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Modulatory Effects of 1,25-Dihydroxyvitamin D₃ on Human B Cell Differentiation

Sheng Chen,*† Gary P. Sims,* Xiao Xiang Chen,† Yue Ying Gu,‡ Shunle Chen,† and Peter E. Lipsky2*‡

1,25-Dihydroxyvitamin D₃ (1,25(OH)₂D₃) can modulate immune responses, but whether it directly affects B cell function is unknown. Patients with systemic lupus erythematosus, especially those with antinuclear Abs and increased disease activity, had decreased 1,25(OH)₂D₃ levels, suggesting that vitamin D might play a role in regulating autoantibody production. To address this, we examined the effects of 1,25(OH)₂D₃ on B cell responses and found that it inhibited the ongoing proliferation of activated B cells and induced their apoptosis, whereas initial cell division was unimpeded. The generation of plasma cells and postswitch memory B cells was significantly inhibited by 1,25(OH)₂D₃, although the up-regulation of genetic programs related to; XBP1, X box-binding protein.

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1,25-Dihydroxyvitamin D₃ (1,25(OH)₂D₃) is the biologically active form of vitamin D₃, which was originally described as an essential hormone for bone and mineral homeostasis, has also been shown to have immunomodulatory effects. Vitamin D₃ can be derived from dietary sources, but the main source of vitamin D₃ is obtained through photosynthesis in the skin. Exposure of skin to UV light (270–300 nm) catalyzes the first step in vitamin D₃ biosynthesis, converting 7-dehydrocholesterol to previtamin D₃, which is followed by a spontaneous and temperature-dependent isomerization into vitamin D₃. To obtain the biologically active metabolite, 1,25(OH)₂D₃, vitamin D₃ must first be hydroxylated by D₃-25-hydroxylase (CYP2D25) in the liver into 25-hydroxyvitamin D₃ (25(OH)D₃). Further hydroxylation of 25(OH)D₃ by 25(OH)D₃-1α-hydroxylase (CYP27B1) occurs mainly in the proximal convoluted tubule cells of the kidney, resulting in the biologically active 1,25(OH)₂D₃ (2). In addition, CYP27B1 is expressed by activated macrophages and dendritic cells (3, 4), but their contribution to systemic vitamin D metabolism is unknown. 1,25(OH)₂D₃ also induces the expression of the major 1,25(OH)₂D₃-degrading enzyme 24-hydroxylase (CYP24A1) (2). Most of the known biological effects of 1,25(OH)₂D₃ are mediated through the vitamin D receptor (VDR), a member of the superfamily of nuclear hormone receptors (5) that is expressed by different cells of the immune system and elsewhere (5, 6).

The immunoregulatory effects of 1,25(OH)₂D₃ are mainly thought to be mediated through its action on APCs, with the most potent reported effects on dendritic cells (DCs) (7, 8). The in vitro differentiation of DCs from monocytes or murine bone marrow-derived precursors is inhibited by 1,25(OH)₂D₃. Moreover, the Ag-presenting function of monocytes and DCs is profoundly inhibited, as is the surface expression of costimulatory molecules and also IL-12 production (9). In addition, expression of the immunosuppressive cytokine IL-10 by DCs, which opposes the Th1-inducing effects of IL-12, is increased by 1,25(OH)₂D₃ (7, 8).

Direct effects of 1,25(OH)₂D₃ on T lymphocytes have also been reported. T cell proliferation and cell cycle progression from G₁a to G₁b are inhibited in vitro by 1,25(OH)₂D₃. Cytokine production by T cells is also modulated, with inhibition of the Th1 cytokine IFN-γ and an increase of the Th2 cytokines IL-4, IL-5, and IL-10 (10). Thus, 1,25(OH)₂D₃ is thought to polarize activated T cells toward a Th2 phenotype. In addition, it has been shown that expression of the Fas ligand by activated T cells could be repressed by 1,25(OH)₂D₃ (11).

The effects of 1,25(OH)₂D₃ on B cell function has not been examined in detail. Published reports have yielded conflicting observations. It has been claimed that 1,25(OH)₂D₃ may directly inhibit B cell function as a consequence of the impairment of CD4 T cell responses or the inhibition of cytokine production by macrophages/microphages (12). Alternatively, a direct inhibitory effect of 1,25(OH)₂D₃ on IgE production by human B cells has also been reported (13).

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*Abbreviations used in this paper: 1,25(OH)₂D₃, 1,25-dihydroxyvitamin D₃; AID, activation-induced cytidine deaminase; ANA, antinuclear Ab; 2-M, β₂-microglobulin; BCL6, B cell lymphoma 6; BLIMP1, B lymphocyte-induced maturation protein 1; DC, dendritic cell; ERN1, endoplasmic reticulum to nucleus signaling; IRF4, IFN regulatory factor 4; PI, propidium iodide; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; SLE-DAL, SLE disease activity index; VDR, vitamin D receptor; XBP1, X box-binding protein.

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Interestingly, decreased 1,25(OH)2D3 serum concentrations have been reported in many autoimmune diseases, including systemic lupus erythematosus (SLE) (14, 15). SLE is an autoimmune disease characterized by immune dysregulation resulting in overproduction of autoantibodies (16). Although the exact cause of SLE remains unknown, recent studies, including a demonstration of the effectiveness of therapeutic B cell depletion, strongly implicate B cells as central players in the pathogenesis of this disease (17–20). We therefore hypothesized that 1,25(OH)2D3 may be important in maintaining B cell homeostasis and that deficiency of 1,25(OH)2D3 might contribute to B cell hyperactivity in SLE.

In this study, we measured 1,25(OH)2D3 and 25(OH)D3 levels in patients with SLE and correlated them with disease activity and the presence of antinuclear Abs (ANA). Subsequently, we investigated the direct effects of 1,25(OH)2D3 on primary human B cells to gain more insight into the potential role of vitamin D on autoimmune disease pathogenesis. Our results provide evidence that vitamin D might be a useful alternative therapy for the B cell hyperactivity characteristic of SLE.

**Materials and Methods**

**Clinical samples**

For the analysis of serological levels of 25(OH)D and 1,25(OH)2D3, peripheral blood samples from patients with SLE, patients with rheumatoid arthritis (RA), and demographically comparable healthy controls were obtained from the Shanghai Clinical Center for Rheumatology, Renji Hospital, Shanghai, China after informed consent. The diagnosis of SLE relied on American College of Rheumatology revised criteria (21). Clinical charts of all patients were reviewed and disease activity was scored according to the SLE disease activity index (SLEDAI) (22). For the analysis of B cell function in vitro, blood samples from normal healthy adult donors were obtained from the Warren G. Magnuson Clinical Center Blood Bank (Bethesda, MD). The collection of samples was approved by the National Institute of Arthritis and Musculoskeletal and Skin Diseases/National Institute of Diabetes & Digestive & Kidney Diseases (NIAMS/NIDDK) Institutional Review Board (Bethesda, MD), and informed consent was obtained according to the Declaration of Helsinki.

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

Serum total 25(OH)D and 1,25(OH)2D3 levels were measured in SLE patients with RA, and healthy donors by RIA or ELISA (both from ALPCO Diagnostics) according to manufacturer’s instructions. Both assays yielded similar results.

