Table II). Histopathology showed that the 1,25-(OH)$_2$D$_3$-fed B6 mice had lesions with inflammatory cell infiltration in 17% of the microscopic fields examined, whereas the placebo-fed controls had lesions in >60% of the fields (Fig. 2). The 1,25-(OH)$_2$D$_3$-treated B6 mice also had 66% fewer spinal cord IFN-γ transcripts than the placebo-treated controls, as determined by real-time PCR (Table III). In contrast, the 1,25-(OH)$_2$D$_3$-treated and placebo-treated mice had equivalent numbers of IFN-γ-producing CD4$^+$ Th1 cells in the lymph nodes (placebo, 16 ± 5%; 1,25-(OH)$_2$D$_3$, 16 ± 6%). Collectively, the clinical, histological, and immunological data establish that 1,25-(OH)$_2$D$_3$ inhibits MOG_{35-55}-induced EAE in B6 mice, as it does MBP-induced EAE in B10.PL mice (9, 10, 29).

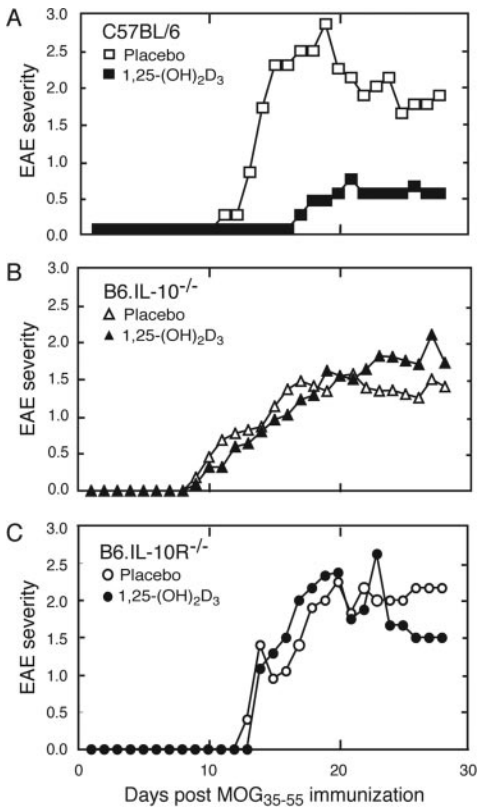

FIGURE 1. Functional *IL-10* and *IL-10R* genes were required for 1,25(OH)$_2$D$_3$-mediated inhibition of EAE. *A*, Clinical EAE disease in B6 mice. Adult female and male mice were fed synthetic diets formulated to provide 0 (□), or 50 ng/day (females) or 100 ng/day (males) (■) of 1,25(OH)$_2$D$_3$ for 5 days. EAE was induced with MOG_{35-55} peptide, synthetic diet feeding was continued, and disease severity was scored daily. *B*, Clinical disease in B6.IL-10$^{-/-}$ mice. *C*, Clinical disease in B6.IL-10R$^{-/-}$ mice. The cumulative mean from three separate experiments is shown in *A* and *B* (22 mice/group up to day 23; 10 mice/group after day 23). The mean from one of two experiments is shown in *C* (6 mice/group). From day 14 onward, the B6 mice fed 1,25-(OH)$_2$D$_3$ had significantly less severe EAE than all other groups ($p < 0.05$; Mann-Whitney *U* test).

In contrast, the 1,25-(OH)$_2$D$_3$ did not inhibit MOG_{35-55}-induced EAE in B6.IL-10$^{-/-}$ mice (Fig. 1 and Table II). The incidence, onset, mortality, peak disease severity, and cumulative disease index were not significantly different for 1,25-(OH)$_2$D$_3$-fed and placebo-fed B6.IL-10$^{-/-}$ mice. Furthermore, the 1,25-(OH)$_2$D$_3$-fed and placebo-fed B6.IL-10$^{-/-}$ mice had equivalent histopathology (Fig. 2) with >60% of the fields showing inflammatory lesions. Lastly, the two groups had equally high numbers of IFN-γ transcripts in the spinal cord (Table III). The 1,25-(OH)$_2$D$_3$ also inhibited MBP-induced EAE in Wt B10.PL mice, but not B10.PL mice lacking *IL-10* gene expression, confirming these results (data not shown). We conclude that a functional *IL-10* gene is necessary for 1,25-(OH)$_2$D$_3$ to inhibit EAE.

