MAJOR ARTICLE

Bimodal Influence of Vitamin D in Host Response to Systemic *Candida* Infection—Vitamin D Dose Matters

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Vitamin D level is linked to susceptibility to infections, but its relevance in candidemia is unknown. We aimed to investigate the in vivo sequelae of vitamin D_3 supplementation in systemic *Candida* infection. Implicating the role of vitamin D in *Candida* infections, we showed that candidemic patients had significantly lower 25-OHD concentrations. *Candida*-infected mice treated with low-dose $1,25(OH)_2D_3$ had reduced fungal burden and better survival relative to untreated mice. Conversely, higher $1,25(OH)_2D_3$ doses led to poor outcomes. Mechanistically, low-dose $1,25(OH)_2D_3$ induced proinflammatory immune responses. This was mediated through suppression of SOCS3 and induction of vitamin D receptor binding with the vitamin D–response elements in the promoter of the gene encoding interferon γ. These beneficial effects were negated with higher vitamin D_3 doses. While the antiinflammatory effects of vitamin D_3 are well described, we found that, conversely, lower doses conferred proinflammatory benefits in *Candida* infection. Our study highlights caution against extreme deviations of vitamin D levels during infections.

Keywords. $1,25(OH)_2D_3$; cytokine; suppressor of cytokine signaling (SOCS); interferon gamma.

Beyond its classical role in calcium and bone metabolism, vitamin D_3 is known to exert a protean influence on the immune system [1, 2]. One such effect described is the propensity of vitamin D_3 to skew host cytokine response away from a proinflammatory profile toward a T helper type 2 (Th2) and regulatory T-cell response [3–5]. It also limits the antigen-presenting capacity of macrophages and directs the differentiation of dendritic cells toward a more tolerogenic phenotype, inducing heightened interleukin 10 (IL-10) levels [6, 7]. With the innate and bridging adaptive immune system forming the critical frontline defense protecting the host against pathogens, it is compelling to speculate whether vitamin D may influence host susceptibility to infections. Candidemia is the fourth most common bloodstream infection (BSI) in hospitals worldwide [8]. The options of antifungal agents available are limited, and patients with *Candida* BSI and invasive candidiasis have mortality rates of up to 40% [9]. This is partly due to the host's inability to mount an effective immune response against the invading fungi [10].

We had earlier investigated the role of $1,25(OH)_2D_3$ on in vitro cytokine production induced by *Candida* stimulation of human peripheral blood mononuclear cells (PBMCs). We found that $1,25(OH)_2D_3$ skewed cytokine response toward an antiinflammatory profile

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with downregulation of interleukin 6, tumor necrosis factor α (TNF-α), and interferon γ (IFN-γ) [11]. However, the biological relevance of these effects was not known. Hence, we aim to investigate and validate the sequelae of the immunomodulatory properties of vitamin D_3 in vivo.

METHODS

Stimuli and Reagents

Candida albicans cells (SC5314) were grown overnight at 30°C in yeast extract–peptone–dextrose broth. Cells were washed twice in phosphate-buffered saline (PBS) and diluted to 5×10^6 microorganisms/mL. Heat-killed *C. albicans* blastoconidia were used at a concentration of 1×10^6 blastoconidia/mL. 1,25 $(OH)_2D_3$ was purchased from Fluka Biochemika, Sigma-Aldrich (St. Louis, Missouri), and dissolved in absolute ethanol. The pharmaceutical preparation of 1,25$(OH)_2D_3$, Calcijex (1 μg/mL ampoule), was from Abbott Laboratories (Quebec, Canada).

Mice

Eight weeks old Balb/c mice weighing 20–24 g were infected with 2.5×10^5 live *C. albicans* (SC5314) via tail vein injection. One hundred microliters of vitamin D_3 (Calcijex) was administered intraperitoneally from day 2 after infection at doses of 0.001 μg/kg, 0.01 μg/kg, 0.1 μg/kg, or 1 μg/kg for 3 days; 3-day administration of PBS served as a control. Weight and survival were charted daily, and the fungal burden in the kidneys was assessed 6 days after infection. The number of viable *Candida* organisms was determined by plating serial dilutions on Sabouraud dextrose agar plates, and the colony forming units (CFUs) were counted after incubation for 24 hours at 37°C. For the survival studies, following *Candida* infection (on day 0), the mice were treated from day 2 onward with the stipulated daily doses of Calcijex for either 3 days or continuously.

PBMC Isolation

Separation and stimulation of PBMCs from healthy volunteers were performed as previously described [12]. Blood specimens were collected into sodium heparin tubes (BD Vacutainer, Franklin Lakes, New Jersey) after informed consent was received. PBMCs were isolated by density centrifugation on Ficoll-Hypaque (GE Healthcare, Uppsala, Sweden). Cells were washed, counted, and adjusted to a concentration of 5×10^6 cells/mL in culture medium (Roswell Park Memorial Institute 1640 medium, Dutch modification, supplemented with gentamicin, L-glutamine, and sodium pyruvate). Stimulation assays were performed in 96-well round-bottomed plates, using 100 μL of PBMCs with *C. albicans* and the respective fractions to achieve a total volume of 200 μL/well. After incubation for 24 hours or 48 hours in a humidified atmosphere (5% CO_2) at 37°C, the supernatants were collected and stored at −20°C until further assay.

Vitamin D Level Measurement

Vitamin D levels were measured using deidentified serum specimens from patients with *Candida* BSI in our hospital against anonymized serum specimens from hospitalized and healthy subjects archived in our hospital's clinical laboratory. The Roche Elecsys vitamin D commercial assay was used to determine 25-hydroxyvitamin D levels in human serum, and the assay revealed that >95% of the vitamin D (25-OHD) measured in serum was vitamin D_3 [13]. This electrochemiluminescence-based immunoassay was read on a Cobas e411 analyzer.