**B cell enrichment, flow cytometry, and cell sorting**

B cells were enriched by negative selection from buffy coats or leukapheresis samples using RosetteSep or StemSep B cell purification Ab mixtures (StemCell Technologies). Enriched B cells were >90 and 95% pure, respectively. Purified B cells were stained with various mAb combinations for 20 min on ice in staining buffer (1% BSA and 5% FCS in PBS). The directly conjugated mAb used were anti-IgD-FITC, anti-CD27-PE, anti-CD40-PE, anti-CD86-FITC, anti-IgG-PE, anti-CD19-allophycocyanin, anti-CD38-allophycocyanin, anti-HLA-DR-PE, (BD Pharmingen), anti-CD19-PerCpCy5.5, and anti-CD38-allophycocyanin (clone HB7) (BD Immunocytometry). Stained cells were resuspended with medium containing 10% Ultra Low IgG FCS (Invitrogen-Life Technologies). Fifty nanograms of isolated RNA was added per reaction with Taqman, 50 ng/ml IL-4 (R&D Systems), and 1,25(OH)2D3 (usually 10 nM) or 25(OH)D3 (usually from Sigma-Aldrich).

**Proliferation assay**

Cells were cultured for 3, 4, or 5 days as described above and pulsed for an additional 16 h with [3H]thymidine (1 μCi, 37 kBq) and then harvested. The [3H]thymidine incorporation was measured with a Top Count microplate scintillation counter (Packard Instruments). Alternatively, proliferation was assessed by CFSE (Cell Sciences), 50 ng/ml IL-21 (Cell Sciences), 50 ng/ml IL-4 (R&D Systems), and 1,25(OH)2D3 (usually 10 nM) or 25(OH)D3 (usually from Sigma-Aldrich).

**ELISPOT assay**

The number of IgG- and IgM-secreting cells was determined with the ELISPOT assay. MultiScreen HTS plates (polyvinylidene difluoride membrane; Millipore) were coated overnight at 4°C with 50 μg/ml diluted in 0.05 M carbonate PBS (Sigma-Aldrich). After washing, unbound surfaces of wells were blocked with culture medium for 1 h at 25°C. Stimulated cells from 7-day cultures were washed three times and resuspended with medium containing 10% Ultra Low IgG FCS (Invitrogen Life Technologies). Serial dilutions of cells were added to wells in triplicate and incubated at 37°C overnight. Medium containing 10% Ultra Low IgG FCS was included in all experiments as a negative control. Thereafter, plates were washed with TBS plus 0.05% Tween 20 four times and incubated with either biotinylated goat-anti-human IgG or IgM (Bethyl Laboratories) with Sigma Fast pNPP alkaline phosphate substrate (Sigma-Aldrich).

**RNA preparation and quantitative RT-PCR**

Total RNA from B cells was purified by using the RNeasy mini kit (Qiagen) according to the manufacturer’s manual. Reverse transcription reactions were prepared using the SuperScript one-step PCR system with platinum Taq polymerase and ROX reference dye (Invitrogen Life Technologies). Fifty nanograms of isolated RNA was added per reaction with 1.2 nM MgSO4. TaqMan Assays-on-Demand gene expression primers were synthesized targeting the gene(s) of interest.

**Table I. Characteristics of the patients with SLE**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>SLE Patients (n = 112)</th>
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<tbody>
<tr>
<td>Age in years, mean ± SEM (range in years)</td>
<td>33.8 ± 1.57 (15–66)</td>
</tr>
<tr>
<td>Female (%)</td>
<td>90.2% (101/112)</td>
</tr>
<tr>
<td>Disease duration in years, mean ± SEM (range in years)</td>
<td>4.5 ± 0.5 (0.01–20)</td>
</tr>
<tr>
<td>SLEDAI score, mean ± SEM (range)</td>
<td>6.5 ± 0.9 (0–22)</td>
</tr>
<tr>
<td>Arthritis percentage (no./total)</td>
<td>31.3% (35/112)</td>
</tr>
<tr>
<td>Rash percentage (no./total)</td>
<td>23.2% (26/112)</td>
</tr>
<tr>
<td>Oral ulceration percentage (no./total)</td>
<td>17.9% (20/112)</td>
</tr>
<tr>
<td>Renal involvement percentage (no./total)</td>
<td>37.5% (42/112)</td>
</tr>
<tr>
<td>Vasculitis percentage (no./total)</td>
<td>7.1% (8/112)</td>
</tr>
<tr>
<td>Hematological abnormalities percentage (no./total)</td>
<td>25% (28/112)</td>
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<tr>
<td>CNS involvement percentage (no./total)</td>
<td>3.6% (4/112)</td>
</tr>
<tr>
<td>Glucocorticoid use, mean ± SEM mg/day (range)</td>
<td>23.2 ± 2.9 (0–160)</td>
</tr>
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**Cell apoptosis and viability assay**

To quantify apoptosis, cultured B cells were double-stained with annexin V-FITC conjugate and propidium iodide (PI) using TACS annexin V kits from Oncogene according to the manufacturer’s directions. The stained B cells were immediately analyzed using a FACS Calibur flow cytometer (BD Biosciences).

**B cell stimulation**

B cells were cultured in 96-well plates at 1 × 106 cells/ml in 100-μl volumes of culture medium (10% FCS in RPMI 1640 medium with l-glutamine and penicillin-streptomycin) alone or supplemented with various cytokines and stimuli: 1 μg/ml anti-CD40 (R&D Systems), 3 μg/ml goat F(ab’)2 anti-IgM (Jackson ImmunoResearch Laboratories), 250 ng/ml IL-21 (Cell Sciences), 50 ng/ml IL-4 (R&D Systems), and 1,25(OH)2D3 (usually 10 nM) or 25(OH)D3 (usually from Sigma-Aldrich).
Probe sets for β2-microglobulin (Hs99999907_m1), VDR (Hs01045840_m1), CYP27B1 (Hs00168017_m1), CYP24A1 (Hs00167999_m1), Pax5 (paired box gene 5; Hs00277134_m1), Bcl6 (B-cell CLL/lymphoma 6, zinc finger protein 51; Hs00153368_m1), BLIMP1 (B-lymphocyte-induced maturation protein 1; Hs00153357_m1), IFN regulatory factor 4 (IRF4; Hs00180031_m1), X box-binding protein 1 (XBP1; Hs00231936_m1), endoplasmic reticulum to nucleus signaling 1 (ERN1; Hs00176385_m1), activation-induced cytidine deaminase (AID; Hs00221068_m1), p27 (cyclin-dependent kinase (CDK) inhibitor 1B; Hs00153277_m1), p21 (CDK inhibitor 1A; Hs00355782_m1), and p18 (CDK inhibitor 2C; Hs00176227_m1) were purchased from Applied Biosystems, and quantitative RT-PCR was performed according to the manufacturer’s instructions. mRNA expression for each gene was calculated in triplicate using the comparative cycle threshold method with efficiency calculations, and all mRNA levels were normalized to H9252 2M.

