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## Vitamin D Protects Against Atherosclerosis via Regulation of Cholesterol Efflux and Macrophage Polarization in Hypercholesterolemic Swine

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### Abstract

**Objective**—Prevalence of vitamin D-deficiency and its association with the risk of cardiovascular disease prompted us to evaluate the effect of vitamin D status on lipid metabolism and atherosclerosis in hypercholesterolemic microswine.

**Approach and Results**—Yucatan microswine were fed with vitamin D-deficient (0IU/d), vitamin D-sufficient (1,000IU/d) or vitamin D-supplemented (3,000IU/d) high cholesterol diet for 48 weeks. Serum lipids and 25(OH)-cholecalciferol levels were measured biweekly. Histology and biochemical parameters of liver and arteries were analyzed. Effect of 1,25(OH)<sub>2</sub>D<sub>3</sub> on cholesterol metabolism was examined in human HepG2 and THP-1 macrophage-derived foam cells. Vitamin D-deficiency decreased plasma HDL levels, expression of liver-X-receptors (LXRs), ATP binding cassette transporter A1 (ABCA1) and ABCG1, and promoted cholesterol accumulation and atherosclerosis in hypercholesterolemic microswine. Vitamin D promoted nascent HDL formation in HepG2 cells via ABCA1-mediated cholesterol efflux. CYP27B1 and VDR were predominantly present in the CD206<sup>+</sup> M2 macrophage foam cell-accumulated cores in coronary artery plaques. 1,25(OH)<sub>2</sub>D<sub>3</sub> increased the expression of LXRs, ABCA1, ABCG1, and promoted cholesterol efflux in THP-1 macrophage-derived foam cells. 1,25(OH)<sub>2</sub>D<sub>3</sub> decreased intracellular free cholesterol and polarized macrophages to M2-phenotype with decreased expression of TNF- $\alpha$ , IL-1 $\beta$ , IL-6 under LPS-stimulation. 1,25(OH)<sub>2</sub>D<sub>3</sub> markedly induced CYP27A1 expression via a VDR-dependent JNK1/2 signaling pathway and increased 27-hydroxycholesterol levels, which induced LXRs, ABCA1 and ABCG1 expression, stimulated cholesterol efflux that was inhibited by VDR antagonist and JNK1/2 signaling inhibitor in THP-1 macrophage-derived foam cell.

**Conclusion**—Vitamin D protects against atherosclerosis in hypercholesterolemic swine via controlling cholesterol efflux and macrophage polarization via increased CYP27A1 activation.

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## Keywords

Atherosclerosis; Cholesterol efflux; Macrophage polarization; Vitamin D

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## Introduction

Atherosclerosis is a chronic inflammatory disease characterized by accumulation of macrophages in the arterial intima, which along with an associated inflammatory response in blood vessel walls initiates the formation and progress of atherosclerotic lesions [1]. In atherosclerotic plaques, macrophage-derived foam cell formation is a hallmark of the progression of atherosclerosis that incites inflammatory rupture of plaques and leads to life threatening cardiovascular complications [2]. It is well established that an atherogenic lipid profile, including increased serum low-density lipoprotein (LDL), promotes the massive accumulation of cholesterol in macrophages, while high-density lipoprotein (HDL) inhibits macrophages-derived foam cell formation via promoting cholesterol efflux and transporting it to the liver for excretion, a process termed reverse cholesterol transport [3]. In atherosclerotic lesions, macrophages are submitted to micro-environmental factors, including cytokines and lipid signals, which might differentiate these cells into morphologically and functionally distinct phenotypes that are usually characterized by the classical pro-inflammatory M1 macrophages and alternatively activated anti-inflammatory M2 macrophages [4]. Several ligand-activated nuclear hormone receptors, such as liver X receptor- $\alpha$  (LXR- $\alpha$ ) and peroxisome proliferator activated receptor- $\beta$ , are important lipid sensors that not only regulate expression of genes involved in lipid metabolism but also regulate macrophage polarization [5].

Vitamin D deficiency has been rising in the general population. Approximately 50% of the population worldwide has low levels of plasma 25-hydroxyvitamin D (25(OH)D<sub>3</sub><20 ng/mL), a stable marker of vitamin D status [6]. Beyond its well-defined role in calcium homeostasis, vitamin D has recently been identified as an important factor in cardiovascular health [7,8]. Clinical studies have indicated that plasma 25(OH) D<sub>3</sub> level below 20 ng/mL are associated with increased risk for coronary heart disease [9]. Several mechanisms including protection of endothelial function, modulation of immune response, inhibition of vascular smooth muscle cell growth, have been proposed to account for the anti-atherosclerotic effect of vitamin D [7]. Recently, several large retrospective studies demonstrated that vitamin D deficiency is associated with atherogenic lipid profile including increased serum LDL and decreased HDL levels [10,11]. High 25(OH)D<sub>3</sub> concentrations in the elderly are associated with low prevalence of metabolic syndrome (MetS) with more beneficial HDL-C levels [12]. 1,25(OH)<sub>2</sub>D<sub>3</sub>, the biologically active form of vitamin D, suppresses foam cell formation by reducing oxidized LDL uptake in diabetic subjects [13]. However, repletion of 25-hydroxyvitamin D levels in the short-term has been reported not to improve the lipid profile in human [14]. Thus, further studies are warranted to define the exact role of vitamin D on the lipid metabolism and atherosclerosis.

In the present study, we found that vitamin D-deficiency decreased plasma HDL levels and promoted the progression of atherosclerosis in hypercholesterolemic swine via impaired

LXRs/ATP-binding membrane cassette transporter A1 (ABCA1) pathway, a crucial regulator in the formation and function of HDL. Our experiments revealed that 1,25(OH)<sub>2</sub>D<sub>3</sub> promotes nascent HDL formation in HepG2 cells via ABCA1-mediated cholesterol efflux. In THP-1 macrophages-derived foam cell, 1,25(OH)<sub>2</sub>D<sub>3</sub> increased the expression of LXRs, ABCA1, ABCG1, and promoted cholesterol efflux, and polarized macrophages to an M2-phenotype with decreased expression of inflammatory cytokines under LPS-stimulation. 1,25(OH)<sub>2</sub>D<sub>3</sub> causes upregulation of CYP27A1 via a VDR-dependent JNK1/2 signaling, which plays a crucial role in activation of LXRs/ ABCA1/ABCG1 pathway.

## MATERIALS AND METHODS

Materials and methods are available in the online-only Data Supplement.