Recruitment and Phagocytosis Assay

Neutrophil and macrophage recruitment were assessed following intraperitoneal injection of mice with 1×10^6 inactivated *Candida* microorganisms/mL at 4 hours and 72 hours, respectively. The peritoneal cells were extracted by injecting 4 mL of ice-cold PBS-heparin into the peritoneal cavity. After washing, cytospin of the sample extract, followed by enumeration of the cell types, was performed under microscopy. To ascertain phagocytosis and killing, peritoneal phagocytes as obtained were washed and counted in a counting chamber. The processes of phagocytosis and intracellular killing were studied in an adherent monolayer of phagocytes [14].

Histology

The mouse kidneys were studied by light microscopy with hematoxylin and eosin and Gomori methenamine silver staining after being fixed in 4% formalin, processed and embedded in paraffin, and sectioned at thicknesses of 3 μm. Sections were assessed via light microscopy for the presence of *Candida* and the locations in the kidney where the organisms were present (in the glomeruli, cortex, medulla, and pelvicalyceal regions).

Stimulation and Cytokine Measurement

Spleen cells (5×10^5 cells/mL) obtained from mice on day 6 of infection were stimulated with heat-killed *C. albicans* (1×10^6 organisms/mL) in 96-well microtiter plates at 37°C. After 24 or 48 hours, supernatant was collected, and cytokine measurements were made using an enzyme-linked immunosorbent assay according to the instructions of the manufacturer (eBioscience, San Diego, California). Detection limits were 20 pg/mL (for interleukin 1b [IL-1b], TNF-α, and IFN-γ) and 10 pg/mL (for interleukin 17 [IL-17] and IL-10).

RNA Isolation and Quantitative Polymerase Chain Reaction (PCR) Analysis

Splenocytes were harvested from mice pretreated with 3 days of vitamin D_3 (or PBS as control) while human PBMCs were preincubated with vitamin D_3 (or PBS as control) for 30 minutes. The effects of both low-dose (0.01 μg/mL) and high-dose (1 μg/mL) 1,25$(OH)_2D_3$ were studied. The cells were then stimulated with heat-inactivated *C. albicans* for 4 hours. RNA was extracted from the splenocytes and PBMCs, using 500 μL of

TRIzol reagent (Sigma, St. Louis, Missouri). Subsequently, 200 μL of chloroform and 500 μL of 2-propanol were used to separate the RNA from DNA and proteins. After washing with 75% ethanol, the dry RNA was dissolved in 50 μL of diethylpyrocarbonate water. To obtain complementary DNA, we reverse transcribed 1 μg of DNase-treated total RNA with oligo(dT) primers (0.01 μg/mL) in a reverse transcription PCR mixture with a total volume of 20 μL. Quantitative PCR was performed using the ABI Prism 7000 Thermocycler and SYBR Green. The primers we used are listed in the Supplementary Materials. Quantification of the PCR signals for each sample was performed by comparing the cycle threshold (Ct) values, in duplicate, for the gene of interest with the Ct values for B2M as a housekeeping gene. Mean relative messenger RNA expression was calculated using the comparative Ct method. Values are expressed as a ratio of fold increase to mRNA levels of unprimed cells.

Chromatin Immunoprecipitation (CHIP)

To investigate vitamin D receptor (VDR) binding to the IFN-γ promoter, PBMCs from healthy volunteers were or were not treated with vitamin D_3 for 30 minutes, followed by stimulation with *C. albicans* for 4 hours. Nuclear proteins were cross-linked to DNA with 1% formaldehyde and incubated at room temperature for 10 minutes on a rocking platform. Cross-linking was stopped by adding glycine to a final concentration of 0.2 M for 5 minutes. The cells were collected by centrifugation and washed twice with ice-cold PBS. The cell pellets were resuspended in 500 μL of sodium dodecyl sulfate lysis buffer, and the lysates were sonicated to yield DNA fragments of 200–500 bp. Cell debris was removed by centrifugation. Five percent of the sonicated chromatin was set aside for input control and the rest was divided equally into 2 tubes, one for anti-VDR antibody and the other for the control immunoglobulin G (IgG). The lysates were diluted 1:10 in ChIP dilution buffer. One microgram of anti-VDR antibody (catalog no. sc-1008; Santa Cruz, Dallas, Texas) was added to one of the tubes, and 1 μg of nonspecific IgG (catalog no. sc-2027; Santa Cruz) was added to the other, and the tubes were incubated overnight at 4°C on a rotating platform. The immunocomplexes were collected using Recombinant Protein A-Sepharose beads (Invitrogen, Frederick, Maryland). The beads were washed sequentially for 10 minutes at 4°C with rotation, using low-salt buffer, high-salt buffer, and LiCl wash buffer. The beads were finally washed twice with TE buffer, and the immune complexes were eluted using 300 μL of elution buffer at room temperature for 20 minutes with rotation. The immune complexes and the input samples were reverse cross-linked at 65°C overnight in the presence of proteinase K (Invitrogen). DNA was extracted with phenol chloroform extraction and ethanol precipitation.

Real-time PCR reactions were performed using Sybr Green qPCR master mix (Applied Biosystems, Warrington, United Kingdom) with genomic primers for the BED promoter regions [15] of the gene encoding IFN-γ (Supplementary Materials), using the ABI Prism 7000 apparatus. The results were normalized with respect to the input. Nonspecific IgG results were subtracted using the formula $2^{-\Delta Ct}*100_{(VDR\ ab)} - 2^{-\Delta Ct}*100_{(IgG)}$, where ΔCt is calculated as $Ct_{(ChIP\ DNA)} - Ct_{(Input)}$, and Ct is the cycle number.

