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$1,25(OH)_2$ Vitamin D Inhibits Foam Cell Formation and Suppresses Macrophage Cholesterol Uptake in Patients With Type 2 Diabetes Mellitus

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***Background*—**Cardiovascular disease is the leading cause of death among those with diabetes mellitus. Vitamin D deficiency is associated with an increased risk of cardiovascular disease in this population. To determine the mechanism by which vitamin D deficiency mediates accelerated cardiovascular disease in patients with diabetes mellitus, we investigated the effects of active vitamin D on macrophage cholesterol deposition.

***Methods and Results*—**We obtained macrophages from 76 obese, diabetic, hypertensive patients with vitamin D deficiency (25-hydroxyvitamin D $<$80 nmol/L; group A) and 4 control groups: obese, diabetic, hypertensive patients with normal vitamin D (group B; n=15); obese, nondiabetic, hypertensive patients with vitamin D deficiency (group C; n=25); and nonobese, nondiabetic, nonhypertensive patients with vitamin D deficiency (group D; n=10) or sufficiency (group E; n=10). Macrophages from the same patients in all groups were cultured in vitamin D—deficient or 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] –supplemented media and exposed to modified low-density lipoprotein cholesterol. $1,25(OH)_2D_3$ suppressed foam cell formation by reducing acetylated or oxidized low-density lipoprotein cholesterol uptake in diabetic subjects only. Conversely, deletion of the vitamin D receptor in macrophages from diabetic patients accelerated foam cell formation induced by modified LDL. $1,25(OH)_2D_3$ downregulation of c-Jun N-terminal kinase activation reduced peroxisome proliferated–activated receptor-γ expression, suppressed CD36 expression, and prevented oxidized low-density lipoprotein–derived cholesterol uptake. In addition, $1,25(OH)_2D_3$ suppression of macrophage endoplasmic reticulum stress improved insulin signaling, downregulated SR-A1 expression, and prevented oxidized and acetylated low-density lipoprotein–derived cholesterol uptake.

***Conclusion*—**These results identify reduced vitamin D receptor signaling as a potential mechanism underlying increased foam cell formation and accelerated cardiovascular disease in diabetic subjects. **(*Circulation*. 2009;120:687-698.)**

Key Words: atherosclerosis ■ diabetes mellitus ■ inflammation ■ nutrition ■ vitamin D

Approximately 20 million Americans have type 2 diabetes mellitus, a disease associated with hypertension and increased risk of cardiovascular disease (CVD). This combination of metabolic abnormalities is the leading cause of morbidity and mortality in industrialized countries.[1] Several studies indicate that in poorly controlled diabetes mellitus, altered insulin signaling and/or hyperglycemia promote unbalanced cholesterol metabolism, which favors oxidized low-density lipoprotein (oxLDL) cholesterol retention in the vascular wall.[2] However, the effects of intensive glucose lowering on macrovascular complications in this population are unpredictable and may result in increased mortality.[3] Therefore, identification of glucose-independent factors that modulate macrophage cholesterol deposition and vascular infiltration is critical to understanding the development of CVD in diabetics.

Clinical Perspective on p 698

Vitamin D deficiency is a largely unacknowledged epidemic associated with CVD. Approximately 1 billion people worldwide have low levels of 25-hydroxyvitamin D [25(OH)D; $<$80 nmol/L], the principal circulating storage

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Table. Demographic Characteristics

Characteristics	Group A: Vitamin D–Deficient Diabetic Subjects (n=76)	Group B: Vitamin D–Sufficient Diabetic Subjects (n=15)	Group C: Obese Vitamin D–Deficient Nondiabetic Subjects (n=25)	Group D: Normal Vitamin D–Deficient Control Subjects (n=10)	Group E: Normal Vitamin D–Sufficient Control Subjects (n=10)
Age, y	55±2.4	59±3	50±3.1	42±4	45±3.4
Women, %	82	80	80	70	95
BMI, kg/m^2	32±1	31.6±1.1	31.9±1.2	22±0.8*†‡	23±1*†‡
Blacks, %	86.9‖	80	84	30	30
Smoker, %	22.3	20	20	10	10
25(OH)D, nmol/L	39±4	93±10*‡§	34±3	32±4	97±10*‡§
Systolic blood pressure, mm Hg	132±2.5	136±4	110±3*†	104±4.2*†	113±4*†
Diastolic blood pressure, mm Hg	85±8.8	74±4	75±1.7	71±1.5	78±5
Duration of diabetes, y	5±2.5	9±0.7	0	0	0
Retinopathy, %	1	0	0	0	0
Nephropathy, %	7	10	0	0	0
Neuropathy, %	10	10	0	0	0
Only diabetic oral medications, %	71	70	0	0	0
Diabetic oral medications plus long-acting insulin, %	29	30	0	0	0
Total cholesterol, mg/dL	173±5	165±13	151±8	152±12	149±12
Triglycerides, mg/dL	150±14	137±16	77±7*	76±11	74±8
Lipid medications, %	33	40	28	20	10
Hemoglobin 1_{AC}, %	8±0.2	7.8±0.3	5.1±0.1*†	5.4±0.1*†	5.2±0.6*†

BMI indicates body mass index. Data are expressed as mean±SEM for continuous variables and as ratios for categorical data.

*$P<0.05$ vs group A, †$P<0.05$ vs group B, ‡$P<0.05$ vs group C, §$P<0.05$ vs group D by 1-way ANOVA with Tukey posttest analysis for parametric variables; ‖$P<0.001$ by χ^2 test for multiple categorical variables.

form of vitamin D, and more than half of middle-aged vitamin D–deficient patients develop CVD.[4,5] In hypertensive patients, low serum vitamin D levels increase the risk of CVD by 60%.[6] In women with type 2 diabetes mellitus, the prevalence of vitamin D deficiency is a third higher than that of control subjects, and low vitamin D levels nearly double the risk of developing CVD compared with diabetic patients with normal vitamin D levels.[7,8] Similarly, in diabetic patients with mild renal failure, low vitamin D levels increase the relative risk of CVD compared with their vitamin D–sufficient counterparts.[9] Finally, intervention with 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$], the active form of vitamin D, or paricalcitol [a less calcemic $1,25(OH)_2D$ analog] decreased cardiovascular mortality in patients with end-stage renal disease, suggesting favorable outcomes from normal vitamin D status in attenuating atherosclerosis.[10] Therefore, understanding the mechanism of the accelerated atherosclerosis induced by vitamin D deficiency may be crucial for treating the epidemic of CVD in diabetics.

Increased proinflammatory cytokines in the vessel wall contribute to immune cell recruitment and modified LDL cholesterol deposition by increasing scavenger receptor expression and cholesteryl ester synthesis and by decreasing cholesterol efflux.[11] Active vitamin D metabolites [$1,25(OH)_2D_3$ or its analogs] promote monocyte/macrophage differentiation and diminish proinflammatory cytokine release by immune mononuclear cells in diabetics, suggesting that $1,25(OH)_2D_3$ signaling may regulate monocyte vascular infiltration and macrophage cholesterol retention in the vessel wall in these patients.[12,13]

Initial studies exploring the influence of $1,25(OH)_2D_3$ on macrophage cholesterol metabolism are contradictory. In human promyelocytic leukemic (HL-60) and monocytic (THP-1) cell lines, $1,25(OH)_2D_3$ decreased scavenger receptor A-1 (SR-A1) expression and acetylated LDL (AcLDL) binding.[14,15] In contrast, in normal subjects, $1,25(OH)_2D_3$ increased cholesteryl ester formation stimulated by AcLDL, but only under conditions of lipid deprivation.[16] The aim of our study was to determine whether vitamin D deficiency contributes to the increase in macrophage-mediated cholesterol deposition seen in patients with diabetes mellitus and to investigate the effects of $1,25(OH)_2D_3$ on macrophage cholesterol deposition in diabetics and nondiabetic matched controls.

Methods

Population

Our study population included 76 obese, hypertensive adult subjects with type 2 diabetes mellitus on medications with an age of 55±2 years, body mass index of 32±1.0 kg/m^2, 25(OH)D of 39±5.9 nmol/L, diabetes duration of 5±2.5 years, and hemoglobin A_{1c} level of 8±0.2% (group A). We excluded recently diagnosed diabetes mellitus, pregnancy, known coronary artery disease, and normal vitamin D levels [serum 25(OH)D ≥80 nmol/L]. This population was compared with 4 different control groups: obese, diabetic, hypertensive patients with normal vitamin D levels [serum 25(OH)D, 94±10 nmol/L; group B; n=15]; obese, nondiabetic, hypertensive patients with vitamin D deficiency [serum 25(OH)D, 34±3 nmol/L; group C; n=25]; and normal-weight control subjects with no history of diabetes mellitus or hypertension with either normal vitamin D levels [serum 25(OH)D, 97±10 nmol/L; group D; n=10] or vitamin D deficiency [serum 25(OH)D, 32±4 nmol/L; group E; n=10] (the Table). Subjects were recruited from the outpatient clinic at Barnes-Jewish Hospital, St Louis (Mo). Participation was voluntary, and each subject provided written informed consent and was approved by the Human Research Protection Office of Washington University School of Medicine.

Isolation and Preparation of Primary Human Monocytes

Peripheral monocytes were isolated by standard Ficoll isolation techniques and selected by CD14 marker positivity (Miltenyi Biotec, Auburn, Calif). CD14$^+$/CD11b$^+$ cell purity reached 97% as assessed by flow cytometry (FACStar Plus, BD, Franklin Lakes, NJ). Each patient's cells were differentiated into macrophages by culturing with 100 ng/mL of macrophage colony-stimulating factor for 7 days in vitamin D–deficient media [deficient in both 25(OH)D and 1,25(OH)$_2$D; obtained by DMEM plus 10% charcoal/dextran-treated FBS] with or without supplementation of 1,25(OH)$_2$D$_3$ at 10^{-8} mol/L (courtesy Adriana Dusso, Washington University). Inhibition of phosphorylation of c-Jun N-terminal kinase (JNKp) was obtained in macrophages cultured in vitamin D–deficient media stimulated with modified LDL cholesterol for 6 hours after preincubation with SP600125 (100 μmol/L) for 2 hours (SA Bioscience Corp, Frederick, Md). Induction of endoplasmic reticulum (ER) stress was obtained by adding thapsigargin (0.25 μmol/L) (Sigma, St Louis, Mo) to cultured macrophages for 24 hours in 1,25(OH)$_2$D-supplemented conditions. In a subgroup of diabetic patients from group A, macrophages in either vitamin D–deficient or 1,25(OH)$_2$D$_3$-supplemented media were cultured in high-glucose (450 mg/dL) (routine culture conditions used in this study) and normal-glucose (100 mg/dL) conditions for 7 days before cholesterol homeostasis and CD36 expression were measured. In these glucose conditions and after stimulation with oxLDL for 6 hours, macrophages were cell sorted by staining with phycoerythrin-labeled anti-CD11b and FITC-labeled anti-CD14 (e-Biosciences, San Diego, Calif). They were then evaluated for membrane CD36 expression by use of a primary anti-CD36 and a secondary IgM CD36 biotin-labeled antibody (BD Bioscience, San Jose, Calif). FACScan flow cytometry was performed in 20 000 cells.

Mouse Peritoneal Macrophages

Mouse peritoneal macrophages from CD36$^{-/-}$, SR-A1$^{-/-}$, and wild-type mice (n=12 per each group) were isolated 3 days after intraperitoneal injection of 4% thioglycollate solution and cultured in vitamin D–deficient or 1,25(OH)$_2$D$_3$-supplemented media (see the online-only Data Supplement).[17] Cholesterol uptake was performed after 6 hours of stimulation with modified cholesterol in mouse macrophages cultured for 7 days in vitamin D–deficient or 1,25(OH)$_2$D$_3$-supplemented media. Foam cell formation in vivo was determined by measuring total cholesterol and triglycerides in peritoneal macrophages 4 hours after isolation from LDR$^{-/-}$ mice fed a vitamin D–deficient (n=5) or –sufficient (n=5) Western diet for 10 weeks (Harlan, TD 07019). This methodology is also described in the online-only Data Supplement.