## RESULTS

### 1. Vitamin D deficiency decreases plasma HDL level and promotes atherosclerosis in hypercholesterolemic swine

To investigate the effect of vitamin D on the lipid metabolism and the progression of atherosclerosis, microswine were fed on vitamin D-deficient (VD Def), vitamin D- sufficient (VD Suf), and vitamin D-supplemented (VD Sup) high cholesterol diets. As shown in Fig. 1A, VD Def diet produced significant vitamin D deficiency, while VD Sup diet increased the serum level of 25(OH)D<sub>3</sub>, a stable marker of vitamin D status. Lipid levels of animals fed with high cholesterol diets were increased with time. Compared with VD Suf group, a significant decrease in HDL-C was observed as early as the end of the 3<sup>th</sup> month in VD Def group (VD Def group, 68.4±7.9 mg/dL; VD Suf group, 87.8±6.2 mg/dL; VD Sup group, 98.5±10.3 mg/dL; p<0.01). Furthermore, the VD Sup group had significantly higher level of HDL-C at month 12 compared with the VD Suf group (VD Sup group, 117.3±12.8 mg/dL; VD Suf group, 98.3±9.6 mg/dL; p<0.05). (Fig. 1B). Compared with VD Suf group, vitamin D deficiency increased while vitamin D supplementation decreased the levels of LDL-C. Nevertheless, there was no statistically significant difference among these groups (Fig. 1B).

To investigate the effect of vitamin D status on the atheroma progression in hypercholesterolemic swine, the histological analyses of fatty streak in thoracic aorta from different treatment groups were compared. As shown in Fig. 1C, relative aortic fatty streak lesion area was 49.35±6.17% for the VD Def group, 34.11±2.56% for the VD Suf group, and 24.28±3.05% for the VD Sup group (p <0.01, VD Def compared to VD Suf or VD Sup; p <0.05, VD Sup compared to VD Suf), suggesting that vitamin D deficiency resulted in a more remarkable progress in aortic fatty streak lesions formation under high-cholesterol diet. To further evaluate the extent of atherosclerosis, common carotid arteries from three dietary groups were analyzed for total cholesterol and cholesteryl ester deposition (Fig. 1D). Total cholesterol contents of the arteries were significantly higher in the VD Def group than in swine from the VD Suf or VD Sup group (p <0.01, 58.9±9.81 vs 36.8±5.28 μmol/g artery or 58.9±9.81 vs 35.5±4.44 μmol/g artery). Free cholesterol content in the common carotid artery was significantly increased by 73.2% or 93.2% in the VD Def group compared to VD Suf or VD Sup groups, respectively; further suggesting that vitamin D deficiency exacerbates the lipid accumulation and atherosclerosis in hypercholesterolemic microswine.

## 2. Vitamin D deficiency decreases 27-hydroxycholesterol (HOC) level in liver of hypercholesterolemic swine

To examine the molecular signaling targets that could explain the difference in HDL levels response to vitamin D status, we first performed microarray analysis to examine differentially expressed genes in liver, which is the major organ for the synthesis of HDL. A total of 24,124 genes were examined using the porcine GeneChip for liver of pigs (n=4 for each treatment groups). A total of 342 mRNAs were identified as being up- or down-regulated more than 2-fold in the VD Def group compared with VD Suf and VD Sup groups. Of these genes, 38 genes were regulated in a VitD dose-dependent manner. Analysis of these genes using Gene Ontology Biological Process revealed differential expression of genes primarily regulates the following biological functions: oxidative stress, lipid metabolism and inflammatory response, and cell signaling and interactions (Table I in the online-only Data Supplement). These results support the concept that in addition to its established role in regulating calcium homeostasis, vitamin D appears to play an important role in the modulation of metabolic inflammatory diseases, including atherosclerosis. Prominent effects of vitamin D on the genes in the lipid metabolism were noted. Interestingly, the transcription of genes encoding oxysterols receptor LXRs pathway, ligand-activated transcription factors that raise HDL-C levels, were significantly decreased in the VD Def group (Table I in the online-only Data Supplement *and* Fig. 2A). After further validation by Western-blot, the key proteins in the LXRs pathway, including LXR- $\alpha$  and LXR- $\beta$ , its target gene ABCA1 and steroid catabolic gene CYP27A1 were decreased in VD Def group compared to VD Suf and VD Sup groups (Fig. 2B). CYP27A1 plays a crucial role in the biogenesis of oxysterols, crucial ligands for the activation of LXRs. To clarify whether the regulated effect of vitamin D on the HDL metabolism is related to the oxysterol metabolism, the levels of steroid catabolic intermediates, including 27-hydroxycholesterol (HOC), desmosterol, and 24,25-epoxycholesterol in the liver were measured in the three groups. Compared with VD Suf and VD Sup groups, vitamin D-deficiency decreased the levels of 27-HOC. However, there was no significant difference in the levels of desmosterol and 24,25- epoxycholesterol in the three groups (Fig. 2C). In the cultured HepG2 cell, 1,25(OH)<sub>2</sub>D<sub>3</sub> (10nM) treatment substantially upregulated the expression of CYP27A1 in a time-dependent manner (Fig. 2D) and increased 27-HOC concentrations in oxLDL-loaded HepG2 cells (Fig. 2E).

The activation of LXRs by oxysterol ligands induces the expression of ABCA1, which interacts with apoA-I and promotes the cholesterol efflux that plays a crucial role in the biogenesis of nascent HDL [15, 16]. Since 1,25(OH)<sub>2</sub>D<sub>3</sub> increases 27-HOC production in HepG2 cells, we investigated whether 1,25(OH)<sub>2</sub>D<sub>3</sub> plays a role in nascent HDL formation. After labeling overnight with [1,2-<sup>3</sup>H(N)]cholesterol, HepG2 cells were treated with LXR agonist T0901317 (5 $\mu$ M), 27-HOC (1 $\mu$ M), and 1,25(OH)<sub>2</sub>D<sub>3</sub> (10nM), respectively, for 18h and then incubated with lipid-free apoA-I (25 $\mu$ g/ml) for 12h. The medium was collected and filtered through a 0.45  $\mu$ m PVDF membrane to separate non-HDL and HDL. The radioactivity in [<sup>3</sup>H]-HDL fraction was determined by liquid scintillation counter. We found that the 1,25(OH)<sub>2</sub>D<sub>3</sub> as well as LXR agonist T0901317 and 27-HOC significantly increased cholesterol efflux to apoA-I (Fig. 2F). Following the incubation, ABCA1 protein expression in HepG2 cells was markedly increased by T0901317, 27-HOC and 1,25(OH)<sub>2</sub>D<sub>3</sub> (Fig. 2G).

Overall, these data suggest that  $1,25(\text{OH})_2\text{D}_3$  promotes the nascent HDL formation in HepG2 cells by upregulating CYP27A1 activity and ABCA1 expression.

