1α,25-Dihydroxyvitamin D$_3$-induced Myeloid Cell Differentiation Is Regulated by a Vitamin D Receptor–Phosphatidylinositol 3-Kinase Signaling Complex

By Zakaria Hmama,* Devki Nandan,*, Laura Sly,*, Keith L. Knutson,*, Patricia Herrera-Velit,* and Neil E. Reiner†

From the *Department of Medicine (Division of Infectious Diseases) and the †Department of Microbiology and Immunology, The University of British Columbia, Faculties of Medicine and Science; The Research Institute of the Vancouver Hospital and Health Sciences Center, Vancouver, British Columbia; Canandaigua VA 3 JS; and the §Laboratoire d’Immunologie, faculté des Sciences d’El Ahrazo, University of Mohamed V, Morocco.

Summary
1α,25-dihydroxyvitamin D$_3$ (D$_3$) promotes the maturation of myeloid cells and surface expressions of CD14 and CD11b, markers of cell differentiation in response to D$_3$. To examine how these responses are regulated, THP-1 cells were grown in serum-free medium and incubated with D$_3$. This was associated with rapid and transient increases in phosphatidylinositol 3-kinase (PI 3-kinase) activity. Furthermore, induction of CD14 expression in response to D$_3$ was abrogated by (a) the PI 3-kinase inhibitors LY294002 and wortmannin; (b) antisense oligonucleotides to mRNA for the p110 catalytic subunit of PI 3-kinase; and (c) a dominant negative mutant of PI 3-kinase. In THP-1 cells, induction of CD11b expression by D$_3$ was also abrogated by LY294002 and wortmannin. Similarly, LY294002 and wortmannin inhibited D$_3$-induced expression of both CD14 and CD11b in peripheral blood monocytes. In contrast to CD14 and CD11b, hormone-induced expression of the Cdk inhibitor p21 in THP-1 cells was unaffected by either wortmannin or LY294002. These findings suggest that PI 3-kinase selectively regulates D$_3$-induced monocyte differentiation, independent of any effects on p21.

Pretreatment of THP-1 cells with antisense oligonucleotides to the vitamin D receptor (VDR) mRNA abrogated both activation of PI 3-kinase in response to D$_3$ and hormone-induced CD14 expression. Moreover, both Western blots and in vitro kinase assays carried out on immunoprecipitates of the VDR showed that D$_3$ treatment brought about formation of a complex containing both PI 3-kinase and the VDR. These findings reveal a novel, nongenomic mechanism of hormone action regulating monocyte differentiation, in which vitamin D$_3$ activates a VDR- and PI 3-kinase–dependent signaling pathway.

Key words: myeloid cell • differentiation • vitamin D$_3$ • phosphatidylinositol 3-kinase

The steroid hormone 1α,25-dihydroxyvitamin D$_3$ (D$_3$) plays critical roles in regulating numerous cellular and physiological responses. In addition to its well-established importance in plasma calcium homeostasis (1, 2) and bone resorption (3, 4), D$_3$ also plays a functional role in the hemopoietic system. D$_3$ modulates the expression of several genes in promonocytic cell lines, thereby regulating their differentiation (5). Indeed, when myeloid leukemia cells such as HL60, U937, THP-1, and M1 are incubated with D$_3$, they differentiate into cells expressing functional properties and differentiation markers of monocytes and/or macrophages, including CD14 and CR3 (6–9).

The pleiotropic effects of D$_3$ are principally mediated through the intracellular vitamin D receptor (VDR). The VDR is a member of the superfamily of nuclear steroid, thyroid, and retinoic acid receptors. As such, it functions as a ligand-dependent transcription factor, regulating the activation of vitamin D$_3$-responsive target genes (10). At the molecular level, the VDR activates its target genes via

Abbreviations used in this paper: D$_3$, 1α,25-dihydroxyvitamin D$_3$; ECL, enhanced chemiluminescence; IGF, insulin-like growth factor; MAP, mitogen-activated protein; MFI, mean fluorescence intensity; PI 3-kinase, phosphatidylinositol 3-kinase; PIP, phosphatidylinositol 3-phosphate; RT, reverse transcription; S-oligo, phosphorothioate-modified oligonucleotide; VDR, vitamin D receptor; VDRE, vitamin D–responsive element.
interactions with specific DNA sequences designated as vitamin D–responsive elements (VDR E) (11–13), a mode of signaling referred to as "genomic action."

CD14 is a 55-kD glycoprotein originally described as a differentiation marker of monocytes (14). Extensive research has demonstrated that CD14 is involved in mediating responses to several bacterial cell wall products, most notably bacterial LPS (15–18). CD14 has other pleiotropic effects including, but not limited to, regulating IL-2–induced monocyte tumoricidal activity (19), monocyte resistance to HIV (20), and the adhesive capacity of the molecule LFA-1 (21, 22). As CD14 is inserted into the cell membrane through a glycosylphosphatidylinositol anchor (23), the mode of signal transduction through this molecule has been intensively investigated. Recent reports indicate a Drosophila Toll-like membrane protein acts as a cell surface coreceptor to mediate signaling after ligand binding to CD14 (24, 25).

