Vitamin D-Binding Protein Directs Monocyte Responses to 25-Hydroxy- and 1,25-Dihydroxyvitamin D

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Background: Serum 25-hydroxyvitamin D (25OHD) is a key factor in determining monocyte induction of the antimicrobial protein cathelicidin, which requires intracrine conversion of 25OHD to 1,25-dihydroxyvitamin D [1,25(OH)2D]. Both vitamin D metabolites circulate bound to vitamin D-binding protein (DBP), but the effect of this on induction of monocyte cathelicidin remains unclear.

Methods: Human monocytes were cultured in medium containing 1) serum from DBP knockout (DBP/H11002/H11002) or DBP/H11001/H11002 mice, 2) serum-free defined supplement reconstituted with DBP or albumin (control), and 3) human serum with different DBP [group-specific component [Gc]] genotypes with varying affinities for vitamin D metabolites. In each case, response to added 1,25(OH)2D3 or 25OHD3 was determined by measuring expression of mRNA for cathelicidin and 24-hydroxylase. Monocyte internalization of DBP was assessed by fluorescent tagging followed by microscopic and flow cytometric analysis of tagged DBP.

Results: Monocytes cultured in DBP−/− serum showed more potent induction of cathelicidin by 25OHD3 or 1,25(OH)2D3 when compared with DBP+/- serum. Likewise, DBP added to serum-free medium attenuated 25OHD3/1,25(OH)2D3 responses. Fluorescently tagged DBP showed low-level uptake by monocytes, but this did not appear to involve a megalin-mediated mechanism. Human serum containing low-affinity Gc2-1S or Gc2-2, respectively, supported 2.75-fold (P = 0.003) and 2.43-fold (P = 0.016) higher induction of cathelicidin by 25OHD relative to cells cultured with high affinity Gc1F-1F.

Conclusion: These data indicate that DBP plays a pivotal role in regulating the bioavailability of 25OHD to monocytes. Vitamin D-dependent antimicrobial responses are therefore likely to be strongly influenced by DBP polymorphisms. (J Clin Endocrinol Metab 95: 0000–0000, 2010)
Recent studies have highlighted a role for vitamin D as a potent modulator of human immune responses (1). In monocytes, Toll-like receptor (TLR)-mediated up-regulation of CYP27b1 catalyzes the activation of precursor 25-hydroxyvitamin D (25OHD) to hormonal 25-dihydroxyvitamin D (1,25(OH)₂D). Coincidental TLR induction of the nuclear receptor for 1,25(OH)₂D [vitamin D receptor (VDR)] then enables the induction of key vitamin D target genes, notably the antimicrobial protein cathelicidin (2–5). At a molecular level, this mechanism ultimately depends on the interaction between the liganded VDR and a specific vitamin D response element within the proximal promoter of the cathelicidin gene (6–8), but, in vivo, other factors are likely to be involved. In particular, we have shown that induction of monocyte cathelicidin is highly dependent on the initial component of the intracrine vitamin D pathway, namely the availability of substrate for CYP27b1, 25OHD (2, 3). Serum levels of 25OHD vary considerably within humans and provide the best circulating marker for vitamin D status (9). As such, serum 25OHD is likely to be a key determinant of vitamin D-mediated immune activity, particularly for individuals who are vitamin D (25OHD) insufficient or deficient (10, 11).

A key factor in regulating the availability of 25OHD to cells is the serum vitamin D-binding protein (DBP), also known as group-specific component (Gc). DBP is an abundant multifunctional protein structurally related to albumin and α-fetoprotein (12, 13). Almost all vitamin D metabolites circulate bound to either DBP (high affinity for vitamin D ligands) or serum albumin (high abundance but low affinity for vitamin D ligands). In the proximal tubule of the kidney (14) and in mammary cells (15), delivery of substrate 25OHD to CYP27b1 occurs through megalin/cubulin-mediated endocytosis of this metabolite bound to DBP (16). However, as yet, it is unclear whether such a mechanism is crucial for synthesis of 1,25(OH)₂D in other cells expressing CYP27b1. We have therefore characterized the effects of DBP on vitamin D-induced cathelicidin in monocytes and the extent to which this may be influenced by DBP gene variants.