The effects of 1,25(OH)2D3 on the expression of 84 cell cycle regulated genes and 84 genes associated with NF-kB-mediated signal transduction were examined using human cell cycle RT2 Profiler PCR array and human NFkB signaling pathway RT2 Profiler PCR array, respectively (SuperArray Bioscience) according to the manufacturer’s instructions. In brief, cDNA was prepared from 1 μg total RNA by using a RT 2 PCR array first strand kit. A total volume of 25 μl of PCR mixture, which included 12.5 μl of RT2 Real-Time SYBR Green/ROX PCR master mix from SuperArray Bioscience (containing HotStart DNA polymerase, SYBR Green dye, and the ROX reference dye), 11.5 μl of double-distilled H2O, and 1 μl of template cDNA, was loaded in each well of the PCR array. PCR amplification was conducted with an initial 10-min step at 95°C followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The fluorescent signal from SYBR Green was detected immediately after the extension step of each cycle, and the cycle at which the product was first detectable was recorded as the cycle threshold. Data were imported into an Excel database and analyzed using the comparative cycle threshold method with normalization of the raw data to housekeeping genes including H9252 2M, hypoxanthine phosphoribosyltransferase 1, ribosomal protein L13a, GAPDH, and ACTB (β-actin).

**FIGURE 1.** Patients with SLE have decreased 25(OH)D and 1,25(OH)2D3 levels. Serum levels of 25(OH)D and 1,25(OH)2D3 were measured by RIA and ELISA. A, 25(OH)D levels in SLE patients (n = 57) compared with normal controls (NC; n = 28) and RA patients (n = 29). Mean levels in SLE patients were significantly different from those in NC and RA patients (p < 0.001). B, 25(OH)D levels in patients with established SLE with a mean duration since diagnosis of 5.11 ± 0.88 years (n = 45) compared with newly diagnosed untreated patients with a mean duration of 25.8 ± 9.2 days (n = 12). There was no significant difference in mean 25(OH)D levels in newly diagnosed or established SLE patients (p > 0.05). C, 1,25(OH)2D3 levels in SLE patients (n = 87) compared with normal controls (NC) (n = 30). The 1,25(OH)2D3 levels were significantly lower in SLE patients (p < 0.001). D, 1,25(OH)2D3 levels in nonactive SLE patients (SLEDAI ≤ 4, n = 35) compared with active patients (SLEDAI > 4, n = 52). Active SLE patients had significantly lower levels of 1,25(OH)2D3 (p < 0.001). E, 1,25(OH)2D3 levels in SLE patients with positive ANA (n = 71) compared with those without ANA (n = 11). Patients without ANA had significantly higher levels of 1,25(OH)2D3 (p < 0.05). F, 1,25(OH)2D3 levels in SLE patients with anti-dsDNA (n = 39) compared with those without anti-dsDNA (n = 43). 1,25(OH)2D3 levels did not differ between these groups. Data are shown as mean ± SEM, and significant differences between the groups are shown (***, p < 0.001; **, p < 0.01; *, p < 0.05).
SLE patients have significantly diminished levels of 25(OH)D and 1,25(OH)2D₃

A total of 112 SLE patients who fulfilled American College of Rheumatology revised criteria (21) were assessed. Their characteristics are shown in Table I. The serum 25(OH)D level was significantly lower in SLE patients (11.5 ± 1.5 ng/ml, n = 57) than in demographically comparable healthy controls (59.2 ± 6.5 ng/ml, n = 28) and RA patients (54.6 ± 5.2 ng/ml, n = 29) (p < 0.001) (Fig. 1A). Notably, the level of 25(OH)D in newly diagnosed SLE patients without any treatment was 11.6 ± 2.1 ng/ml (n = 12), which was not statistically different from that in SLE patients with established disease (11.8 ± 1.8 ng/ml, n = 45) (p > 0.05) (Fig. 1B). No correlation was found between the level of 25(OH)D and various clinical parameters including renal involvement, disease activity assessed by SLEDAI (22), and glucocorticoid use. The serum level of 1,25(OH)₂D₃ in SLE patients (14.5 ± 1.2 pg/ml, n = 87) was also significantly lower than in normal controls (29.8 ± 1.5 pg/ml, n = 30, p < 0.001) (Fig. 1C). 1,25(OH)₂D₃ levels were significantly lower in patients with active SLE as indicated by a SLEDAI score of >4 (12.2 ± 1.6 pg/ml, n = 52) compared with those with a SLEDAI score of ≤4 (19.4 ± 1.9 pg/ml, n = 36, p < 0.001) (Fig. 1D). Additional analysis demonstrated that the level of 1,25(OH)₂D₃ was significantly lower in patients with ANA compared with those without ANA (14.4 ± 1.3 pg/ml, n = 71 vs 22.0 ± 4.2 pg/ml, n = 11, p < 0.05), but no significant difference was observed in patients with or without anti-dsDNA (15.0 ± 1.8 pg/ml, n = 39 vs 13.5 ± 1.7 pg/ml, n = 43) (p > 0.05) (Fig. 1, E and F). The data indicated that patients with SLE had decreased levels of 25(OH)D and 1,25(OH)₂D₃, and the latter was related to disease activity and ANA production. These results prompted us to examine the impact of vitamin D on B cell function.

1,25(OH)₂D₃ inhibits proliferation of activated B cells

Initially, the effect of 1,25(OH)₂D₃ on B cell survival was examined. 1,25(OH)₂D₃ did not affect the survival of unstimulated B cells in culture (Fig. 2A). We next assessed the effect of 1,25(OH)₂D₃ on B cell proliferation. As shown in Fig. 2B, 1,25(OH)₂D₃ exerted a significant inhibitory effect on B cell proliferation, especially in cultures activated with a combination of stimuli, e.g., anti-IgM/anti-CD40 (p < 0.05), anti-CD40/IL-21, or anti-IgM/anti-CD40/IL-21 (p < 0.01). B cells stimulated with either anti-CD40 or anti-IgM alone had a limited proliferative capacity and, as a consequence, the effect of 1,25(OH)₂D₃ was less consistent.

CFSE labeling was also used to examine the effect of 1,25(OH)₂D₃ on the proliferation of B cells. After 3 days in culture some CFSE dilution was observed. Notably, cultures with and without 1,25(OH)₂D₃ exhibited a similar frequency of cells with modestly diluted CFSE (Fig. 2C). As the culture period was prolonged, cells with increasing dilution of CFSE were observed. However, the extent of CFSE dilution was strikingly diminished in 1,25(OH)₂D₃-containing cultures compared with control cultures. These results were consistent with the conclusion that
1,25(OH)2D3 had little effect on initial cell division, whereas ongoing proliferation of stimulated B cells was suppressed.

**1,25(OH)2D3 induces apoptosis of activated B cells**

To determine whether the inhibitory effect of 1,25(OH)2D3 on proliferation was associated with the induction of apoptosis, we examined the percentage of apoptotic cells by annexin V/PI staining. During 4 days in culture without stimulation 1,25(OH)2D3 treatment had no significant effect on survival or apoptosis (Fig. 3A). Stimulation with anti-IgM/anti-CD40 decreased the frequency of apoptotic cells, but in cultures of activated B cells, 1,25(OH)2D3 significantly increased the percentage of early apoptotic cells at day 4 (27.5% vs 37.5%, p < 0.01). This was time dependent, as this difference was not apparent at earlier time points (Fig. 3B).