Macrophage Cholesterol Homeostasis

Foam cell formation (Oil Red O stain), cholesteryl ester formation, and cholesterol uptake, binding, and efflux were assessed in macrophages after stimulation with oxLDL or AcLDL from same subjects' monocytes cultured in vitamin D–deficient or 1,25(OH)$_2$D$_3$-supplemented media for 7 days. A detailed description is presented in the online-only Data Supplement.

Plasmids and siRNA

Macrophages obtained from diabetic subjects were cultured for 7 days in vitamin D–deficient or 1,25(OH)$_2$D$_3$-supplemented media and then infected with lentivirus containing either peroxisome proliferated–activated receptor-γ (PPARγ)–siRNA, vitamin D receptor (VDR)–siRNA hairpin, or control siRNA for 48 hours. Protein, mRNA, and cholesterol uptake was determined 48 hours after recovering from viral infection. Descriptions of lentivirus generation are included in the online-only Data Supplement.

Gene Expression, Western Blot Analysis, and JNK Activity

Quantitative reverse-transcription polymerase chain reaction analyses were performed by Sybergreen methodologies. Results were normalized to the housekeeping gene L32. Western blot analyses from macrophage protein extracts were normalized to β-actin expression. Phosphorylated AKT and AKT were determined in 12-hour serum-starved macrophages before or after insulin incubation (100 nmol/L). A detailed description is included in the online-only Data Supplement. JNKp also was determined by a cell-based ELISA kit (SA Bioscience).

Statistical Analysis

Experiments were carried out with duplicate or triplicate samples. All data are expressed as mean±SEM for continuous variables and as ratios for categorical data. Gaussian distribution of continuous variables was verified by Kolmogorov-Smirnov distance. Statistical significance of differences was calculated with the paired t test for parametric data involving 2 groups and ANOVA for parametric data with Tukey test for multiple groups. Two-way ANOVA was performed to test the main effects of each factor and to test for interaction between variables. A χ^2 test was used for multiple group analysis for categorical variables. Differences were considered statistically significant at $P \leq 0.05$.

Results

Population

In group A, we studied 76 obese adult subjects, primarily black women, with type 2 diabetes mellitus and a concurrent diagnosis of hypertension (80%). Twenty-nine percent of patients were on oral hypoglycemics and long-acting insulin; the remainder took oral medications only. All groups were similar with respect to age, gender, total cholesterol, lipid medications, and tobacco use. Control groups B and C were similar to group A with regard to body mass index, ethnicity, cholesterol levels, and lipid medications, and group C had a lower hemoglobin A$_{1C}$ and better blood pressure control compared with groups A and B. Control groups D and E had significantly lower body mass index and more ethnic diversity than all other groups. Groups D and E had lower systolic blood pressure compared with groups A and B. All parametric variables tested were normally distributed (the Table).

1,25(OH)$_2$D$_3$ Prevents Foam Cell Formation

In obese diabetics (group A), macrophages cultured in vitamin D–deficient media exhibited a significant increase in foam cell formation induced by oxLDL and AcLDL compared with macrophages cultured in 1,25(OH)$_2$D$_3$-supplemented conditions (see arrows in Figure 1A). In group A, 1,25(OH)$_2$D$_3$-treated macrophages exposed to AcLDL or oxLDL had almost 50% less cholesteryl ester formation than macrophages maintained on vitamin D–deficient media ($P<0.01$ for both; Figure 1B). However, in macrophages obtained from nondiabetic controls (group C), AcLDL- or oxLDL-induced cholesteryl ester formation was reduced by 1,25(OH)$_2$D$_3$ but not significantly compared with macrophages cultured in vitamin D–deficient media ($P=0.1$ and $P=0.09$, respectively; Figure 1B).

To determine the influence of vitamin D status on macrophage foam cell formation in vivo, we extracted peritoneal macrophages from LDLR$^{-/-}$ mice fed a vitamin D–deficient or –sufficient high-fat diet for 10 weeks. Mice on both diets

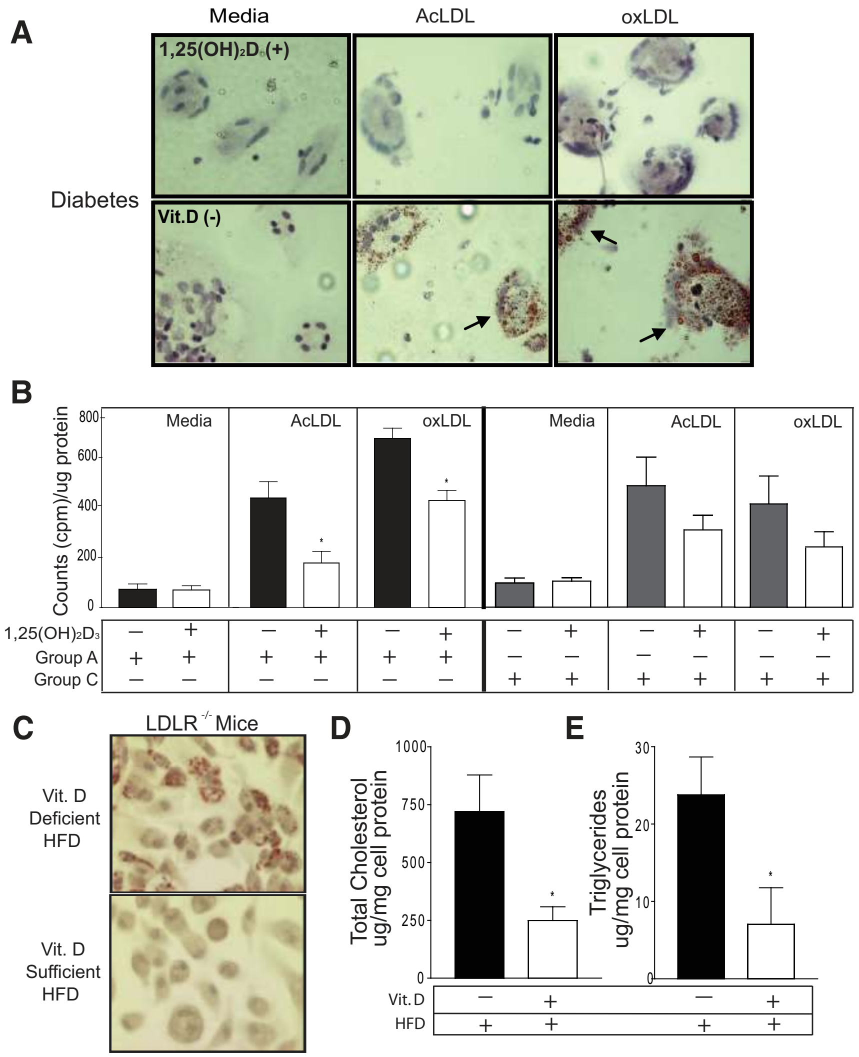

Figure 1. $1,25(OH)_2D_3$ prevents foam cell formation. Macrophages stained with Oil Red O. A, Diabetic subjects (group A). Top, $1,25(OH)_2D_3$-treated cells; bottom, vitamin D–deficient cells. Arrowheads indicate foam cells. B, Cholesteryl ester formation in macrophages from diabetics (group A) incubated in vitamin D–deficient (black bars) or $1,25(OH)_2D_3$-supplemented (white bars) media or in macrophages from nondiabetic, vitamin D–deficient nondiabetic controls (group C) ($n=8$ per group) incubated in vitamin D–deficient (gray bars) or $1,25(OH)_2D_3$-supplemented (white bars) media (*$P<0.01$ vs vitamin D deficient). C, Oil Red O stain. D, Cholesterol. **E,** Triglycerides from peritoneal macrophages from $LDR^{-/-}$ mice fed vitamin D–deficient or –sufficient high-fat diet ($n=5$ per group) (*$P<0.05$ vs vitamin D deficient).

had similar serum cholesterol (1117±56 versus 1244±73 mg/dL; $P=0.2$) and triglyceride (312±27 versus 312±42 mg/d; $P=0.8$) levels, but serum 25(OH)D levels were significantly lower in mice fed the high-fat, vitamin D–deficient diet (19±3.4 versus 87±10 nmol/L; $P<0.01$). Macrophages isolated from vitamin D–sufficient hypercholesterolemic mice exhibited fewer Oil Red O droplets and lower total cholesterol and triglycerides compared with macrophages isolated from vitamin D–deficient mice (Figure 1C through 1E). These observations suggest that a normal vitamin D status may be sufficient to inhibit foam cell formation in vivo.

$1,25(OH)_2D_3$ Decreases Macrophage Cholesterol Uptake

To investigate the mechanism underlying the reduction of foam cell formation induced by vitamin D in diabetics, we assessed cholesterol uptake and efflux in macrophages cultured in either vitamin D–deficient or $1,25(OH)_2D_3$-supplemented media. Confocal microscopy after fluorescence-labeled 1,1′dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine-labeled (Dil)-oxLDL or Dil-AcLDL stimulation showed that diabetes-derived macrophages (group A) cultured in $1,25(OH)_2D_3$-supplemented media had decreased oxLDL and AcLDL cholesterol uptake both qualitatively and quantitatively by 40% to 50%, respectively, compared with macrophages cultured in vitamin D–deficient media ($P<0.01$ for both; Figure 2A and 2B). Of note, macrophages from a subgroup of multiple patients from group A that were cultured in $1,25(OH)_2D_3$ at concentrations of 10^{-8} mol/L showed activation of CYP24 expression (VDR receptor target gene) and suppression of cholesterol uptake after stimulation with oxLDL or AcLDL in contrast to $1,25(OH)_2D_3$ concentrations of 10^{-10} or 10^{-12} mol/L (Figure IA, IB, and IC of the online-only Data Supplement). Incubation with $1,25(OH)_2D_3$ at a concentration of 10^{-8} mol/L also suppressed macrophage cholesterol binding induced by Dil-oxLDL or Dil-AcLDL by ≈20% ($P<0.03$ for both conditions; Figure 2C and 2D).

In human macrophages, high glucose upregulates and HMG-CoA reductase inhibitors downregulate cholesterol uptake of oxLDL and scavenger receptor expression of CD36.[18,19] In diabetic patients (group A), $1,25(OH)_2D_3$ suppression of oxLDL cholesterol uptake was independent of the glucose conditions ($P=0.2$). $1,25(OH)_2D_3$ suppresses oxLDL cholesterol uptake by 30% and 40% in macrophages cultured in high- and low-glucose conditions, respectively, compared with macrophages cultured in vitamin D–deficient media