The precise mechanism for 1,25-(OH)$_2$D$_3$-mediated inhibition of EAE is debated. It was recently suggested that elevated serum calcium may be necessary and sufficient to inhibit EAE in female mice, and that 1,25-(OH)$_2$D$_3$ may have no functional role other than to elevate serum calcium (37). To investigate this point, we analyzed serum calcium before and during the study shown in Fig. 1. The serum calcium levels were equivalent in all groups before the study (Table IV). The 1,25-(OH)$_2$D$_3$-treated B6 mice had no increase in the serum calcium on day 0, but elevated serum calcium by day 53. Therefore, elevated calcium correlated with decreased EAE in these mice. However, the 1,25-(OH)$_2$D$_3$-treated B6.IL-10$^{-/-}$ mice had a significant increase in serum calcium on day 0, and a further increase by day 53 (Table IV), but no decrease in any measure of EAE disease (Table II). Thus, elevated serum calcium was not sufficient to inhibit EAE in the B6.IL-10$^{-/-}$ female mice. Moreover, the high vitamin D$_3$ diet inhibited all measures of EAE disease in females without causing hypercalcemia (Table I and Ref. 26). Thus elevated serum calcium was neither necessary nor sufficient to inhibit EAE.

Table II. *The 1,25-$(OH)_2D_3$ delayed the onset and reduced the severity of EAE in B6 but not B6.IL-$10^{-/-}$ or B6.IL-$10R^{-/-}$ mice*[a]

Strain	Treatment	Incidence[b] (%)	Onset (day)	Mortality (%)	Peak Clinical Score	Cumulative Disease Index[c]
C57BL/6	Placebo	95	16 ± 4	5	2.3 ± 1.2	15 ± 8
C57BL/6	1,25$(OH)_2D_3$	41**	20 ± 6	0*	0.7 ± 0.7***	2 ± 4***
B6.IL-$10^{-/-}$	Placebo	95	12 ± 3	9	2.1 ± 1.1	16 ± 6
B6.IL-$10^{-/-}$	1,25$(OH)_2D_3$	100	13 ± 3	27	2.6 ± 1.6	15 ± 6
B6.IL-10R$\beta^{-/-}$	Placebo	100	18 ± 3	20	2.5 ± 0.7	14 ± 6
B6.IL-10R$\beta^{-/-}$	1,25$(OH)_2D_3$	100	17 ± 2	17	3.0 ± 1.2	15 ± 6

[a] The experiment was performed as described in the Fig. 1 legend. The clinical data shown are the composite mean ± SD of three independent experiments for B6 and B6.IL-$10^{-/-}$ mice (22 per group). The clinical data for B6.IL-10R$\beta^{-/-}$ mice (six per group) were obtained in two experiments performed after the B6 and B6.IL-$10^{-/-}$ studies. The statistical tests performed on the data were the χ^2 test (incidence and mortality) and the Student's t test (disease onset, severity, and cumulative disease index). For the comparison between the 1,25-$(OH)_2D_3$-treated B6 mice and all other groups; *, $p < 0.05$; **, $p < 0.001$; and ***, $p < 0.0001$.

[b] Mice with a clinical score ≥1 for 2 consecutive days were considered to have EAE.

[c] A cumulative disease index for each mouse was calculated as the sum of the mouse's daily clinical scores for the first 28 days postimmunization; the data shown are the cumulative disease indices for each group (mean ± SD).

1,25-$(OH)_2D_3$ did not inhibit EAE in IL-$10R^{-/-}$ mice

We reasoned that a fully functional IL-10-IL-10R pathway might be necessary for 1,25-$(OH)_2D_3$ to inhibit EAE. To test this hypothesis, we repeated the prevention study using B6.IL-10R$\beta^{-/-}$ mice (with a nonfunctional IL-10Rβ-signaling chain) in place of B6.IL-$10^{-/-}$ mice (20). The results showed that 1,25-$(OH)_2D_3$ did not inhibit MOG_{35-55}-induced EAE in B6.IL-10R$\beta^{-/-}$ mice (Fig. 1*C*). The EAE incidence, onset, mortality, peak disease severity, and cumulative disease index were not significantly different for 1,25-$(OH)_2D_3$-fed and placebo-fed B6.IL-10R$\beta^{-/-}$ mice (Table II). Collectively, our data show for the first time that the CNS-specific, 1,25-$(OH)_2D_3$-activated, neuroprotective mechanism depends on a functional IL-10-IL-10R pathway.