VDR and 24-hydroxylase can be induced by 1,25(OH)2D3 on B cells

We next examined the expression of the VDR and the known vitamin D responsive gene 24-hydroxylase (CYP24A1) by quantitative PCR. Freshly isolated B cells constitutively expressed very low levels of VDR mRNA. Compared with day 0 expression, the mean fold increase of VDR expression by B cells after 3 days of culture with anti-IgM/anti-CD40, anti-CD40/IL-21, anti-IgM/anti-CD40/IL-21, or without stimulation was 1.45, 2.88, 4.44, and 1.46-fold respectively. 1,25(OH)2D3 increased VDR expression in both activated and unstimulated B cells (Fig. 4A), but not earlier than day 3 (data not shown). In contrast, CYP24A1 expression was not detectable in either resting or activated B cells. However, it

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**FIGURE 3.** Vitamin D (VitD) induces apoptosis of activated human peripheral B cells. Purified B cells were cultured with no stimulus (nil), anti-IgM (aIgM), and anti-CD40 (aCD40) in the presence (VitD) or absence (Nil) of 1,25(OH)2D3 (10 nM). A. After 4 days in culture, cells were washed and stained with annexin V and PI and analyzed by flow cytometry. The numbers in the upper left quadrants represent the percentage of apoptotic cells in culture. B. Percentages of apoptotic cells in 3- and 4-day cultures with no stimulus (nil) or anti-IgM (aIgM) and anti-CD40 (aCD40) and with or without the addition of 1,25(OH)2D3 (VitD; 10 nM). Data are mean ± SEM of triplicate determinations from one of three independent experiments. Asterisks indicate the statistical significance of the difference between vitamin D and untreated B cells (**, p < 0.01).

**FIGURE 4.** Vitamin D (VitD)-inducible genes are up-regulated on activated B cells. Purified B cells were stimulated in various conditions in the presence (VitD) or absence (nil) of 1,25(OH)2D3 (10 nM) as indicated. Total RNA was extracted from cells at day 0 and after 3 days in culture. Gene expression was detected by quantitative RT-PCR in triplicate. A. Expression of VDR relative to β2M. Left panel shows results from stimulated vs fresh B cells, and the right panel shows results from B cells culture without stimulation (aIgM, anti-IgM; aCD40, anti-CD40). B. Expression of CYP24A1 relative to β2M. Left panel shows results from stimulated vs fresh B cells, and the right panel shows results from B cells cultured without stimulation. Data are the mean ± SEM from triplicates from one of two representative experiments. Significant differences between vitamin D (VitD) and control (nil) responses are shown (*, p < 0.05; **, p < 0.01; ***, p < 0.001).
was significantly induced by 1,25(OH)\(_2\)D\(_3\) (Fig. 4B). The results demonstrate that the VDR is expressed and inducible in primary B cells. Activation signals or 1,25(OH)\(_2\)D\(_3\) up-regulated VDR expression, whereas CYP24A1 was only induced by 1,25(OH)\(_2\)D\(_3\) but not by stimulation.

**1,25(OH)\(_2\)D\(_3\) specifically increases CD38 expression on B cells**

It was previously reported that 1,25(OH)\(_2\)D\(_3\) could increase CD38 expression by lymphocytes (23, 24). To confirm this and to examine the effect of 1,25(OH)\(_2\)D\(_3\) on other B cell markers, we activated purified B cells with various stimuli with and without 1,25(OH)\(_2\)D\(_3\), and then stained the cells for various surface markers. The expression of CD38 on activated B cells was significantly increased by 1,25(OH)\(_2\)D\(_3\). This difference was not observed with unstimulated B cells or cultures activated with anti-CD40 or anti-IgM alone, but it was apparent in B cells stimulated with the combination of anti-IgM/anti-CD40, anti-CD40/IL-21, or anti-IgM/anti-CD40/IL-21. In contrast, the expressions of IgD, CD21, CD23, CD27, CD28, CD86, and HLA-DR were altered by activating stimuli but not further affected by 1,25(OH)\(_2\)D\(_3\) (Fig. 5A). Similarly, 1,25(OH)\(_2\)D\(_3\) had no effect on the expression of CD19 or CD20 (data not shown). To examine the expression of CD40, we used anti-IgM and IL-4 to activate B cells and up-regulate CD40 expression. Using these conditions, 1,25(OH)\(_2\)D\(_3\) again increased CD38 expression (data not shown), but CD40 expression was not altered by 1,25(OH)\(_2\)D\(_3\). These results show that 1,25(OH)\(_2\)D\(_3\) specifically increases CD38 expression by activated B cells but has no effect on the expression of a variety of other lineage, differentiation, or activation markers.

Increased expression of CD38 is commonly used as a phenotypic marker of plasma cells (25). To determine whether the 1,25(OH)\(_2\)D\(_3\)-mediated increase in CD38 expression was associated with plasma cell differentiation, we used a method of in vitro stimulation known to induce plasma cells from peripheral B cells (anti-CD40 and IL-21 with or without anti-IgM) and examined the effect of 1,25(OH)\(_2\)D\(_3\) (26). The induction of CD38 resulting from 1,25(OH)\(_2\)D\(_3\) was not associated with an increase in CD27 expression and, indeed, fewer CD38\(^{\text{high}}\)/CD27\(^{\text{high}}\) plasma cells were generated in the presence of 1,25(OH)\(_2\)D\(_3\) (Fig. 5B). Both the increase of CD38 expression and the decrease of plasma cell generation occurred in a concentration-dependent manner, with the maximum effect noted reproducibly at 10 nM or less (Fig. 5C).

1,25(OH)\(_2\)D\(_3\) inhibits plasma cell differentiation and Ig secretion

A more detailed analysis showed that 1,25(OH)\(_2\)D\(_3\) inhibited plasma cell differentiation and the secretion of Ig (Fig. 6). The generation of plasma cells was inhibited throughout the culture period after day 5 and 1,25(OH)\(_2\)D\(_3\) caused significant decreases in
both the percentage (Fig. 6A, left panel) and absolute number of plasma cells (data not shown). In multiple experiments, the generation of phenotypically defined CD19<sup>+</sup> CD27<sup>high</sup>CD38<sup>high</sup> plasma cells was inhibited by 23–34% (Fig. 6A, right panel). Similar results were noted when B cells purified by cell sorting and 98% pure were examined (data not shown).

Analysis of culture supernatants revealed that 1,25(OH)<sub>2</sub>D<sub>3</sub> also significantly decreased the secretion of Ig. Stimulation with anti-CD40 and IL-21 with or without anti-IgM induced considerable Ig secretion by day 5 that increased through day 9 (Fig. 6B). 1,25(OH)<sub>2</sub>D<sub>3</sub> significantly inhibited IgG secretion. The percent inhibition progressively increased as the cultures were prolonged with the maximal inhibition noted at day 9 (p < 0.001). A significant albeit smaller reduction in IgM production in 1,25(OH)<sub>2</sub>D<sub>3</sub> cultures was only apparent at day 9. The effect of 1,25(OH)<sub>2</sub>D<sub>3</sub> on the number of Ig-secreting cells generated in these cultures was determined by ELISPOT. Data are the mean ± SEM from triplicate determinations and are representative of three independent experiments. For further details, see Methods. A significant reduction in IgG-secreting cells was observed as early as day 8 (p < 0.05).