Materials and Methods

Cell culture

Ficoll-isolated peripheral blood mononuclear cells (PBMCs) derived from anonymous healthy donors (screened in accordance with standard transfusion medicine protocols) were obtained from the Center for AIDS Research Virology Core/BSL3 Facility (supported by National Institutes of Health award AI-28697 and by the UCLA AIDS Institute and the UCLA Council of Bioscience Resources). Briefly, monocytes were enriched by adherence by incubating 5 × 10⁶ PBMCs per well in 12-well plates for 2 h in RPMI (Invitrogen, Carlsbad, CA) with 1% fetal bovine serum (FBS) for DBP knockout mouse serum studies or macrophage serum-free (SF) medium (Invitrogen) for DBP add-back and Gc genotype studies. Adherent monocytes were then washed in SF RPMI and cultured overnight in RPMI with 10% FBS (Omega, Tarzana, CA) or in macrophage SF medium depending on experiment type as outlined below. After overnight incubation, cells were washed with SF RPMI and then 1) recultured in RPMI plus 5% DBP −/− mouse serum or 5% DBP −/− mouse serum for 6 h; 2) recultured in RPMI plus BSA (Calbiochem, San Diego, CA) plus human DBP (Calbiochem) for 6 h, with varying amounts of DBP and BSA used for treatments to normalize all samples to 10 μM of added protein; or 3) recultured in RPMI plus 5% human donor serum (Innovative Research, Novi, MI; or provided by Drs. Martinez and Wilkinson in accordance with local ethical approval) for 6 h. All culture media were supplemented with 10 U/ml granulocyte-macrophage colony-stimulating factor (Immunex, Seattle, WA). Serum was collected from C57BL/6 mice heterozygous (+/−) or homozygous (−/−) for the DBP/Gc gene. Monocytes were treated with the vitamin D₃ form of 25OHD (25OHD₃; 2–200 nM) or 1,25(OH)₂D [1,25(OH)₂D₃; 0.02–2 nM (Biomial, Plymouth Meeting, PA) or with vehicle (0.2% ethanol) for 6 h. Megalin-expressing BN16 cells (rat yolk sac carcinoma) were propagated in DMEM plus 10% FBS (kind gift of Dr. T. Willnow, Max-Delbrueck-Center for Molecular Medicine, Berlin, Germany) (17). In some cases, monocytes were treated with IL-15 (R&D Systems, Minneapolis, MN).

Quantitative real-time PCR

RNA was isolated by Trizol (Invitrogen) extraction and cDNA synthesized by SuperScript reverse transcriptase III (Invitrogen) according to the manufacturer’s protocol using random hexamers as previously described (2). Quantitative PCR analysis was performed on a Stratagene MX-3005P instrument using TaqMan reagents from Applied Biosystems (Foster City, CA). Specifically, we used FAM-labeled TaqMan gene expression assay probe/primer sets Hs00167999_m1 (CYP24A1) and Hs00189038_m1 (cathelicidin) in conjunction with VIC/MGB probe/primer for 18S rRNA (endogenous control) (part 4319413E). All cDNAs were amplified under the following conditions: 50 C for 2 min and then 95 C for 10 min followed by 45 cycles of 95 C for 15 sec and 60 C for 1 min. All reactions were performed in triplicate and reported as difference in cycle threshold (ΔΔCt) values (ΔCt value for vehicle-treated control − ΔCt for treated sample).

Fluorescent tagging of DBP

One milligram of human Gc-globulin (Calbiochem) was fluorescently tagged using Alexa-Fluor 488 protein labeling kit (cat. no. A-10235; Molecular Probes/Invitrogen, Carlsbad, CA) reagents for 30 min at room temperature and separated from free dye through size exclusion resin provided with the kit according to manufacturer’s instructions. The resulting fluorescently labeled DBP is referred to as Alexa-DBP.

25OHD₃–DBP-binding assays

Aliquots (0.2 μg) of Alexa-DBP were incubated with 40,000 cpm [³²P]25OHD₃ (NEN Life Science Products/PerkinElmer, Waltham, MA) in 200 μl PBS plus 0.1% gelatin for 30 min at room temperature. Dextran-coated charcoal buffer (200 μl) was
then added and incubated for 30 additional minutes on ice. Non-specific binding was determined using the same assay conditions but with 100 nM unlabeled 25OHD₃ as competitor. DBP-bound [³H]25OHD₃ was then obtained as a supernatant by centrifugation (Sorval H6000A) at 3500 rpm for 30 min at 4°C and measured by scintillation counting.