CD14 is undetectable on the surface of monocyctic precursors, and increases dramatically during their differentiation into monocytes (26–28). Therefore, surface expression of CD14 is an excellent model in which to study the mechanisms of myeloid cell maturation regulated by D$_3$. Induction of CD14 expression in response to D$_3$ occurs at the level of gene transcription and requires new protein synthesis (28, 29). However, a curious paradox of this and other systems is that VDRE sequences have not been identified in the promoter regions of many D$_3$-inducible genes, including CD14 (10, 28). Hence, the regulatory events leading to CD14 expression in response to D$_3$ remain largely unknown.

The $\beta_2$ integrin CR3 is another classical marker of monocyte differentiation that is involved in cell adhesion and also functions as a complement receptor (6–9). CD11b, the $\alpha$ subunit of CR3, is a 160-kD glycoprotein that associates noncovalently with a $\beta_2$ subunit partner, CD18. CD11b–CD18 is expressed by mature myeloid cells, and is widely used as an early monocyte differentiation marker (30). Vitamin D$_3$ has been shown to increase cell surface expression of CD11b by HL-60 (30, 31), U937 (31), and THP-1 cells (8).

In various attempts to define the molecular mechanisms regulating cellular responses to D$_3$, efforts have been made to identify alternative signaling pathways to the classical mode of genomic action. Indeed, during the past decade, experimental evidence for "nongenomic" signaling has accumulated. Efforts have been made to identify alternative receptor systems involved in monocyte tumoricidal activity (19), monocyte resistance to HIV (20), and the adhesive capacity of the molecule LFA-1 (21, 22). As CD14 is inserted into the cell membrane through a glycosylphosphatidylinositol anchor (23), the mode of signal transduction through this molecule has been intensively investigated. Recent reports indicate a Drosophila Toll-like membrane protein acts as a cell surface coreceptor to mediate signaling after ligand binding to CD14 (24, 25).

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In various attempts to define the molecular mechanisms regulating cellular responses to D$_3$, efforts have been made to identify alternative signaling pathways to the classical mode of genomic action. Indeed, during the past decade, experimental evidence for "nongenomic" signaling has challenged the concept of exclusive VDR-mediated genomic action. For example, D$_3$ has been shown to stimulate the rapid formation of second messengers including ceramides, cAMP, inositol, and calcium, and to activate a variety of protein kinases including protein kinase C, Raf, mitogen-activated protein (MAP) kinase, and Src family kinases (10, 32–34). However, the physiological importance of nongenomic signaling by itself or relative to VDR-mediated genomic action is still unclear. Moreover, it is not known whether D$_3$ uses the classical VDR for nongenomic signaling, or whether an alternative receptor system is involved.

Phosphatidylinositol 3-kinase (PI 3-kinase), a lipid kinase composed of a Src homology 2 domain-containing regulatory subunit (p85) and a 110-kD catalytic subunit (p110), catalyzes the formation of inositol phospholipids phosphorylated at the D3 position of PI. Although PI 3-kinase is known to be important in a wide variety of cellular processes, including intracellular trafficking, organization of the cytoskeleton, cell growth and transformation, and prevention of apoptosis (35, 36), its potential role in D$_3$-induced monocyte differentiation remains largely unknown. In this regard, in a recent series of experiments from this laboratory that examined monocyte differentiation, it was observed that expression of a dominant negative mutant of PI 3-kinase in U937 cells abrogated D$_3$-induced CD14 expression (Herrera-Velit, P., Z. Hmama, and N. Reiner, unpublished data). This finding provided direct evidence to suggest that monocyte differentiation in response to D$_3$ may be PI 3-kinase dependent. This study investigated the role of PI 3-kinase and the VDR in regulating monocyte differentiation in response to D$_3$. The results obtained show that D$_3$-induced expression of the monocyte differentiation markers CD14 and CD11b requires PI 3-kinase, and that hormone treatment induces formation of a signaling complex in which the VDR associates with PI 3-kinase. Activation of PI 3-kinase represents a novel pathway for D$_3$ signaling regulating monocyte differentiation, and suggests a mechanism of action for the VDR distinct from classical genomic signaling.

Materials and Methods

Reagents and Chemicals. RPMI 1640, HBSS, and penicillin/streptomycin were from StemCell Technologies, Inc. Wortmannin, 1-$\alpha$-phosphatidylinositol, PM SF, leupeptin, pepstatin A, and apronin were purchased from Sigma Chemical Co. LY294002 and microcystin were from Calbiochem Corp. Protein A–agarose and electrophoresis reagents were purchased from Bio-Rad Laboratories. [g-$^{32}$P]ATP was from Nycorne Amersham Plc.

mAbs. The following mAbs were used: 3C10 (mouse IgG2b, anti–CD14 mAb; a gift from Dr. W. C. Van Voorhis, University of Washington, Seattle, WA); W6/32 (mouse IgG$_2a$, anti–HLA class I mAb; American Type Culture Collection); U937-3 (mouse mAb to PI 3-kinase; Upstate Biotechnology Inc.) and 9A7 (rat IgG$_2a$, anti–VDR; Chemicon International). Other Abs used included anti-CD14 and anti-CD11b, both murine IgG$_1$ (SC-7328 and SC-1186, respectively; Santa Cruz Biotechnology, Inc.),Murine IgG$_1$, isotype control M G100 was from Caltag Laboratories.

