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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*²

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 of activated B cells and induced their apoptosis, whereas initial cell division was unimpeded. The generation of plasma cells

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 G₁ to G₂/M 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 indirectly inhibit B cell function as a consequence of the impairment of CD4⁺ T cell responses or the inhibition of cytokine production by monocytes/macrophages (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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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 antibasal ANA (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 Warren G. Magnuson Clinical Center Blood Bank (Bethesda, MD). The collection of samples was approved by the National Institute of Diabetes and Digestive and Kidney Diseases (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 mixes (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-IdG-FITC, anti-CD27-PE, anti-CD40-PE, anti-CD86-FITC, anti-IgM/PE, anti-CD19-allophycocyanin, anti-HLA-DR-PE, (BD Pharmingen), anti-CD19-PerCpCy5.5, and anti-CD38-allophycocyanin (clone HB7) (BD Immunocytometry). Stained cells were counted using a CTL Series 3B analyzer (Cellular Technology). Ig-secreting cells appeared as blue spots, which were alkaline phosphatase substrate kit III (Vector Laboratories) after washing and bound Ig was detected using alkaline phosphatase-conjugated streptavidin (Sigma-Aldrich) in TBS plus an alkaline phosphate substrate (Sigma-Aldrich).

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 FACScalibur 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 μM 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 (both from Sigma-Aldrich).

Proliferation assay

B 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 (Invitrogen Life Technologies) dilution. Briefly, 1 × 107 B cells were incubated with 1 μM of CFSE for 15 min at room temperature and then cultured with an equal volume of 100% FCS for 2 min and washed. CFSE-labeled cells were cultured for 3 to 6 days as mentioned above and data were collected by FACS analysis.

Ig ELISA

Culture supernatants were incubated on goat antihuman IgG and IgM-coated (Bethyl Laboratories) Maxisorp plates (Nalge Nunc International) and bound Ig was detected using alkaline phosphatase-conjugated goat anti-human IgG or IgM (Bethyl Laboratories) with Sigma Fast pNPP alkaline phosphatase substrate (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 (Invitrogen) diluted 1/2000 in TBS plus 0.05% Tween 20 at 25°C for 3 h. Plates were then washed in TBS plus 0.05% Tween 20 and incubated with 0.5 μg/ml alkaline phosphatase-conjugated streptavidin (Sigma-Aldrich) in TBS plus 0.05% Tween 20 for 1 h at 25°C. All plates were developed with the alkaline phosphatase substrate kit III (Vector Laboratories) after washing in distilled water. Ig-secreting cells appeared as blue spots, which were counted using a CTL Series 3B analyzer (Cellular Technology).

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 mM MgSO4. TaqMan Assays-on-Demand gene expression primers/
Probe sets for 2-microglobulin (H9252), 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. 1,25(OH)2D3 levels in newly diagnosed or established SLE patients (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).
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)D3 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).

FIGURE 3. Vitamin D (VitD) induces apoptosis of activated human peripheral B cells. Purified B cells were cultured with no stimulus (nil), anti-IgM (αlgM), and anti-CD40 (αCD40) 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 (αlgM) and anti-CD40 (αCD40) 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).

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

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 (αlgM, anti-IgM; αCD40, 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, 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⁺CD27⁺CD38⁺ 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)₂D₃ 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)₂D₃ 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)₂D₃ cultures was only apparent at day 9. The effect of 1,25(OH)₂D₃ on the number of Ig-secreting cells generated in these cultures was determined by ELISPOT assay. In agreement with the phenotype and Ig production data, 1,25(OH)₂D₃ significantly reduced the number of IgG-secreting cells in cultures of activated B cells. The number of IgM secreting cells was also significantly decreased by 1,25(OH)₂D₃ in anti-CD40/IL-21 cultures (Fig. 6C).
Notably, when 1,25(OH)₂D₃ 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)₂D₃ inhibits the generation of plasma cells but not their subsequent persistence or Ig secretion.

1,25(OH)₂D₃ inhibits the generation of postswitched memory B cells from naive B cells

To determine whether the inhibitory effect of 1,25(OH)₂D₃ 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)₂D₃-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)₂D₃ inhibits generation of both postswitch memory cells and plasma cells.

1,25(OH)₂D₃ 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)₂D₃ 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)₂D₃ exerts its effects remained unclear. To gain further insight into the nature of B cell inhibition by 1,25(OH)₂D₃, 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)2D3 had no significant effect on the expression of PAX5, BCL6, AID, BLIMP1, IRF4, and ERN1. However, even at this relatively early time point the expressions of XBP1 and ERN1 were significantly reduced but not eliminated by 1,25(OH)2D3 (Fig. 8). The lack of a consistent effect of 1,25(OH)2D3 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)2D3 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)2D3 on B cells, we initially screened an array of NF-kB-mediated signal transduction pathway genes. As shown in Table II, a few mRNAs were up- or down-regulated by 1,25(OH)2D3, but no general pattern of gene regulation was observed consistent with a major effect on the NF-kB signaling pathway. We next examined the effect of 1,25(OH)2D3 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)2D3. In contrast, the CDK inhibitor p27 was significantly up-regulated by 1,25(OH)2D3, whereas most genes, including CDK inhibitor p21,
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