Flow cytometric analysis of DBP cellular uptake

PBMCs (2.5 × 10⁶) were added to 24-well plates in RPMI containing 1% FBS and incubated for 2 h, and nonadherent cells were aspirated to enrich for monocytes. Cells were cultured with RPMI plus 10% FBS plus granulocyte-macrophage colony-stimulating factor (10 U/ml) and then washed with SF RPMI. Parallel cultures of megalin-positive BN16 cells were also used. Both types of cell were pretreated with 0.4 μM receptor-associated protein (RAP) (Calbiochem), a megalin-cubilin antagonist for 15 min. Aliquots of Alexa-DBP (0.4 or 0.1 μM in RAP antagonist studies) were added to cells in SF RPMI and incubated for 15 min at 37°C. Cells were then placed on ice for 5 min, IgG-R-phycoerythrin isotype control or CD14-R-phycoerythrin (Caltag/Invitrogen, Carlsbad, CA) was added and incubated for 10 min on ice. The cells were then washed with ice-cold PBS, removed from culture wells by scraping in ice-cold PBS, collected by centrifugation, washed again in ice-cold PBS, and then fixed with 4% paraformaldehyde in PBS (pH 7.4). Flow cytometric analysis was carried out using a Beckman Coulter (Brea, CA) Cyan ADP with Summit 4.3 software.

DBP genotype (Gc) analysis

Twenty age-matched (30–39 yr) and sex-matched (male) human serum samples from Black and white donors were purchased from a commercial source (Innovative Research), and 16 donor serum samples from uninfected patients of Gujarati ethnicity were obtained from Dr. Martineau’s United Kingdom clinic, the latter in accordance with local ethical approval. Allelic forms of DBP were determined by DNA sequencing (18) or by serum isoelectric focusing as described previously (19).

Statistical analyses

Data are expressed as mean ± SD unless otherwise stated. Statistical analysis of dose response and single treatment was determined by two-tailed unpaired equal-variance Student’s t test. Statistical analysis of variations between sera with different DBP genotypes treatment studies was carried out by Kruskal-Wallis one-way ANOVA with the Dunn method as a post hoc multiple-comparison procedure applied to raw ΔCt values from RT-PCR assays (SigmaPlot 9.0 software; Systat Inc., San Jose, CA).

Results

DBP attenuates 25OHD₃- and 1,25(OH)₂D₃-induced cathelicidin expression in monocytes

Initial experiments were carried out to determine whether monocyte responses to vitamin D metabolites are influenced by DBP. Data in Fig. 1 show that for monocytes cultured in medium containing mouse serum lacking DBP (DBP⁻/⁻), expression of cathelicidin and the classical vitamin D target gene 24-hydroxylase (CYP24A1) was induced when cells were treated for 6 h with 25OHD₃ (2–200 nM) or 1,25(OH)₂D₃ (0.02–2 nM), or vehicle control (C, 0.2% ethanol) for 6 h. RNA from the resulting cells were then analyzed by RT-PCR for cathelicidin (panel A) and 24-hydroxylase (CYP24A1) (panel B). Data are shown as mean (n = 3) changes in RT-PCR ΔCt values relative to vehicle-treated cells. *, Statistically different from DBP⁺/⁺ serum cells at P < 0.001.
the addition of DBP to SF cultures. The DBP was added with BSA in different combinations to provide a final concentration of 10 μM protein per well to prevent any bias from variable total protein levels. Data indicated that doses of DBP as low as 0.1 μM were sufficient to completely suppress induction of cathelicidin or CYP24A1 by 25OHD3. This squelching response to DBP was much less pronounced in cells treated with 1,25(OH)2D3: 2 μM DBP moderately suppressed responses to a physiologically normal level of 1,25(OH)2D3 (0.2 nM), whereas 0.5 μM DBP was sufficient to completely inhibit responses to a physiologically high level of 25OHD3 (200 nM).