Analysis of culture supernatants revealed that 1,25(OH)<sub>2</sub>D<sub>3</sub> also significantly decreased the secretion of Ig. Stimulation with anti-CD40 and IL-21 with or without anti-IgM induced considerable Ig secretion by day 5 that increased through day 9 (Fig. 6B). 1,25(OH)<sub>2</sub>D<sub>3</sub> significantly inhibited IgG secretion. The percent inhibition progressively increased as the cultures were prolonged with the maximal inhibition noted at day 9 (p < 0.001). A significant albeit smaller reduction in IgM production in 1,25(OH)<sub>2</sub>D<sub>3</sub> cultures was only apparent at day 9. The effect of 1,25(OH)<sub>2</sub>D<sub>3</sub> on the number of Ig-secreting cells generated in these cultures was determined by ELISPOT. Data are the mean ± SEM from triplicate determinations and are representative of three independent experiments. For further details, see Methods. A significant reduction in IgG-secreting cells was observed as early as day 8 (p < 0.05).
Notably, when 1,25(OH)$_2$D$_3$ was added at day 5 of culture, no effect on the maintenance of plasma cells or on Ig secretion was noted over the next 5 days (Fig. 6D). This result indicates that 1,25(OH)$_2$D$_3$ inhibits the generation of plasma cells but not their subsequent persistence or Ig secretion.

1,25(OH)$_2$D$_3$ inhibits the generation of postswitched memory B cells from naive B cells

To determine whether the inhibitory effect of 1,25(OH)$_2$D$_3$ was specific for plasma cell generation, we also examined its role in regulating the generation of postswitched memory B cells. To address this, naive B cells (CD19$^+$ IgG$^-$/CD27$^+$) were purified by sorting (>99% purity) as shown in Fig. 7A and stimulated in a manner known to induce class switch recombination (26). As shown in Fig. 7B, the combination of anti-CD40 and IL-21 with or without anti-IgM was able to induce postswitched cells expressing surface IgG. In the absence of IL-21, few cells expressed surface IgG. Notably, the frequency of IgG positive cells was significantly lower in the 1,25(OH)$_2$D$_3$-containing cultures (Fig. 7, B and C), whereas there were more B cells expressing surface IgM (Fig. 7B). This reduction in class switching correlated with reduced plasma cell numbers and IgG production in parallel 7-day cultures (Fig. 7D). These data demonstrate that 1,25(OH)$_2$D$_3$ inhibits generation of both postswitch memory cells and plasma cells.

1,25(OH)$_2$D$_3$ down-regulates the expression of XBP1 and ERN1 mRNA but not that of PAX5, B cell lymphoma 6 (BCL6), AID, B lymphocyte-induced maturation protein 1 (BLIMP1), MTA3, and IRF4

Thus far, the results clearly demonstrated that 1,25(OH)$_2$D$_3$ reduced B cell proliferation, the generation of postswitched memory B cells, plasma cell differentiation, and Ig secretion. However, the stage at which 1,25(OH)$_2$D$_3$ exerts its effects remained unclear. To gain further insight into the nature of B cell inhibition by 1,25(OH)$_2$D$_3$, we examined the expression of key transcription factors involved in B cell maturation and plasma cell differentiation. We compared the expression of genes involved in germinal center reactions, including PAX5, BCL6, and AID as well as those
involved in plasma cell differentiation, including BLIMP1, IRF4, MTA3, XBP1, and ERN1 (27). All of the analyses were conducted at day 3 before significant effects on the frequency of memory B cells and plasma cells or Ig secretion were evident. Interestingly, 1,25(OH)_{2}D_{3} had no significant effect on the expression of PAX5, BCL6, AID, BLIMP1, MAT3, or IRF4. However, even at this relatively early time point the expressions of XBP1 and ERN1 were significantly reduced but not eliminated by 1,25(OH)_{2}D_{3} (Fig. 8). The lack of a consistent effect of 1,25(OH)_{2}D_{3} on the genetic programs regulating the differentiation of memory cells or plasma cells suggests that it may inhibit their maturation by other means.

1,25(OH)_{2}D_{3} increases the expression of p27 mRNA but not that of p21 and p18

To determine the mechanism involved in the inhibitory effect of 1,25(OH)_{2}D_{3} on B cells, we initially screened an array of NF-κB-mediated signal transduction pathway genes. As shown in Table II, a few mRNAs were up- or down-regulated by 1,25(OH)_{2}D_{3}, but no general pattern of gene regulation was observed consistent with a major effect on the NF-κB signaling pathway. We next examined the effect of 1,25(OH)_{2}D_{3} on cell cycle-related gene expression by B cells (shown in Table III). We found a number of genes, including cyclin D1, cyclin D2, cyclin T1, cyclin T2, CDK4, and CDK6, were significantly down-regulated by 1,25(OH)_{2}D_{3}. In contrast, the CDK inhibitor p27 was significantly up-regulated by 1,25(OH)_{2}D_{3}, whereas most genes, including CDK inhibitor p21,