Flow Cytometry

Splenocytes were harvested and washed twice with 1 × PBS supplemented with 0.2% (w/v) bovine serum albumin, stained with CD4-APC/CD25-PE fluorochromes, and fixed and permeabilized using the FoxP3 staining kit (catalog no. 320018; Biolegend, San Diego, California). The cells were then intracellularly stained using Foxp3-Alexa Fluor 488 or isotype control and were analyzed on the BD LSRII Flow Cytometer (BD Biosciences, San Jose, California). Data analysis was performed using FlowJo software (version 7.6.5; Tree Star, Ashland, Oregon).

Statistics

Results were pooled from at least 3 sets of experiments (unless otherwise stated) and are presented as means ± standard error of the mean (SEM). Statistical analysis was performed using the Mann–Whitney *U* test or the paired Wilcoxon signed rank test, when relevant. *P* values of <.05 were considered statistically significant.

Ethics Statement

Institutional review board approval (from the Domain Specific Review Boards, National Healthcare Group, Singapore) was obtained to perform the studies involving human subjects. Ethics approval for conduct of the animal study had been attained through the Biological Resource Centre Institutional Animal Care and Use Committee (Singapore).

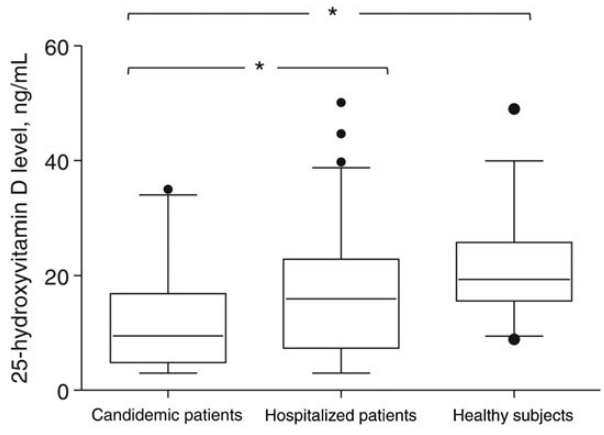

Figure 1. Sera 25-hydroxyvitamin D levels in candidemic patients, hospitalized patients without candidemia, and healthy subjects. *P < .05 for comparisons between the indicated groups.