1,25-$(OH)_2D_3$ did not alter IL-10 or IL-10R in the CNS

We next looked for possible 1,25-$(OH)_2D_3$-mediated enhancement of *IL-10* gene expression. The experiment shown in Fig. 1*A* was repeated, and the IL-10-producing $CD4^+$ T cells were analyzed by flow cytometry 28 days postimmunization. The IL-10-producing $CD4^+$ T cells in the CNS cell suspension were too infrequent to measure. In the cervical lymph node cell suspension, the two groups had equivalent IL-10-producing $CD4^+$ T cell percentages (placebo, 15 ± 8%; 1,25-$(OH)_2D_3$, 12 ± 4%) and fluorescence intensities (data not shown). Furthermore, when 1,25-$(OH)_2D_3$ or a placebo was administered to mice with acute EAE, the spinal cord and the lymph nodes had equivalent IL-10 transcripts 6-h posttreatment (placebo 0.4 ± 0.2 and 1,25-$(OH)_2D_3$ 0.3 ± 0.1 IL-10 transcripts per 10^3 GAPDH transcripts). We have shown that direct, 1,25-$(OH)_2D_3$-mediated changes in gene expression can be detected using the 6 h treatment (29). Thus, neither protein nor transcript studies showed 1,25-$(OH)_2D_3$-mediated enhancement of IL-10 synthesis. However, the data do not rule out 1,25-$(OH)_2D_3$-mediated effects on minor cell populations like regulatory lymphocytes.

We also looked for 1,25-$(OH)_2D_3$-mediated enhancement of *IL-10R* gene expression. The 1,25-$(OH)_2D_3$ or a placebo was administered to mice with acute EAE, and flow cytometry was performed on spinal cord cells collected 6 h later. We have established that no hormone-mediated changes in the CNS cell populations occur within this time period (29). The nonimmunized control mice had microglial cells expressing an intermediate level of CD11b and the IL-10R (Fig. 3*A*) (38–40). The placebo-treated mice with EAE had the $CD11b^{int}$IL-$10R^+$ microglial cells, plus a $CD11b^+$IL-$10R^-$ cell population not found in the nonimmunized control mice (Fig. 3*B*). These $CD11b^+$IL-$10R^-$ myeloid lineage cells appear to be infiltrating, activated macrophages. To our knowledge, this is the first report that CNS-infiltrating, activated macrophages do not express the IL-10R. We found no differences in the IL-$10R^+$ or IL-$10R^-$ cell percentages or the IL-10R density between the two treatment groups (Fig. 3, *B* and *C*, and data not shown). Thus, we found no evidence for a direct effect of 1,25-$(OH)_2D_3$ on IL-10R expression. However, we cannot rule out effects on minor cell

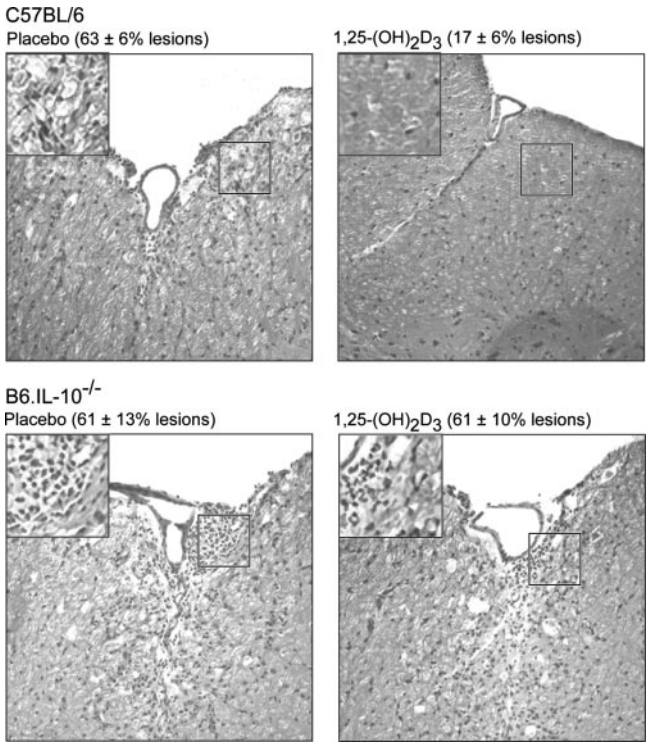

FIGURE 2. Dietary 1,25-$(OH)_2D_3$ reduced the inflammatory cell infiltration and lesion formation associated with EAE in B6 but not B6.IL-$10^{-/-}$ or B6.IL-$10R^{-/-}$ mice. The experiment was performed as described in the Fig. 1 legend. Spinal cord samples (three per group) were obtained on day 22 post-MOG injection and a histopathological analysis was performed. Representative H&E-stained sections are shown. The nuclei of infiltrating, inflammatory cells appear dark blue. The percentage of sections with lesions (in parentheses) in the 1,25-$(OH)_2D_3$-treated B6 mice differed significantly ($p < 0.05$; Mann-Whitney U test) from all other groups.