### 3. Vitamin D increases cholesterol efflux and M2 polarization in coronary artery of hypercholesterolemic swine

To determine the exact mechanism of vitamin D on the inhibition of atherosclerotic progress, we stained coronary plaques from hypercholesterolemic swine. As shown in Fig. 3A, vitamin D metabolism, signal-related proteins including CYP27B1 and VDR were predominantly present in the macrophage foam cell-accumulated cores of plaques in coronary artery. Vitamin D<sub>3</sub> and its biologically active form,  $1,25(\text{OH})_2\text{D}_3$ , have been confirmed to have crucial effects on macrophage function via binding to its receptor VDR<sup>[7]</sup>. Therefore, this data suggest that vitamin D-mediated inhibition of atherosclerosis may be the result from the direct effect of vitamin D on arterial wall besides its effect on HDL levels. Macrophages, with extreme polarization phenotypes M1 and M2 macrophages, are the major cell components in atherosclerotic lesion that contribute to the foam cell formation and secretion of inflammatory factors. In order to clarify the exact role of vitamin D in macrophage function, we compared the staining intensity between M1 marker CCR7 and M2 marker mannose receptor (CD206) in the regions of CYP27B1-positive and VDR-positive cores in plaques. We found significantly greater staining of M2 macrophage marker, CD206, in VDR-positive area, while pro-inflammatory M1 macrophage marker CCR7 showed minimal immunoreactivity in this area (Fig. 3A), suggesting that vitamin D may play a role in the regulation of macrophage differentiation and inflammatory response in atherosclerotic lesion. To verify this hypothesis, we examined the expression of pro-inflammatory cytokines (TNF- $\alpha$  and IL-1 $\beta$ ) in the left common carotid arteries. As shown in Fig. 3B, there was significantly increased level of pro-inflammatory cytokines in the VD Def group compared to VD Suf or VD Sup groups ( $p < 0.05$ , VD Def vs VD Suf or VD Sup).

As shown in Fig. 3C, ABCA1 and ABCG1, another LXR $\alpha$  target genes primarily expressed in macrophages, are mainly co-expressed with VDR at the sites of M2 macrophage foam cells in coronary atherosclerotic lesions of hypercholesterolemic swine, suggesting that the ABCA1/ABCG1 in macrophage might be regulated targets for the vitamin D treatment. Therefore, we next examined the effect of vitamin D on the expression of LXR $\alpha/\beta$ , ABCA1, and ABCG1 in macrophages. Western-blot results in Fig. 3D showed that the vitamin D level positively correlated to the expression of ABCA1, ABCG1 and LXR $\alpha/\beta$  in artery of hypercholesterolemic swine, suggesting that the enhanced effect of vitamin D deficiency on atherosclerosis might be related to the impaired cholesterol efflux in the arterial wall.

### 4. $1,25(\text{OH})_2\text{D}_3$ promotes cholesterol efflux and M2 polarization in macrophage-derived foam cells via upregulating 27HOC

To further determine whether  $1,25(\text{OH})_2\text{D}_3$  altered cholesterol efflux in macrophages, we analyzed the effect of  $1,25(\text{OH})_2\text{D}_3$  on ABCA1-mediated cholesterol efflux in THP-1 macrophage-derived foam cells. As shown in Fig. 4A, oxLDL-induced macrophage-derived foam cells cultured in  $1,25(\text{OH})_2\text{D}_3$  deficient media exhibited a significantly greater staining in oil red after treatment with apoA-I compared with macrophage-derived foam cells cultured in  $1,25(\text{OH})_2\text{D}_3$ -supplemented media. Next, we examined the effect of

1,25(OH)<sub>2</sub>D<sub>3</sub> on cholesterol content and cholesterol efflux in THP-1 macrophage-derived foam cells. As shown in Fig. 4B–C, the ability of apoA-I to decrease cholesterol content and to promote cholesterol efflux in THP-1 macrophage-derived foam cell was significantly increased in 1,25(OH)<sub>2</sub>D<sub>3</sub>-supplemented media compared with 1,25(OH)<sub>2</sub>D<sub>3</sub>-deficient media. To determine whether the increased ability of apoA-I to promote cholesterol efflux in 1,25(OH)<sub>2</sub>D<sub>3</sub>-supplemented media is related to cholesterol transporters, we analyzed the expression of ABCA1 and ABCG1 in these cells. Analysis of protein levels by Western blotting demonstrated a 1,25(OH)<sub>2</sub>D<sub>3</sub>-induced upregulation in the levels of ABCA1, and ABCG1 expression in THP-1 macrophage-derived foam cells (Fig.4D).

Macrophages-derived foam cells from atherosclerotic subjects have been shown to exhibit classic (M1) pro-inflammatory phenotype [5,17]. LXR- $\alpha$  activation and increasing ABCA1 levels have been found to inhibit inflammation and induce expression of IL-10, a marker of the M2 macrophage phenotype [18,19]. To investigate whether the increased activation of LXR- $\alpha$  induced by 1,25(OH)<sub>2</sub>D<sub>3</sub> results in the macrophage phenotypic polarization, we compared the expression of M1 or M2 marker in basal, interleukin (IL)-4 or LPS condition. Compared with 1,25(OH)<sub>2</sub>D<sub>3</sub>-deficient media, the macrophages from 1,25(OH)<sub>2</sub>D<sub>3</sub>-supplemented media have a significant increase in IL-4 stimulated mRNA expression of CD206 and MGL-1 (Fig. 4E). Cells were analyzed by fluorescence activated cell sorter using anti-mannose receptor CD206 antibody. As shown in Fig. 4F, 1,25(OH)<sub>2</sub>D<sub>3</sub> robustly increased IL-4-induced expression of CD206, suggesting 1,25(OH)<sub>2</sub>D<sub>3</sub> promotes the M2 polarization of macrophage-derived foam cells. In vitro stimulation with LPS demonstrated that macrophages from 1,25(OH)<sub>2</sub>D<sub>3</sub>-supplemented media produced significantly less pro-inflammatory cytokines, including TNF- $\alpha$  and IL-1 $\beta$ , compared with macrophages from 1,25(OH)<sub>2</sub>D<sub>3</sub>-deficient media (Fig. 4G). These results support the concept that 1,25(OH)<sub>2</sub>D<sub>3</sub> in macrophage is anti-atherogenic via promotion of cholesterol efflux and anti-inflammatory macrophage polarization by upregulating LXR- $\alpha$  pathway.