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**Table II. (Continued)**

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<td>Toll-like receptor 3</td>
<td>1.47</td>
<td>0.26</td>
</tr>
<tr>
<td>Toll-like receptor 4</td>
<td>1.04</td>
<td>0.46</td>
</tr>
<tr>
<td>Toll-like receptor 6</td>
<td>0.83</td>
<td>0.07</td>
</tr>
<tr>
<td>Toll-like receptor 7</td>
<td>0.65</td>
<td>0.01</td>
</tr>
<tr>
<td>Toll-like receptor 8</td>
<td>0.72</td>
<td>0.34</td>
</tr>
<tr>
<td>Toll-like receptor 9</td>
<td>0.69</td>
<td>0.22</td>
</tr>
<tr>
<td>Transmembrane emp24 protein transport domain containing 4</td>
<td>0.99</td>
<td>0.48</td>
</tr>
<tr>
<td>Tumor necrosis factor (TNF superfamily, member 2)</td>
<td>0.48</td>
<td>0.07</td>
</tr>
<tr>
<td>Tumor necrosis factor, α-induced protein 3</td>
<td>0.74</td>
<td>0.24</td>
</tr>
<tr>
<td>Tumor necrosis factor receptor superfamily, member 10a</td>
<td>0.56</td>
<td>0.05</td>
</tr>
<tr>
<td>Tumor necrosis factor receptor superfamily, member 10b</td>
<td>0.82</td>
<td>0.24</td>
</tr>
<tr>
<td>Tumor necrosis factor receptor superfamily, member 1A</td>
<td>0.53</td>
<td>0.01</td>
</tr>
<tr>
<td>Tumor necrosis factor receptor superfamily, member 7</td>
<td>0.95</td>
<td>0.44</td>
</tr>
<tr>
<td>Tumor necrosis factor (ligand) superfamily, member 10</td>
<td>1.16</td>
<td>0.28</td>
</tr>
<tr>
<td>Tumor necrosis factor (ligand) superfamily, member 14</td>
<td>1.82</td>
<td>0.23</td>
</tr>
<tr>
<td>TNFRSF1A-associated via death domain</td>
<td>1.28</td>
<td>0.10</td>
</tr>
<tr>
<td>Toll-like receptor adaptor molecule 1</td>
<td>1.39</td>
<td>0.10</td>
</tr>
</tbody>
</table>

**Table III. Effect of 1,25(OH)2D3 on cell cycle regulatory gene expression**

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Mean of Fold Change</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclin-dependent kinase 6</td>
<td>0.83</td>
<td>0.12</td>
</tr>
<tr>
<td>Cyclin-dependent kinase 7 (MO15 homolog, Xenopus laevis, cdk-activating kinase)</td>
<td>0.99</td>
<td>0.43</td>
</tr>
<tr>
<td>Cyclin-dependent kinase 8</td>
<td>0.79</td>
<td>0.13</td>
</tr>
<tr>
<td>Cyclin-dependent kinase inhibitor 1A (p21, Cip1)</td>
<td>0.74</td>
<td>0.08</td>
</tr>
<tr>
<td>Cyclin-dependent kinase inhibitor 1B (p27, Kip1)</td>
<td>2.44</td>
<td>0.03</td>
</tr>
<tr>
<td>Cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)</td>
<td>1.65</td>
<td>0.38</td>
</tr>
<tr>
<td>Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)</td>
<td>0.60</td>
<td>0.11</td>
</tr>
<tr>
<td>Cyclin-dependent kinase inhibitor 3 (CDK2-associated dual specificity phosphatase)</td>
<td>0.96</td>
<td>0.35</td>
</tr>
</tbody>
</table>

The Journal of Immunology
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. Previously, 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 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 regulated 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 following the incubation of human B cells with 1,25(OH)₂D₃. As opposed to the VDR, CYP24A1 was not altered by B cell activation. 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)D₂-1-α-hydroxylase (CYP27B1), the enzyme responsible for the final hydroxylation and activation of 25(OH)D₂ 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)D₂, had similar effects on purified B cells compared with the active form, albeit at higher concentrations. Therefore, 25(OH)D₂ 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 macrophages and dendritic cells (3, 4). 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 effect on B cell proliferation. By using CFSE analysis, initial B cell division was not affected by 1,25(OH)₂D₃ whereas subsequent ongoing proliferation was progressively inhibited. This effect coincides with activation and 1,25(OH)₂D₃-mediated up-regulation of the VDR and suggests that a threshold level of VDR engagement might be required for the antiproliferative effect of the occupied VDR to become apparent. Notably, 1,25(OH)₂D₃-mediated inhibition of proliferation was associated with apoptosis of the activated and dividing B cells. These results suggest that differentiative events that require the initial expansion of B cells might be eliminated as a result of the 1,25(OH)₂D₃-mediated death of proliferating B cells.

The inhibition of proliferation and the induction of apoptosis are likely to result in the significant reduction in plasma cell differentiation and Ig production that we observed. Previous studies have
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
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)2D3 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 G1/S transition, depending on the cell type (38–40). In vivo, 1,25(OH)2D3 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 increasing 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 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 G1 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)2D3 in activated human B cells. These results suggest the possibility that 1,25(OH)2D3 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)2D3 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)2D3-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)2D3 in Chinese lupus patients. Consistent with some previous findings, both 25(OH)D and 1,25(OH)2D3 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)2D3 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)2D3 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)2D3 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)2D3 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)2D3 is consistent with this possibility.

Besides the inhibitory effect on plasma cell differentiation and Ig production, our data also demonstrated that 1,25(OH)2D3 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)2D3 or its analog has been shown to decrease proteinuria or increase survival in a spontaneous mouse lupus model (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.

Acknowledgments
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Disclosures
The authors have no financial conflict of interest.

References