Table III. *Supplementary 1,25-(OH)$_2$D$_3$ inhibited IFN-γ production in the CNS of B6 but not B6.IL-10$^{-/-}$ mice*[a]

Strain	1,25(OH)$_2$D$_3$	IFN-γ Transcripts in the Spinal Cord[b] (copies/10^6 GAPDH)
C57BL/6	−	1.8 ± 1.3
C57BL/6	+	0.6 ± 0.5*
B6.IL-10$^{-/-}$	−	3.6 ± 5.8
B6.IL-10$^{-/-}$	+	3.9 ± 5.3

[a] The experiment was performed as described in the Fig. 1 legend. The individual spinal cord samples were collected 22 days after MOG$_{35-55}$ immunization.

[b] A real-time PCR analysis for IFN-γ transcripts was performed on individual spinal cord RNA samples (nine female and male mice/group). The Mann-Whitney test gave $p = 0.052$ (*) for the comparison between the 1,25-(OH)$_2$D$_3$-treated B6 mice and each other group (36).

populations like regulatory lymphocytes, astrocytes, or neurons that are not represented in the cell suspension.

Regulatory CD4$^+$CD25$^+$ T cells that express Foxp3 and suppress EAE by an IL-10-dependent mechanism have been described (11–14, 41–43). To see whether 1,25-(OH)$_2$D$_3$ increased Foxp3, the prevention study was repeated, and spinal cord Foxp3 transcripts/10^3 GAPDH transcripts were quantified 4 wk postimmunization. The placebo (24 ± 6) and 1,25-(OH)$_2$D$_3$-fed mice (29 ± 17) had equivalent Foxp3 transcripts, ruling out direct effects on Foxp3.

Reciprocal BM chimera studies

Having established that a functional IL-10-IL-10R pathway is necessary for the 1,25-(OH)$_2$D$_3$-activated protective mechanism, we next sought to determine the source of the essential IL-10. The T and B lymphocytes, infiltrating macrophages, microglial cells, and astrocytes all have the capacity to produce IL-10 (15, 44). Reciprocal irradiation BM chimeras were constructed by transferring Wt BM into lethally irradiated, *IL-10$^{-/-}$* recipients (B6.CD45.1→B6.IL-10$^{-/-}$) and vice versa (B6.IL-10$^{-/-}$→B6.CD45.1). Cells with a Wt *IL-10* gene carried the CD45.1 allotype, whereas cells with an *IL-10$^{-/-}$* genotype carried the CD45.2 allotype as described (34, 35). Control B6.CD45.1→B6 and B6.IL-10$^{-/-}$→B6.IL-10$^{-/-}$ chimeras were also constructed. At 6 wk post-BM transplantation, PBLs were >85% donor origin. At the end of each study, the splenocytes and splenic CD3$^+$ T cells were 85–91% donor origin (Table V and data not shown). These CD45 allotype data indicated that all animals were chimeras.

Chimeric mice were fed synthetic diets with or without 1,25-(OH)$_2$D$_3$, immunized with MOG$_{35-55}$, and evaluated for EAE