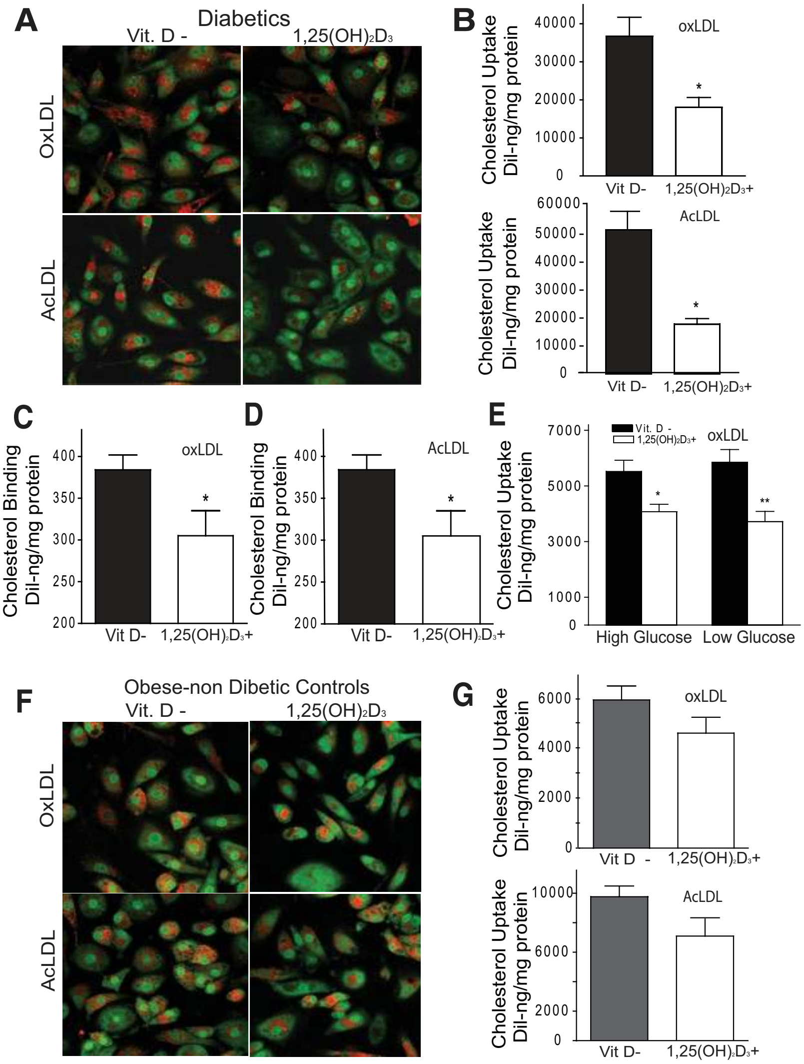

Figure 2. 1,25(OH)$_2$D$_3$ decreases macrophage cholesterol uptake in diabetic subjects. A, Macrophage cholesterol uptake assessed by confocal microscopy in diabetic subjects (group A). Red represents labeled cholesterol uptake after 6 hours of stimulation with Dil-oxLDL (top) or Dil-AcLDL (bottom); green fluorescence, nuclear counterstains. B, Quantification of cholesterol uptake in the same patients' macrophages cultured in vitamin D–deficient (black bars) and 1,25(OH)$_2$D$_3$-supplemented (white bars) media. Mean fluorescence absorbance after Dil-oxLDL (top) (n=24 subjects) or after Dil-AcLDL (bottom) (n=12 subjects) stimulation (*$P<0.01$ vs vitamin D deficient). C and D, Cholesterol binding in macrophages stimulated by Dil-oxLDL or Dil-AcLDL at 4°C for 2 hours (n=8 per experiment) (*$P<0.03$ vs vitamin D deficient). E, Quantification of cholesterol uptake induced by oxLDL in macrophages in high- or normal-glucose conditions while on vitamin D–deficient or 1,25(OH)$_2$D$_3$-supplemented media (n=6 per condition) (*$P<0.02$ and **$P<0.01$ vs vitamin D deficient). F and G, Nondiabetic subjects (group C). F, Cholesterol uptake assessed by confocal microscopy. Red represents labeled cholesterol uptake after 6 hours of stimulation with Dil-oxLDL (top) or AcLDL (bottom). G, Quantification of cholesterol uptake after incubation with Dil-oxLDL (top) or Dil-AcLDL (bottom) in the same patients' macrophages cultured in vitamin D–deficient (gray bars) and 1,25(OH)$_2$D$_3$-supplemented media (white bars) (n=25 subjects).

with high- and low-glucose conditions ($P<0.02$ and $P<0.01$, respectively; Figure 2E). In vitamin D–deficient diabetics from group A on HMG-CoA reductase inhibitors, 1,25(OH)$_2$D$_3$ suppresses oxLDL-stimulated cholesterol uptake by 45% compared with macrophages cultured in vitamin D–deficient conditions (Figure II of the online-only Data Supplement). These data suggest that 1,25(OH)$_2$D$_3$ regulation of cholesterol metabolism is independent of macrophage glucose conditions and in these cultured conditions is not influenced by a patient's intake of HMG-CoA reductase inhibitors.

In macrophages from vitamin D–sufficient diabetics (group B), culture in 1,25(OH)$_2$D$_3$-supplemented media also elicited a reduction in oxLDL- and AcLDL-induced cholesterol uptake of ≈45% compared with macrophages cultured in vitamin D–deficient conditions ($P<0.01$ and $P<0.05$, respectively; Figure IIIA and IIIB of the online-only Data Supplement). However, in macrophages from vitamin D–deficient, nondiabetic controls (group C), 1,25(OH)$_2$D$_3$ did not significantly reduce cholesterol uptake after induction with oxLDL or AcLDL compared with macrophages maintained on vitamin D–deficient media ($P=0.07$ and $P=0.1$, respectively; Figure 2F and 2G). Similarly, 1,25(OH)$_2$D$_3$ did not suppress macrophage oxLDL or AcLDL cholesterol uptake in vitamin D–deficient (group D) or –sufficient (group E) normal volunteers (Figure IIIC and IIID of the online-only Data Supplement). These findings indicate clear differences between control subjects and diabetic subjects in 1,25(OH)$_2$D$_3$ regulation of macrophage cholesterol metabolism.

Cholesterol efflux was determined in macrophages from diabetic subjects from group A after incubation for 24 hours with labeled oxLDL. 1,25(OH)$_2$D$_3$ supplementation did not regulate passive, high-density lipoprotein–stimulated, or apolipoprotein AI–stimulated macrophage cholesterol efflux (Figure IVA of the online-only Data Supplement). 1,25(OH)$_2$D$_3$ supplementation did decrease macrophage ABCA1 mRNA expression by 30% ($P<0.05$) but did not suppress ABCG1 and SR-B1 mRNA expression compared with cells on vitamin D–deficient media (Figure IVB of the online-only Data Supplement).

Decrease in Macrophage Cholesterol Uptake Induced by 1,25(OH)$_2$D$_3$ Is CD36 and SR-A1 Dependent

Membrane scavenger receptors SR-A1 and CD36 are essential for recognition and internalization of modified LDL particles.[20] In diabetes-derived macrophages (group A) cultured in high and normal glucose, macrophages supplemented

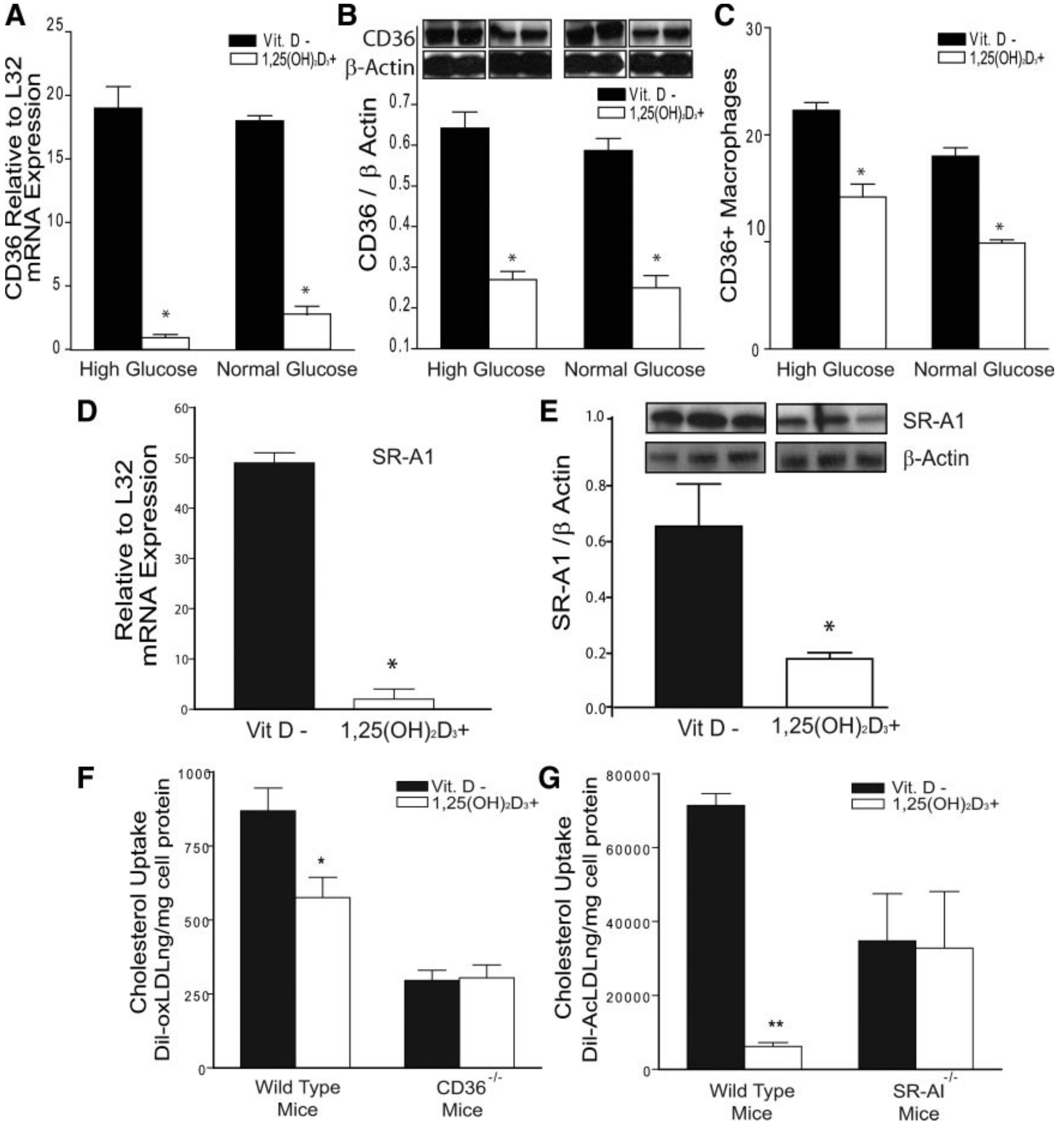

Figure 3. $1,25(OH)_2D_3$ suppression of cholesterol uptake is CD36 and SR-A1 dependent. Macrophages from diabetic subjects (group A) cultured in vitamin D–deficient (black bars) or $1,25(OH)_2D_3$-supplemented media (white bars) with high- or normal-glucose conditions were used to prepare RNA and protein. A through C, After stimulation for 6 hours with oxLDL. A, Quantitative polymerase chain reaction of CD36 mRNA expression relative to L32 expression (n=10) (*$P<0.0001$ vs vitamin D deficient). B, Densitometric analysis of CD36 expression normalized to β-actin (n=4) (*$P<0.002$ vs vitamin D deficient). C, Percent of $CD14^+/CD11b^+$ macrophages positive for CD36 via flow cytometry (n=4) (*$P<0.001$ vs vitamin D deficient). D and E, In high-glucose conditions after stimulation for 6 hours with AcLDL. D, Quantitative polymerase chain reaction of SR-A1 mRNA relative to L32 expression (n=10) (*$P<0.0001$ vs vitamin D deficient). E, Densitometric analysis of SR-A1 expression normalized to β-actin (*$P<0.001$ vs vitamin D deficient). F and G, Quantification of cholesterol uptake after stimulation with oxLDL or AcLDL in thioglycollate-elicited macrophages from $CD36^{-/-}$, $SR\text{-}A1^{-/-}$, and wild-type littermates cultured in vitamin D–deficient or $1,25(OH)_2D_3$-supplemented media. Mean fluorescence of DiI absorbance was measured after 6 hours of incubation with DiI-oxLDL or DiI-AcLDL (n=12 per group per condition) (*$P<0.03$, **$P<0.001$ vs vitamin D deficient).

with $1,25(OH)_2D_3$ had ≈6-fold lower CD36 mRNA and ≈40% decreased total and membrane-associated CD36 protein expression after oxLDL stimulation in both glucose conditions compared with macrophages cultured in vitamin D–deficient media ($P<0.01$ for all; Figure 3A through 3C). $1,25(OH)_2D_3$ effects on CD36 mRNA, protein, and membrane-associated protein were independent of glucose concentrations ($P=0.3$, $P=0.7$, and $P=0.3$, respectively). $1,25(OH)_2D_3$ also decreased macrophage SR-A1 mRNA 20-fold and reduced SR-A1 protein expression after AcLDL stimulation compared with macrophages cultured in vitamin D–deficient media ($P<0.001$ for both; Figure 3D and 3E). However, in macrophages from vitamin D–deficient, nondiabetic controls (group C), $1,25(OH)_2D_3$ did not significantly suppress macrophage CD36 or SR-A1 protein expression (Figure VA and VB of the online-only Data Supplement).

