Cholecalciferol Supplementation Alters Calcitriol-Responsive Monocyte Proteins and Decreases Inflammatory Cytokines in ESRD

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ABSTRACT

In vitro, monocyte 1α-hydroxylase converts 25-hydroxyvitamin D [25(OH)D] to 1,25-dihydroxyvitamin D to regulate local innate immune responses, but whether 25(OH)D repletion affects vitamin D–responsive monocyte pathways in vivo is unknown. Here, we identified seven patients who had 25(OH)D insufficiency and were undergoing long-term hemodialysis and assessed changes after cholecalciferol and paricalcitol therapies in both vitamin D–responsive proteins in circulating monocytes and serum levels of inflammatory cytokines. Cholecalciferol therapy increased serum 25(OH)D levels four-fold, monocyte vitamin D receptor expression three-fold, and 24-hydroxylase expression; therapy decreased monocyte 1α-hydroxylase levels. The CD16+ “inflammatory” monocyte subset responded to 25(OH)D repletion the most, demonstrating the greatest increase in vitamin D receptor expression after cholecalciferol. Cholecalciferol therapy reduced circulating levels of inflammatory cytokines, including IL-8, IL-6, and TNF. These data suggest that nutritional vitamin D therapy has a biologic effect on circulating monocytes and associated inflammatory markers in patients with ESRD.


There are two sources of vitamin D in humans: Dietary sources, including ergocalciferol (D2) and cholecalciferol (D3), and endogenous production by the skin in response to sunlight (D3 only). To become active, vitamin D (D2 and D3) must undergo hydroxylation in the liver to form 25-hydroxyvitamin D [25(OH)D], a prehormone that is further hydroxylated via the 1α-hydroxylase (CYP27B1) enzyme in the kidney to generate 1,25-dihydroxyvitamin D [1,25(OH)2D], the active form of this hormone. A separate enzyme, 24-hydroxylase (CYP24), is a deactivating enzyme that forms an inactive metabolite of this hormone that is excreted in the bile. As patients with chronic kidney disease (CKD) progress to ESRD, renal CYP27B1 activity decreases and the formation of 1,25(OH)2D becomes impaired, resulting in hypocalcemia and secondary hyperparathyroidism in many of these patients. Previous attempts to counteract these changes in mineral metabolism with nutritional vitamin D therapy have been unsuccessful, because these patients are expected to lack sufficient residual renal CYP27B1 activity to support circulating serum 1,25(OH)2D levels. For this reason, therapeutic approaches to treat vitamin D deficiency in patients with ESRD have shifted away from the use of nutritional vitamin D forms to favor the use of calcitriol or its associated analogues.

Although calcitriol and its analogues have proved to be important therapies for the treatment of disordered mineral metabolism in patients with...
ESRD, the sole use of these compounds for the correction of vitamin D deficiency in this setting has the potential for adverse effects and may be a fundamentally flawed treatment strategy. It is well established that vitamin D has both “classical” actions to affect mineral metabolism and “nonclassical” actions at various other tissues unrelated to mineral metabolism, including the heart, prostate, and monocytes, among others. The existence of nonclassical actions of vitamin D is supported by the presence of vitamin D regulatory enzymes, such as CYP27B1 and CYP24, within these nonclassical tissues, where it is hypothesized that circulating 25(OH)D serves as a necessary substrate for the local generation of 1,25(OH)2D and the regulation of tissue-specific, biologic pathways. It is believed that the local production of 1,25(OH)2D in these nonclassical tissues forms a microenvironment for the regulation of vitamin D–responsive pathways at the cellular level, ultimately leading to biologic effects that are independent of circulating 1,25(OH)2D. Existing associations between vitamin D deficiency and various disease processes, including cancer, diabetes, infection, and cardiovascular health, have provided evidence for nonclassical actions of 1,25(OH)2D.