To clarify whether the regulated effect of 1,25(OH)<sub>2</sub>D<sub>3</sub> on macrophage is dependent on 27HOC, we investigated the effect of 1,25(OH)<sub>2</sub>D<sub>3</sub> on macrophage CYP27A1 expression. 1,25(OH)<sub>2</sub>D<sub>3</sub> treatment substantially increased the expression of CYP27A1 in THP-1 macrophage-derived foam cells (Fig. 4H). We also examined the effect of 27-HOC on the expression of ABCA1 and ABCG1, and found that 27-HOC induced ABCA1 and ABCG1 expression in a dose-dependent manner in THP-1 macrophage-derived foam cells (Figure I in the online-only Data Supplement). These results suggest that the 27-HOC/LXR- $\alpha$  pathway plays a crucial role in the regulation of macrophage cholesterol efflux and anti-inflammatory effect of 1,25(OH)<sub>2</sub>D<sub>3</sub>.

### 5. 1,25(OH)<sub>2</sub>D<sub>3</sub> induces 27-hydroxycholesterol in a VDR-dependent JNK signal manner

CYP27A1 expression is controlled by activated JNK signal [20,21]. In multiple cell types, the JNK pathway has been demonstrated to be activated by 1,25(OH)<sub>2</sub>D<sub>3</sub> [20–22]. After treatment with 1,25(OH)<sub>2</sub>D<sub>3</sub> for 30 min, the phosphorylation of JNK1/2 (p-JNK1/2) in THP-1 macrophage-derived foam cells was detected, and it was further enhanced up to 3h (Fig. 5A). We further used JNK signal inhibitor SP600125 to determine whether 1,25(OH)<sub>2</sub>D<sub>3</sub> is exerting its CYP27A1 induction effects through activated JNK signal. THP-1 macrophage-

derived foam cells were exposed to SP600125 and/or 1,25(OH)<sub>2</sub>D<sub>3</sub>. SP600125 almost completely blocked 1,25(OH)<sub>2</sub>D<sub>3</sub>-induced expression of p-JNK1/2 in THP-1 macrophages (Fig. 5B). The ability of 1,25(OH)<sub>2</sub>D<sub>3</sub> to induce CYP27A1, ABCA1 and ABCG1 mRNA expression in THP-1 macrophage-derived foam cells was significantly impaired by SP600125 (Fig. 5C), suggesting that 1,25(OH)<sub>2</sub>D<sub>3</sub>-induced CYP27A1 expression is mediated by the JNK signaling pathway.

To test the role of VDR on the effect of 1,25(OH)<sub>2</sub>D<sub>3</sub> on CYP27A1 in THP-1 macrophage-derived foam cells, we first incubated cells with 100 nM VDR siRNA, and the expression of VDR was almost completely inhibited by siRNA (Fig. 5D). The ability of 1,25(OH)<sub>2</sub>D<sub>3</sub> to induce expression of CYP27A1 in THP-1 macrophage-derived foam cells was also significantly inhibited in VDR siRNA-treated cells (Fig. 5E). We then confirmed a role of VDR in the 1,25(OH)<sub>2</sub>D<sub>3</sub>-mediated increase of cholesterol efflux in VDR knockdown cells. The effect of 1,25(OH)<sub>2</sub>D<sub>3</sub> on ABCA1, ABCG1 and LXR $\alpha$  expression in VDR siRNA cells was significantly abolished compared with control siRNA-untreated cells (Fig. 5F), indicating a VDR-dependent effects of 1,25(OH)<sub>2</sub>D<sub>3</sub> on LXR $\alpha$  and its target genes. We further examined the role of VDR in the 1,25(OH)<sub>2</sub>D<sub>3</sub>-induced cholesterol efflux using liquid scintillation counter. The ability of 1,25(OH)<sub>2</sub>D<sub>3</sub>-induced cholesterol efflux in VDR siRNA cells was markedly decreased compared to control siRNA-treated cells (Fig. 5G).

## DISCUSSION

Although clinical studies suggest that vitamin D deficiency is related to a higher risk for cardiovascular disease, the exact role of vitamin D in the progression of cardiovascular diseases has not been well defined [23]. Because a higher plasma vitamin D concentration is usually associated with a healthier lifestyle, it is unclear whether vitamin D status is causally related to disease or is merely a marker of health [8,10]. In this study, we fed hypercholesterolemic swine with different vitamin D diet resulting in different plasma 25(OH)D levels that provide the direct evidence for the effect of vitamin D status on the progress of the disease. We found that vitamin D deficiency promoted the progress of atherosclerosis and decreased plasma HDL-C level, a crucial cardiovascular protective factor, under hypercholesterolemic condition. Supplementation of vitamin D<sub>3</sub> in the diet (3,000 IU per day  $\times$  24 weeks) significantly prevented the development of atherosclerosis and increased the level of HDL-C, suggesting the beneficial effect of vitamin D<sub>3</sub> supplementation in modifying lipid profile and decreasing cardiovascular disease. In a randomized clinical study including 57 postmenopausal vitamin D deficient women, Catalano et al. found that the supplementation with 25(OH)D<sub>3</sub> (calcifediol) for 24 weeks significantly increased HDL-C level even after adjustment for age, baseline BMI, 25(OH)D<sub>3</sub> and lipid levels [24]. In another randomized double-blind placebo-controlled clinical trial including 70 participants with type 2 diabetes it was found that the supplementation of calcitriol (1,25(OH)<sub>2</sub>D<sub>3</sub>, 0.5  $\mu$ g per day) for 12 weeks increased the HDL-C level compared to the placebo group [25]. However, Ponda et al. have reported no improvement in the lipid profile following short-term supplementation of vitamin D<sub>3</sub> (50,000 IU of vitamin D<sub>3</sub> weekly for 8 Weeks) in vitamin D-deficient adults with elevated risk for cardiovascular disease, suggesting that the dose and duration of treatment determine the outcomes and this remains to be elucidated.

Oxysterols are the endogenous ligands for the LXRs, ligand-activated transcription factors that can regulate a crucial checkpoint in cholesterol homeostasis [26]. The sterol 27-hydroxylase (CYP27A1) is a mitochondrial cytochrome P450 and is involved in the synthesis of oxysterols, 27-hydroxycholesterol (HOC), 3 $\beta$ -hydroxy-5-cholestenic acid and 25(OH)D<sub>3</sub>, which play a crucial role in cholesterol, bile acids and vitamin D metabolism [27]. It was reported previously that vitamin D<sub>3</sub> and its biologically active form, 1,25(OH)<sub>2</sub>D<sub>3</sub>, can regulate expression of various cytochromes P450 (CYP) [28–30]. Utilizing a microarray method, we found that vitamin D-deficiency induces a response for the expression of the mRNA of genes associated with oxidative stress, lipid metabolism, and inflammation pathways in the liver. Vitamin D-supplementation increased liver mRNA levels of oxidation-reduction and oxysterol-related CYP genes, including CYP1A1, CYP1A2, CYP2J34, and CYP27A1. In addition, we found that vitamin D upregulates the expression of LXRs and ABCA1 in liver in a dose-dependent manner.