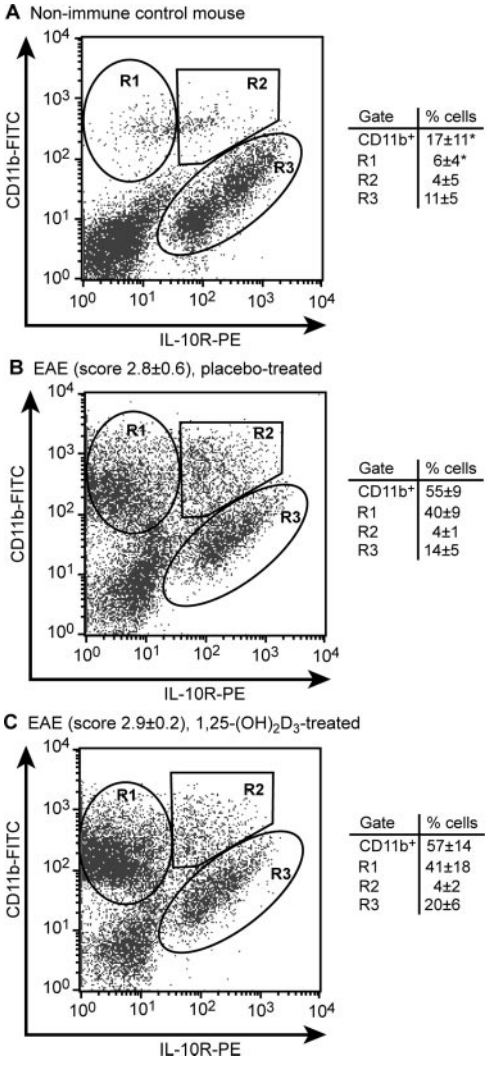

FIGURE 3. The 1,25-(OH)$_2$D$_3$ had no rapid, direct effect on IL-10R expression in the inflamed CNS. *A*, Spinal cord cells from a nonimmune control mouse. *B*, Spinal cord cells from a mouse that had EAE and was placebo treated. *C*, Spinal cord cells from a mouse that had EAE and was treated with 1,25-(OH)$_2$D$_3$. B6 mice with MOG$_{35-55}$-induced EAE (clinical score 2.5–3.0) were injected with a placebo or with 1,25-(OH)$_2$D$_3$. Six hours later, the spinal cord cells were collected, and separated on a Percoll gradient, stained with fluorescent Ab to CD11b and to IL-10R. Cells in the live cell gate were analyzed by flow cytometry for CD11b and IL-10R expression. The results shown are representative of two separate experiments with four to five female and male mice per group. The samples from the experimental mice with EAE were compared with the naive control sample; *, $p < 0.05$ (Mann-Whitney *U* test).

Table IV. *Long-term 1,25-(OH)$_2$D$_3$ supplementation resulted in elevated serum calcium levels without concomitant EAE inhibition in mice with a disrupted IL-10 gene*[a]

Strain	Sex	1,25-(OH)$_2$D$_3$ (ng/day)	Serum Ca^{2+} (mM/L)[a] Day −5	Day 0	Day 53
C57BL/6	Female	0	2.9 ± 0.2	3.1 ± 0.2	3.0 ± 0.3
		50		3.3 ± 0.5	3.8 ± 0.6**
	Male	0	2.8 ± 0.2	2.9 ± 0.1	3.6 ± 0.4
		100		3.2 ± 0.5	5.0 ± 0.3**
B6-IL-10$^{-/-}$	Female	0	2.8 ± 0.5	2.7 ± 0.3	3.2 ± 0.4
		50		3.4 ± 0.2**	4.0 ± 0.5**
	Male	0	2.7 ± 0.3	2.6 ± 0.2	3.3 ± 0.3
		100		3.2 ± 0.2*	4.2 ± 0.7*

[a] The experiment was performed as described in the Fig. 1 legend. The serum calcium was analyzed before 1,25-(OH)$_2$D$_3$ (day −5), on the day of priming to induce EAE (day 0), and 53 days after priming. The results shown are the mean ± SD for 5 (male) or 11 (female) mice per group on the day indicated relative to MOG$_{35-55}$ immunization; *, $p < 0.05$ and the **, $p < 0.01$ for the comparison between the 1,25(OH)$_2$D$_3$-treated group and the respective placebo-treated group.

Table V. *The 1,25-$(OH)_2D_3$ inhibited EAE only if the radio-sensitive hemopoietic cells and the radio-resistant brain parenchymal cells were capable of producing IL-10*[a]

Chimera	1,25-$(OH)_2D_3$	Donor Spleen Cells[b] (%)	Incidence[c] (%)	Onset (Day)	Peak Clinical Score	Cumulative Disease Index[d]
B6.CD45.1→B6	−	91 ± 1	100	13 ± 0	3.0 ± 0.3	6.5 ± 3.5
B6.CD45.1→B6	+	89 ± 3	25	22###	0.4 ± 0.6###	1.8 ± 3.5###
B6.IL-$10^{-/-}$→B6.IL-$10^{-/-}$	−	n.d.	100	9 ± 1***	3.1 ± 0.2	9.2 ± 2.0*
B6.IL-$10^{-/-}$→B6.IL-$10^{-/-}$	+	n.d.	100	10 ± 2	2.6 ± 0.7	7.0 ± 2.8
B6.CD45.1→B6.IL-$10^{-/-}$	−	88 ± 2	100	14 ± 1	2.0 ± 0.9	4.6 ± 2.5
B6.CD45.1→B6.IL-$10^{-/-}$	+	85 ± 4	73	13 ± 1	2.4 ± 0.6	5.4 ± 2.8
B6.IL-$10^{-/-}$→B6.CD45.1	−	89 ± 4	100	13 ± 1	2.7 ± 1.0	7.7 ± 2.3†††
B6.IL-$10^{-/-}$→B6.CD45.1	+	89 ± 4	92	12 ± 1	2.4 ± 0.8	7.7 ± 3.1†††