Table II. Effect of 1,25(OH)_{2}D_{3} on NF-κB regulatory gene expression

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Mean of Fold Change</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiotensinogen (serpin peptidase inhibitor,</td>
<td>0.55</td>
<td>0.09</td>
</tr>
<tr>
<td>clade A, member 8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>v-sal murine thymoma viral oncogene homolog 1</td>
<td>1.10</td>
<td>0.34</td>
</tr>
<tr>
<td>Activating transcription factor 1</td>
<td>1.19</td>
<td>0.11</td>
</tr>
<tr>
<td>B cell CLL/Lymphoma 10</td>
<td>1.16</td>
<td>0.11</td>
</tr>
<tr>
<td>B cell CLL/Lymphoma 3</td>
<td>2.12</td>
<td>0.16</td>
</tr>
<tr>
<td>B factor, properdin</td>
<td>0.68</td>
<td>0.05</td>
</tr>
<tr>
<td>Baculoviral IAP repeat-containing 2</td>
<td>0.73</td>
<td>0.12</td>
</tr>
<tr>
<td>Caspase recruitment domain family, member 4</td>
<td>0.79</td>
<td>0.07</td>
</tr>
<tr>
<td>Caspase 1, apoptosis-related cysteine peptidase</td>
<td>0.85</td>
<td>0.29</td>
</tr>
<tr>
<td>Caspase 8, apoptosis-related cysteine peptidase</td>
<td>0.87</td>
<td>0.35</td>
</tr>
<tr>
<td>Chemokine (C-C motif) ligand 2</td>
<td>0.09</td>
<td>0.12</td>
</tr>
<tr>
<td>CD40 antigen (TNF receptor superfamily member 5)</td>
<td>0.85</td>
<td>0.20</td>
</tr>
<tr>
<td>CASP8 and FADD-like apoptosis regulator</td>
<td>1.29</td>
<td>0.12</td>
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<tr>
<td>Conserved helix-loop-helix ubiquitous kinase</td>
<td>1.08</td>
<td>0.42</td>
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<tr>
<td>Colony-stimulating factor 2 (granulocyte-</td>
<td>0.51</td>
<td>0.27</td>
</tr>
<tr>
<td>macrophage)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colony stimulating factor 3 (granulocyte)</td>
<td>0.63</td>
<td>0.16</td>
</tr>
<tr>
<td>Solute carrier family 44, member 2</td>
<td>1.45</td>
<td>0.00</td>
</tr>
<tr>
<td>EDAR-associated death domain</td>
<td>0.48</td>
<td>0.04</td>
</tr>
<tr>
<td>Endothelial differentiation, lymphophasphatic</td>
<td>0.89</td>
<td>0.39</td>
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<tr>
<td>acid G protein coupled receptor, 2</td>
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<td></td>
</tr>
<tr>
<td>Early growth response 1</td>
<td>1.37</td>
<td>0.22</td>
</tr>
<tr>
<td>ELK1, member of ETS oncogene family</td>
<td>0.88</td>
<td>0.30</td>
</tr>
<tr>
<td>Coagulation factor II (thrombin) receptor</td>
<td>0.59</td>
<td>0.10</td>
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<tr>
<td>Fas (TNFRSF6)-associated via death domain</td>
<td>0.88</td>
<td>0.25</td>
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<tr>
<td>Fas ligand (TNF superfamily, member 6)</td>
<td>0.84</td>
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</tr>
<tr>
<td>v-fos FBJ murine osteosarcoma viral oncogene</td>
<td>0.83</td>
<td>0.32</td>
</tr>
<tr>
<td>homolog</td>
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<td></td>
</tr>
<tr>
<td>Gap junction protein, α, 43 kDa (connexin 43)</td>
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<td>0.20</td>
</tr>
<tr>
<td>Heme oxygenase (decycling) 1</td>
<td>1.36</td>
<td>0.21</td>
</tr>
<tr>
<td>3-Hydroxytryptamine (serotonin) receptor 2B</td>
<td>0.64</td>
<td>0.04</td>
</tr>
<tr>
<td>Interleukin cell adhesion molecule 1 (CD54),</td>
<td>0.91</td>
<td>0.40</td>
</tr>
<tr>
<td>human rhinovirus receptor</td>
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<td></td>
</tr>
<tr>
<td>Interferon, α</td>
<td>0.61</td>
<td>0.16</td>
</tr>
<tr>
<td>Interferon, β, fibroblast</td>
<td>0.55</td>
<td>0.14</td>
</tr>
<tr>
<td>Interferon, γ</td>
<td>0.80</td>
<td>0.39</td>
</tr>
<tr>
<td>Inhibitor of κ light polypeptide gene enhancer</td>
<td>1.11</td>
<td>0.39</td>
</tr>
<tr>
<td>in B cells, kinase β</td>
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<td></td>
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<tr>
<td>Inhibitor of κ light polypeptide gene enhancer</td>
<td>0.86</td>
<td>0.33</td>
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<tr>
<td>in B cells, kinase e</td>
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<tr>
<td>Inhibitor of κ light polypeptide gene enhancer</td>
<td>0.81</td>
<td>0.04</td>
</tr>
<tr>
<td>in B cells, kinase γ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interleukin 10</td>
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<td>0.27</td>
</tr>
<tr>
<td>Interleukin 1, α</td>
<td>0.70</td>
<td>0.01</td>
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<tr>
<td>Interleukin 1, β</td>
<td>0.38</td>
<td>0.05</td>
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<tr>
<td>Interleukin 1 receptor, type I</td>
<td>0.71</td>
<td>0.14</td>
</tr>
<tr>
<td>Interleukin 6 (interferon, β2)</td>
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<td>0.01</td>
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<tr>
<td>Interleukin 8</td>
<td>0.76</td>
<td>0.22</td>
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<tr>
<td>Interleukin-1 receptor-associated kinase 1</td>
<td>0.66</td>
<td>0.19</td>
</tr>
<tr>
<td>Interleukin-1 receptor-associated kinase 2</td>
<td>3.09</td>
<td>0.09</td>
</tr>
<tr>
<td>v-jun sarcoma virus 17 oncogene homolog (avian)</td>
<td>0.93</td>
<td>0.36</td>
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<tr>
<td>Lymphotxin α (TNF superfamily, member 1)</td>
<td>0.60</td>
<td>0.15</td>
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<tr>
<td>Lymphotxin β receptor (TNFR superfamily,</td>
<td>0.96</td>
<td>0.47</td>
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<tr>
<td>member 3)</td>
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<tr>
<td>Mucosa-associated lymphoid tissue lymphoma</td>
<td>0.88</td>
<td>0.24</td>
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<tr>
<td>translocation gene 1</td>
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<td></td>
</tr>
<tr>
<td>Mitogen-activated protein kinase kinase 1</td>
<td>1.25</td>
<td>0.23</td>
</tr>
<tr>
<td>Myeloid differentiation primary response gene</td>
<td>1.01</td>
<td>0.48</td>
</tr>
<tr>
<td>(88)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NACHT, leucine rich repeat and PYD containing</td>
<td>0.26</td>
<td>0.19</td>
</tr>
<tr>
<td>12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nuclear factor of κ light polypeptide gene</td>
<td>0.95</td>
<td>0.40</td>
</tr>
<tr>
<td>enhancer in B-cells 1 (p105)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nuclear factor of κ light polypeptide gene</td>
<td>1.00</td>
<td>0.50</td>
</tr>
<tr>
<td>enhancer in B-cells 2 (p49/p100)</td>
<td></td>
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</tbody>
</table>

(Table continues)
remained unchanged. We verified these findings using quantitative PCR and found that p27, but not p21 or p18 mRNA, was significantly up-regulated by 1,25(OH)2D3 in activated B cells (Fig. 9).

### B cells can directly metabolize 25(OH)D3 into a biologically active molecule

It has been shown that the precursor of 1,25(OH)2D3, 25(OH)D3, is present in serum and is converted to the active form by 1α-hydroxylase (CYP27B1), which is mainly expressed in kidney and also can be found in monocytes/macrophages (3, 4). The expression of 1α-hydroxylase (CYP27B1) mRNA by human peripheral B cells was therefore investigated by RT-PCR. As shown in Fig. 10A, B cells constitutively expressed 1α-hydroxylase mRNA, and it was up-regulated following stimulation but not further induced by 1,25(OH)2D3. To determine whether this up-regulation had a
plasma cell generation and IgG production in a concentration-de-
through binding to the VDR. We clearly demonstrate that VDR
1,25(OH)2D3 that can inhibit B cell function.

Functional consequence, we added the precursor, 25(OH)D3, to
purified B cells stimulated with anti-IgM, anti-CD40, and IL-21 with (VitD) or without (nil) 1,25(OH)2D3 (10 nM) for 3 days. The mRNA expressions of p27, p21, and p18 were determined by SuperArray and/or quantitative PCR. A. The re-

duction. Reduced levels of 1,25(OH)2D3 in SLE patients, partic-
ularly in those patients with high disease activity scores and
ANAs, suggest that vitamin D-dependent B cell regulation may
might be required for the antiproliferative effect of the occupied
the VDR and suggests that a threshold level of VDR engagement
is thought to be the case for macrophages and dendritic cells (3).
In conditions such as SLE, in which there is diffuse B cell activation (20), systemic vitamin D metabolism might therefore be significantly influenced.

The capacity of 1,25(OH)2D3 to inhibit proliferation has been
reported in lymphocytes and in a variety of human cancer cell lines
(31). We showed that 1,25(OH)2D3 also exerted an inhibitory ef-
ficacy, and also the functional activity of 1,25(OH)2D3 clearly show that
the VDR is expressed by human peripheral B cells. Moreover, our
data clearly demonstrate that VDR expression by B cells is regu-
ulated both by 1,25(OH)2D3, as it is on other cells (5), but also by
activation signals. These results indicate that vitamin D may exert
differential effects on activated vs resting B cells and also may
have different effects in individuals with different levels of serum
1,25(OH)2D3.