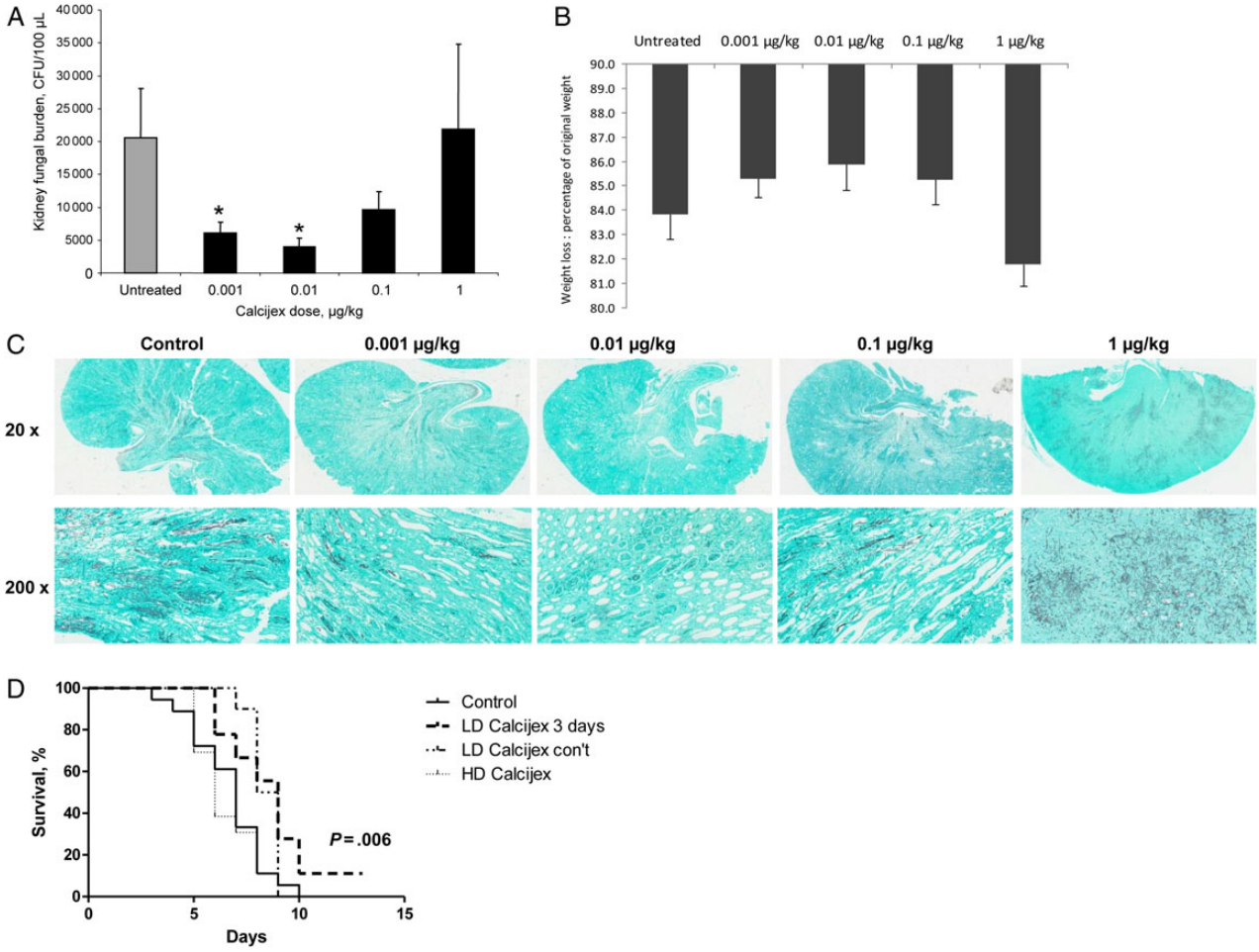

Figure 2. *A*, Kidney fungal burden in *Candida*-infected mice treated with 0.001–1 µg/kg of Calcijex and untreated mice. *B*, Trending of weight loss in *Candida*-infected mice treated with 0.001–1 µg/kg of Calcijex and untreated mice (administered an equivolume of phosphate-buffered saline [PBS]). Each experiment consisted of 5 mice per condition. The experiment was repeated 3 times, and the results were pooled. *C*, Histological sections of kidneys from *Candida*-infected mice treated with 0.001–1 µg/kg of Calcijex and from untreated mice, stained with Gomori methenamine silver. *D*, Survival rate among *Candida*-infected mice treated with daily continuous low-dose Calcijex (LD Calcijex con't) or a 3-day Calcijex treatment regimen (LD Calcijex 3 days), each at a dose of 0.01 µg/kg, or with high-dose Calcijex (HD Calcijex, 1 µg/kg) for 3 days, compared with that among untreated mice (who were administered an equivolume of PBS). *$P < .05$, compared with the untreated group. Abbreviation: CFU, colony forming units.

RESULTS

Candidemia Patients Have Lower Vitamin D Levels

To underline the role of vitamin D in candidemia in the clinical setting, we measured 25-OHD levels in sera obtained from 28 hospitalized patients with *Candida* BSI and compared them to levels in anonymized sera from 75 hospitalized patients in our hospital. As shown in Figure 1, patients with *Candida* BSI had significantly lower 25-OHD levels (mean [±SEM], 12.31 ± 1.69 ng/mL vs 17.04 ± 1.28 ng/mL; $P = .045$). The mean 25-OHD level (±SEM) measured in a cohort of 30 healthy volunteers was 20.88 ± 1.51 ng/mL, and this was significantly higher than in candidemic patients ($P < .01$) but was not significantly different from values for the anonymized hospitalized patients.

$1,25(OH)_2D_3$ Reduces *Candida* Fungal Burden but Not at High Dose

In the following experiments, we assessed whether vitamin D might influence susceptibility to *Candida* infection in vivo. Over a treatment range of 0.001–1 µg/kg, *Candida*-infected mice that received 0.01 or 0.1 µg/kg of Calcijex had significantly decreased kidney fungal burden, compared with untreated mice (Figure 2*A*). The weight loss trends mirrored that of fungal burden in the lower-dose vitamin D_3–treated mice (Figure 2*B*). On the other hand, mice that received high dose of Calcijex (1 µg/kg) had increased *Candida* growth. Findings in histological sections of the mouse kidneys corresponded to the CFUs obtained through cultures. There were fewer germinating yeast in mice treated with 0.01 or 0.1 µg/kg of Calcijex (Figure 2*C*). In terms of overall survival (Figure 2*D*), *Candida*-infected