Cell Lines. The promonocytic cell line THP-1 was from the American Type Culture Collection. The promonocytic cell line U937 was transfected with CDNA, encoding the entire coding region of either wild-type bovine PI 3-kinase subunit p85$_{\alpha}$ (Wp85$_{\alpha}$) or mutant bovine p85$_{\alpha}$ (Ap85$_{\alpha}$) has been described (37). The mutant has a deletion of 35 amino acids from residues 479–513 of bovine p85$_{\alpha}$ and the insertion of two other amino acids (Ser-Arg) in the deleted position. Mutant p85$_{\alpha}$ competes with native p85 for binding to essential signaling proteins, thereby acting as a dominant negative mutant (38). THP-1 and U937 cell lines were cultured in RPMI 1640 supplemented with 10% FCS (HyClone), 2 mM l-glutamine, penicillin (100 U/ml), and streptomycin (100 $\mu$g/ml). Cell density was maintained at a concentration of $<5 \times 10^{5}$/ml.
Isolation and Culture of Human Monocytes. Peripheral blood mononuclear cells were isolated as described previously (39). Monocytes were allowed to adhere for 1 h at 37°C in a humidified atmosphere with 5% CO₂. Nonadherent cells were removed by three washes with HBSS. Adherent cells were immediately treated and used for cell surface phenotype analysis.

Cell Surface Phenotype Analysis. To measure the expression of surface molecules, cells were incubated with specific mouse mAb or irrelevant isotype-matched IgG (10 μg/ml) for 30 min, then washed twice and labeled with FITC-conjugated F(ab')₂ sheep anti-mouse IgG (Sigma Chemical Co.) for 30 min. Cells were then washed twice and fixed in 2% paraformaldehyde in staining buffer. All staining and washing procedures were performed at 4°C in HBSS containing 0.1% NaN₃ and 1% FCS. Cell fluorescence was analyzed using a Coulter Elite flow cytometer. Relative fluorescence intensities of 5,000-10,000 cells were recorded as single-parameter histograms (log scale, 1,024 channels, 4 log decades), and the mean fluorescence intensity (MFI) was calculated for each histogram. Results are expressed as MFI indices which correspond to MFI of cells + specific Ab/MFI of cells + irrelevant isotype-matched IgG.

In Vitro PI 3-Kinase Assay. Cell lysates for analysis of PI 3-kinase were prepared in 20 mM Tris pH 8.0, 1% Triton X-100, 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM Na₂VO₃, 5 mM NaF, 100 nM microcystin, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 10 μg/ml aprotinin. Aliquots of lysates adjusted for protein concentration (300-500 μg protein) were incubated for 2–4 h at 4°C with UB93-3 mAb (anti-PI 3-kinase), and immune complexes were adsorbed onto protein A–agarose for 30–60 min. The complexes were washed twice with lysis buffer and three times with 10 mM Tris-HCl, pH 7.4. PI 3-kinase activity was measured as described (22, 40). In brief, immunoprecipitates were incubated for 10 min at 4°C with 10 μg of sonicated (3 times for 20 s in an ultrasonic cell disrupter; Branson Sonic Power Co.) I-α-phosphatidylinositol in 10 μl of 30 mM Hepes, to which was added 40 μl of kinase assay buffer (30 mM Hepes, 30 mM MgCl₂, 200 μM adenosine, 50 μM ATP, and 10 μCi of [γ-³²P]ATP). Reactions were carried out for 15 min at room temperature, and stopped by the addition of 100 μl of 1 N HCl and 200 μl of chloroform/methanol (1:1, vol/vol). Lipids were separated on oxalate-treated silica TLC plates using a solvent system of chloroform/methanol/water/28% ammonium (45:35:7.5:2.5, vol/vol/vol/vol). Plates were exposed to X-ray film at −70°C. Incorporation of radioactivity into lipids was measured by excising the corresponding portions of the TLC plate, followed by liquid scintillation counting. Alternatively, cell lysates were incubated overnight at 4°C with 9A7 mAb (anti-VDR), and immune complexes were adsorbed onto protein A–agarose, washed in lysis buffer containing 200 mM NaCl and 1% FCS. Cell fluorescence was analyzed using a Coulter Elite flow cytometer. Resulting PI 3-kinase activity was measured.

RNA Isolation and Reverse Transcriptase PCR. RNA isolation, cDNA synthesis, and PCR conditions were as described previously (41). Sequences (5’ to 3’) of oligonucleotide primers used in PCR amplifications were as follows: CD14 sense, CCC AAG CTT GGG CAG AGG TTT GGA AGA CTT ATC G; CD14 antisense, GGG GTA CCC CTT GAC GTG ACG ATA CTG CC (29); Cdk inhibitor p21 sense, TTT TTT TTT TTT TCT TCT C; p21 antisense, TCT ACT CCC CCA TCA TAT ACC; β-actin sense, CAC CCC GTG CTG CTT GAC ACC GGC; β-actin antisense, CCA CAC GGA GTA CTT GGC CTC AGG (41). Controls included in the reverse transcription (RT)-PCR reactions were no RNA and RNA without RT, and different cycle numbers of PCR reactions were performed to ensure linear cDNA amplification.