Active 1,25(OH)2D3 is mainly degraded by 24-hydroxylase
(CYP24A1) (2). Microarray analysis from human colon carcinoma
and ovarian cancer cell lines revealed that CYP24A1 was the most
inducible gene responsive to 1,25(OH)2D3 (29, 30). In this report
we show that 24-hydroxylase was significantly up-regulated fol-
lowing the incubation of human B cells with 1,25(OH)2D3. As
opposed to the VDR, CYP24A1 was not altered by B cell activa-
tion. These results further demonstrate that human B cells can
respond to 1,25(OH)2D3 directly. In addition, the results suggest
that the activity of vitamin D on B cells might be influenced not
only by VDR expression but also by the capacity to degrade the
active molecule. The increased susceptibility of activated B cells
to many of the effects of vitamin D might therefore reflect the up-
regulation of VDR but not CYP24A1 by these cells.

25(OH)D3-1-α-hydroxylase (CYP27B1), the enzyme responsible
for the final hydroxylation and activation of 25(OH)D3 into
1,25(OH)2D3, is mainly found in the proximal convoluted tubule
cells of the kidney (2). Interestingly, we found that CYP27B1
mRNA was also expressed by resting B cells and could be further
induced by stimulation, but not by 1,25(OH)2D3. Moreover, we
found that the precursor, 25(OH)D3, had similar effects on purified
B cells compared with the active form, albeit at higher concentra-
tions. Therefore, 25(OH)D3 might be metabolized to 1,25(OH)2D3
by B cells themselves and may represent a source for the extrarenal
synthesis of 1,25(OH)2D3 as is thought to be the case for macro-
phages and dendritic cells (3). In conditions such as SLE, in
which there is diffuse B cell activation (20), systemic vitamin D
metabolism might therefore be significantly influenced.

Discussion

In this study we show that 1,25(OH)2D3 has potent direct effects
on B cell responses, inhibiting proliferation, generation of class-
switched memory B cells, plasma cell differentiation, and Ig pro-
duction. Reduced levels of 1,25(OH)2D3 in SLE patients, partic-
ularly in those patients with high disease activity scores and
ANAs, suggest that vitamin D-dependent B cell regulation may
play an important role in maintaining normal B cell homeostasis
and that decreased levels of 1,25(OH)2D3 may contribute to B cell
hyperactivity in SLE. Finally, because activated B cells express
many of the molecules involved in vitamin D metabolism, their
characteristic hyperactivity in SLE could contribute to the de-
creased levels of 1,25(OH)2D3 found in these patients.

The varied functions of 1,25(OH)2D3 are mainly mediated
through binding to the VDR. We clearly demonstrate that VDR
mRNA is constitutively expressed in human primary B cells at low
levels and is up-regulated following stimulation and enhanced in
the presence of 1,25(OH)2D3 in a time-dependent manner. Previ-
ously, the expression of VDR on B cells has been controversial. It
has been claimed that resting B cells do not contain detectable
amounts of VDR (13). However, it has also been reported that
VDR is constitutively expressed on human tonsil B cells and can
be further up-regulated by activation (13, 24) and that VDR is
expressed on the B cell lymphoma cell lines SUDHL4 and
SUDHL5 (28). Our data analyzing the expression of VDR mRNA
and the functional activity of 1,25(OH)2D3 clearly show that
the VDR is expressed by human peripheral B cells. Moreover, our
data clearly demonstrate that VDR expression by B cells is regu-
ulated both by 1,25(OH)2D3, as it is on other cells (5), but also by
activation signals. These results indicate that vitamin D may exert
differential effects on activated vs resting B cells and also may
have different effects in individuals with different levels of serum
1,25(OH)2D3.

In this study we show that 1,25(OH)2D3 has potent direct effects
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switched memory B cells, plasma cell differentiation, and Ig pro-
duction. Reduced levels of 1,25(OH)2D3 in SLE patients, partic-
ularly in those patients with high disease activity scores and
ANAs, suggest that vitamin D-dependent B cell regulation may
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and that decreased levels of 1,25(OH)2D3 may contribute to B cell
hyperactivity in SLE. Finally, because activated B cells express
many of the molecules involved in vitamin D metabolism, their
characteristic hyperactivity in SLE could contribute to the de-
creased levels of 1,25(OH)2D3 found in these patients.

The varied functions of 1,25(OH)2D3 are mainly mediated
through binding to the VDR. We clearly demonstrate that VDR

FIGURE 9. The effect of vitamin D (VitD) on cell cycle-regulated
genes. RNAs were extracted from freshly isolated B cells and B cells
 cultured with anti-IgM, anti-CD40, and IL-21 with (VitD) or without (nil)
1,25(OH)2D3 (10 nM) for 3 days. The mRNA expressions of p27, p21, and
p18 were determined by SuperArray and/or quantitative PCR. A. The re-
elative expression of p21 and p27 determined by SuperArray. Data represent
the mean ± SEM of three independent experiments. B. The relative ex-
pression of p21, p27, and p18 as determined by quantitative PCR. Data
represent the mean ± SEM of three independent experiments. Significant
differences between vitamin D-treated and control B cells are shown
(*, p < 0.05).
shown that plasma cell differentiation requires initial B cell proliferation (32) and increases with the number of cell divisions (33). We have previously shown that the combination of IL-21 and anti-CD40 stimulation with or without BCR crosslinking is a potent inducer of proliferation and plasma cell differentiation and that all of the plasma cells generated had undergone extensive proliferation (26). Using this system, we demonstrated that 1,25(OH)2D3 had inhibitory effects on plasma differentiation and Ig production. Interestingly, this effect is not evident when the B cells are treated with 1,25(OH)2D3 after 5 days in culture, indicating that 1,25(OH)2D3 inhibits the generation of plasma cells but not their subsequent persistence and lends support to the conclusion that the inhibition of B cell proliferation by 1,25(OH)2D3 is responsible for the reduction of Ig-secreting cells and Ig production.

Several transcription factors regulate different stages of B cell maturation and plasma cell differentiation (34). The most notable finding of the current study was that expression of most of these transcriptional regulators was unaffected by 1,25(OH)2D3, including PAX5, BCL6, AID, BLIMP1, MTA3, and IRF4. These results provide no support for the possibility that 1,25(OH)2D3 inhibits memory cell or plasma cell differentiation by directly affecting the expression of any of these regulators of B cell maturation. However, the expression of XBP1 was modestly but significantly down-regulated by 1,25(OH)2D3. In addition, mRNA for ERN1, which is required for processing XBP1 mRNA to a spliced form that encodes a more stable and active protein (35), was also down-regulated by 1,25(OH)2D3. The inhibitory effect of 1,25(OH)2D3 on the expressions of XBP1 and ERN1 mRNA levels may explain the greater inhibitory effect of vitamin D on Ig secretion and detection of plasma cells by ELISPOT compared with its more modest inhibition of the differentiation of phenotypically defined plasma cells. Although inhibition of the expressions of XBP1 and ERN1 mRNA by 1,25(OH)2D3 may contribute to a reduced amount of Ig secretion per phenotypically defined plasma cell, it is unlikely to explain the inhibition of the generation of both plasma cells and postswitched memory cells as well as the ongoing proliferation of activated B cells by vitamin D.