ABCA1 is a lipid transport protein transcriptionally regulated by nuclear receptors LXR $\alpha$ , which facilitates cellular cholesterol efflux to lipid-poor apoAI and plays a key role in the formation and function of HDL [31]. In mice, specific lack of ABCA1 in hepatocytes almost totally reduces the circulating HDL-C level [32]. Mutations of ABCA1 in human underlie the HDL deficiency syndrome, Tangier disease, suggesting that primarily hepatic ABCA1 determines the generation of nascent HDL particles [31,32]. To clarify whether the regulated effect of vitamin D on the HDL metabolism is related to the ABCA1 expression, we investigated the effect of 1,25(OH)<sub>2</sub>D<sub>3</sub> on the expression of ABCA1 and nascent HDL formation in hepatocytes, and found 1,25(OH)<sub>2</sub>D<sub>3</sub> promoted cellular cholesterol efflux to lipid-poor apoAI, a major component of nascent HDL via upregulating ABCA1 expression. In several cholesterol-loaded cells, including hepatocytes and macrophages, LXR- $\alpha$ /ABCA1 pathway has been found to be upregulated to promote the cholesterol efflux via a 27-HOC dependent manner [33,34]. In this study, we have shown for the first time that 1,25(OH)<sub>2</sub>D<sub>3</sub> facilitates the upregulation of LXR- $\alpha$ /ABCA1 in cholesterol-loaded hepatocytes and macrophages via promoting the expression of CYP27A1. This effect could be supported by a recent clinical data, showing a significant positive correlation between the levels of 27-OHC and 25(OH)D<sub>3</sub> under hypercholesterolemic condition [35]. Interestingly, the LXR target genes such as ABCG5/8, SREBP-1c, and SR-B1 were not found to be regulated by 1,25(OH)<sub>2</sub>D<sub>3</sub> in hepG2 cells (Figure II in the online-only Data Supplement). The conserved consensus cis-acting element DR4-dependent transcription is necessary for LXR- $\alpha$ -induced ABCA1 expression in HepG2 cells [36]. The activation of VDR by 1,25(OH)<sub>2</sub>D<sub>3</sub> has been found to recruit co-activators at DR4-type response element that leads to the expression of target genes [37,38]. These results suggest that the recruitment of co-activators at the response element induced by 1,25(OH)<sub>2</sub>D<sub>3</sub> may be playing a critical role in supporting the transactivation of LXR- $\alpha$ /ABCA1 pathway, though the exact mechanisms remain to be further examined.

A number of studies have shown that 1,25(OH)<sub>2</sub>D<sub>3</sub> can regulate the differentiation, maturation and function of macrophages [7,39,40], which prompted us to investigate whether the anti-atherogenic effects of vitamin D results from the direct effect of vitamin D on the arterial wall. In this study, we found VDR and CYP27B1, a key enzyme to control the synthesis of 1,25(OH)<sub>2</sub>D<sub>3</sub>, are mainly expressed at the M2 macrophage foam cell-



accumulated cores of plaques in the coronary artery. Vitamin D regulates the macrophage M1/M2 phenotype in diabetic nephropathy rats<sup>36</sup>. In vitro, 1,25(OH)<sub>2</sub>D<sub>3</sub> has also been found to switch high glucose-induced M1/M2 polarization to suppress cholesteryl ester formation and to enhance cholesterol efflux in M2 macrophages<sup>[41,42]</sup>. In atherosclerotic lesion, we found that VDR is primarily co-expressed with ABCA1/ABCG1 at M2 macrophage-derived foam cell. In vitro, 1,25(OH)<sub>2</sub>D<sub>3</sub> has been found to promote the cholesterol efflux and polarize macrophages to pro-inflammatory M2-phenotype. In macrophages, ABCA1 and ABCG1, another ABC family of transporter, play critical role in mobilizing cholesterol out of macrophages and onto extracellular HDL<sup>[43]</sup>. In addition to its lipid transport function, ABCA1 and ABCG1 have been found to play a crucial role in the regulation of cytokine-triggered macrophage polarization programs and inflammatory response<sup>[44,45]</sup>. Several studies have shown that the cholesterol export activity of ABCA1/ABCG1 could account for its potent anti-inflammatory properties<sup>[46,47]</sup>. Knockdown of ABCA1 in macrophage results in an increase of cell membrane cholesterol and lipid raft content, which facilitate toll-like receptor 4 - MyD88-mediated pro-inflammatory response<sup>[48]</sup>. In this study, we found 1,25(OH)<sub>2</sub>D<sub>3</sub> markedly induced the expression of ABCA1 and ABCG1 in macrophages, suggesting that enhanced cholesterol efflux may play a crucial role in anti-inflammatory effect of vitamin D.

Previously, JNK/c-jun pathway has been reported to upregulate the expression of CYP27A1 in hepatocytes<sup>[20]</sup>. 1,25(OH)<sub>2</sub>D<sub>3</sub> activates the JNK/c-jun pathway in various cell lines<sup>[21,22,49]</sup>. Consistently, our study demonstrated that 1,25(OH)<sub>2</sub>D<sub>3</sub> upregulates the phosphorylation of JNK in macrophages. Furthermore, inhibitor of JNK activation blocks 1,25(OH)<sub>2</sub>D<sub>3</sub>-induced upregulation of CYP27A1 and 27HOC content, suggesting that JNK/c-Jun activation plays an important role in anti-atherogenic activity of vitamin D. Both VDR and LXR $\alpha$  are crucial lipid-activated nuclear receptors that shape macrophage function<sup>[50]</sup>. Earlier studies have shown that VDR is regulated by LXR $\alpha$  through its ability to modulate sterol regulatory element binding protein present in the promoter region of VDR gene<sup>[51]</sup>. In our study, we found that LXR $\alpha$  pathway is also regulated by VDR through its ability to modulate CYP27A1 and 27HOC (Fig. 6), suggesting the existence of crosstalk between LXR and VDR that stimulates the intracellular pathway to exert the anti-atherogenic effect.