Results

D₃-induced CD14 Expression in THP-1 Cells. Initial experiments involving immunofluorescence and flow cytometric analysis defined the experimental conditions for D₃-induced CD14 expression by THP-1 cells. THP-1 cells maintained in complete medium expressed nearly undetectable levels of CD14 on the surface, and treatment with hormone caused a dose- and time-dependent increase in CD14 expression (Fig. 1). A response was obtained in the presence of as little as 0.1 nM D₃ (Fig. 1 A), and at 100 nM, virtually all cells expressed CD14 at an average of 2 separate experiments) after 48 h. Since a 24-h exposure to 100 nM D₃ was sufficient to induce a high level of surface CD14 (53-fold increase in MFI index, average of 2 separate experiments) in nearly 100% of cells, subsequent experiments were carried out under these conditions.
Other experiments examined whether the CD14 response of cells to D3 (100 nM, 24 h) was serum dependent. Results obtained in three independent experiments (Fig. 1C) using RPMI 1640 alone showed that THP-1 cells were fully responsive for CD14 induction (MFI index = 104.4 ± 5.1, mean ± SEM). Addition of FCS had only a limited additive effect (MFI index = 125.0 ± 14.1). Conversely, addition of insulin growth factor (IGF)-I, a serum component known to influence cell differentiation (30), to D3 minimally inhibited CD14 expression. These observations suggest that in THP-1 cells, D3 is capable of inducing CD14 in the absence of any serum factors, and that D3 delivery to cell surface is independent of serum vitamin D binding protein (45, 46).

Treatment of THP-1 cells with D3 activates PI 3-kinase. In light of the important roles played by PI 3-kinase in regulating the differentiation of a variety of cell types (47-49), the possibility that D3-induced monocytic differentiation involves PI 3-kinase was investigated. As shown in Fig. 2A, incubation of serum-starved THP-1 cells with 10 nM D3 brought about a significant increase in PI 3-kinase activity (3.07 ± 1.17 fold increase, mean ± SEM, n = 3), with a maximum response observed at 1 μM (6.59 ± 1.47 fold increase). Although PI 3-kinase activity was also significantly increased in IGF-I (100 ng/ml)-treated cells (Fig. 2A), this was not associated with increased CD14 expression (Fig. 1C). Time course experiments using 100 nM D3 (Fig. 2B) showed that PI 3-kinase activation was detectable by as early as 5 min and reached a maximum level (4.94 ± 0.72 fold increase) by 20 min.

D3-induced expression of CD14 and CD11b are PI 3-kinase dependent. To address whether PI 3-kinase activation is required for D3-induced monocytic differentiation, serum-starved THP-1 cells were incubated with either wortmannin or LY294002 for 20 min before addition of hormone. Inhibitors were used at concentrations known to be relatively selective for inhibition of PI 3-kinase (48, 50). Incubation with inhibitors alone had no effect on basal expression of CD14 (data not shown). Preincubation with 5 nM wortmannin led to 65.7 ± 5.6% inhibition of D3-induced CD14 expression (mean ± SEM, n = 3; Fig. 3A). Increasing the concentration of wortmannin to 50 nM inhibited CD14 expression by 86.0 ± 8.5%. LY294002, an inhibitor of PI 3-kinase that acts via a distinct mechanism from
of wortmannin, when used at 1.5 μM reduced D3-induced CD14 expression by 66.8 ± 11.9%. CD14 expression decreased further in the presence of 15 μM LY 294002 (88.1 ± 5.1%). In contrast to abrogation of D3-induced CD14, neither wortmannin nor LY 294002 had significant effects on the expression of HLA class I molecules (data not shown), indicating that the effects of neither wortmannin nor LY 294002 were due to nonspecific toxicity. The PI 3-kinase inhibitors LY 294002 and wortmannin also attenuated D3-induced cell surface expression of CD11b by THP-1 cells. While incubation with inhibitors alone had no effect on basal levels of CD11b expression, preincubation with 5 nM wortmannin inhibited the response to D3 by 41 ± 7% (Table I). A higher concentration of 50 nM increased inhibition to 100 ± 12%. LY 294002 inhibited D3-induced CD11b surface expression by 48 ± 10% at a concentration of 1.5 μM, and by 88 ± 14% at a concentration of 15 μM (Table I). Surface expression of CD14 and CD11b was also examined in human peripheral blood monocytes. As shown in Table II, LY 294002 and wortmannin markedly inhibited D3-induced expression of both CD14 and CD11b in these cells.