Several important intracellular pathways have been reported to be inhibited by 1,25(OH)2D3, such as NF-κB activation and cell cycle progression. The suppressive effect of 1,25(OH)2D3 on the NF-κB signaling pathway was previously observed in T cells, monocytes or macrophages (9, 36, 37), and could have affected the expression of various essential cell surface and secreted molecules. The NF-κB pathway, however, did not appear to be generally suppressed by 1,25(OH)2D3 in activated B cells as assessed by the analysis of expression of various genes influenced by this signaling pathway. Because 1,25(OH)2D3 directly inhibited the proliferation of activated B cells, we therefore examined whether vitamin D might suppress the expression of a variety of cell cycle regulators and found that 1,25(OH)2D3 increased the expression of p27 and

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**FIGURE 10.** B cells can convert inactive 25(OH)D3 into the active form of vitamin D. A, CYP27B1 is constitutively expressed by fresh B cells and is induced by stimulation. B cells were analyzed either immediately after isolation or were incubated for 3 days with the indicated stimuli with (1,25(OH)2D3) or without (nil) 1,25(OH)2D3 (10 nM). The expression of CYP27B1 was determined by RT-PCR relative to β2M mRNA (×107). The means ± SEM of one of three independent experiments are shown. Significant differences between vitamin D-treated and control B cells are shown (*, p < 0.05). Significant differences between day 0 and stimulated B cells are shown (**, p < 0.01) (αlgM, anti-IgM; αCD40, anti-CD40). B, Purified B cells were stimulated with anti-CD40 and IL-21 with either 10 nM 1,25(OH)2D3 or different concentrations of 25(OH)D3 ranging from 0 to 1000 nM. After 7 days in culture, the cells were washed and stained for expression of CD19, CD27, and CD38. The geometric (Geom.) mean fluorescence of CD38 and the frequency of plasma cells (CD27highCD38high) were determined. The culture supernatants were analyzed for IgG production. The 25(OH)D3 dose response (solid line) is compared with 10 nM 1,25(OH)2D3 (dashed line).
decreased the mRNA levels of CDK4 and 6 and cyclin D. Cell cycle progression is under the control of CDK, the activity of which is dependent on the expression of specific CDK inhibitors. Previous studies have shown that 1,25(OH)_{2}D_{3} inhibits the proliferation of cancer cells by inducing gene transcription of the CDK inhibitors p21 and/or p27, which can inhibit cell cycle progression at the G_{1}/S transition, depending on the cell type (38–40). In vivo, 1,25(OH)_{2}D_{3} administration can effectively restore p27 accumulation in cancer cells as detected by immunohistochemistry and reduce the tumor burden (41). Several mechanisms have been reported to be involved, including increased p27 transcription mediated by VDR-Sp1 interactions with the p27 promoter (42), stabilization of the protein through VDR-induced reduction of CDK2 activity and Skp2 abundance (the main factors responsible for p27 degradation), or induction of PTEN, a phosphatase that dephosphorylates p27 (40). Other CDK inhibitors have been shown to play an essential role in B cell responses. In this regard, p18 has been shown to be required for B cells to terminate proliferation and differentiate into functional plasma cells (38, 43, 44). Previous studies have confirmed that p18 and p27 were involved in regulating different steps of B cell proliferation. Whereas p27 was thought to regulate cell cycle entry by forming a ternary complex with CDKs and cyclins, p18 was directly involved in induction of the G_{1} cell cycle arrest by blocking the association of CDK4 and CDK6 with cyclin D (44). Interestingly, our data demonstrated that the mRNA level of p27, but not of p21 or p18, was up-regulated by 1,25(OH)_{2}D_{3} in activated human B cells. These results suggest the possibility that 1,25(OH)_{2}D_{3} could inhibit B cell proliferation by up-regulating p27 and thereby inhibit the cell cycle entry of previously cycling B cells. Because 1,25(OH)_{2}D_{3} also decreased mRNA levels of CDK4 and CDK6 as well as cyclin D, the effect of p27 on ongoing B cell proliferation might be more profound. The 1,25(OH)_{2}D_{3}-mediated induction of p27 may limit ongoing B cell proliferation and thereby play an important role in the control of B cell responses. These findings suggest the possibility that the major effects of vitamin D on plasma cell and memory cell differentiation may result from the suppression of ongoing B cell proliferation, which is required before these differentiation steps can occur (32). Additional experiments will be necessary to test this hypothesis.

Decreased vitamin D concentrations have been reported in many autoimmune diseases such as multiple sclerosis, type 1 diabetes mellitus, RA, fibromyalgia, and SLE (14, 15, 45, 46). We therefore examined the serum levels of 25(OH)D and 1,25(OH)_{2}D_{3} in Chinese lupus patients. Consistent with some previous findings, both 25(OH)D and 1,25(OH)_{2}D_{3} levels were significantly lower in patients with SLE compared with healthy donors (14, 46). However, another study reported significantly lower 25(OH)D, but not 1,25(OH)_{2}D_{3} levels in SLE patients (47). The explanation for the differences in these studies is not clear but could relate to ethnicity or disease duration (46). Although previous studies suggested a possible relationship between depressed levels of 25(OH)D and the presence of lupus renal disease (46), we were able to demonstrate that lower levels of 1,25(OH)_{2}D_{3} significantly correlated with increased disease activity. Moreover, significantly decreased vitamin D levels were detected in 12 newly diagnosed patients before any treatment was administered, indicating that the deficiency may be present at the onset of lupus and possibly before. This could explain, in part, why African Americans who are often chronically vitamin D deficient (46, 48, 49) also have a higher risk and severity of SLE (50). It is known that SLE may be preceded by autoantibody production that may exist for many years before diagnosis (51), and it is possible that vitamin D deficiency may contribute to this. The current results are consistent with the conclusion that vitamin D is involved in maintaining normal B cell homeostasis and that vitamin D deficiency in SLE patients may contribute to B cell hyperactivity, the breakdown of B cell tolerance, and the generation of some autoantibodies.

Consistent with this, we also found that those SLE patients with positive ANA titers had lower levels of 1,25(OH)_{2}D_{3} than those without ANA. Interestingly, such a difference was not seen between the patients who were positive or negative for dsDNA Abs. The persistent and therapy-resistant presence of ANA in SLE even during the clinically quiescent phases has been explained by the production of these autoantibodies from long-lived plasma cells (52). In contrast, the presence of the dsDNA Ab is usually correlated with SLE activity and decreases after immunosuppressive or B cell-depletion therapy, suggesting that these autoantibodies originate from short-lived dividing plasmablasts (20, 53). Thus, 1,25(OH)_{2}D_{3} may play a role in regulating the generation of long-lived plasma cell and be less potent in suppressing the rapid production of autoantibodies from dividing plasmablasts. The kinetics of the in vitro inhibition of B cell responses by 1,25(OH)_{2}D_{3} is consistent with this possibility.

Besides the inhibitory effect on plasma cell differentiation and Ig production, our data also demonstrated that 1,25(OH)_{2}D_{3} exerted its inhibitory effects on memory B cell differentiation. Relative increases in memory cells and plasma cells are both characteristic B cell abnormalities in lupus (54). In contrast to naive B cells and plasma cells, memory B cells are not targeted by conventional immunosuppressive therapies and may contribute to disease flares (55). Thus, vitamin D therapy may offer a new opportunity to induce remission in SLE by preventing or decreasing the differentiation of memory cells. This possibility is supported by murine studies in which administration of 1,25(OH)_{2}D_{3} or its analog has been shown to decrease proteinuria or increase survival in a spontaneous mouse lupus model, MRL/lpr (56).

In conclusion, 1,25-dihydroxyvitamin D3 may play an important role in the maintenance of B cell homeostasis. Correction of vitamin D deficiency may be useful not only to prevent osteopenia but also in the suppression of B cell overactivity in autoimmune disorders such as SLE.

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Disclosures
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