To clarify the role of CD36 and SR-A1 expression in the prevention of foam cell formation by $1,25(OH)_2D_3$, we measured cholesterol uptake after modified LDL stimulation in peritoneal macrophages from wild-type, CD36$^{-/-}$, and SR-A1$^{-/-}$ mice cultured in vitamin D–deficient or 1,25(OH)$_2$D$_3$-supplemented media. 1,25(OH)$_2$D$_3$ suppression of oxLDL- and AcLDL-induced cholesterol uptake was dependent on mouse genotype ($P<0.01$ for each genotype). In wild-type mice, 1,25(OH)$_2$D$_3$ suppressed cholesterol uptake induced by oxLDL (Figure 3F) and AcLDL (Figure 3G) compared with macrophages on vitamin D–deficient media ($P<0.03$ and $P<0.001$, respectively). However, the effect of vitamin D deficiency on cholesterol uptake was blunted by the absence of CD36 or SR-A1 in macrophages (Figure 3F and 3G). These results suggest that 1,25(OH)$_2$D$_3$ suppression of oxLDL and AcLDL cholesterol uptake is at least partially mediated by CD36 and SR-A1.

1,25(OH)$_2$D$_3$ Suppression of JNKp Prevents Foam Cell Formation

Stress-related JNK is highly activated in human atherosclerotic plaques and is known to mediate CD36- and SR-A1–dependent foam cell formation in mice.[21,22] OxLDL activates several mitogen-activated protein kinases, including extracellular signal-regulated kinases, JNK, and p38 mitogen-activated protein kinase, but the role of these pathways in vitamin D regulation of oxLDL or AcLDL uptake is unknown.[21,23] In vitamin D–deficient diabetics (group A), macrophages cultured in 1,25(OH)$_2$D$_3$-supplemented media have decreased phosphorylation of JNK1, JNK2, and JNK3 before and after oxLDL or AcLDL stimulation. However, no changes in activation of p38 or extracellular signal-regulated kinase-1 phosphorylation were found in these subjects (Figure 4A and 4B). In this population, JNKp analysis by ELISA confirmed that macrophages cultured in 1,25(OH)$_2$D$_3$-supplemented media have 50% lower JNKp levels after either oxLDL or AcLDL stimulation compared with macrophages cultured in vitamin D–deficient media ($P<0.002$ and $P<0.03$, respectively; Figure 4C and 4D). No change in activation of mitogen-activated protein kinase family members was present in nondiabetic controls (group C; Figure VC and VD of the online-only Data Supplement). In macrophages from diabetics (group A), the suppressive effects of JNK inhibition on cholesterol uptake induced by oxLDL or AcLDL were dependent on vitamin D status ($P<0.01$ for both). In macrophages cultured in vitamin D–deficient media, incubation with JNKp inhibitor (SP600125) decreased cholesterol uptake stimulated by oxLDL (Figure 4E) and AcLDL (Figure 4F) by 50% compared with vitamin D–deficient macrophages not exposed to the JNK inhibitor ($P<0.03$ and $P<0.01$, respectively). No additional JNKp downregulation (data not shown) or cholesterol uptake was observed after SP600125 was added to macrophages cultured in 1,25(OH)$_2$D$_3$-supplemented media (Figure 4E and 4F). These data suggest that vitamin D downregulation of JNKp is a unifying signaling pathway that suppresses oxLDL and AcLDL cholesterol uptake in diabetic patients.

1,25(OH)$_2$D$_3$ Downregulation of JNKp Suppresses Macrophage oxLDL Cholesterol Uptake via PPARγ

PPARγ is expressed in foam cells of human atherosclerotic lesions.[24] PPARγ can be activated by oxLDL and controls macrophage CD36 expression.[20] In diabetics (group A), macrophages cultured in 1,25(OH)$_2$D$_3$-supplemented media had significantly less PPARγ protein expression after oxLDL stimulation compared with macrophages cultured in vitamin D–deficient media. Addition of JNKp inhibitor to vitamin D–deficient or 1,25(OH)$_2$D$_3$-supplemented media almost abolished oxLDL-stimulated PPARγ protein expression compared with macrophages without JNK inhibitor (Figure 5A). These data suggest that 1,25(OH)$_2$D$_3$-mediated downregulation of JNKp suppresses PPARγ expression.

In vitamin D–deficient conditions, macrophages from diabetic patients (group A) infected with PPARγ-siRNA lentivirus had almost totally suppressed PPARγ and CD36 expression without altering JNKp compared with control siRNA-infected cells (Figure 5B). Reduction of PPARγ significantly suppressed oxLDL-stimulated cholesterol uptake induced by vitamin D deficiency ($P<0.01$; Figure 5C). However, no interaction between PPARγ inhibition and vitamin D status was identified ($P=0.3$). These data suggest that 1,25(OH)$_2$D$_3$-mediated downregulation of JNKp reduces macrophage PPARγ and CD36 expression and suppresses oxLDL-stimulated cholesterol uptake in diabetic patients. PPARγ downregulation did not alter SR-A1 expression or AcLDL-induced cholesterol uptake (data not shown).

1,25(OH)$_2$D$_3$ Downregulation of ER Stress Prevents Modified LDL-Stimulated Macrophage Cholesterol Uptake and Suppresses SR-A1 and CD36 Expression

Defective macrophage insulin signaling induces the accumulation of misfolded proteins in the ER lumen, causing stress.[2] Persistent ER stress leads to increased SR-A1 expression and JNK activation.[25] In diabetic patients (group A), 1,25(OH)$_2$D$_3$-supplemented media improved macrophage insulin signaling by increasing insulin-induced AKT phosphorylation (Figure 5D). In addition, 1,25(OH)$_2$D$_3$ significantly suppressed the expression of ER stress protein markers (GADD34 and CHOP; Figure 5E) and reduced CD36 and SR-A1 expression (Figure 3A through 3E). Conversely, induction of ER stress with thapsigargin in 1,25(OH)$_2$D$_3$-treated macrophages increased SR-A1, CD36, PPARγ, GAD34, and CHOP protein expression and promoted JNK activation compared with macrophages cultured in 1,25(OH)$_2$D$_3$-supplemented media without thapsigargin (Figure 5F). Thapsigargin-induced ER stress blunted the 1,25(OH)$_2$D$_3$ suppression of oxLDL- and AcLDL-induced cholesterol uptake compared with macrophages cultured in 1,25(OH)$_2$D$_3$-supplemented media without thapsigargin ($P<0.03$ and $P<0.01$, respectively; Figure 5G and 5H). By improving insulin signaling and ER stress in macrophages from diabetic patients, 1,25(OH)$_2$D$_3$ modulates JNK activity and PPARγ expression and suppresses modified LDL cholesterol uptake.

Activation of VDR Signaling Prevents Foam Cell Formation

1,25(OH)$_2$D$_3$ acts mostly through the VDR, a member of the nuclear receptor superfamily of transcriptional regulators, but also through rapid, nongenomic actions on binding to several

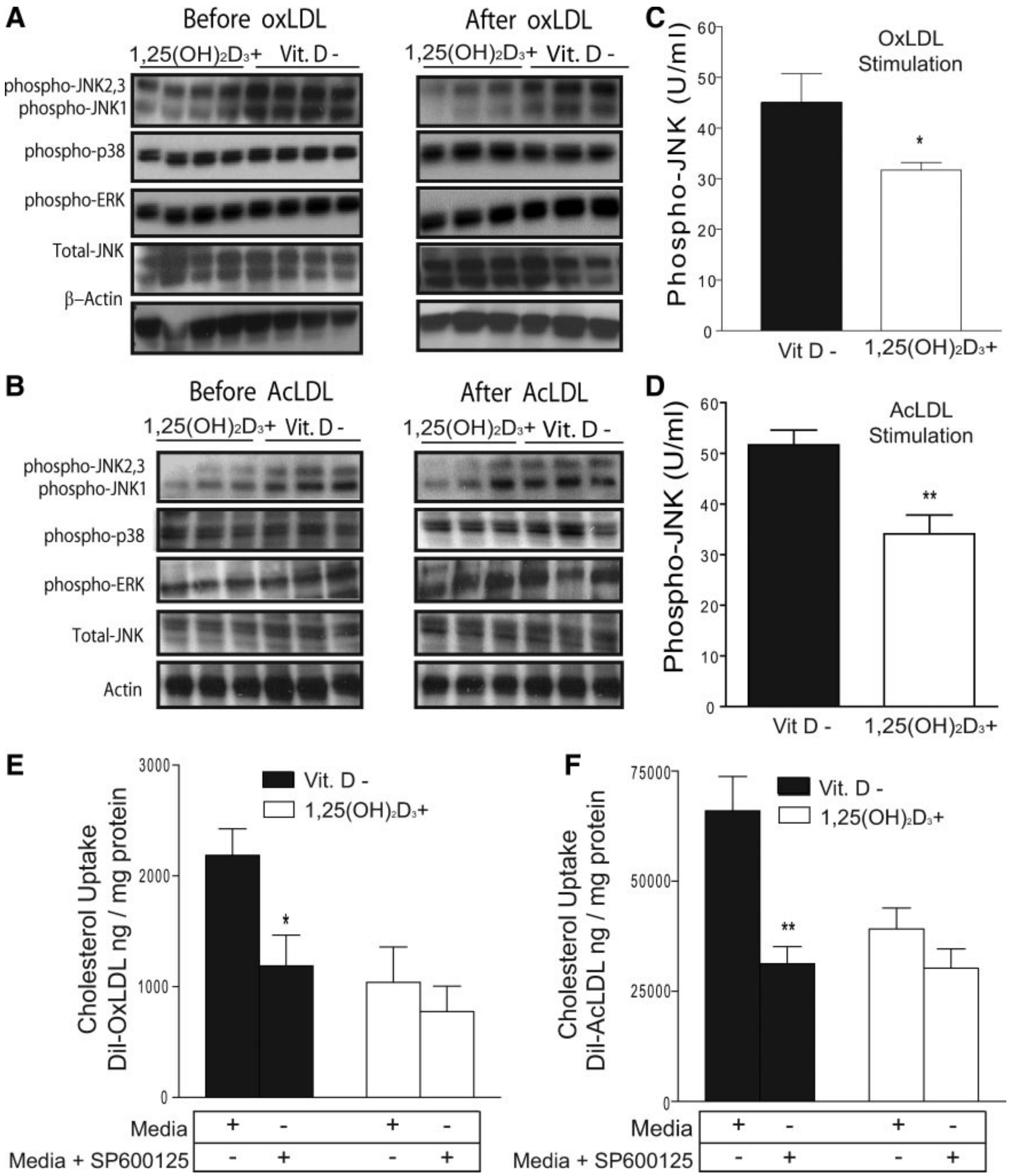

Figure 4. JNK phosphorylation is required for 1,25(OH)$_2$D$_3$ suppression of foam cell formation. A and B, Western blot from macrophages from diabetic subjects (group A) cultured in vitamin D–deficient or 1,25(OH)$_2$D$_3$-supplemented media before and after stimulation with oxLDL or AcLDL. Expression of total JNK and β-actin was used as a loading control. C and D, ELISA of JNKp normalized to total JNK in macrophages cultured in vitamin D–deficient (black bars) and 1,25(OH)$_2$D$_3$-supplemented (white bars) media after oxLDL (n=10) or AcLDL (n=6) (*$P<0.002$, ** $P<0.03$ vs vitamin D deficient). E and F, Cholesterol uptake in macrophages cultured with or without JNK inhibitor in vitamin D–deficient (black bars) or 1,25(OH)$_2$D$_3$-supplemented (white bars) media after stimulation with Dil-oxLDL or Dil-AcLDL (n=8 per group per condition) (*$P<0.03$,**$P<0.02$ vs no treatment with JNKp inhibitor).

other proteins near the plasma membrane of target cells.[26] To identify whether the 1,25(OH)$_2$D$_3$-suppressive effects on cholesterol uptake are VDR dependent, we infected diabetes-derived macrophages (group A) cultured in 1,25(OH)$_2$D$_3$-supplemented media with lentivirus containing either siRNA VDR hairpins or control siRNA. VDR-siRNA–infected macrophages showed an 80% reduction in VDR mRNA and protein levels and a 6-fold reduction in the mRNA levels of a classic VDR target gene, the 24-hydroxylase (CYP24), compared with control siRNA-infected macrophages ($P<0.001$; Figure 6A through 6C).