### Table II. Effects of PI 3-kinase Inhibitors on Vitamin D3–induced Surface Expression of CD14 and CD11b by Peripheral Blood Monocytes

| Treatment | CD14 (MFI index) | CD11b (MFI index) |
|-----------|------------------|------------------|
| Control   | 1.09             | 1.27             |
| VIT D3    | 1.98             | 1.80             |
| 15 μM LY alone | 1.03             | 1.08             |
| 50 nM wortm alone | 1.21             | 1.25             |
| 1.5 μM LY + vit D3 | 1.37             | 1.63             |
| 5.0 nM wortm + vit D3 | 1.54             | 1.54             |
| 15 μM LY + vit D3 | 1.07             | 1.24             |
| 50 nM wortm + vit D3 | 1.20             | 1.20             |

Peripheral blood monocytes were allowed to adhere to plastic dishes for 1 h at 37°C and 5% CO2 in serum-free medium. Cells were then washed with warm HBSS, and the remaining adherent monocytes were incubated for 20 min at 37°C and 5% CO2 in either medium alone, wortmannin (wrtm), or LY 294002 (LY). D3 (vit D3 [100 nM]) was then added for 24 h at 37°C. Cells from each treatment were washed with staining buffer, labeled with anti-CD11b mAb or irrelevant mAb followed by FITC-conjugated secondary Abs, and flow cytometric analyses were performed. The results shown are the means ± SEM of values obtained in three separate experiments. Results are expressed as MFI indices which correspond to MFI of cells + specific Ab/MFI of cells + irrelevant isotype-matched IgG.
Inhibition of PI 3-kinase has been shown to affect the transport of some cell surface molecules (51), and thus was a possible mechanism to explain the effects of PI 3-kinase inhibitors on D3-induced CD14 expression. However, when total RNA was isolated and RT-PCR was performed using primers for CD14 and β-actin, the results showed that mRNA levels for CD14 were markedly reduced in cells incubated with either wortmannin or LY294002 (Fig. 3 B). These findings indicate that inhibition of PI 3-kinase results in attenuation of D3-induced CD14 gene expression at a pretranslational level.

Treatment of immature myeloid cells with D3 induces the expression of the Cdk inhibitor p21, and the latter has been shown to regulate gene expression and to promote myeloid cell differentiation (52). Experiments were carried out to examine whether the induction of p21 in response to hormone also involved PI 3-kinase. As shown in Fig. 3 C, THP-1 cells expressed low levels of p21 mRNA in the basal state. As expected, p21 expression was significantly induced in response to CD14 and β-actin. The results shown in Fig. 3 C, THP-1 cells expressed low levels of p21 mRNA in the basal state. As expected, p21 expression was significantly induced in response to incubation of cells with D3. However, in contrast to the findings with CD14 and CD11b, preincubation of cells with either wortmannin or LY294002 had no effect on hormone-induced expression of p21. These findings suggest that PI 3-kinase selectively regulates monococyte differentiation, independent of any effects on p21.

The role of PI 3-kinase in regulating monocyte differentiation was investigated further by inhibiting the synthesis of the p110 catalytic subunit of PI 3-kinase. THP-1 cells were incubated in the presence of antisense S-oligo complementary to the p110 translation initiation region (including the ATG initiation codon), and then assayed for both PI 3-kinase activation and CD14 expression in response to D3. Flow cytometric analysis of cells exposed to fluorescein-modified antisense S-oligo, under the same conditions used for unmodified S-oligos, revealed that THP-1 cells readily incorporated foreign DNA (data not shown). As shown in Fig. 4 A, antisense S-oligo to p110 m RNA significantly attenuated D3-induced PI 3-kinase activity (5 inhibition = 87.1 ± 5.6, mean ± SEM, n = 3), whereas at the same concentration, the control sense S-oligo had no apparent effect on the PI 3-kinase response. In parallel with this effect on PI 3-kinase activation, pretreatment with antisense S-oligo (and not with the control, sense oligo) almost completely inhibited hormone-induced surface expression of CD14 (% inhibition = 91.7 ± 3.9, mean ± SEM of three experiments; Fig. 4 B). In addition, RT-PCR results shown in Fig. 4 C demonstrated that D3-induced mRNA levels for CD14 were markedly diminished in cells treated with antisense S-oligo to p110 mRNA.

The PI 3-kinase requirement for D3-induced CD14 expression was also examined in U937 cells transfected with a dominant negative mutant of p85 (Δp85). Stable transfection of these cells with Δp85 resulted in a significant reduction of stimulated PI 3-kinase activity (22, 37). Exposure of Δp85 U937 to D3 (100 nM, 48 h) led to only a marginal increase in surface expression of CD14 above baseline. In contrast, cells transfected with wild-type p85 showed significant induction of CD14 expression (Fig. 5 A). In addition, as observed with THP-1 cells, RT-PCR experiments showed that attenuation of PI 3-kinase in U937 cells resulted in markedly reduced response to D3 for induction of CD14 mRNA (Fig. 5 B). Taken together, these findings described in Materials and Methods. The upper rectangle shows PI 3-kinase activities (means ± SEM of values obtained in three separate experiments), calculated as described in the legend to Fig. 2. (B) Fractions of control or S-oligo-treated cells (~1 × 10^6) were incubated in medium alone or with D3 (100 nM, 24 h), washed with staining buffer, and then labeled with anti-CD14 mAb or irrelevant mAb, followed by FITC-conjugated secondary Abs. Flow cytometric analyses were performed, and MFI indices were determined as described in the legend to Fig. 1. Results are expressed as histograms of fluorescence intensity. Histograms displaced to the right represent cells stained with anti-CD14, and histograms on the left represent cells stained with irrelevant IgG2b. Bold, italicized values are means ± SEM (n = 3) of MFI indices, calculated as described in the legend to Fig. 1. C Control or S-oligo-treated cells (~3 × 10^6) were incubated with D3 (100 nM, 24 h). Total RNA was extracted and RT-PCR was carried out for CD14 and β-actin as described (reference 41). RT-PCR controls, as described in the legend to Fig. 3, were included. The data shown are from one of two independent experiments that yielded similar results.
strongly suggest that PI 3-kinase plays a central role in D3-induced monocyte differentiation, and indicate that PI 3-kinase activation is required to induce CD14 expression.