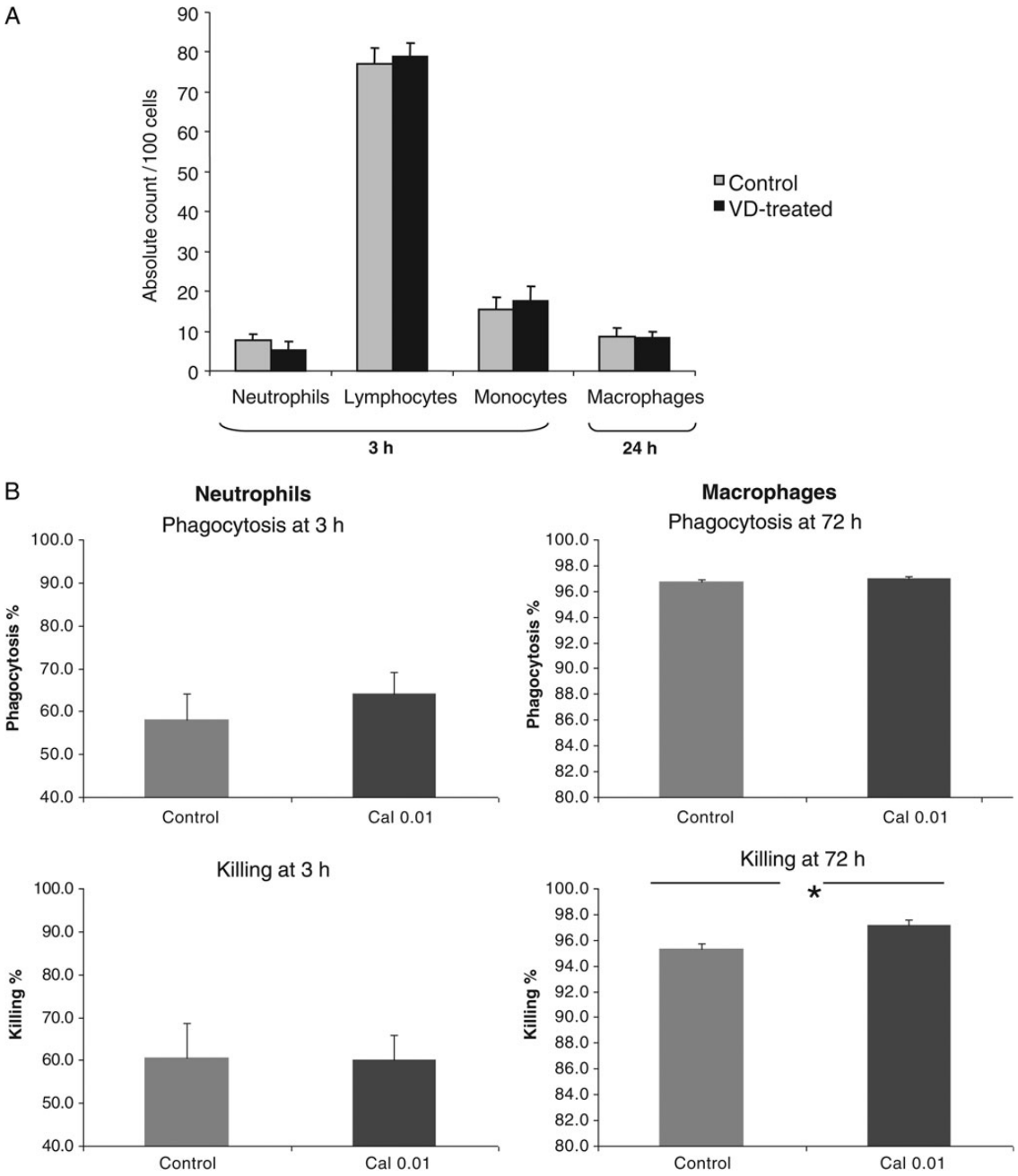

Figure 3. *A*, Recruitment of neutrophils, lymphocytes, monocytes (at 3 hours), and macrophages (at 24 hours) to the peritoneal cavity following intraperitoneal injection of *Candida albicans* in mice pretreated with 0.01 μg/kg Calcijex for 2 days and in control mice, as measured by cytospin assay. *B*, Phagocytosis and killing capacity of extracted neutrophils (at 3 hours) and macrophages (at 72 hours) from Calcijex-treated and control (untreated) mice. Data are pooled from 2 sets of experiments. *$P < .05$, compared with the untreated group.

mice that received 3 daily doses of $1,25(OH)_2D_3$ had increased survival, compared with untreated mice ($P = .006$). The administration of daily doses of vitamin D_3 (0.01 μg/kg of Calcijex intraperitoneally) did not confer an additional survival benefit, compared with the 3-day Calcijex regimen described above at the same dose ($P = .5$). Not unexpectedly, mice given a high dose of Calcijex (1 μg/kg) showed poor survival, consistent with the increased fungal burden observed.

Recruitment Capability and Phagocytic Function

To investigate whether vitamin D_3 might influence the influx kinetics of inflammatory cells and phagocyte function, cells were harvested from the peritoneal cavity 3 and 72 hours after intraperitoneal injection with *Candida* in control mice and $1,25(OH)_2D_3$-treated mice. There was no significant difference in the recruitment capacity of neutrophils, monocytes, lymphocytes, and macrophages in mice treated with low-dose