In conclusion, the findings in this study revealed a novel mechanistic link between vitamin D deficiency and cardiovascular risk. Vitamin D supplementation significantly increased the levels of plasma HDL, and the expression of 27HOC/LXR $\alpha$  pathway, which contributes to alleviate cholesterol toxicity and macrophage pro-inflammatory polarization, resulting in protection against the development of atherosclerosis.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations

<b>ABCA1</b>	ATP binding cassette transporter A1
<b>ABCG1</b>	ATP binding cassette transporter G1
<b>CYP</b>	cytochrome P450
<b>HDL</b>	high density lipoprotein
<b>HOC</b>	27-hydroxy cholesterol
<b>LDL</b>	low density lipoprotein
<b>LXR</b>	liver-X-receptor
<b>MetS</b>	metabolic syndrome
<b>VD</b>	vitamin D
<b>VD-Def</b>	vitamin D deficient
<b>VD-Suf</b>	vitamin D sufficient
<b>VDR</b>	vitamin D receptor

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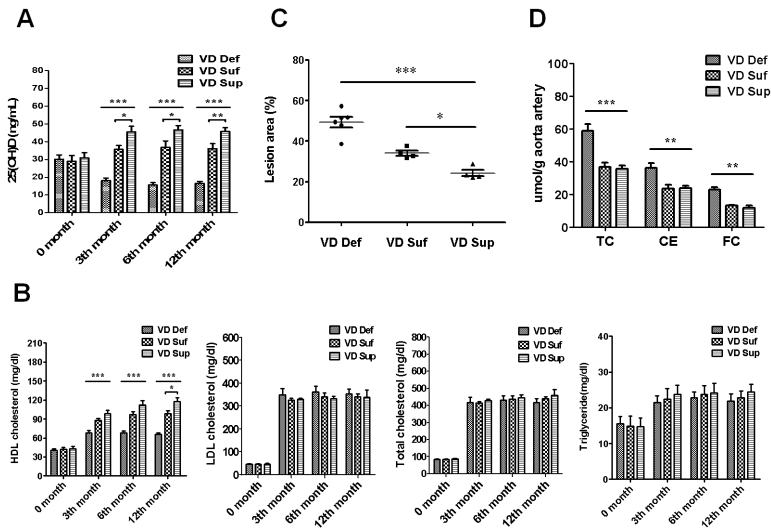
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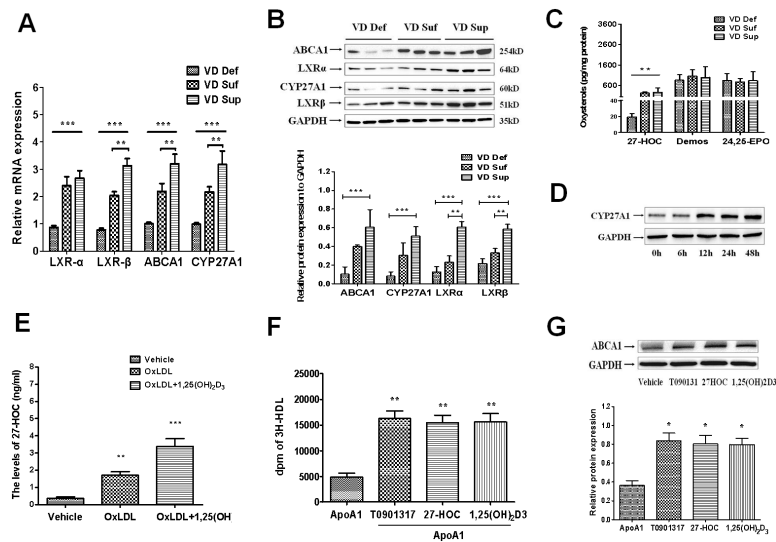
### Significance

Epidemiological studies suggest that vitamin D deficiency is related to a pro-atherogenic lipid profile and inflammation. However, the exact role of vitamin D in the progress of atherosclerosis has not been fully elucidated. Here, we found that vitamin D deficiency accelerated the development of atherosclerosis in hypercholesterolemic swine, which is very similar to the lipid metabolism and the progress of atherosclerosis in human. Vitamin D deficiency results in impaired cholesterol efflux in the liver and artery that lead to decreased high-density lipoprotein (HDL) level, cholesterol accumulation and M1 macrophage polarization in vascular wall. The  $1,25(\text{OH})_2\text{D}_3$  markedly induced CYP27A1 expression and increased 27HOC levels, which induced cholesterol efflux. These results suggest that vitamin D supplementation might serve as a novel therapeutic modality to increase HDL level and decrease severity of atherosclerosis in patients with cardiovascular disease risk.



**Figure 1. Vitamin D deficiency decreases plasma HDL level and promotes atherosclerosis in hypercholesterolemic swine**

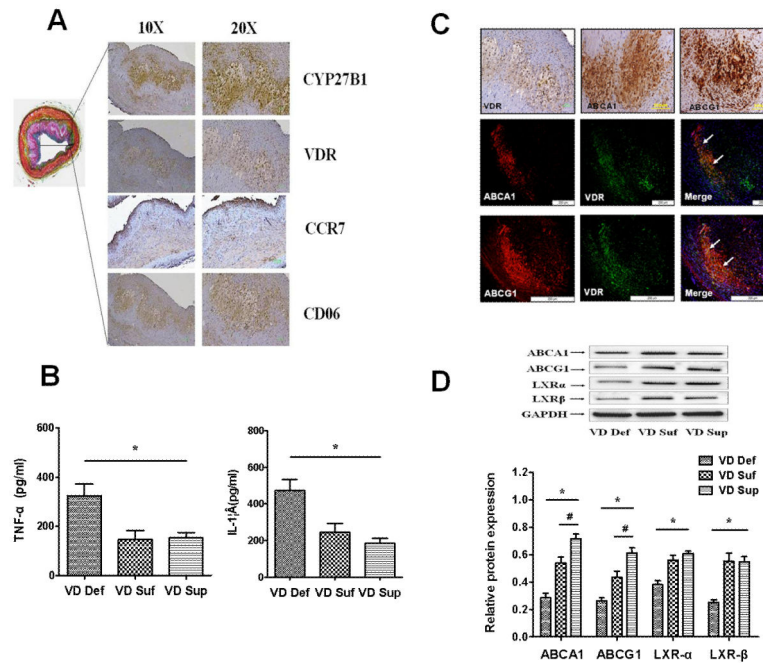
**Fig. 1A:** Plasma 25(OH)-cholecalciferol (D) and **Fig. 1B:** lipid levels in vitamin D deficient (VD Def, n=6), vitamin D sufficient (VD Suf, n=4) and vitamin D supplemented (VD Sup, n=4) hypercholesterolemic swine. **Fig. 1C:** Scatter plot showing lesion areas on the portion of the thoracic aorta of swine fed with the VD def (circle), VD Suf (square), and VD Sup (triangles) diet. **Fig. 1D:** Chemical analysis of cholesterol and cholesteryl ester in the common carotid arteries of swine. Data are expressed as means ± SEM. Statistical differences between groups were detected by one-way ANOVA with post-hoc analysis using Tukey's test. (\*\*\*) p < 0.01, VD Def compared to VD Suf or VD Sup; \*\* p < 0.01, VD Sup compared to VD Suf; \* p < 0.05, VD Sup compared to VD Suf).