Induction of CD14 in response to D3 requires the VDR. Many responses to D3, such as induction of expression of osteopontin, osteocalcin, calbindin, and 24-hydroxylase, are brought about by a mechanism involving binding of the VDR to a specific VDRE in the corresponding promoters (53). Since no VDRE has been identified within the CD14 gene promoter (28), this raised the question as to whether the VDR plays any role in regulating D3-induced CD14 expression. To address this question, THP-1 cells were incubated overnight with antisense S-oligo specific to VDR mRNA. This resulted in significant attenuation of the level of VDR protein in THP-1 cells as detected by Western blotting (Fig. 6 A). In contrast, pretreatment of cells with the control, sense S-oligo had no apparent effect on the level of VDR protein. In parallel with the reduction of VDR protein, D3-induced surface expression of CD14 was markedly attenuated (Fig. 6 B; in two separate experiments an average decrease of 86.8% was observed). In addition, cells were treated with S-oligo antisense specific for VDR mRNA before D3, followed by RNA extraction and RT-PCR. The results showed that the induction of CD14 mRNA was markedly attenuated in antisense-treated cells (Fig. 6 C). Taken together, these findings strongly suggest that the VDR is essential for D3-induced CD14 gene expression.

Figure 5. CD14 response to D3 in U937 cells transfected with wild-type bovine p85α (WP85) or dominant negative mutant Δp85α (Δp85). (A) Cells were stimulated with either 100 nM D3 or medium alone for 48 h. Cells were washed with staining buffer, and then labeled with anti-CD14 mAb or irrelevant mAb, followed by secondary FITC-conjugated Abs. Flow cytometric analyses were performed, and MFI indices were determined as described in the legend to Fig. 1. The data shown are the means ± SEM of values obtained in three separate experiments. (B) Cells were stimulated with D3 or medium alone for 24 h, then total RNA was extracted and RT-PCR was carried out for CD14 and β-actin as described (reference 41). No signals were obtained with controls consisting of no RNA and RNA without RT (data not shown). The data shown are from one of two independent experiments that yielded similar results.

Figure 6. VDR antisense S-oligo inhibits D3-induced CD14 expression. 5 × 10⁶ THP-1 cells were suspended in 500 μl of 2.5% lipofectAMINE/RPMI 1640 alone (control), or containing 5 μM of either sense (S) or antisense (AS) S-oligo to the VDR. Cells were then incubated on a rotary shaker for 4 h at 37°C. The volume was then brought up to 7 ml, and cells were cultured for an additional 18 h at 37°C and 5% CO2. (A) 0.5 × 10⁶ cells were boiled in Laemmli buffer and subjected to SDS-PAGE and immunoblotting with mAb to VDR. Membranes were developed by ECL as described (reference 44). The data shown are from one of two independent experiments that yielded similar results. (B) Aliquots of control or S-oligo–treated cells (~1 × 10⁶) were incubated with D3 (100 nM, 24 h), washed with staining buffer, and then labeled with anti-CD14 mAb or irrelevant mAb followed by FITC-conjugated, secondary Abs. Flow cytometric analyses were performed, and MFI indices were determined as described in the legend to Fig. 1. Results are expressed as histograms of fluorescence intensity. Histograms displaced to the right represent cells stained with anti-CD14, and histograms on the left represent cells stained with irrelevant IgG2b. Bold, italicized values in each frame are averages (n = 2) of MFI indices, determined as in the legend to Fig. 1. (C) Control or S-oligo–treated cells (~3 × 10⁶) were incubated with D3 (100 nM, 24 h). Total RNA was extracted and RT-PCR was carried out for CD14 and β-actin as described (reference 41). Controls consisting of no RNA and RNA without RT were included, and no signals were obtained (data not shown). The data shown are from one of two independent experiments that yielded similar results.
Cell lysates were then prepared and assayed for PI 3-kinase activity. The results in Fig. 7 A show that pretreatment with antisense to VDR almost completely abrogated (92.9% inhibition, average of two separate experiments) PI 3-kinase activation in response to D3. In contrast, treatment with control sense S-oligos to VDR had no inhibitory effect.