We have reported previously that even in the absence of treatments known to induce CYP27b1 such as TLR ligands (2, 3) or IL-15 (4), monocytes at day 1 of culture exhibit baseline conversion of 25OHD3 to 1,25(OH)2D3 which can act in an intracrine fashion to induce expression of genes such as cathelicidin (2). Thus, in the current study, cells were cultured without added immune activators to minimize confounding effects that might influence responses to DBP and vitamin D metabolites. However, in a series of parallel experiments we showed that suppression of 25OHD3 action by DBP was also observed in monocytes cultured for up to ten days to generate a more macrophage-like phenotype (Supplemental Fig. 1, published on The Endocrine Society’s Journals Online web site at http://jcem.endojournals.org), and in monocytes treated with IL-15 to stimulate CYP27b1 expression (Supplemental Fig. 2).

**Internalization of DBP by human monocytes**

Previous reports have shown that DBP can be internalized by cells via a megalin-mediated endocytic pathway in kidney (14) and mammary (15) tissue. In both cases, receptor-mediated uptake of DBP facilitated local conversion of 25OHD3 to 1,25(OH)2D3 and downstream VDR responses. To determine whether the effects of DBP on monocyte responses to 25OHD3 or 1,25(OH)2D3 also involve an internalization pathway, further studies were carried out using fluorescently tagged DBP. Initial ligand-binding analyses confirmed that Alexa-DBP was able to specifically bind [3H]25OHD3, albeit at a slightly lower level than unlabeled DBP (Fig. 3A). Alexa-DBP was then incubated with monocytes and uptake assessed by fluorescent microscopy (Fig. 3B) and flow cytometry (Fig. 3, C–E). Microscopy indicated that Alexa-DBP was internalized by monocytes in a similar fashion to megalin-positive BN16 cells, (Fig. 3B). To demonstrate similar cytosolic patterns of DBP expression in the two cell lines, longer exposure times were required for monocytes, indicating higher levels of DBP uptake in BN16 cells. This was further underlined by flow cytometric analyses that showed higher mean fluorescence intensity for BN16 cells (Fig. 3D, middle panel) compared with monocytes (Fig. 3E, middle panel). Nevertheless, measurable uptake of Alexa-DBP was detectable in CD14-positive monocytes at both d 1 and 7 of culture (Fig. 3C). To determine whether this uptake was mediated via megalin, cells were preincubated with RAP, a megalin antagonist. RAP reduced Alexa-DBP uptake in BN16 cells (Fig. 3D) but had no effect on Alexa-DBP uptake in monocytes (Fig. 3E). In additional studies, RT-PCR analysis revealed no significant mRNA expression for either megalin or its coreceptor cubulin in monocytic cells (data not shown).

**Induction of monocyte cathelicidin by 25OHD3 varies with DBP genotype**

Having demonstrated the importance of DBP as a determinant of vitamin D-induced responses in monocytes, we then sought to determine whether established genetic differences in DBP provide an additional level of variation to this effect. To assess the impact of DBP gene variants on 25OHD3-induced cathelicidin, monocytes were cultured in medium containing 5% human serum from donors with the six different combinations of the three major allelic
variants of DBP: Gc1F, Gc1S, and Gc2. Initial dose-response studies showed more sensitive responses to 25OHD$_3$ using serum from Gc2-1S donors compared with those with the Gc1F-1F genotype (Fig. 4).

Further analysis by ANOVA of combinations of Gc alleles using 36 serum samples from donors with different ethnic backgrounds confirmed a statistically significant ($P < 0.01$) increase in cathelicidin induction after treatment with a single dose of 25OHD$_3$ in monocytes cultured with Gc2-1S ($P = 0.003$) or Gc2-2 ($P = 0.016$) serum compared with Gc1F-1F serum (Table 1). Indeed, a straightforward comparison between donors with at least one allele of Gc1F (Gc1F-1F, Gc1F-1S, and Gc1F-2) and those without Gc1F (Gc1S-1S and Gc2-1S, and Gc2-2) showed that monocytes cultured in the latter were 1.97-fold more sensitive to 25OHD$_3$ ($P = 0.002$). Likewise, cells cultured in serum from donors with at least one Gc2 allele showed a 1.73-fold higher sensitivity to 25OHD$_3$ than cells cultured in serum from donors without a Gc2 allele ($P = 0.011$). Analysis of a subset of the sera revealed no statistical difference in levels of 25OHD or 1,25(OH)$_2$D between donors with different Gc types (Supplemental Table 1).