An overwhelming amount of data supporting nonclassical actions of 1,25(OH)2D exists, but it remains unclear whether these actions are related to the local production of this hormone or from circulating 1,25(OH)2D produced by the kidneys. Despite an overwhelming amount of ex vivo data suggesting the importance of local vitamin D production by cells not involved in mineral metabolism, in vivo assessments of the autocrine and paracrine functions of these cells have been difficult and are confounded by the concurrent systemic generation of 1,25(OH)2D by the kidneys with nutritional vitamin D therapy. This controversy of local versus systemic regulation of the nonclassical actions of vitamin D has the potential for profound consequences in patients who have chronic kidney disease (CKD) and are treated with calcitriol analogues, because these patients often have a deficiency of both 25(OH)D and 1,25(OH)2D. Although this scenario poses a difficult problem for clinicians developing treatment strategies for these patients, it affords a unique opportunity to study the effects of extrarenal production of calcitriol in the setting of minimal renal CYP27B1 activity.

Thus, there is a gap in our knowledge about the effects of nutritional vitamin D (D2 or D3) supplementation on cellular functions in peripheral tissues unrelated to mineral metabolism, and it remains unclear whether this supplementation has any measurable physiologic effects in the setting of ESRD. To address this question, we set out to investigate the physiologic impact of nutritional vitamin D repletion on monocyte protein expression and monocyte-derived inflammatory cytokine levels in patients who have ESRD and are on hemodialysis. Our findings demonstrate that the administration of nutritional vitamin D in the form of cholecalciferol to patients with nonfunctioning kidneys does indeed have biologic effects on circulating monocytes and decreases circulating levels of multiple inflammatory cytokines that have been linked to increased morbidity and mortality in this population. Our findings may serve as early evidence for in vivo, nonclassical effects of cholecalciferol therapy in patients with ESRD and suggest a potential benefit of 25(OH)D repletion in this patient population.

**RESULTS**

**Cholecalciferol Therapy Increases 1,25(OH)2D Production in Patients with ESRD**

We measured changes in the serum markers of mineral metabolism before and after cholecalciferol therapy. We found that both 25(OH)D and 1,25(OH)2D levels significantly increased from the precholecalciferol to postcholecalciferol time points (Table 1). Not only did these levels rise in response to therapy, but also the final 1,25(OH)2D levels fell within the reference range of “normal” values for the general population. Despite a significant rise in both 25(OH)D and 1,25(OH)2D levels, cholecalciferol therapy resulted in no significant change in the other markers of mineral metabolism, including calcium, phosphorus, fibroblast growth factor 23 (FGF-23), and parathyroid hormone (PTH). Two weeks after the discontinuation of cholecalciferol therapy, the levels of 25(OH)D remained significantly elevated compared with pretreatment values.

**Cholecalciferol Effect on CYP27B1, CYP24, Vitamin D Receptor, Toll-Like Receptor 2, and Cathelicidin Expression in Monocytes**

We used flow cytometry analysis to assess changes in monocyte CYP27B1 and CYP24 expression as a measure of the intracel-

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**Table 1. Serum biochemistries for patients who had ESRD and were treated with cholecalciferol and paricalcitol**

<table>
<thead>
<tr>
<th>Serum Variable</th>
<th>Treatment Time Point</th>
<th>Before Cholecalciferol</th>
<th>After Cholecalciferol</th>
<th>After Paricalcitol Restart</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphorus (mg/dl)</td>
<td>4.9 ± 0.5</td>
<td>4.6 ± 0.3</td>
<td>5.3 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>Calcium (mg/dl)</td>
<td>8.6 ± 0.3</td>
<td>8.4 ± 0.4</td>
<td>8.5 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>25(OH)D (ng/ml)</td>
<td>13.9 ± 2.2</td>
<td>53.9 ± 3.3*</td>
<td>47.5 ± 3.9*</td>
<td></td>
</tr>
<tr>
<td>1,25(OH)2D (pg/ml)</td>
<td>9.4 ± 1.1</td>
<td>23.2 ± 1.2*</td>
<td>16.9 ± 3.4c</td>
<td></td>
</tr>
<tr>
<td>Intact PTH (pg/ml)</td>
<td>366.5 ± 86.1</td>
<td>358.9 ± 94.3</td>
<td>351.4 ± 102.8</td>
<td></td>
</tr>
<tr>
<td>FGF-23 (pg/ml)</td>
<td>6841.1 ± 5686.6</td>
<td>5816.1 ± 4968.4</td>
<td>12369.1 ± 11292.5</td>
<td></td>
</tr>
</tbody>
</table>