The evidence that the D3 receptor was required for both PI 3-kinase activation and CD14 expression in response to D3 suggested the possibility that this might involve a signaling complex containing both the VDR and PI 3-kinase. To test this hypothesis, THP-1 cells were incubated with D3, and immunoprecipitates prepared with mAb to the VDR were assayed for PI 3-kinase activity. The results shown in Fig. 7 B indicate that immunoprecipitates of the VDR prepared from cells activated with D3 contained PI 3-kinase activity. To examine further whether the VDR associates with PI 3-kinase upon D3 stimulation, aliquots from anti-VDR and anti-p85 (PI 3-kinase) immunoprecipitates of D3-treated cells were subjected to SDS-PAGE and Western blotting. Blots were probed with mAb to p85 and developed by ECL. The results shown in Fig. 7 C indicate that Abs to p85 reacted in anti-VDR immunoprecipitates with a band that presumably corresponds to the p85 subunit of PI 3-kinase. This association was only observed in cells treated with hormone, and not in untreated cells. Taken together, the findings suggest that D3 treatment induces the formation of a signaling complex containing the VDR and PI 3-kinase. This results in activation of the lipid kinase, and is required for monocyte differentiation and induction of CD14 expression.

Discussion
The steroid hormone vitamin D3 is known to induce immature, myeloid precursor cells to differentiate into mature monocytic cells. This process is accompanied by high-level expression of mRNA and protein for CD14, and other markers such as CD11b (27–29). How these D3-initiated events are regulated is not completely understood. Cellular responses to D3 have primarily been attributed to activation of the VDR, which acts as a transcription factor modulating the expression of a variety of genes (33). In contrast to genomic signaling, several reports have implicated alternative, nongenomic mechanisms of action for D3 involving initiation of signaling at the cell membrane (54–56). The objective of this study was to examine signaling events in response to D3 in order to define further how this hormone regulates myeloid cell differentiation.

The principal conclusions drawn from the experiments reported are that D3-induced expression of CD14 and CD11b requires both PI 3-kinase and the VDR. Moreover, this process appears to involve the formation of a PI 3-kinase-VDR signaling complex. The conclusion that D3-induced CD14 expression is PI 3-kinase dependent is based on several lines of evidence, including: (a) in vitro kinase assays with immunoprecipitated PI 3-kinase, showing that incubation of cells with D3 leads to activation of the enzyme (Fig. 2); (b) D3-induced CD14 expression is abrogated in THP-1 cells incubated with the PI 3-kinase inhibitors, wortmannin and LY294002 (Fig. 3); (c) an antisense strategy to downregulate PI 3-kinase also attenuated D3-induced CD14 expression (Fig. 4); and (d) expression of a dominant negative mutant of PI 3-kinase (Δp85) in U937 cells also completely abrogated D3-induced expression of both CD14 mRNA and protein (Fig. 5). Similarly, CD11b induction in response to D3 was also attenuated by wortmannin and LY294002 in both THP-1 cells and in human peripheral blood monocytes. Taken together, these findings establish that D3-induced CD14 and CD11b expres-
been shown to be critical for CD14 expression in response to D3 was markedly attenuated in cells treated with VDR antisense S-oligo to the CD14 promoter, particularly from bp −128 to −70, has been shown to be critical for CD14 expression in response to D3 (28). However, this region does not contain a canonical VDRE (28). Rather, the CD14 promoter contains two GC boxes that bind the nonreceptor, transcription factor Sp1, and this interaction is believed to be essential for CD14 expression (28, 57). In light of this molecular data, activation of Cdk4 transcription would not be expected to be a direct response to D3 signaling through the VDR. Nevertheless, experiments that used VDR antisense S-oligo to downregulate expression of the endogenous VDRE before D3 stimulation showed that the VDR is required for D3-induced expression of CD14 mRNA (Fig. 6). The obligate requirement for the VDR in this response was also supported by the finding that activation of PI 3-kinase in response to D3 was markedly attenuated in cells treated with VDR antisense (Fig. 7 A). Moreover, a signaling complex containing both the VDR and PI 3-kinase was identified in D3-treated cells (Fig. 7, B and C), suggesting that a functional cytosolic VDR is a prerequisite for PI 3-kinase activation in response to D3. These findings appear to identify a novel, nongenomic mechanism of action for the VDR. It is of interest to note that the hormone-induced formation of a VDR–PI 3-kinase signaling complex is reminiscent of the finding that activation of the Raf-MAP kinase pathway by D3 in keratinocytes involves an association of the VDR with the adaptor protein p66hsc (58). Together, these findings suggest that other models of nongenomic signaling involving the VDR may yet be identified.

The observations that PI 3-kinase regulates CD14 expression in response to D3 and that the VDR is directly involved in this process do not exclude the possibility of a component of genomic action by the VDR, despite the absence of a canonical VDRE within the CD14 promoter. One possibility to consider is that upon incubation of cells with D3, the VDR may translocate to the nucleus towards D3-responsive elements, and enhance the activity of the known CD14 transcription factor Sp1. Two observations lend support to this hypothesis: (a) the finding of transcriptional synergism between the VDR and Sp1 using a construct of the VDRE, the binding sites for Sp1, and a luciferase reporter gene (59); and (b) the presence of a VDRE-like sequence at the distal Sp1 site within the CD14 gene (60).