**Discussion**

Serum levels of 25OHD vary considerably within humans and provide the best circulating marker for vitamin D status (9). As such, serum 25OHD is likely to be a key determinant of many vitamin D functions, notably its antibacterial effects, which are known to be mediated via intracrine/autocrine mechanisms (2, 3). However, we hypothesized that biological responses to 25OHD will also be strongly influenced by serum proteins that bind vitamin D metabolites, in particular DBP. In data presented here, we confirm that DBP plays a pivotal role in modulating monocyte responses to 25OHD$_3$. 

**FIG. 3.** DBP is internalized by monocytes via a non-megalin-mediated mechanism. A, Binding of [³H]25OHD$_3$ to unlabeled DBP and fluorescently tagged DBP (Alexa-DBP). Nonspecifically bound (NSB) [³H]25OHD$_3$ was determined in the presence of a molar excess of nontritiated 25OHD$_3$. Total binding (TB) data are shown as picomoles 25OHD$_3$ bound per microgram DBP. B, Fluorescence microscopy of megalin-positive BN16 cells and monocytes showing that DBP is internalized by human monocytes. Longer exposure time was used to demonstrate DBP uptake by monocytes. C, Flow cytometric analysis of DBP uptake by monocytes at different stages of differentiation. Aliquots (0.4 µM) of Alexa-DBP were incubated with d-1 or -7 cultures of human monocytes. Flow cytometry was then carried out to identify cells with coexpression of CD14 and Alexa-DBP; dual-label data are shown as mean fluorescence intensity (MFI). D and E, Effect of the megalin inhibitor RAP on BN16 cells (D) and monocytes (E) (d 7). Cells were pretreated with 0.4 µM RAP, a megalin antagonist and then incubated with Alexa-DBP (0.1 µM). Data show flow cytometric data for Alexa-DBP uptake in untreated (control) cells and cells treated with Alexa-DBP in the absence or presence of RAP. Data in C–E show representative flow cytometry plots for each cell treatment incorporating 40,000 cells per plot. Flow cytometry assays were repeated at least twice with similar results. *, Statistically different from unlabeled DBP at $P < 0.05$. FITC, Fluorescein isothiocyanate.
sensitive to 1,25(OH)2D3 when compared with DBP- cultured in medium lacking DBP were shown to be more
features of DBP. Notably, osteoblastic MC3T3-E1 cells
of 1,25(OH)2D in peripheral tissues (21), but to date, there
shown that DBP
ethnic backgrounds

In serum, vitamin D metabolites bind primarily to
DBP but may also associate with other abundant circu-
lating proteins such as albumin. The affinity of albumin
for 25OHD3 (dissociation constant, Kd = 1.7 μM) or
1,25(OH)2D3 (Kd = 19 μM) is substantially lower than
that observed for DBP and 25OHD3 (Kd = 1.4 nM) or
1,25(OH)2D3 (Kd = 25 nM) (23, 24). However, because of
the relative abundance of albumin in serum (650 μM)
compared with DBP (5 μM), the potential remains for some
vitamin D metabolites to be transported in the circulation
by albumin. Despite this, it is generally assumed that vi-
tamin D metabolites are biologically active when un-
bound, even though this fraction is likely to be very small
(24, 25). Indeed, the free-hormone hypothesis has been
proposed as a general mechanism for the cellular uptake of
steroid-like molecules because they are highly lipophilic
and therefore have the potential to passively diffuse across
cell membranes (26, 27). The concentrations of 25OHD3
and 1,25(OH)2D3 used in the current study were chosen to
represent physiological to supraphysiological levels of
these metabolites (9). Under such conditions, one would
predict that the proportion of free (relative to DBP-bound)
will be greater for 1,25(OH)2D3 than 25OHD3. This is
endorsed by data in Figs. 1 and 2 showing that DBP is less
effective at attenuating monocyte responses to 1,25(OH)2D3
and provides further support for the free-hormone
hypothesis.