Data are means ± SEM (n = 7); cholecalciferol therapy = 8 wk; paricalcitol therapy = 2 wk.

*P < 0.0001 versus precholecalciferol levels.

*P < 0.01 versus postcholecalciferol levels.

*P < 0.05 versus precholecalciferol levels.
lular conversion of 25(OH)D to 1,25(OH)₂D in response to cholecalciferol and paricalcitol therapies in patients with ESRD. In addition, we assessed changes in vitamin D receptor (VDR), Toll-like receptor 2 (TLR2), and cathelicidin expression by this same method to evaluate the potential impact of 25(OH)D repletion and paricalcitol therapy on monocyte VDR expression and downstream VDR-responsive pathways. Our flow cytometry analysis of monocytes in these patients revealed a decrease in the CYP27B1 expression after both 25(OH)D repletion with cholecalciferol and the restart of paricalcitol (Figure 1A). In addition, CYP24 expression increased after cholecalciferol therapy, whereas the CYP24 expression returned to baseline levels after the discontinuation of cholecalciferol and restart of paricalcitol therapy (Figure 1A). Combining these measurements to generate the CYP27B1-to-CYP24 ratio demonstrated a decrease in this ratio after both 25(OH)D repletion and paricalcitol therapy (Figure 1A). Our analysis of changes in VDR, TLR2, and cathelicidin expression revealed a significant increase in VDR expression after cholecalciferol administration and a further rise in VDR expression in response to the restart of paricalcitol (Figure 1B). There was no significant change in TLR2 expression, whereas the cathelicidin expression slightly decreased in response to cholecalciferol and paricalcitol therapy (Figure 1B).

**Differential Impact of Cholecalciferol Therapy on Monocyte Subpopulations**

After cholecalciferol administration, flow cytometry analysis of monocytes demonstrated the emergence of a subpopulation of cells expressing very high levels of VDR (Figure 2A). To characterize further this monocyte subpopulation, we performed additional staining for the CD16 monocyte marker, which is expressed in a subset of monocytes exhibiting an inflammatory phenotype. We found that this subpopulation expressing high levels of VDR was strongly positive for CD16 (Figure 2B), whereas the larger population of monocytes demonstrating lesser degrees of VDR expression contained both CD16⁺ and CD16⁻ cells. Reinitiating paricalcitol treatment in 25(OH)D-replete patients resulted in further expansion of this CD16⁺, high-VDR monocyte subpopulation (Figure 2A).

**Differential Expression of TLR2, Cathelicidin, and CYP24 in High- and Low-VDR Monocyte Subpopulations**

After discovering the presence of two separate populations of monocytes in response to 25(OH)D repletion, one with very high VDR expression and one with lower VDR expression, we performed a subanalysis on TLR2, cathelicidin, and CYP24 expression in each of these populations after cholecalciferol therapy. We found that TLR2, cathelicidin, and CYP24 expression were significantly higher in the high-VDR monocyte subset (Figure 3).

**Cholecalciferol Therapy Alters Serum Inflammatory Cytokine Levels**

To evaluate the impact of 25(OH)D repletion on the inflammatory phenotype of these patients, we measured serum changes in inflammatory cytokines at three time points: Before cholecalciferol, after cholecalciferol and after paricalcitol re-start. We observed a decrease in IL-6, IL-8, and TNF-α after...
25(OH)D repletion with cholecalciferol (Figure 4). After the discontinuation of cholecalciferol and reinitiation of paricalcitol therapy, serum levels of IL-8 and TNF-α remained low, whereas IL-6 levels returned to levels near baseline.