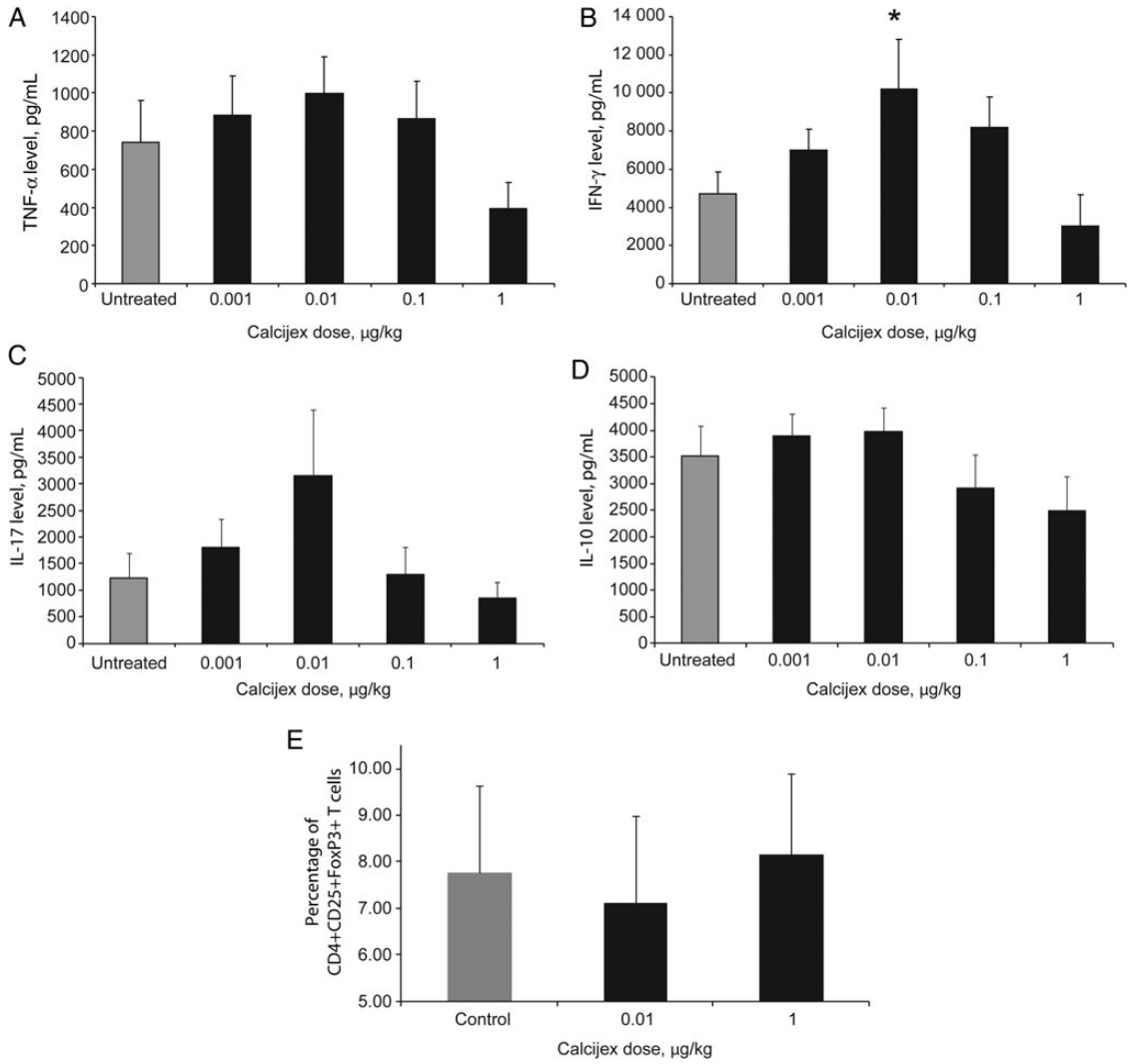

Figure 4. Levels of tumor necrosis factor α (TNF-α; *A*), interferon γ (IFN-γ; *B*), interleukin 17 (IL-17; *C*), and interleukin 10 (IL-10; *D*) after stimulation of splenocytes from *Candida*-infected mice treated with 0.001–1 μg/kg of Calcijex and untreated mice. *E*, Expression of $CD4^+CD25^+FoxP3^+$ T-regulatory cells in splenocytes from *Candida*-infected mice treated with 0.01 or 1 μg/kg of Calcijex, compared with untreated mice. $^*P < .05$, compared with the untreated group.

Calcijex (Figure 3*A*). In the $1,25(OH)_2D_3$-treated group, there was a trend toward increased phagocytosis of *Candida*, but overall, there was no significant enhancement of neutrophil function. A marginal but significant increase in the killing of *Candida* was observed in macrophages extracted from mice treated with $1,25(OH)_2D_3$ (Figure 3*B*). However, the enhanced anti-*Candida* capabilities observed were modest and less likely to be of relevant physiological significance.

Vitamin D_3 Dose-Related Cytokine Response to *Candida*

Using splenocytes from infected control mice and mice primed with 3 daily doses of $1,25(OH)_2D_3$, we studied cytokine induction by *C. albicans*. The splenocytes of mice that received the lower Calcijex doses of 0.01 and 0.1 μg/kg showed a significantly elevated IFN-γ response (Figure 4*B*). At the higher dose of 1 μg/kg, levels of cytokines were largely suppressed. IL-17 levels showed a dose-related trend similar to that of IFN-γ and a maximal response with 0.01 μg/kg Calcijex (Figure 4*C*). The induction of TNF-α and IL-10 by low-dose $1,25(OH)_2D_3$ was limited. Evaluation of whether vitamin D_3 has an influence on regulatory T cells revealed no significant changes in expression of $CD4^+CD25^+FoxP3^+$ cells in splenocytes of $1,25(OH)_2D_3$-treated mice (Figure 4*E*).

Modulation of STAT and SOCS mRNA Transcription by Vitamin D_3

In view of the unanticipated proinflammatory response induced by $1,25(OH)_2D_3$ during *Candida* infection, we studied the