[a] The experiment was performed as described in the Fig. 4 legend. The clinical data shown are the composite mean ± SD from two separate experiments (10–12 female mice/group for mixed bone marrow chimeras; 3–4 female mice/group for control chimeras). The statistical tests performed on the data were the χ^2 test (incidence) and the Student t test (disease onset, peak disease severity, and cumulative disease index). ###, $p < 0.001$ for comparisons between the placebo and 1,25-$(OH)_2D_3$-treated B6.CD45.1→B6 chimeras. *, $p < 0.05$ and the ***, $p < 0.01$ for comparisons between the B6.CD45.1→B6 and the B6.IL-$10^{-/-}$→B6.IL-$10^{-/-}$ chimeras ingesting identical diets. †††, $p < 0.01$ for comparisons between the B6.CD45.1→B6.IL-$10^{-/-}$ and the B6.IL-$10^{-/-}$→B6.CD45.1 chimeras ingesting identical diets.

[b] Splenocytes were harvested at the end of the study, stained with fluorescent Abs to CD3 and to CD45.1 or CD45.2, and analyzed on the flow cytometer.

[c] Mice with a clinical score ≥1 were considered to have EAE.

[d] The cumulative disease index for each mouse is the sum of the mouse's daily clinical scores for 7 consecutive days beginning with the day the first signs appeared. This method of calculating the cumulative disease index was used because the experiments were done at different times, and there was interexperiment variability in the day of onset. The mixed bone marrow chimeric mice were followed for only 15 days postpriming because they became moribund, requiring euthanasia according to the animal protocol. The data shown are the mean ± SD for each group.

disease. As expected, the 1,25-$(OH)_2D_3$-treated B6.CD45.1→B6 mice had a lower incidence of EAE, a later onset, a lower peak clinical score, and a lower cumulative disease index than the placebo-treated B6.CD45.1→B6 mice (Fig. 4*A* and Table V). Also as expected, the 1,25-$(OH)_2D_3$-treated and placebo-treated B6.IL-$10^{-/-}$→B6.IL-$10^{-/-}$ mice did not differ in the incidence of EAE, the day of onset, the peak clinical score, or the cumulative disease index (Fig. 4*B* and Table V). These data confirm that a functional IL-10-IL-10R pathway is necessary for 1,25-$(OH)_2D_3$-mediated protection from EAE.

Most of the chimeric mice with limited *IL-10* gene expression developed such severe and rapidly progressive EAE disease that the experiment was terminated 15 days post immunization. The placebo-treated B6.IL-$10^{-/-}$→B6.CD45.1 chimeric mice had significantly more severe clinical EAE disease on days 13 and 14, and a higher cumulative EAE disease index on day 15, than the placebo-treated B6.CD45.1→B6.IL-$10^{-/-}$ chimeric mice. These data suggest that hemopoietic cell-produced IL-10 is especially critical for protection from EAE.

Surprisingly, the 1,25-$(OH)_2D_3$ did not inhibit EAE in B6.CD45.1→B6.IL-$10^{-/-}$ or B6.IL-$10^{-/-}$→B6.CD45.1 mice (Fig. 4, *C* and *D*; Table V). Thus, limiting IL-10 synthesis to either the radio-sensitive or the radio-resistant cells abrogated 1,25-$(OH)_2D_3$-mediated protection. These data suggest that IL-10 synthesis from both cell types is essential for optimal 1,25-$(OH)_2D_3$-mediated protection. In fact, there may be an anti-inflammatory amplification loop involving bidirectional IL-10 signaling.

Discussion

The present experiments investigated how vitamin D_3 and 1,25-$(OH)_2D_3$ inhibit EAE induction, in an effort to understand how this sunlight-derived hormone might mediate sunlight's protective effects in MS. The results supported the hypothesis that the protective mechanism requires a functional IL-10-IL-10R pathway. The vitamin D_3 and 1,25-$(OH)_2D_3$ inhibited MOG_{35-55}-induced EAE in B6 female mice, and 1,25-$(OH)_2D_3$ also inhibited EAE in male mice, consistent with our published data on MBP_{Ac1-11}-induced EAE in B10.PL female and male mice (9, 26). Importantly, our data show for the first time that vitamin D_3 and 1,25-$(OH)_2D_3$ failed to inhibit EAE in B6 mice with targeted disruptions of either the *IL-10* or the *IL-10R* genes. Extending this finding, we also show that 1,25-$(OH)_2D_3$ failed to inhibit EAE in reciprocal BM chimeric mice with restricted *IL-10* gene expression. These studies