Confocal microscopy and quantification of cholesterol uptake confirmed that 1,25(OH)$_2$D$_3$ decreased macrophage cholesterol uptake induced by AcLDL and oxLDL by 50% and 60%, respectively, only in macrophages with intact VDR signaling pathways; this response was blunted in macrophages lacking a VDR signaling pathway ($P<0.001$ and $P<0.02$, respectively; Figure 6D and 6E). In addition,

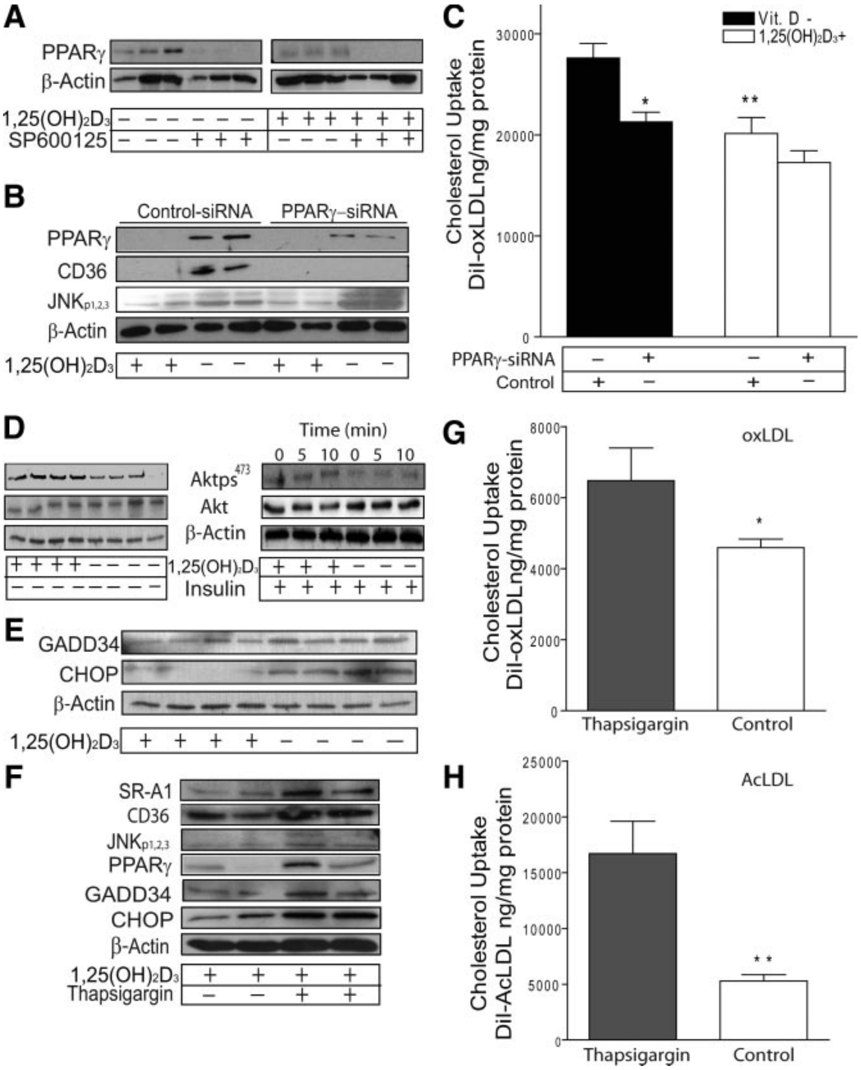

Figure 5. 1,25(OH)$_2$D$_3$ suppresses macrophage ER stress and PPARγ expression in diabetic subjects (group A). A, PPARγ expression from macrophages cultured in vitamin D–deficient or 1,25(OH)$_2$D$_3$-supplemented media before and after the addition of JNK inhibitor (representative of n=6). B, PPARγ, CD36, and JNKp expression from macrophages cultured in vitamin D–deficient or 1,25(OH)$_2$D$_3$-supplemented media after infection with PPARγ-siRNA or control siRNA lentivirus (representative of n=8).C, Cholesterol uptake in macrophages cultured in vitamin D–deficient (black bars) or 1,25(OH)$_2$D$_3$-supplemented (white bars) media after stimulation for 6 hours with Dil-oxLDL and infection with siRNA lentivirus (n=8) (*$P<0.01$, **$P<0.02$ vs vitamin D–deficient control siRNA-infected cells). D, Phosphorylated AKT from macrophages cultured in vitamin D–deficient or 1,25(OH)$_2$D$_3$-supplemented media before (representative of n=8) and after (representative of n=4) insulin stimulation. Expression of total AKT and β-actin was used for loading control. E, GADD34 and CHOP expression from macrophages cultured in vitamin D–deficient or 1,25(OH)$_2$D$_3$-supplemented media. β-Actin expression was used for loading control (representative of n=8). F, SR-A1, CD36, JNKp, PPARγ, GADD34, and CHOP expression from macrophages cultured in 1,25(OH)$_2$D$_3$-supplemented media with or without ER induction by thapsigargin (representative of n=8). G and H, Quantification of cholesterol uptake in macrophages cultured in 1,25(OH)$_2$D$_3$-supplemented media with (black bars) or without (white bars) thapsigargin. Mean fluorescence absorbance after 6 hours of incubation with Dil-oxLDL (G; n=8) or Dil-AcLDL (H; n=8) (*$P<0.03$, **$P<0.01$ vs thapsigargin-treated cells).

1,25(OH)$_2$D$_3$ downregulated CD36, SR-A1, and PPARγ expression, as well as JNKp, in the presence of intact VDR signaling, but these effects were reduced in VDR-siRNA–infected macrophages (Figure 6F). These data confirm the importance of the activation of VDR signaling in the regulation of both scavenger receptors and cell signaling pathways involved in macrophage foam cell formation (Figure 6G).

Discussion

Despite aggressive lipid-lowering strategies aimed at type 2 diabetics, CVD remains the leading cause of mortality for these individuals. In this study, we demonstrate that activation of vitamin D receptor signaling prevents foam cell formation by reducing modified LDL cholesterol uptake in macrophages from diabetic patients. Through suppression of ER stress and JNK activation, 1,25(OH)$_2$D$_3$ downregulates 2 critical scavenger receptors involved in macrophage cholesterol deposition. Impairment of VDR signaling confirmed acceleration of foam cell formation in diabetics. Taken together, these results suggest that modulation of vitamin D signaling is a potential therapeutic target to prevent vascular disease progression.

25(OH)D has minimal intrinsic activity and needs to be converted into 1,25(OH)$_2$D to activate VDR. The direct relationship between 25(OH)D replacement and increased serum 1,25(OH)$_2$D in anephric patients demonstrates that increased local production of 1,25(OH)$_2$D occurs in extrarenal tissues, particularly macrophages.[27,28] Therefore, increased local macrophage conversion of 25(OH)D to its active form by vitamin D replacement is a potential therapeutic target to suppress foam cell formation and vascular disease progression in diabetics.

Macrophage scavenger receptors play a decisive role in transforming macrophages into foam cells.[20] Targeted disruption of the SR-A1 or CD36 in diet-induced insulin-resistant mouse models confirms the importance of both receptors in the development of atherosclerosis.[29] During hyperglycemia and/or an insulin-resistant state, increased scavenger receptor expression promotes foam cell formation and is considered a link between diabetes mellitus and atherosclerosis.[18,30] Previous studies indicated the importance of 1,25(OH)$_2$D$_3$ downregulation of SR-A1 receptor expression in tissue plasminogen activator–treated THP-1 macrophages.[15] In this study, we provide evidence that 1,25(OH)$_2$D$_3$ activation of VDR de-

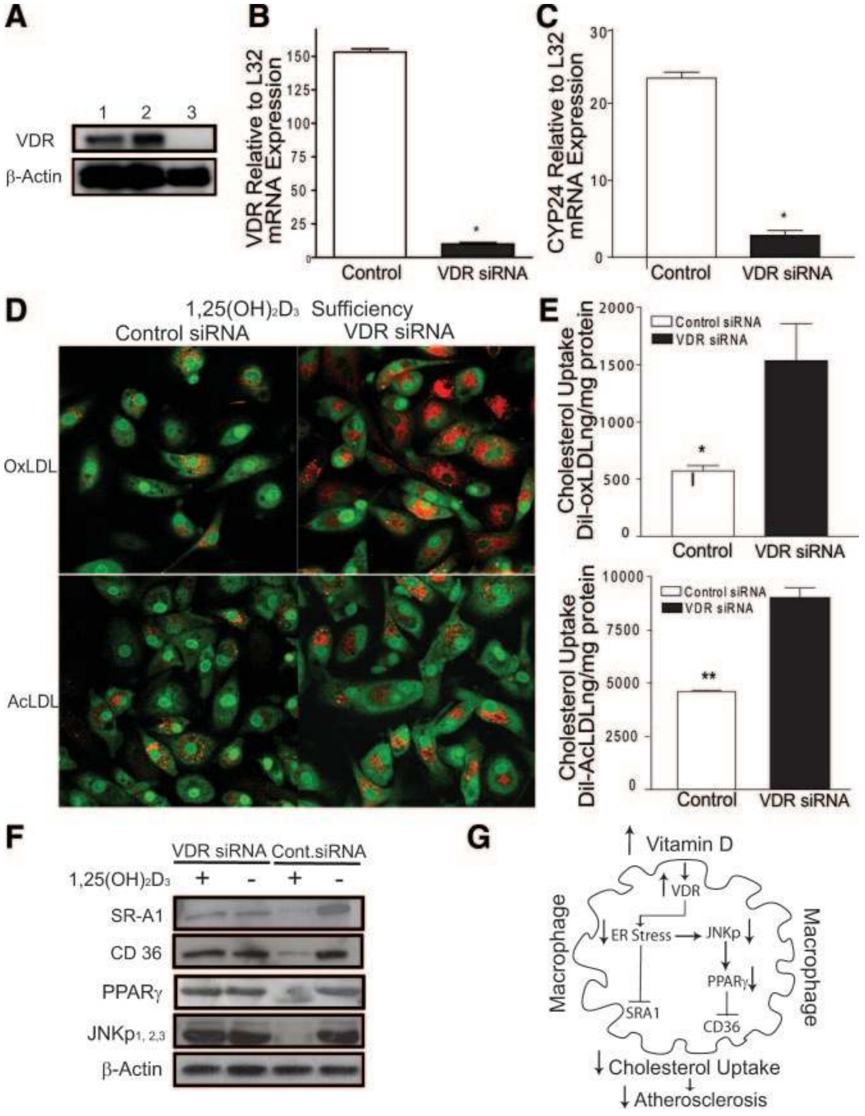

Figure 6. 1,25(OH)$_2$D$_3$ activation of VDR signaling prevents macrophage cholesterol uptake in diabetics (group A). A, VDR (top) and β-actin (bottom) expression. Lines 1 and 2, siRNA-control–infected cells; line 3, VDR-siRNA–infected cells. B and C, Quantitative polymerase chain reaction of VDR mRNA and CYP24 mRNA expression in response to 1,25(OH)$_2$D$_3$ supplementation in macrophages infected with either VDR-siRNA (black bars) or control siRNA (white bars) lentivirus, respectively (n=8 per group) (*P<0.0001 vs control siRNA macrophages). D, Cholesterol uptake assessed by confocal microscopy. Red represents labeled cholesterol uptake after DiI-oxLDL (top) or DiI-AcLDL (bottom) stimulation; green fluorescence, nuclear counterstains. E, Cholesterol uptake after incubation with DiI-oxLDL (top) or DiI-AcLDL (bottom) in macrophages cultured in 1,25(OH)$_2$D$_3$-supplemented media after infection with siRNA lentivirus (n=5) (*P<0.01, *P<0.02 vs VDR-siRNA macrophages). F, SR-A1, CD36, PPARγ, and JNKp expression from macrophages infected with either VDR-siRNA or control siRNA lentivirus cultured in vitamin D–deficient media or 1,25(OH)$_2$D$_3$-supplemented media. G, Mechanistic pathways involved in 1,25(OH)$_2$D$_3$ suppression of foam cell formation.

creases macrophage cholesterol uptake by reducing CD36 and SR-A1 expression in diabetics. Furthermore, deletion of macrophage VDR interrupts 1,25(OH)$_2$D$_3$ downregulation of CD36 and SR-A1 expression and accelerates oxLDL and AcLDL cholesterol uptake. These data suggest that activation of VDR regulates a unifying cell signaling pathway that suppresses both scavenger receptor expression and uptake of modified LDL cholesterol.