**DISCUSSION**

Monocytes are hypothesized to use locally produced 1,25(OH)₂D to regulate vitamin D–responsive intracellular pathways affecting immune function.¹⁰,¹⁶–²¹ *In vitro* and *ex vivo* studies have shown that cells in the monocyte lineage are exquisitely sensitive to vitamin D analogs through VDR-mediated pathways, and as terminal differentiation occurs toward the macrophage phenotype, these cells exhibit an enhanced ability to synthesize 1,25(OH)₂D from the 25(OH)D precursor.²²–²⁴ Whereas nutritional vitamin D deficiency has been widely associated with disease processes affecting several vita-
min D–responsive tissues in patients with normal renal function, the impact in patients with ESRD, who exhibit a diminished capacity for renal 1,25(OH)\(_2\)D production yet an intact capacity for local production, remains ill-defined.\(^{14,25–27}\) With this study, we administered cholecalciferol (D\(_3\)) to patients with ESRD and 25(OH)D deficiency and evaluated in vivo changes in 1,25(OH)\(_2\)-responsive monocyte protein expression. Whereas previous in vivo investigations of the nonclassical effects of vitamin D therapy in patients on hemodialysis have focused on defining monocyte responses to 1,25(OH)\(_2\)D administration,\(^{28,29}\) to our knowledge, this is the first prospective, in vivo investigation of the nonclassical, biologic effects of nutritional vitamin D (D\(_2\) or D\(_3\)) therapy in patients with ESRD.

Because we examined patients who had ESRD and lacked significant residual renal function, our a priori assumption was that this unique population would afford us the opportunity to study the effects of locally generated 1,25(OH)\(_2\)D from monocytes in response to 25(OH)D repletion; however, we observed an unexpected rise in serum 1,25(OH)\(_2\)D levels with 25(OH)D repletion in our patients (Table 1), a response previously described by others.\(^{30–32}\) As a result, we are unable to distinguish between changes in monocyte protein expression that are due to locally generated 1,25(OH)\(_2\)D from possible effects of the circulating hormone. The observed local upregulation of 1,25(OH)\(_2\)D in our patients with ESRD. Although such a theory may explain why a previous study by other investigators found that nephrectomized rats lack peripheral conversion of 25(OH)D to 1,25(OH)\(_2\)D may not contribute to circulating calcitriol levels. Alternatively, it is possible that the kidneys possess residual CYP27B1 activity and, in the face of adequate 25(OH)D substrate, are the source of the increased circulating 1,25(OH)\(_2\)D in our patients with ESRD. Because calcitriol administration has been shown to increase FGF-23 production by bone in animal models,\(^{34}\) it was not unexpected that we observed a rise in mean FGF-23 levels in patients with intact renal function found that circulating 25(OH)D levels did not correlate with monocyte cathelicidin mRNA expression.\(^{38}\)