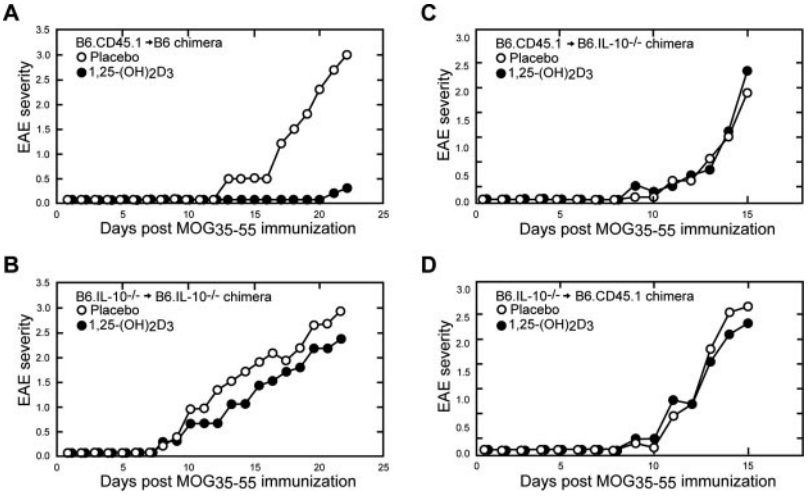

FIGURE 4. Radiosensitive hemopoietic cells and radioresistant brain parenchymal cells must express IL-10 for 1,25$(OH)_2D_3$-mediated inhibition of EAE. Female B6.IL-$10^{-/-}$ or B6.CD45.1 BM was transferred into irradiated B6.CD45.1 or B6.IL-$10^{-/-}$ female mice to produce four chimeras: B6.CD45.1→B6 (*A*); B6.IL-$10^{-/-}$→B6.IL-$10^{-/-}$ (*B*); B6.CD45.1→B6.IL-$10^{-/-}$ (*C*); and B6.IL-$10^{-/-}$→B6.CD45.1 (*D*). The chimeric mice were fed synthetic diets containing 0 (○) or 50 ng/day (●) 1,25$(OH)_2D_3$ for 5 days. Eight weeks post-transplantation, EAE was induced with MOG_{35-55} peptide, synthetic diet feeding was continued, and disease severity was scored daily. The cumulative mean from two separate experiments is shown (10–12 mice/group). On days 13 and 14, the B6.IL-$10^{-/-}$→B6.CD45.1 mice had significantly more severe EAE than the B6.CD45.1→B6.IL-$10^{-/-}$ mice, regardless of the diet group ($p < 0.05$; Mann-Whitney U test). Other differences were not significant.

suggest that both the radio-sensitive hemopoietic cells and the radio-resistant brain parenchymal cells must contribute IL-10 for optimal 1,25-$(OH)_2D_3$-mediated protection. Others reported that adding 1,25-$(OH)_2D_3$ and dexamethasone to cultures of D011.10 transgenic T cells with APC and peptide increased the frequency of IL-10-producing, regulatory T cells (12). However, our data showed no 1,25-$(OH)_2D_3$-induced increases in Foxp3 transcripts as a marker of regulatory T cells in the CNS. It is possible that the 1,25-$(OH)_2D_3$ stimulated IL-10-producing regulatory cells that do not express Foxp3. Our data also did not show an effect of 1,25-$(OH)_2D_3$ on IL-10 or IL-10R expression in freshly explanted spinal cord cells. However, these data do not rule out an effect on regulatory lymphocytes, astrocytes, or neurons that would have comprised $<5\%$ of the cell suspension.

Several caveats apply to our interpretation of the present data. First, we may have missed minor IL-10-independent protective effects of the 1,25-$(OH)_2D_3$, if the major protective activities were IL-10 dependent and determined the experimental results. We may also have missed IL-10-independent protective effects occurring at a later stage of EAE than was studied here. With respect to the chimera studies, because chimerism was 85–91%, a small percentage of the hemopoietic cells in the B6.IL-$10^{-/-}$→B6.CD45.1 chimeric mice had the Wt *IL-10* gene. Furthermore, because a minor percentage of microglial cells in irradiation BM chimeras derived from hemopoietic stem cells (45, 46), some brain parenchymal cells in the B6.CD45.1→B6.IL-$10^{-/-}$ chimeric mice would likely have had the Wt *IL-10* gene. Nevertheless, in both cases, the IL-10 producers were insufficient to enable 1,25-$(OH)_2D_3$-mediated protection, further supporting the concept of a bidirectional IL-10 amplification loop.