Several mechanisms may be involved in the ability of 1,25(OH)$_2$D$_3$ to suppress macrophage cholesterol ester accumulation in diabetics, but JNK is particularly important. JNK is activated by stressors such as oxidative stress, fatty acids, and inflammatory cytokines, which are commonly present in insulin-resistant tissues.[31] In apolipoprotein E–null mice, pharmacological inhibition of JNK activity and genetic JNK2 deficiency decreased atherosclerosis, in part because of the inhibition of CD36- and SR-A1–dependent foam cell formation.[17,22] 1,25(OH)$_2$D$_3$ modulates JNK signaling activation in response to extracellular stress stimulation.[32] In concert, p38/JNK activation regulates VDR gene expression, further supporting the interaction between this signaling pathway and vitamin D.[33] In this study, we found that 1,25(OH)$_2$D$_3$ is a natural inhibitor of macrophage JNKp in diabetics. 1,25(OH)$_2$D$_3$ downregulation of the JNK pathway suppresses cholesterol uptake by the scavenger receptors CD36 and SR-A1. Furthermore, targeted deletion of VDR interrupts the ability of 1,25(OH)$_2$D$_3$ to inhibit foam cell formation and JNK activation. These data suggest that downregulation of JNK stress signaling by VDR activation is a unifying mechanism for both scavenger receptor–induced foam cell formation and possibly atherogenesis.

PPARγ expression is induced in foam cells of human atherosclerotic lesions.[24] PPARγ plays a critical role in maintaining macrophage cholesterol homeostasis by positively regulating the expression of genes involved in cholesterol storage and efflux.[2,19,34] Previous observations indicate that 1,25(OH)$_2$D$_3$ is capable of repressing PPARγ expression in adipocytes.[35] Consistent with this possibility, we find that 1,25(OH)$_2$D$_3$ downregulation of JNK activation suppresses PPARγ and CD36 expression, reducing oxLDL-derived cholesterol uptake. Conversely, inhibition of macrophage PPARγ expression suppresses oxLDL-derived cholesterol uptake induced by culturing macrophages in vitamin D–deficient media. No interaction was identified between PPARγ inhibition and vitamin D status, but we suspect this was secondary to the small sample size. PPARγ inhibition did not prevent 1,25(OH)$_2$D$_3$ suppression of SR-A1 expression and AcLDL-derived cholesterol uptake, suggesting that 1,25(OH)$_2$D$_3$ downregulation of JNKp-PPARγ-CD36 only partially explains the 1,25(OH)$_2$D$_3$ effects on foam cell formation.

In insulin-resistant mouse models, persistent metabolic stress activates ER stress regulation of SR-A1 expression and JNK2 activation in macrophages.[25,36] Here, we show that 1,25(OH)$_2$D$_3$ couples ER stress to regulation of SR-A1 expression and JNK activation in macrophages from diabetic patients. ER stress activation blunts the 1,25(OH)$_2$D$_3$ suppression of JNKp and modified LDL cholesterol uptake, suggesting that the prevention of ER stress by 1,25(OH)$_2$D$_3$ is critical for limiting macrophage cholesterol accumulation. Previous studies indicated that increased cholesterol trafficking to the ER induces macrophage apoptosis and leads to plaque instability. The p38-CHOP and JNK2 signaling pathways are known apoptotic pathways triggered by ER stress.[25] Increased CHOP is also shown in macrophages in advanced atherosclerotic lesions in humans.[37] Thus, 1,25(OH)$_2$D$_3$ suppression of ER stress and foam cell formation led us to speculate that 1,25(OH)$_2$D$_3$ potentially influences not only the initiation of foam cell formation but also the progression of the atherosclerotic plaque.

This study shows clear differences between control subjects and diabetic subjects in 1,25(OH)$_2$D$_3$ regulation of macrophage cholesterol metabolism. In a previous study with normal, nondiabetic subjects, 1,25(OH)$_2$D$_3$ increased cholesteryl ester formation in monocytes stimulated with AcLDL only after 24 hours of lipid deprivation.[16] In our study, in the absence of lipid deprivation, 1,25(OH)$_2$D$_3$ did not induce a significant effect on cholesterol metabolism in obese, nondiabetic, hypertensive control subjects. In contrast, robust 1,25(OH)$_2$D$_3$ suppression of foam cell formation in diabetic subjects was observed. In diabetic subjects and insulin-resistant mice models, defective insulin signaling and elevated JNK activity promote foam cell formation.[17,18,36] Induction of insulin sensitivity reverses abnormal cholesterol metabolism in macrophages.[34] In this study, we showed that induction of insulin sensitivity and/or downregulation of ER stress–JNK activity by 1,25(OH)$_2$D$_3$ may represent the potential mechanisms whereby 1,25(OH)$_2$D$_3$ suppresses cholesterol metabolism in diabetic subjects.

This study reveals a novel mechanistic link between vitamin D deficiency in macrophages and foam cell formation in type 2 diabetics. Interventional studies are needed to assess the effects of vitamin D status on CVD in diabetic subjects and the impact of diabetes mellitus on the macrophage conversion of 25(OH)D to 1,25(OH)$_2$D$_3$.

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Disclosures

None.

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CLINICAL PERSPECTIVE

Cardiovascular disease (CVD) is the leading cause of death among diabetics. Intensive glucose-lowering effects on macrovascular complications in this population are unpredictable and may result in increased mortality. Therefore, identification of glucose-independent factors modulating macrophage cholesterol deposition is critical to our understanding of the development of CVD in diabetics. Approximately 1 billion people worldwide have 25-hydroxyvitamin D deficiency or insufficiency, and more than half of middle-aged vitamin D–deficient patients develop CVD. In hypertensive patients, low serum vitamin D levels increase the risk of CVD by 60%. In women with type 2 diabetes mellitus, the prevalence of vitamin D deficiency is a third higher than in control subjects, and low vitamin D levels nearly double the risk of developing CVD compared with diabetic patients with normal vitamin D levels. Therefore, understanding the mechanism of accelerated atherosclerosis induced by vitamin D deficiency may be crucial for treating CVD in diabetics. In this study, we demonstrate that active vitamin D suppresses foam cell formation by reducing acetylated or oxidized low-density lipoprotein cholesterol uptake in diabetics. Through downregulation of macrophage stress-related c-Jun N-terminal kinase signaling and suppression of endoplasmic reticulum stress, active vitamin D reduces peroxisome proliferated–activated receptor-γ expression, suppresses CD36 and scavenger receptor A-1 expression, and prevents macrophage cholesterol deposition. Deletion of vitamin D receptor confirmed acceleration of foam cell formation. This study reveals a novel mechanistic link between vitamin D deficiency in macrophages and foam cell formation in type 2 diabetics and suggests that modulation of vitamin D signaling is a potential therapeutic target to prevent vascular disease progression.

SUPPLEMENTAL MATERIAL

Supplemental Methods.

Mouse Peritoneal Macrophage Isolation

Mouse peritoneal macrophages lacking either CD36 (provided by Roy L. Silverstein at Cleveland Clinic) or SR-A1 (Jackson Laboratory) and macrophages from WT mice were isolated 3 days after intraperitoneal injection of 4% thioglycollate solution, as previously described. [1] Cells were selected by fluorescence-activated cell sorting for F4/80 (e-Biosciences, San Diego, CA) and CD11b (BD Bioscience, San Jose, CA) antigen expression. Macrophages (0.5×10^6 cells per well in 12-well plates) were cultured in vitamin D-deficient or $1,25(OH)_2D_3$-supplemented (10^{-8} M) media. Macrophages were subsequently evaluated for cholesterol uptake and harvested to isolate RNA or protein according to standard methods. Foam-cell formation in vivo was determined in peritoneal macrophages 4h after isolation from $LDR^{-/-}$ mice fed a vitamin D-deficient (n=5) or -sufficient (n=5) western diet for 10 weeks (Harlan, TD 07019). Macrophages were homogenized in chloroform:methanol (2:1 vol/vol) and extracts analyzed using Thermo DMA triglyceride and cholesterol reagents (Thermo Electron Corp, Waltham, MA). [2]

Macrophage Cholesterol Homeostasis

Macrophages derived from same patients' peripheral blood mononuclear cells were cultured for 7 days with or without $1,25(OH)_2D_3$-supplementation. To assess foam-cell formation, macrophage slides were fixed with 5% paraformaldehyde for 15 minutes and stained with Oil-red-O and hematoxylin. [3] Analysis of cholesteryl ester formation was performed as previously described. [4, 5] Macrophages were incubated with a mix of either oxLDL (200 μg/mL) or AcLDL (200 μg/mL) with ^{3}H oleic acid (0.1 mM) (American Radiolabeled Chemicals Inc.) for 6 hours. Lipids were extracted, dried under nitrogen, and separated by TLC. Spots representing the cholesteryl ester and free oleic acid were counted. Results were normalized to total cell protein concentrations.

Cholesterol uptake was performed as previously described.[6] Macrophages (0.5 × 10^6 cells/well) in 12-well plates cultured with or without 1,25(OH)$_2$D$_3$-supplementation were incubated with 10 μg/mL oxLDL labeled with 1,1'-dioctadecyl-3,3,3',3'-tetramethyl indocarbocyanine percholate (Invitrogen) for 6 hours. Results were normalized to total cell protein concentrations. Cholesterol efflux was performed as previously described.[7] Macrophages (0.5 × 10^6 cells/well) in 12-well plates cultured with or with 1,25(OH)$_2$D$_3$ supplementation were incubated for 24 hours with labeled oxLDL (300 μg/mL) preincubated with 5 mCi of ^{3}H cholesterol (American Radiolabeled Chemical, Inc.). Free cholesterol efflux was initiated by the replacement of the medium with serum-free medium alone or media containing apolipoprotein AI (25 μg/mL) or HDL (50 μg/mL). Supernatant fluid and cells were assessed for radioactivity. Efflux of ^{3}H cholesterol from the cells into the medium was calculated as percent of total ^{3}H cholesterol incorporated in the cells.

Gene Expression

qPCR analyses were done in a GeneAmp 7700 Sequence Detector (Applied Biosystems) as previously described.[8] RNA not subjected to reverse transcription was included in each assay as a negative control. We used the following oligonucleotides: *VDR* forward, 5'-ACCCTGGTGACTTTGACCG -3'; *VDR* reverse, 5'-GGCAATCTCCAT TGAAGGGG-3'; *SR-A1* forward, 5'-TATGGCACAGTGGGATGACTTTC -3'; *SR-A1* reverse, 5'- GAGGAAGCAAAGCTGTCACTGAG-3'; *CD36* forward, 5'- TGTAAC CCAGGACGCT GAGG; *CD36* reverse, 5'- GAAGGTTCGAAGATGG CACC-3'; *ABCA1* forward, 5'- AACAGCAGTTGGATGGCTTAGA-3'; *ABCA1* reverse, 5'- CACAGAACCAT TACTGGACTGGA-3'; *ABCG1* forward, 5'-CAGGAAGATTAGACACTGTGG-3'; *ABCG1* reverse, 5'-GAAAGGGGAATGGAGAGAAGA-3'; *CYP24* forward, 5'- CTGCCCC ATTGACAAAAGGC-3'; *CYP24* reverse, 5'- CTAACCGTCGGTC ATCAGC-3'; *L32* forward, 5'- GAAGATTCAAGGGCCAGATCC-3'; *L32* reverse, 5'- GTGGACCAGAAACTTCCGGA - 3'; Results were normalized to the housekeeping gene *L32*.