The next significant observation in this investigation was an effect of cholecalciferol therapy to affect preferentially specific monocyte subpopulations. In this regard, we found that 25(OH)D repletion resulted in the emergence of two discrete monocyte subsets, one exhibiting very high levels of VDR and the other demonstrating lower levels of VDR expression (Figure 2A). Further characterization of these subpopulations revealed the high-VDR subset to be exclusively CD16\(^+\), whereas the low-VDR subset consisted of both CD16\(^+\) and CD16\(^−\) monocytes (Figure 2B). In addition, we observed both TLR2 and cathelicidin expression to be significantly higher in the high-VDR monocyte subset compared with the monocyte population expressing lower VDR levels (Figure 3). Because an evaluation of monocyte CD16 expression was added to our protocol only after observing the emergence of the new high-VDR monocyte population that resulted from cholecalciferol treatment, we were unable to determine whether activation of the monocyte VDR resulted in a differentiation of CD16\(^+\) monocytes into a CD16\(^+\) phenotype or existing CD16\(^+\) monocytes simply demonstrated a greater degree of VDR upregulation after 25(OH)D repletion. Because patients with ESRD are known to exhibit higher levels of circulating CD16\(^+\) monocytes,\(^{39,40}\) which have been hypothesized to contribute to the pathogenesis of cardiovascular disease and other inflammatory comorbidities in this setting,\(^{41–45}\) it will be important to define further the downstream functional changes that result from the observed VDR upregulation in the CD16\(^+\) monocyte sub-
population with cholecalciferol therapy. The future characterization of changes in VDR-dependent pathways with this therapy may allow us to understand better the emerging association between 25(OH)D deficiency and adverse cardiovascular outcomes in patients with ESRD.46,47

Finally, 25(OH)D repletion caused a decline in serum inflammatory cytokine levels, including IL-8, IL-6, and TNF-α (Figure 4). We observed an approximate 55, 30, and 60% reduction in serum IL-8, IL-6, and TNF-α levels, respectively, after cholecalciferol therapy. Both IL-8 and TNF-α levels remained suppressed after discontinuation of cholecalciferol and restart of paricalcitol, whereas IL-6 levels seemed to increase back to the baseline levels. Alterations in monocytes and monocyte-derived cytokines have been implicated in the inflammatory pathology that exists in ESRD.39,41,44,48,49 IL-8, IL-6, and TNF-α are possible direct mediators in the pathogenesis of atherosclerosis.50–58 In addition, there seems to be a significant correlation between the level of these various cytokines and patient outcomes, with multiple studies suggesting that elevations in IL-8, IL-6, and TNF-α levels are associated with increased morbidity and mortality in patients with ESRD.49,59–62 Regardless, the observed reductions in serum cytokine profiles that occurred with 25(OH)D repletion in these patients further support our hypothesis that the correction of nutritional vitamin D deficiency may improve the inflammatory phenotype of patients with ESRD through nonclassical effects on circulating monocytes and possibly other tissues.

We acknowledge that there are several limitations to this investigation. First, the small number of patients and short-term nature of this study make it difficult to draw definitive conclusions regarding benefit or harm of this therapy. It is possible that 25(OH)D deficiency in the setting of ESRD represents an adaptive response to prevent unwanted physiologic changes that occur with failing kidneys and that repletion of 25(OH)D levels could have detrimental effects for these patients. Prospective, long-term studies focusing on the functional changes in monocytes, progression of comorbidities, and outcomes measures are needed for further assessment of the potential impact of this therapy. Likewise, we acknowledge that acute changes in cellular function may not translate to chronic alterations within these cell populations. It is quite possible that the changes in monocyte protein expression and serum cytokine levels that were witnessed in this study were a response to the acute administration of cholecalciferol and paricalcitol, which may differ substantially from the long-term administration of vitamin D derivatives. Furthermore, it remains unclear whether 25(OH)D repletion has any salutary effects at the local cellular level that would extend beyond the effects of systemic repletion of 1,25(OH)₂D with calcitriol analogue therapies. Prospective analyses comparing long-term functional changes in monocyte subpopulations in response to nutritional vitamin D versus calcitriol analogue therapy or combination therapy with these two agents would provide important information to help guide future therapeutic strategies for vitamin D replacement in the setting of ESRD.