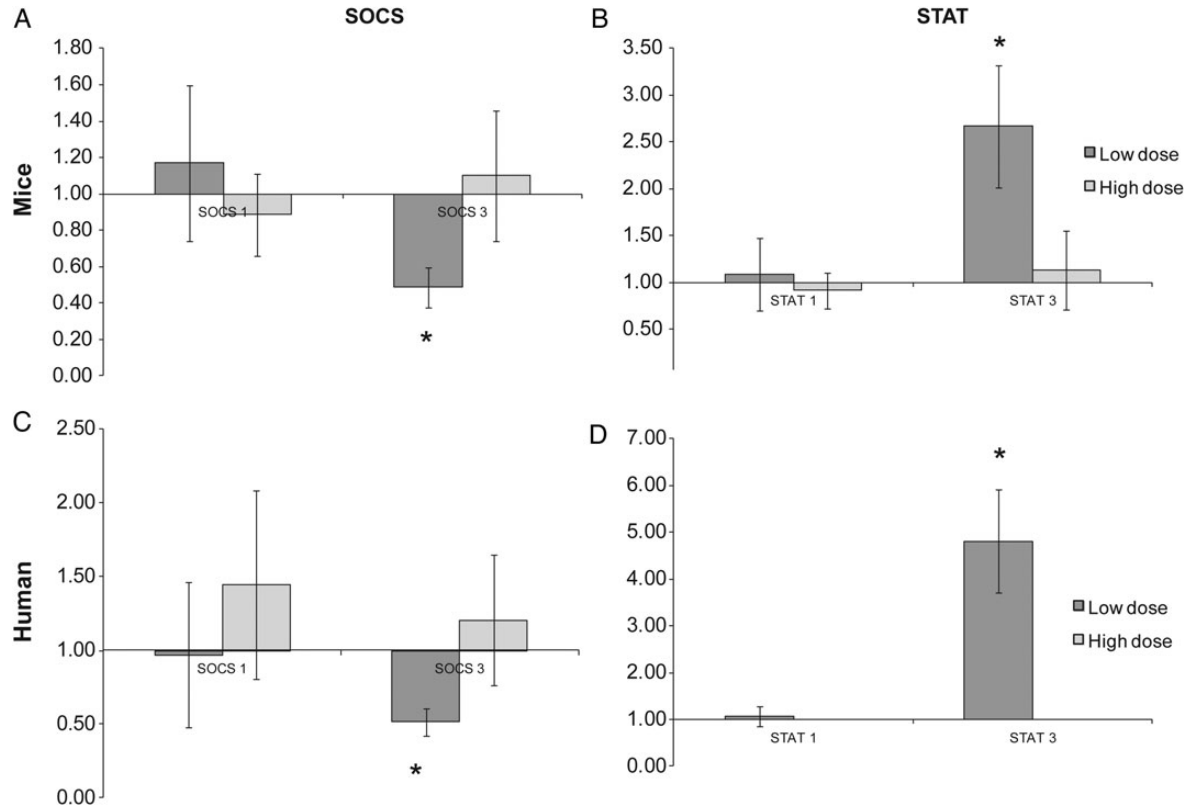

Figure 5. *A* to *D*, SOCS1, SOCS3, STAT1, and STAT3 relative messenger RNA expression in mice splenocytes from *Candida*-infected mice treated with 0.01 μg/kg or 1 μg/kg of Calcijex and human peripheral blood mononuclear cells treated with low-dose (0.01 μg/mL) and high-dose (1 μg/mL) Calcijex. Data are expressed as fold changes relative to levels in untreated controls. **P* < .05, compared with untreated controls.

possible mechanisms through which this response may be mediated. In particular, the STAT and SOCS pathways are known to be central mediators of the host type 1 T helper cell (Th1) response. Vitamin D_3 exerted a differential dose-related effect on SOCS3 transcription: only low-dose vitamin D_3 inhibited SOCS3 mRNA transcription. Correspondingly, STAT-3 mRNA expression was enhanced (Figure 5*A*). On the other hand, there was loss of effect of vitamin D_3 at higher doses. There was also no influence on the STAT1 and SOCS1 pathway (Figure 5*B*). These effects were seen in both the human PBMC system and in mouse splenocytes (Figure 5*C* and 5*D*).

VDR Binding to the Promoter Region of the Gene Encoding IFN-γ

The accentuated proinflammatory response, particularly the IFN-γ response, induced by vitamin D_3 was not mediated to a significant extent by the STAT1/SOCS1 pathway. Binding and activation of the VDR could induce its interaction with specific vitamin D response elements present in the BED promoter of the gene encoding IFN-γ [15]. Using ChIP-PCR, we showed that low-dose $1,25(OH)_2D_3$ induced increased VDR binding to the IFN-γ BED promoter during *Candida* infection in PBMCs (Figure 6*A*). In contrast, high-dose vitamin D_3 led to reduced binding. Cells treated with low-dose $1,25(OH)_2D_3$ had increased IFN-γ production downstream, validating the sequelae of the mechanistic modulation that we had elucidated above (Figure 6*B*).

DISCUSSION

In the present study, we showed that vitamin D_3 exerts a bimodal effect on host immune response against *Candida*. At low doses, vitamin D_3 conferred resistance against candidemia. On the other hand, supplementation of the host with high-dose vitamin D_3 could be detrimental. The beneficial effects observed with low-dose vitamin D_3 were mediated through the enhancement of the host immune response. This was through potentiation of the proinflammatory cytokine response, among which IFN-γ, TNF-α, and IL-17 are known to have important roles in antifungal host defense [16, 17]. Mechanistically, low doses of vitamin D_3 suppressed SOCS3 at the level of mRNA transcription and correspondingly increased STAT3 activity. Furthermore, we showed that vitamin D_3 also exerted an effect upstream through increased binding to the IFN-γ promoter BED to induce IFN-γ production. The vitamin D nuclear receptor was involved in mediating these responses.

These findings are both novel and unanticipated. The reported observations to date generally describe the propensity of

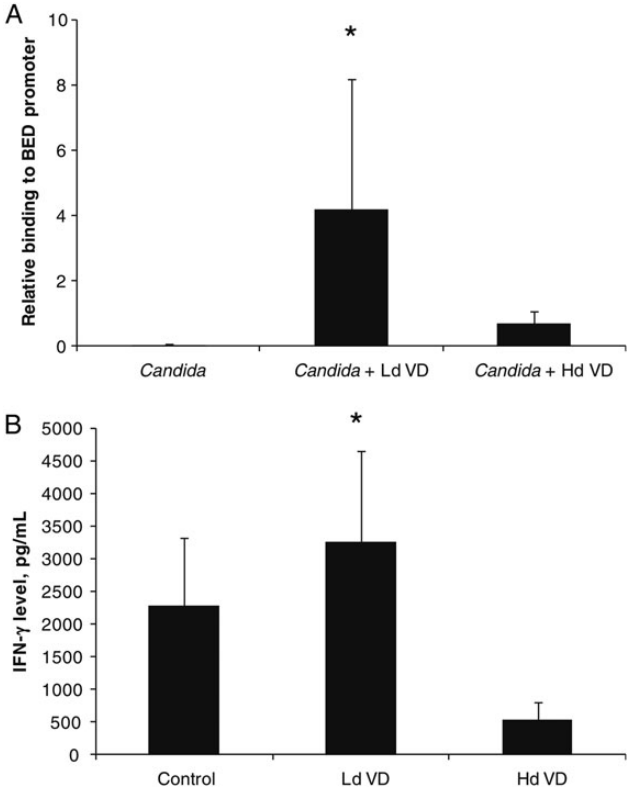

Figure 6. *A*, Binding of activated vitamin D receptor (VDR) to the BED promoter of the gene encoding interferon γ (IFN-γ). Results are from quantitative polymerase chain reaction analysis of the chromatin immunoprecipitation product from peripheral blood mononuclear cells (PBMCs) treated with low-dose (Ld VD; 1 nM) and high-dose (Hd VD; 100 nM) $1,25(OH)_2D_3$ and stimulated with heat-inactivated *Candida albicans*. Results are expressed relative to VDR binding to the BED promoter with *C. albicans* in the absence of $1,25(OH)_2D_3$ and are normalized to 1. **P* < .05, compared with non–$1,25(OH)_2D_3$-treated control. *B*, PBMCs that were not treated with $1,25(OH)_2D_3$ (control) and those that were treated with Ld VD or Hd VD were stimulated with *C. albicans*. Production of IFN-γ was measured in each group (n = 5 subjects). Results were pooled from 2 sets of experiments, **P* < .05, compared with untreated controls.