Western Blot Analysis and c-Jun N-Terminal Kinase Activity

Macrophages were homogenized in RIPA lysis buffer containing protease and phosphatase inhibitors. Lysates were clarified, centrifuged, and resolved by SDS-PAGE. Samples were transferred overnight to nitrocellulose membranes that were subsequently probed with the following antibodies for protein and phosphoprotein detection: AKT/p-Ser473 (Santa Cruz Biotechnology Inc. Santa Cruz, CA), JNK/p-Thr183/Tyr185 (Cell Signaling Technology Beverly, MA), p-p38 -Tyr 182 (Cell Signaling Tech, Beverly, MA), ERK/p-Thr 218/Tyr 220 (Cell Signaling Tech, Beverly, MA), total AKT (Cell Signaling Tech, Beverly, MA), CD36 (Abcam, Cambridge, MA), SR-A1, CHOP and GADD34 (Santa Cruz Biotechnology, Santa Cruz, CA). All protein expression was corrected to β-actin protein expression.

Plasmids and siRNA

siRNA hairpins against VDR and a control siRNA were expressed from a lentiviral vector under the control of the U6 human promoter and were generated by using PLKopuro.1 and

PLKoneo.1 (provided by Sheila Stewart, Washington University). Complementary siRNA oligos were annealed and cloned into vectors digested with AgeI and EcoRI and confirmed by sequence analysis. The sense siRNA oligonucleotide probes were as follows: VDR sense; CCGGTTGGCTGATCTTGTCAGTTACTCGAGTAACTGACAAGATCAGCCATTTTTG; VDR antisense; AATTCAAAAATGGCTGATCTTGTCAGTTACTCGAGTAACT GACAAGATCAGCCAA. PPARγ-siRNA was obtained from Sigma, St. Louis, MO. A plasmid expressing siRNA against luciferase was used as a control. Recombinant lentiviruses were generated in 293 T cells. [9]

Supplemental Figure Legends

Supplemental Figure 1. 1,25(OH)$_2$D$_3$ decreases macrophage cholesterol uptake in vitamin D-deficient diabetics only at concentrations of 10^{-8}M. Quantification by mean fluorescence absorbance of cholesterol uptake in vitamin D-deficient diabetics (group A) after incubation for 6h with (**a**) Dil-oxLDL or (**b**) Dil-AcLDL in macrophages cultured in 1,25(OH)$_2$D$_3$-supplemented media at concentrations of 10^{-8} M (*white-bars*), 10^{-10} M (*gray-bars*), 10^{-12} M (*hatched-bars*), or in vitamin D-deficient media (*black-bars*) (n=12) (*$p < 0.01$). (**c**) CYP24 mRNA from macrophages cultured in 1,25(OH)$_2$D$_3$ -supplemented media at concentrations of 10^{-8} M (*white-bar*), 10^{-10} M (*gray-bar*), or in vitamin D-deficient media (*black-bar*) (n=12). qPCR normalized to *L32* expression (*$p < 0.001$).

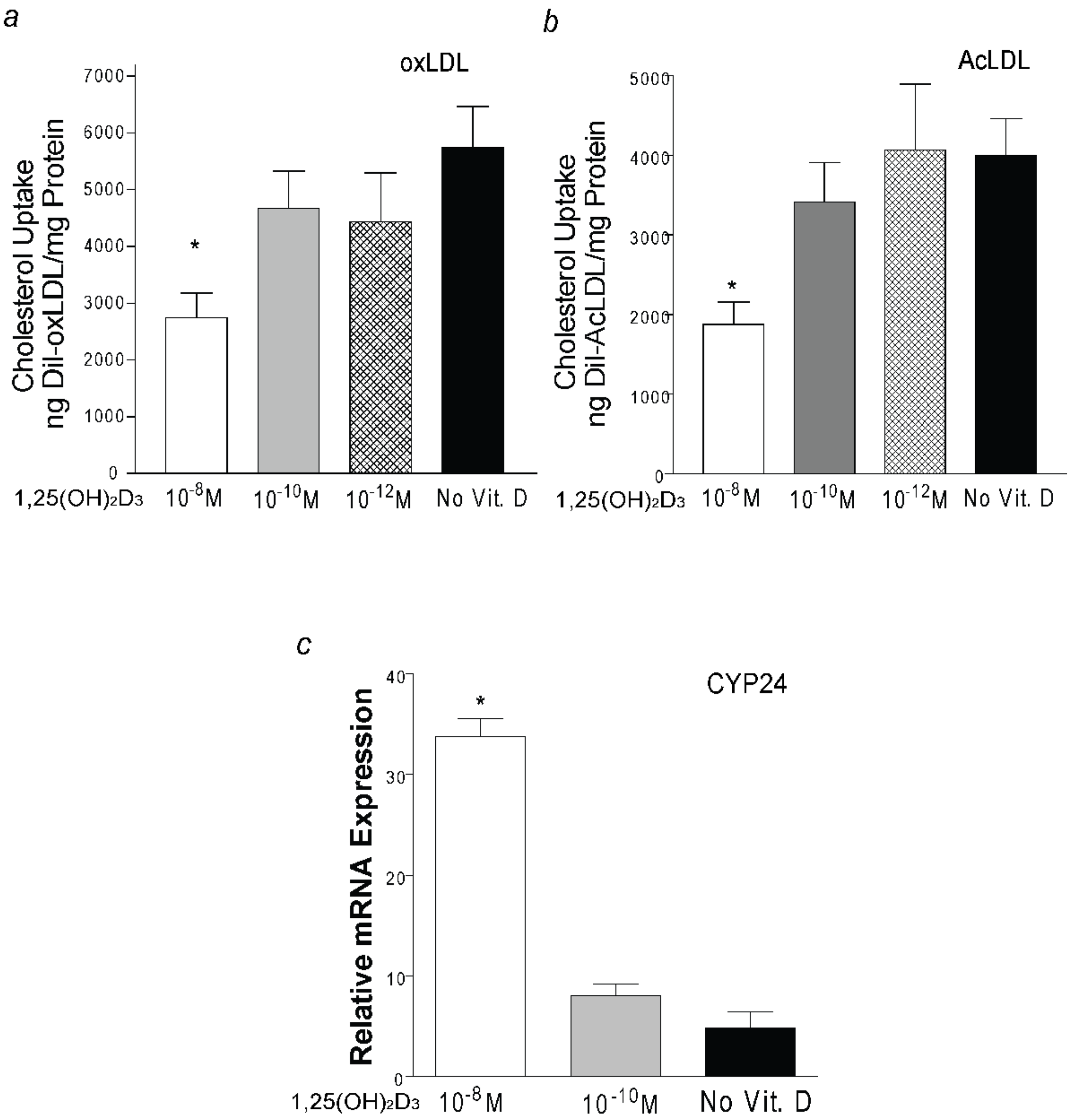
Supplemental Figure 2. 1,25(OH)$_2$D$_3$ decreases macrophage cholesterol uptake in vitamin D-deficient diabetics on HMG-CoA reductase inhibitors. Quantification of cholesterol uptake in macrophages (group A) cultured in vitamin D-deficient (*black-bars*) and 1,25(OH)$_2$D$_3$-supplemented (*white-bars*) media. Mean fluorescence absorbance after Dil-oxLDL (n=10) (*$p < 0.03$ vs. vitamin D-deficient).

Supplemental Figure 3. 1,25(OH)$_2$D$_3$ effects on cholesterol uptake in control subjects. Quantification of cholesterol uptake in vitamin D-sufficient diabetics (group B) after incubation for 6h with (**a**) Dil-oxLDL or (**b**) Dil-AcLDL in macrophages cultured in vitamin D-deficient (*black-bars*) and 1,25(OH)$_2$D$_3$-supplemented (*white-bars*) media (n=10) (* $p < 0.01$ and **$p < 0.05$ vs. vitamin D-deficient). Quantification of cholesterol uptake in macrophages cultured in vitamin D-deficient (*black-bars*) and 1,25(OH)$_2$D$_3$-supplemented (*white-bars*) media (n=10) after incubation for 6h with Dil-oxLDL from (**c**) vitamin D-deficient (group D) (*p* = 0.3 vs. vitamin D–deficient) or (**d**) vitamin D-sufficient (group E) subjects (*p* = 0.7 vs. vitamin D–deficient).

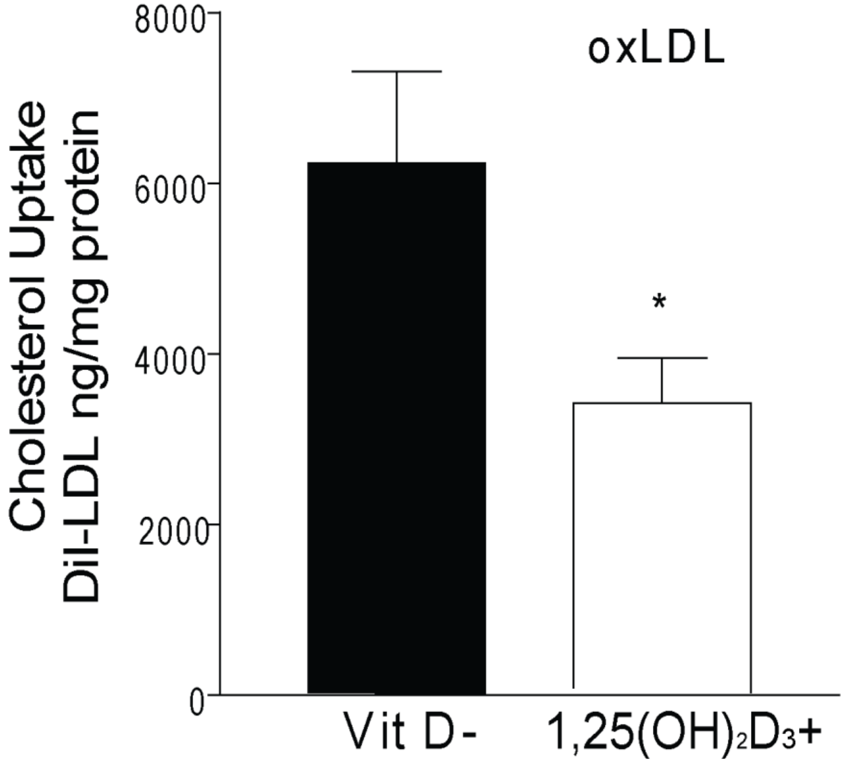
Supplemental Figure 4. 1,25(OH)$_2$D$_3$ effects on macrophage cholesterol efflux. (**a**) Functional cholesterol efflux assay in macrophages (group A) cultured in vitamin D-deficient (*black-bars*) or 1,25(OH)$_2$D$_3$-supplemented (*white-bars*) media after incubation for 24 hours with ^{3}H cholesterol and stimulation with HDL and apolipoprotein AI (n=8). (**b**) ABC1, ABCG1, and SR-B1 mRNA from macrophages as described in (**a**) after 24h stimulation with HDL and apolipoprotein AI (n=10). qPCR normalized to *L32* expression ($^*p < 0.05$).

Supplemental Figure 5. **1,25(OH)$_2$D$_3$ effects on scavenger receptor expression in macrophages from non-diabetic patients with vitamin D deficiency (group C)**. (**a and b**) Densitometric analysis of Western blots for CD36 and SR-A1 receptor normalized to β-actin in macrophages stimulated for 6h with oxLDL (**a**) or AcLDL (**b**) and cultured either in vitamin D–deficient (*gray-bar*) or 1,25(OH)$_2$D$_3$-supplemented (*white-bar*) media (*p* = 0.2, *p* = 0.08, respectively vs. macrophages in 1,25(OH)$_2$D$_3$-supplemented media) (n=3 per group). (**c**) Western blots using a specific antibody for JNKp, ERK, or p38 normalized to β-actin in macrophages obtained from group C patients cultured in vitamin D-deficient or 1,25(OH)$_2$D$_3$-supplemented media before and after oxLDL stimulation for 6h. (**d**) Densitometric analysis of Western blots for JNK-p receptor normalized to total JNK in macrophages cultured either in vitamin D–deficient (*gray-bar*) or 1,25(OH)$_2$D$_3$-supplemented media (*white-bar*) (n=3 per group) (*p* = 0.2 vs. vitamin D deficient).