Taken together, our findings provide in vivo evidence that 25(OH)D repletion exerts biologic effects on circulating monocytes and may alter the inflammatory phenotype of patients with ESRD. In addition, our data suggest that specific monocyte subpopulations may exhibit unique responses to this therapy. Our study also provides evidence for a separate and possibly additive effect of cholecalciferol and paricalcitol therapy on VDR-responsive cellular pathways, thereby potentially allowing reduced administration of more expensive calcitriol analogues to activate nonclassical VDR-dependent functions. Future comparative testing of the separate in vivo responses of monocyte subsets to cholecalciferol versus calcitriol analogue therapy, however, is needed to justify the incorporation of 25(OH)D repletion into treatment recommendations for patients with ESRD. As our therapeutic options advance for the treatment of secondary hyperparathyroidism in patients with ESRD, it is imperative to develop studies that compare the nonclassical, biologic effects of nutritional vitamin D to calcitriol analogue therapy for the treatment of vitamin D deficiency associated with progressive kidney disease.

CONCISE METHODS

Study Design
In a prospective study involving seven patients who had nutritional vitamin D insufficiency and ESRD and were on hemodialysis, we investigated changes in the expression of several vitamin D–responsive proteins within circulating monocytes after 25(OH)D repletion with cholecalciferol (Biotech Pharmacal, Fayetteville, AR) 50,000 U twice per week. After initial screening of medical history and current health, patients were enrolled according to predetermined inclusion and exclusion criteria. Inclusion criteria were long-term hemodialysis treatment at University of Kansas Medical Center outpatient dialysis unit three times per week, dialysis vintage >6 mo, and 25(OH)D level <25 ng/ml. Exclusion criteria were active infection, acute illness within last month, intact PTH >600 pg/ml, history of chronic inflammatory disease (e.g., inflammatory bowel disease, lupus, rheumatoid arthritis), cinacalcet therapy, allergy to cholecalciferol or paricalcitol, previous parathyroidectomy, previous renal transplantation, active treatment with immunosuppressant medications, and noncompliance with hemodialysis prescription. After consent was obtained, patients underwent a washout period from paricalcitol for 4 wk. After washout, patients had the first of three blood draws collected for monocyte isolation and subsequent measurement of monocyte VDR, CYP27B1, CYP24, cathelicidin, and TLR2 expression by flow cytometry. In addition to the monocyte analysis, patients had serum samples collected for measurement of cytokine levels and markers of mineral metabolism, including 25(OH)D, 1,25(OH)₂D, phosphorus, calcium, FGF-23, and PTH levels before the initiation of cholecalciferol therapy. Patients were subsequently started on cholecalciferol 50,000 U twice weekly. 25(OH)D levels were monitored at 3 and 6 wk of therapy, and dosing adjustments were made according to these levels. At the 3-wk time point, patients who experienced a slow correction of 25(OH)D levels (as defined by levels <30 ng/ml) had their cholecalciferol dos-
ing increased to 50,000 U thrice weekly, whereas patients who demonstrated a more rapid correction (>45 ng/ml at 3 wk) had their cholecalciferol dosing decreased to 50,000 U once weekly. At the 6-wk time point, 25(OH)D levels were again reevaluated and cholecalciferol dosing was adjusted on the basis of the following parameters: Patients who had levels <45 ng/ml at 6 wk had their dosing increased to 50,000 U thrice weekly, whereas patients exhibiting a more rapid correction (levels >60 ng/ml) had their cholecalciferol dosing decreased to 50,000 U once weekly. After a total of 8 wk of cholecalciferol therapy, the second blood draw was taken for monocyte isolation and processing, as well as the identical serum measurements that were performed before the beginning of treatment. At this point, cholecalciferol therapy was stopped and patients were placed back on their original paricalcitol dosing regimen. A third and final monocyte draw was obtained 2 wk after the restart of paricalcitol therapy, and serum measurements were once again performed. This study was approved by the institutional review board of the University of Kansas Medical Center, and all participants provided written consent before study enrollment.