The findings that D3-induced monocyte differentiation for CD14 and CD11b expression requires PI 3-kinase are consistent with previous reports in which this lipid kinase has been implicated in regulating the differentiation of various cell types, including immature myeloid cells (49, 61–63). For example, indirect data, based solely on the use of inhibitors, suggested a potential role for PI 3-kinase in regulating the differentiation of myeloid leukemia cells HL-60 (63). In addition, when FDC-P1 myeloid cells that express the M-CSF receptor c-fms were treated with M-CSF, there occurred the rapid formation of a complex between c-fms and several signal transduction proteins, including PI 3-kinase (49). Other studies have suggested that the differentiation of FDC-P1 cells is regulated by the activities of both phospholipase C-γ and PI 3-kinase in response to M-CSF stimulation (61). In addition, stimulation of type III receptor tyrosine kinases in the same cell line was observed to lead to PI 3-kinase-dependent monocytic differentiation (62). Thus, the steroid hormone D3 appears to share with M-CSF the property of PI 3-kinase activation during the induction of monocyte differentiation.

It is of interest to compare D3 with phorbol esters that also act to promote monocyte differentiation. For example, PMA induces the expression of the monocyte differentiation markers CR3 (CD11b/CD18) and p150,95 (64), and modulates several functional properties of myeloid precursor cells, such as intracellular adhesion molecule 1-dependent adhesion (22), phagocytosis (44, 65), and bactericidal activity (66). However, PMA does not activate PI 3-kinase (Hanna, M., and N. Reiner, unpublished data), and PMA-induced cell differentiation is resistant to PI 3-kinase inhibition (22). Consistent with the findings presented above, although low concentrations of D3 induce an ∼50-fold increase in CD14 surface expression, optimal doses of PMA resulted in only marginal increases (Hanna, M., and N. Reiner, unpublished data). Regarding signaling mechanisms regulating PMA-induced cell differentiation, several reports have implicated the protein kinase C–Raf-MAP kinase pathway (33, 67, 68). MAP kinase activity has been also shown to be activated by D3 in keratinocytes, enterocytes, and in the promyelocytic cell lines HL60 and NB4 (34, 58, 69). To examine whether the MAP kinase pathway may be involved in THP-1 cell differentiation, D3-treated cells were examined for tyrosine phosphorylation of p42 and p44 MAP kinase isoforms, and assayed for MAP

1591  Hmama et al.
kinase activity using myelin basic protein as substrate. The results obtained (data not shown) indicated that MAP kinase is not activated in response to D₃ in THP-1 cells.

The mechanism by which PI 3-kinase becomes activated in a complex with the VDR is not presently known. At least one well-established mechanism for activation of PI 3-kinase is known to involve interactions of the Src homology 2 domain of the p85 regulatory subunit with tyrosine phosphorylated proteins, including both receptor- and nonreceptor-protein tyrosine kinases (35). In the VDR, the only potential sites of phosphorylation correspond to Ser⁵₁(70) and Ser²⁰⁸(71), and the relevance of these residues in VDR-mediated D₃ signal transduction is unknown. Thus, it is not presently clear how a direct interaction of the VDR with the p85 subunit may lead to activation of PI 3-kinase. On the other hand, a multimolecular complex involving the VDR and a tyrosine-phosphorylated adaptor protein could potentially be involved in D₃-induced activation of PI 3-kinase.

At present, it is not clear how VDR-activated PI 3-kinase would be targeted to the membrane compartment to mediate cellular responses, given the cytosolic and nuclear localization of the VDR. One possibility to consider is that there is a small, membrane-associated population of VDR molecules that induces translocation of PI 3-kinase to the membrane compartment. A second possibility is that once activated, the kinase may simply diffuse to the vicinity of the inner leaflet of the plasma membrane. Third, it is also possible that VDR-activated PI 3-kinase may act on nonmembrane-associated substrates to mediate its effects.

During the course of these studies, it was observed that IGF-I failed to induce CD14 surface expression (Fig. 1 C), despite its ability to activate PI 3-kinase (Fig. 2). Therefore, PI 3-kinase activation in response to D₃ appears to be necessary, but not sufficient to bring about CD14 expression. These findings suggest the possibility that induction of CD14 in response to D₃ may involve VDR-mediated signals that bifurcate to involve nongenomic effects of PI 3-kinase, perhaps acting in concert with a genomic mechanism of action. In this respect, cross-talk between nuclear receptor-mediated signaling and nongenomic actions have been described in D₃-treated osteoblasts (72).

In summary, the findings presented demonstrate a novel pathway in which D₃ signaling for myeloid cell differentiation involves PI 3-kinase activation and signal complex formation with the VDR, which is itself shown to be required for cell differentiation. This pathway is essential for D₃-induced expression of CD14 and CD11b, and attributes important functional roles to PI 3-kinase and the VDR in monocyte differentiation.

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Address correspondence to Neil E. Reiner, Division of Infectious Diseases, University of British Columbia, Rm. 452D, 2733 Heather St., Vancouver, BC, Canada, V5Z 3J5. Phone: 604-875-4011; Fax: 604-875-4013; E-mail: ethan@interchange.ubc.ca

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Vitamin D₃, PI 3-Kinase, and Myeloid Cell Differentiation

1594