Supplemental Fig. 1



Supplemental Fig . 2



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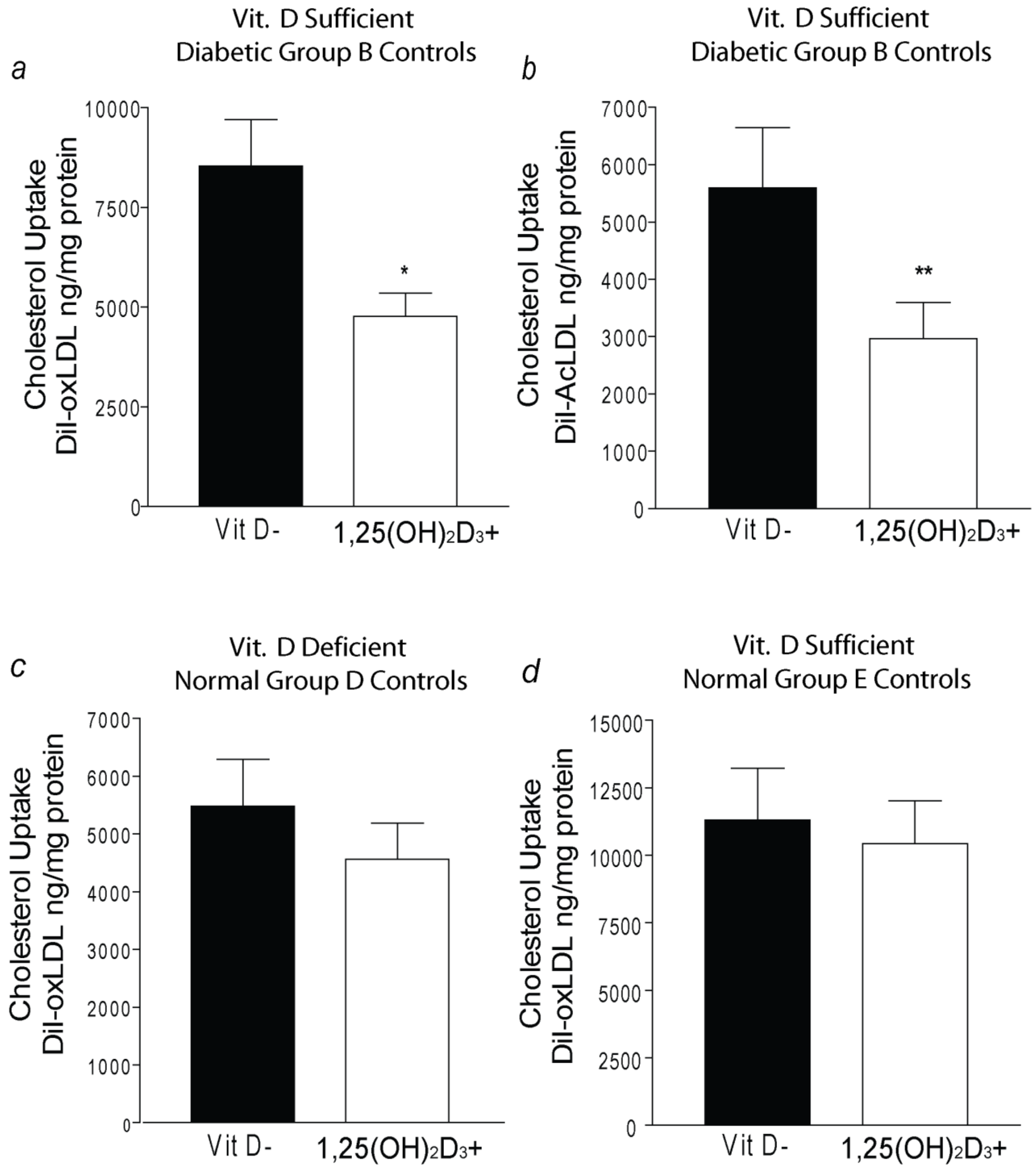

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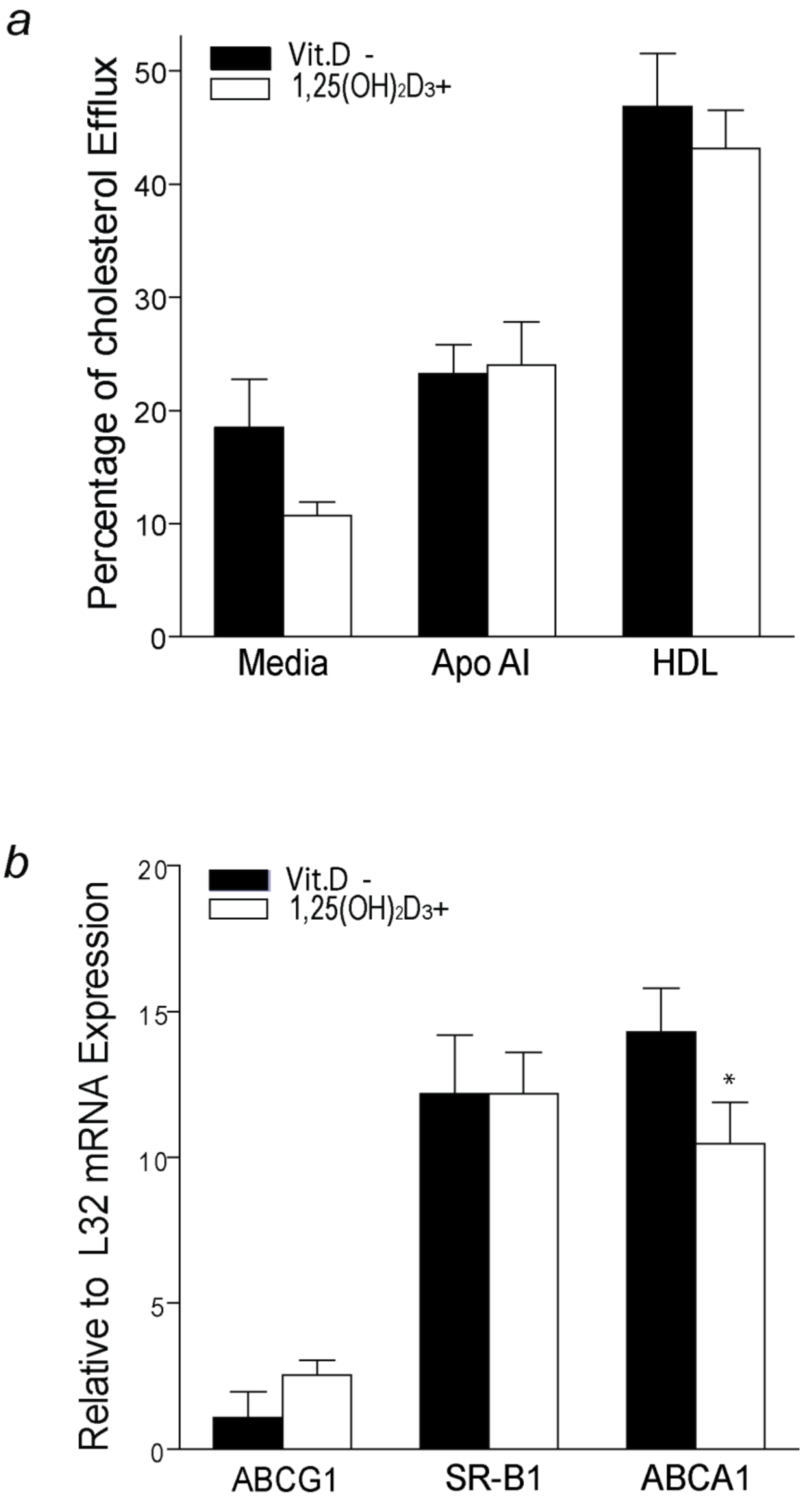
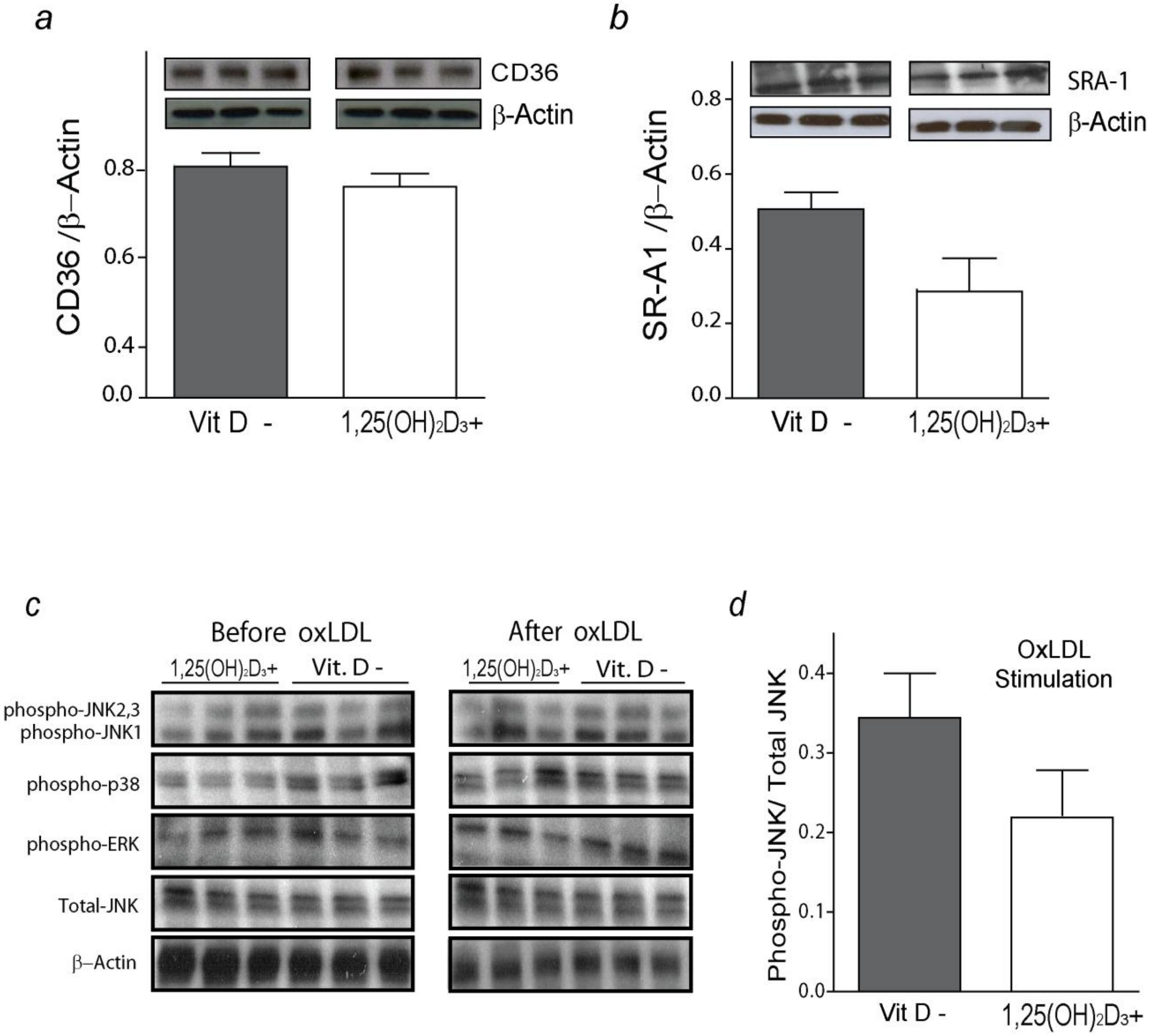


Supplemental Figure 5



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