**Figure 2. Vitamin D deficiency decreases 27 hydroxycholesterol (HOC) level in liver of hypercholesterolemic swine**

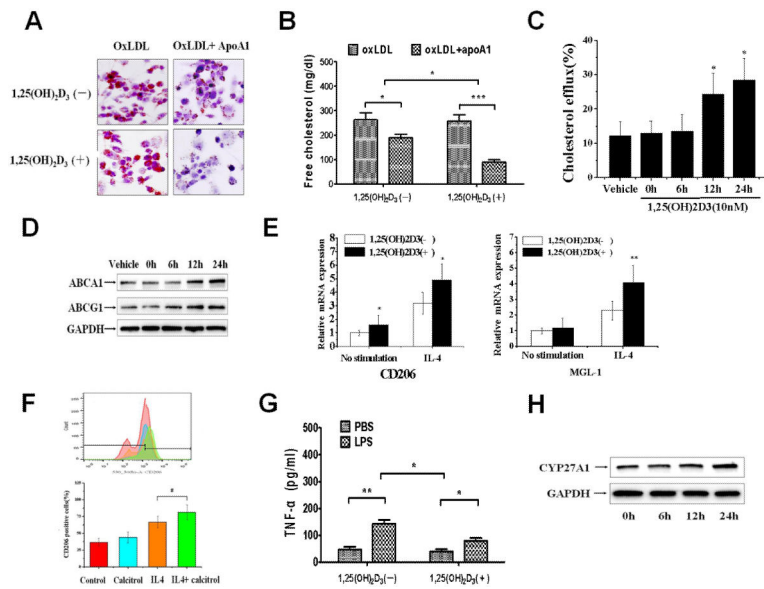
**Fig. 2A:** The mRNA expression of LXR- $\alpha$ , LXR- $\beta$ , ABCA1, and CYP27A1 in liver were determined by quantitative real-time PCR. **Fig. 2B:** The expression of LXR- $\alpha$ , LXR- $\beta$ , ABCA1, and CYP27A1 in liver were determined by Western-blot. **Fig. 2C:** The oxysterol levels in liver of hypercholesterolemic swine were determined by HPLC. Statistical differences between groups were detected by one-way ANOVA and Student's t test (\*\* $p < 0.01$ , VD Def compared to VD Suf or VD Sup; \* $p < 0.05$ , VD Sup compared to VD Suf,  $n=3$ ). **Fig. 2D:** HepG2 cells were cultured on six-well plates. Cells were treated with either vehicle (ethanol) or 1,25(OH) $_2$ D $_3$  (10nM) for 0, 3h, 6h, 12h and 24h. The protein was isolated for western-blot analysis of CYP27A1 expression. **Fig. 2E:** Cells were treated with either vehicle (ethanol), oxLDL (50 $\mu$ g/ml) and oxLDL (50 $\mu$ g/ml) + 1,25(OH) $_2$ D $_3$  (10nM) for 24h. The 27-HOC level in HepG2 cells was determined by HPLC (\*\* $p < 0.01$ , oxLDL +1,25(OH) $_2$ D $_3$  compared to oxLDL only or vehicle; \* $p < 0.05$ , 1,25(OH) $_2$ D $_3$  compared to vehicle,  $n=3$ ). **Fig. 2F:** HepG2 cells were treated with LXR agonist T0901317 (5 $\mu$ M), 27-HOC (1 $\mu$ M), and 1,25(OH) $_2$ D $_3$  (10nM), respectively, for 18h and then incubated with lipid free apoA-I (25 $\mu$ g/ml) for 12h. The radioactivity in [ $^3$ H]-HDL fraction was determined by liquid scintillation counting (\*\*  $p < 0.05$ , compared to apoA-I alone treatment,  $n=3$ ). **Fig. 2G:** The expression of ABCA1 in HepG2 cells was assessed by Western blot analysis. Values are means  $\pm$  SEM for  $n=3$  per group. (\*  $p < 0.05$ , compared to apoA-I alone treatment,  $n=3$ ).





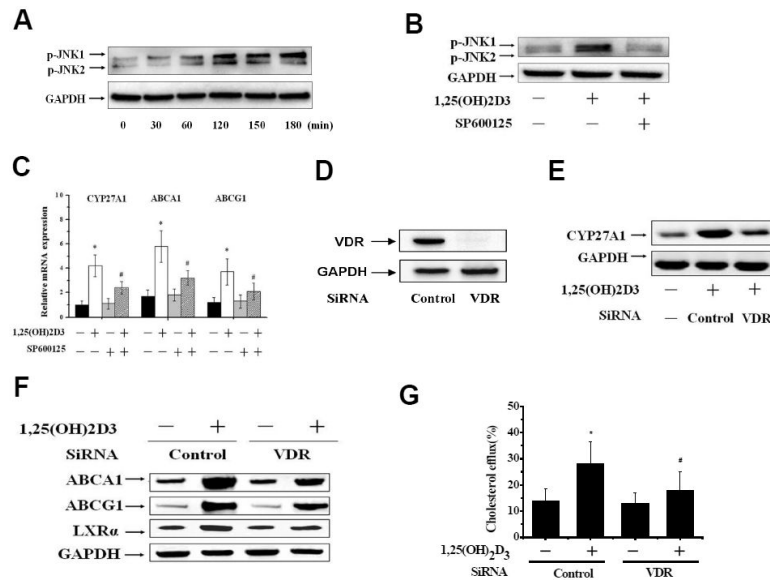
**Figure 3. Vitamin D increases cholesterol efflux and M2 macrophage polarization in artery of hypercholesterolemic swine**

**Fig.3A:** Vitamin D signal-related CYP27B1 and VDR are expressed at sites of M2 macrophage (CD206 positive) foam cells in coronary atherosclerotic lesions of hypercholesterolemic swine (Left= Movat stain, Right = Immunohistochemistry); **Fig. 3B:** Proteins from the common carotid arteries of different treatment groups of swine were isolated and analyzed to determine the levels of inflammatory cytokines TNF- $\alpha$  and IL-1 $\beta$  by ELISA. **Fig. 3C:** ABCA1/ABCG1 are co-expressed with VDR at sites of M2 macrophage foam cells in coronary atherosclerotic lesions of hypercholesterolemic swine; **Fig. 3D:** Western-blot analysis of ABCA1, ABCG1, LXR $\alpha$  and LXR  $\beta$  expression in the common carotid arteries of different hypercholesterolemic swine. Values are means  $\pm$  SEM for n=3 animals per group. Statistical differences between groups were detected by one-way ANOVA (\*p < 0.05, VD Def compared to VD Suf or VD Sup; #p < 0.05, VD Sup or VD Suf, n=3)