The free-hormone hypothesis is not universal. For some
cell types such as renal proximal tubule cells (14, 16) and

FIG. 4. Dose-responsive induction of cathelicidin by 25OHD3 in
monocytes cultured in medium supplemented with serum from donors
with different DBP genotypes. Human monocytes cultured in medium
supplemented with 5% serum from 36 donors with different DBP allelic combinations and different
ethnic backgrounds [African-American (Black), Caucasian-American (white), United Kingdom Gujarati (Asian)] were cultured in culture media with 5–10% FBS is unrepresentative of con-
vitro and in vitro models is
complex. Animals lacking DBP are viable and physi-
ologically normal under conditions of vitamin D suffi-
ciency but have greatly reduced circulating levels of
25OHD and 1,25(OH)2D (20). More recent studies have
shown that DBP+− mice also have normal accumulation
of 1,25(OH)2D in peripheral tissues (21), but to date, there
has been no similar analysis of 25OHD. Studies of DBP
in vitro are complicated by the fact that supplementation
of culture media with 5–10% FBS is unrepresentative of con-
ditions in vivo. However, in vitro comparisons using the
same cells cultured with similar levels of serum from
DBP+− and DBP−− mice has shed light on some key
features of DBP. Notably, osteoblastic MC3T3-E1 cells
cultured in medium lacking DBP were shown to be more
sensitive to 1,25(OH)2D3 than when compared with DBP-
positive equivalents (21). Data presented here using
CYP27b1-positive monocytes (Figs. 1 and 2) confirm
these previous observations while also showing that the
regulatory effects of DBP are more pronounced for re-
sponses to 25OHD, the major circulating form of vitamin
D. These observations, coupled with the fact that 25OHD
has a higher binding affinity for DBP than 1,25(OH)2D
(22), suggest that it is free rather than DBP-bound vitamin
D that is biologically active in monocytes, at least in vitro.

<TABLE 1>
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<tr>
<th>DBP genotype</th>
<th>Black</th>
<th>White</th>
<th>Asian</th>
<th>Mean ΔCt</th>
<th>SD</th>
<th>Fold change relative to Gc1F-1F</th>
<th>P value relative to Gc1F-1F</th>
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<td>0</td>
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<td>1</td>
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<td>1.53</td>
<td>1.03</td>
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<tr>
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<td>0.88</td>
<td>1.27</td>
<td>0.407</td>
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<tr>
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<td>1.62</td>
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<tr>
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<td>0</td>
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<td>0.43</td>
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Human monocytes were cultured in medium supplemented with 5% serum from 36 donors with different DBP allelic combinations and different ethnic backgrounds [African-American (Black), Caucasian-American (white), United Kingdom Gujarati (Asian)]. Each monocyte culture was then treated with either vehicle (0.1% ethanol) or a single dose of 25OHD3 (112.5 nM) for 6 h. RNA from the resulting cells was then analyzed by RT-PCR for cathelicidin. Data are shown as mean increase in cathelicidin mRNA (ΔΔCt) for donor DBP genotype relative to the Gc1S-1S genotype (equal to zero) and the fold change in expression relative to Gc1F-1F.
that the DBP genotype with the highest affinity/abundance was associated with the highest levels (19). It was therefore interesting to observe that Gc2 is the least abundant and Gc1F exhibits the highest affinity/abundance linked to varying concentrations of DBP in serum, with 160 nM, and Gc2

![Image](image_url)

**FIG. 5.** Evolution of low-affinity forms of DBP enhances monocyte responses to vitamin D. Schematic representation of the impact of DBP genotype (Gc type) on monocyte responses to 25OHD_3, the major circulating form of vitamin D is shown. The left part of the panel shows primary DBP peptide sequence variations corresponding to the three major GC forms. The right part of the panel shows proposed variations in sensitivity to the immunomodulatory effects of 25OHD_3 for low- or high-affinity Gc forms.

breast epithelial cells (15, 28), uptake of 25OHD_3, and subsequent conversion to 1,25(OH)_2D_3 has been shown to involve endocytosis of DBP mediated via the megalin and cubilin receptors. Flow cytometry suggests that monocytes are also able to internalize DBP (see Fig. 3). However, the magnitude of this uptake was relatively low when compared with cells known to express megalin and cubilin. Moreover, we were unable to detect transcripts for megalin/cubilin in monocytes (data not shown), and inhibition of these receptors using RAP had no effect on monocyte uptake of DBP. The functional significance of non-megalin-mediated DBP uptake by monocytes is unclear but may be related to the alternative function of DBP as an actin-binding protein (29). This facet of DBP has been shown to be a highly effective mechanism for disrupting the intracellular translocation of infectious agents such as *Listeria monocytogenes*, which occurs via hijack of host actin filaments (30). DBP can also act as a macrophage-activating factor (Maf) precursor after posttranslational modification involving glycosidase enzymes found in T and B cells (31). Although DBP-Maf has been reported to act as a pluripotent cytokine affecting a variety of biological systems (32–34), these effects do not appear to be influenced by binding of vitamin D metabolites.