To envision how an 1,25-$(OH)_2D_3$-activated, *IL-10*- and *IL-10R*-dependent, *Rag-1*-dependent protective mechanism could limit encephalitogenic $CD4^+$ Th1 cells in the CNS, it is important to consider which CNS cells produce IL-10, and how IL-10 protects the CNS during inflammation. The major IL-10-producing brain parenchymal cells are the microglial cells and astrocytes (44, 47). The IL-10 promotes neuron and glial cell survival by blocking apoptotic signaling. More specifically, IL-10R stimulation regulates the Jak1/Stat3, PI3-kinase, MAP-kinase, suppressor of cytokine signaling (SOCS), and NF-κB life- and death-signaling pathways, ultimately inhibiting both proapoptotic cytokine-induced and mitochondrial-induced apoptosis in neurons and glial cells. The IL-10 also blocks proinflammatory cytokine induction and signaling. Specifically, IL-10R-mediated activation of SOCS reduces proinflammatory cytokine synthesis and cytokine receptor expression and activation. Finally, IL-10 inhibits costimulation of brain-infiltrating T cells via the CD28-CD80/86 pathway, thereby favoring anergy induction.

A model explaining how a 1,25-$(OH)_2D_3$-activated, *IL-10*- and *IL-10R*-dependent, *Rag-1*-dependent protective mechanism could limit encephalitogenic $CD4^+$ Th1 cell activation in the CNS must also take into account which cells express the vitamin D receptor (VDR) (48). Myeloid cells, lymphoid cells, astrocytes, microglial cells, neurons, and oligodendrocytes all express the VDR (reviewed in (49). The T cells and type 2 astrocytes increase their VDR expression when activated, whereas the myeloid lineage cells diminish their VDR expression when activated (49). Our present and previous data rule out a direct effect of 1,25-$(OH)_2D_3$ on APCs and on naive encephalitogenic $CD4^+$ T cells in the absence of additional *Rag-1*-dependent lymphocytes in the EAE model (10). Therefore, it is most likely that the 1,25-$(OH)_2D_3$ acted directly on regulatory lymphocytes and/or on CNS-resident cells like astrocytes, neurons, and/or microglia. The requirement for *IL-10* gene expression in both hemopoietic cells and brain parenchymal cells suggests that 1,25-$(OH)_2D_3$ may be acting on both types of cells. The model that is most consistent with our data is bi-directional IL-10 signaling. The 1,25-$(OH)_2D_3$ could act directly on regulatory lymphocytes to increase their IL-10-dependent suppressive functions, and the regulatory lymphocyte-produced IL-10 could act on the brain parenchymal cells, altering their function such that they stimulate potentially encephalitogenic, autoreactive T cells to apoptose or to become anergic. To complete the bi-directional IL-10-signaling, the 1,25-$(OH)_2D_3$ could also be acting directly on the brain parenchymal cells to increase their IL-10-dependent suppressive functions. Further experimentation will be required to test this model. In particular, it will be important to demonstrate that there are direct effects of the 1,25-$(OH)_2D_3$ on regulatory lymphocytes and on brain parenchymal cells like neurons, astrocytes, and microglia that result in an immunosuppressive environment in the CNS.

Given the importance of IL-10 for the protection of the CNS during inflammation (44), it is not surprising that IL-10 deficiency correlates strongly with susceptibility to EAE and MS. The IL-10-deficient mice were highly susceptible to EAE (18, 19). Similarly, IL-10 deficiency appears to be an MS risk factor in humans. The IL-10-secreting T cell frequency was lower in MS patients than controls (50). Also, MS patients had less serum IL-10 protein (51), blood cell IL-10 mRNA (52–54), and blood cell IL-10 protein (55) than controls. Finally, the *IL-10* allele with the low expresser promoter polymorphism was more common in MS patients than controls (56, 57). These correlations suggest a possible cause and effect relationship between IL-10 deficiency and EAE in mice and MS in humans.

Our data showing that IL-10 deficiency in mice eliminated the protective effects of 1,25-$(OH)_2D_3$ in EAE has very important implications for MS. Specifically, our data suggest that IL-10 deficiency in humans may diminish the protective effects of sunlight and vitamin D_3 in MS-susceptible individuals. There may be synergy between a genetic MS risk factor, IL-10 deficiency, and an environmental MS risk factor, insufficient vitamin D_3 synthesis due to inadequate sunlight exposure, increasing the risk of MS by compromising regulatory lymphocyte functions. If this hypothesis is correct, then it will be important to correct the IL-10 deficiency in MS-susceptible individuals for sunlight and vitamin D_3 prevention and treatment strategies to be beneficial.

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Disclosures

The authors have no financial conflict of interest.

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