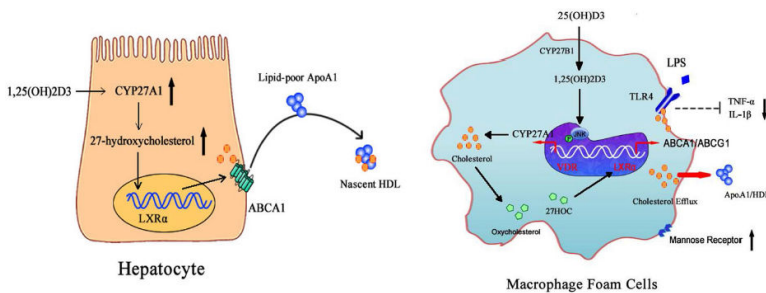


**Figure 4. 1,25(OH)<sub>2</sub>D<sub>3</sub> promotes cholesterol efflux and M2 polarization in macrophage-derived foam cells via upregulating 27HOC**

**Fig. 4A:** THP-1 macrophages were treated with oxLDL (50 µg/ml) for 48 h, then cultured with apoA1(25 µg/ml) under 1,25(OH)<sub>2</sub>D<sub>3</sub>-deficient medium (*Top*) or 1,25(OH)<sub>2</sub>D<sub>3</sub> (10nM)-supplement medium (*bottom*) for 24h. Macrophages were stained with Oil Red O. **Fig. 4B:** The intercellular free cholesterol (FC) was determined by the cholesterol quantitation kit. n = 3; \*\*\*p<0.001, \*\*p<0.01, \*p<0.05, two-way analysis of variance (ANOVA) with Bonferroni's post-hoc test. **Fig. 4C:** Liquid scintillation counting was performed to determine the intercellular cholesterol efflux. **Fig. 4D:** THP-1 macrophage-derived foam cells were treated with 1,25(OH)<sub>2</sub>D<sub>3</sub> (10nM) for 0, 6h, 12h and 24h, the expression of ABCA1 and ABCG1 were determined by western-blot. **Fig. 4E:** After cultured with 1,25(OH)<sub>2</sub>D<sub>3</sub> (10 nM) for 24h, THP-1 macrophage-derived foam cells were treated with IL-4 (20ng/ml) for 6h. The M2 marker (CD206 and MGL-1) mRNA levels in cells were determined by quantitative real-time PCR. **Fig. 4F:** Representative histograms of CD206/mannose receptor (MR) in THP-1 macrophage-derived foam cells exposed to vehicle (Ctrl), 1,25(OH)<sub>2</sub>D<sub>3</sub> (10 nM), IL-4 (20ng/ml), or IL-4+1,25(OH)<sub>2</sub>D<sub>3</sub>. **Fig. 4G:** After cultured with 1,25(OH)<sub>2</sub>D<sub>3</sub> (10nM) for 24h, THP-1 macrophage-derived foam cells were treated with LPS (10ng/ml) for 12h. The level of TNFα was quantified by ELISA. n = 3; \*\*p<0.01, \*p<0.05, two-way analysis of variance (ANOVA) with Bonferroni's post-hoc test. **Fig. 4H:** THP-1 macrophage derived foam cells were treated with either vehicle (ethanol) or 1,25(OH)<sub>2</sub>D<sub>3</sub> (10 nM) for 0, 6, 12, 24h. The expression of CYP27A1 was determined by Western-blot.



**Figure 5. 1,25(OH)<sub>2</sub>D<sub>3</sub> induces CYP27A1 in a VDR-dependent JNK/SAPK signal manner**  
**Fig. 5A:** THP-1 macrophage-derived foam cells were cultured with either 1,25(OH)<sub>2</sub>D<sub>3</sub> (10nM) or vehicle at the indicated time point. Cells were harvested and subjected to Western blot analysis to monitor the levels of p-JNK1/2. **Fig. 5B:** THP-1 macrophages were treated with 1,25(OH)<sub>2</sub>D<sub>3</sub> (10nM) or JNK inhibitor SP600125 (2µM) for 3h. Total proteins were subjected to immunoblot analyses with antibody against p-JNK1/2. **Fig. 5C:** THP-1 macrophage-derived foam cells were treated with SP600125 (2µM) for 30 min, followed by stimulation with 1,25(OH)<sub>2</sub>D<sub>3</sub> (10nM) for 24 h. Total RNA were subjected to real-time PCR for CYP27A1, ABCA1 and ABCG1 (\*p<0.01 compared to untreated groups, #p<0.01 compared to 1,25(OH)<sub>2</sub>D<sub>3</sub> groups,. **Fig. 5D:** THP-1 macrophage-derived foam cells were transfected with control or VDR siRNA for 48h, protein samples were immunoblotted with anti-VDR antibody. **Fig. 5E:** THP-1 macrophage-derived foam cells transfected with control (WT) or VDR siRNA (VDR<sup>-/-</sup>) were treated with 1,25(OH)<sub>2</sub>D<sub>3</sub> for 24h. CYP27A1 was analyzed by Western-blot. **Fig. 5F:** ABCA1, ABCG1, and LXR-α expression were analyzed by Western-Blot; **Fig. 5G:** The intercellular cholesterol efflux was determined by liquid scintillation counting. \*p<0.01 compared to other groups. The data represent the mean ± SE from three separate experiments with triplicate samples.



**Figure 6. Schematic representation of vitamin D regulation of nascent HDL formation and macrophage polarization via promoting cholesterol efflux in hepatocytes and macrophage-derived foam cells**

In hepatocytes (**Left**), the  $1,25(\text{OH})_2\text{D}_3$  increases CYP27A1 expression as well as the production of the endogenous LXR ligand, 27-hydroxycholesterol(27HOC), which stimulated ABCA1-mediated cholesterol efflux and nascent HDL formation. In macrophages-derived foam cells (**Right**),  $1,25(\text{OH})_2\text{D}_3$  markedly induced CYP27A1 expression and increased 27HOC levels, which induced LXR $\alpha$ , ABCA1 and ABCG1 expression, stimulated intercellular cholesterol efflux, reduced the cholesterol accumulation and attenuated LPS induced M1-polarization via a VDR-dependent JNK/SAPK signal manner.

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