**Monocyte Isolation**

For monocyte isolation, 30 ml of whole blood was collected in phlebotomy tubes containing EDTA anticoagulant and subsequently processed using Ficoll-Paque (Stem Cell Technologies, Vancouver, British Columbia, Canada) centrifugation to obtain the lymphocyte fraction. Ficoll-Paque centrifugation was performed by first mixing 15 ml of whole blood with 15 ml of 1× PBS supplemented with 2% FBS, then layering this mixture over 15 ml of Ficoll-Paque solution in a 50-ml conical bottom tube. Next, this mixture was centrifuged at 400 × g for 35 min at 20°C. After centrifugation, the middle lymphocyte layer was transferred to a new conical bottom tube. This layer was diluted with PBS supplemented with FBS, vortexed to remove any cell aggregates, and centrifuged at 300 × g for 10 min. The supernatant was removed, and cells were suspended in PBS with FBS and centrifuged at 200 × g for 10 min. This step was repeated one additional time to help remove platelets and any remaining Ficoll-Paque solution from the lymphocyte sample. The final supernatant was removed, and cells were resuspended in plain 1× PBS. Isolated cells were further processed by negative selection for the CD14 cell surface marker using a labeled magnetic bead kit (Stem Cell Technologies) to isolate the monocyte fraction.

**Antibody Labeling of Target Proteins**

After an unstained fraction of monocytes was reserved in a separate tube, 10 μl of PECy7-labeled CD14 antibody (cat. no. 557907; BD Biosciences, San Jose, CA) was added to the remaining monocytes and incubated for 15 min in the dark at 4°C. Cells were washed with 1× PBS and centrifuged at 1000 rpm for 3 min. The supernatant was discarded, and 500 μl of Cytofix/Cytoperm solution (BD Biosciences) was added to the cells and incubated for 20 min at 4°C. After incubation, cells were washed twice with 1 ml of Perm/Wash buffer (BD Biosciences). After final centrifugation, the supernatant was again discarded and the final pellet was resuspended in 100 μl of 1× PBS. The monocytes were then subdivided for staining with antibodies specific for VDR, CYP27b1, CYP24, and cathelicidin (cat. nos. sc-13133, sc-49642, sc-66851, and sc-21578; Santa Cruz Biotechnology, Santa Cruz, CA) and TLR2 (cat. no. 56-9922-73; eBioscience, San Diego, CA). The VDR, CYP27b1, CYP24, and cathelicidin antibodies were labeled according to package directions using Zenon antibody labeling kit (cat. nos. Z25113, Z25608, Z25341, and Z25602; Invitrogen, Carlsbad, CA). Labeled antibodies were applied immediately to the monocytes and read by flow cytometry (LSRII; BD Biosciences, Diva6 software). CD16 staining was performed on a separate aliquot of cells using 20 μl of FITC-labeled CD16 antibody (cat. no. 55407; BD Biosciences) added after the CD14 labeling step outlined already.

**Flow Cytometric Analysis**

Monocytes labeled with individual antibodies were used for initial LSRII setup, and fluorescence minus one protocol was used to establish gates for monocyte analysis. Gating strategy, voltages, and compensation remained constant throughout the experiment. Zenon kits demonstrated consistent results in consecutive labeling for the experiment duration.

**Serum Analysis**

For serum samples, 4 ml of whole blood was collected in serum isolation tubes and centrifuged at 3700 rpm for 7 min. Serum calcium and phosphorus were measured by a Unicel Dxc 800 (Beckman Coulter). Intact serum PTH was measured by human intact PTH ELISA kit (Calbiotech, Spring Valley, CA). 25(OH)D and 1,25(OH)2D levels were measured by RIA per kit instructions (DiaSorin, Saluggia, Italy). Serum FGF-23 levels were measured by an intact FGF-23 ELISA (Kainos, Tokyo, Japan). IL-8, IL-6, and TNF-α levels were measured in duplicate by cytokine bead array per package instructions (cat. no. 551811; BD Biosciences).

**Statistical Analysis**

We evaluated differences between groups by one-way ANOVA for multiple group comparisons and t test for two-group comparisons. All values are expressed as means ± SD. P < 0.05 was considered statistically significant. All computations were performed using the GraphPad Prism 5 software (GraphPad Software, San Diego, CA).

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**DISCLOSURES**

None.

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