A key feature of DBP is that polymorphic variants of this protein were first described as long ago as 1959 (35). The ancestral DBP allele, Gc1F, has undergone two amino acid changes: a D416E change to form Gc1S and a T420K change to form Gc2 (see Fig. 5). These alterations in amino acid sequence have been shown to affect the affinity of DBP for vitamin D ligands. The reported K_d values for 25OHD_3 with Gc1F = 0.9 nM, with Gc1S = 1.7 nM, and with Gc2 = 2.8 nM (22). Similar K_d variations have also been reported for 1,25(OH)_2D_3: Gc1F = 56 nM, Gc1S = 160 nM, and Gc2 = 240 nM (22). Gc alleles have also been linked to varying concentrations of DBP in serum, with Gc2 being the least abundant and Gc1F exhibiting the highest levels (19). It was therefore interesting to observe that the DBP genotype with the highest affinity/abundance (Gc1F-1F allelic combination) exhibited a significantly weaker cathelicidin response to 25OHD_3 than the low-affinity/abundance Gc2-1S genotype (Table 1 and Fig. 4). This contrasts the endocrine function of the DBP variants, where Gc1F-1F is better able to maintain serum levels of vitamin D metabolites as a consequence of more efficient retention of 25OHD_3 and 1,25(OH)_2D_3 after megalin-mediated uptake of DBP by kidney cells (36).

Data presented here suggest that in humans, the ancestral form of DBP (Gc1F) has undergone loss-of-function amino acid changes that nevertheless confer important biological advantages with respect to the antibacterial actions of vitamin D. As outlined in Fig. 5, alterations in DBP would have had little or no effect on vitamin D physiology under conditions of high serum 25OHD status. However, with lower levels of 25OHD, loss of the Gc1F allele would be advantageous with respect to improved induction of antimicrobial agents such as cathelicidin. Maintenance of vitamin D function is considered to have been a pivotal factor in the adaptive changes in skin pigmentation that occurred as early humans migrated toward Northern latitudes (37). Under these conditions, individuals with darker skin pigmentation would have been less able to generate vitamin D via the epidermal action of UV light. As a consequence, early humans may have experienced a decline in serum 25OHD levels sufficient to compromise biological functions such as innate immune antibacterial activity. Kappelman and colleagues (38) have postulated that this may have been the underlying basis for their detection of tuberculosis infection in 500,000-yr-old hominin fossils discovered in Western Turkey. With this in mind, it is tempting to speculate that evolutionary changes in DBP may have occurred against a backdrop of increased bacterial infection and decreased vitamin D status as early humans migrated out of Africa.

Insufficient serum levels of 25OHD have been correlated with a wide range of detrimental health outcomes including infectious diseases (11, 39–41). However, data presented here suggest that the DBP genotype of individuals is also an important determinant of the bioavailability of vitamin D metabolites to key target cells such as monocytes. It is therefore interesting to note studies showing association between the Gc1F allele and increased risk of chronic obstructive pulmonary disease (42) and syphilis infection (43). Paradoxically, a more recent study has linked the Gc2 allele with susceptibility to active tuberculosis (18). There are several potential explanations for this. Notably, the Gc2 form of DBP lacks the threonine at amino acid 420 required for posttranslational modifica-
tion of DBP to DBP-Maf. Individuals with a Gc2-2 genotype will thus be unable to exhibit this facet of DBP action, although the impact of this with respect to innate immune response to infection is unclear. It should also be noted that association between the Gc2-2 genotype and active tuberculosis was observed only for a specific cohort of Gujarati origin living in London, in which the mean serum levels of 25OHD were extremely low (approximately 20 nM). Moreover, the association was lost for those patients with 25OHD levels greater than 20 nM. With this in mind, it is interesting to note that in patients with chronic obstructive pulmonary disease, the Gc2-2 allelic combination has been linked to low serum 25OHD levels (44). Thus, it is important to recognize that variations in DBP will affect both local (intracrine) and systemic (endocrine) actions of vitamin D. We therefore postulate that future studies of vitamin D and human disease will need to incorporate analysis of both serum vitamin D status and DBP genotype.

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