vitamin D_3 to skew the immune response away from a Th1 type response toward interleukin 4– and IL-10–driven Th2 responses [3, 18]. This has been attributed to the capacity of vitamin D_3 to inhibit T-cell proliferation through suppression of interleukin 2 through the NFATp/AP-1 complex [19, 20]. Similarly, vitamin D_3 inhibits IL-12 in dendritic cells [21]. This results in suppression of not just Th1, but also Th17 inflammatory responses. We and others have attributed this partly to vitamin D_3's capacity to downregulate transcription and expression of surface pattern-recognition receptors [11, 22]. However, many of those in vitro experiments, including our own earlier studies on the immunomodulatory effects of vitamin D_3, had used higher doses of vitamin D_3 that, in retrospect, might be supraphysiological in vivo [23].

On reassessment of our published data in *Candida*-infected human PBMCs, we observed a trend toward increased IFN-γ production with low-dose $1,25(OH)_2D_3$ and cytokine suppression at higher doses [11]. As such, our current in vivo and ex vivo results were largely consistent with our earlier observation. Besides the bimodal cytokine trends elicited in the *Candida*-stimulated splenocytes, more important was our ability to demonstrate the corresponding sequelae of such immunomodulation by vitamin D_3 in the host, as evidenced by the fungal burden trends (both microbiologically and histologically). While the recruitment of inflammatory cells during infection and phagocytic function did not seem significantly altered, there was a marginal increase in intracellular *Candida*-killing capacity. We showed, in turn, that low-dose vitamin D_3 enhanced host inflammatory response most clearly through IFN-γ in the anti-*Candida* Th1 effector arm and likely through induction of Th17 responses. This was mediated through the suppression of SOCS3 transcription. A resultant increase in IFN-γ could be accounted by the reported capacity of SOCS3 to inhibit Th1 differentiation [24]. The corresponding upregulation of STAT3 expression is seen with the trend toward increased production of IL-17, as seen in our study.

The VDR played a central role in mediating these responses. As a member of the nuclear receptor superfamily, the VDR can be activated by low concentrations of $1,25(OH)_2D_3$ [25]. The VDR is a nuclear receptor that can act as a transcription factor to bind to specific vitamin D response elements within the regulatory regions of specific primary target genes [26]. To further account for the increased IFN-γ immunogenicity induced by vitamin D_3, we demonstrated that low-dose vitamin D_3 exerted a more profound effect on IFN-γ production upstream. We showed that $1,25(OH)_2D_3$ induced the increased binding of VDR to the BED promoter region of the gene encoding IFN-γ and that this led to increased production of IFN-γ.

This observation of a bimodal effect suggests the differential susceptibility to modulation of the various immune targets and pathways by $1,25(OH)_2D_3$. SOCS3 transcription, for instance, could be suppressed by low doses of $1,25(OH)_2D_3$. However, it is only with higher doses that we saw general attenuation or even inactivation of other target genes of interest. This reasonably accounts for the differences between results of our study and those of other studies performed earlier. Most of such earlier studies on the immunomodulatory effects of $1,25(OH)_2D_3$ had been derived from in vitro studies using higher doses of $1,25(OH)_2D_3$ that might be supraphysiological. Thus, while the consensus remained in that the effects of high concentrations of $1,25(OH)_2D_3$ are largely antiinflammatory [27], the intrinsic higher susceptibility of some immune targets (eg, SOCS3) to low levels of vitamin D_3 and the increased $1,25(OH)_2D_3$-induced VDR binding to the IFN-γ BED promoter warrant highlighting.

The above observations are very relevant in the clinical context. Vitamin D_3 deficiency has been well described to be associated with various disease states, especially susceptibility to

infections [28–31], as we have also demonstrated here in the context of candidemia. However, epidemiological data studying the association between vitamin D levels, supplementation, and specific infectious diseases, such as tuberculosis and viral infections, have been inconclusive [32–38]. This may be attributable to the differences in the supplemental doses used, as our findings suggest that the immunomodulatory effect of vitamin D_3 is dose dependent. Nonetheless, the bimodal effect as demonstrated in our animal studies raises the questions about what constitutes a low dose of vitamin D_3 (with its potentially beneficial effects of supplementation) and what constitutes a higher dose of vitamin D_3 (with its perhaps excessively immunosuppressive effects) in the human host. Indeed, while a higher vitamin D_3 level with its anticipated immunosuppressive influence may be a requisite against inflammatory conditions such as multiple sclerosis or inflammatory diseases, immunomodulation at such levels may not be appropriate in the context of infections. It remains to be determined as to what ought to be the optimal physiological level of vitamin D_3 in the body, although a recent recommendation had been to increase circulating $25(OH)D_3$ concentration to >75 nmol/L [39]. In light of this study, however, a more prudent approach and more-specific in vivo studies need to be conducted to answer this question.

In conclusion, we have demonstrated that patients with candidemia have lower levels of vitamin D. In turn, limited supplementation of vitamin D augments host immunity and enhances resistance against *C. albicans* infection. On the other hand, excessive high-dose vitamin D suppresses the host immune response. On the background of the findings of this study and the continued debate over recommendations for vitamin D supplementation [40] against morbidity and mortality risk reduction, the balance between vitamin D supplementation and potential caution against hypervitaminosis D needs to be highlighted.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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