Deletion of macrophage vitamin D receptor promotes insulin resistance and monocyte cholesterol transport to accelerate atherosclerosis in mice

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Deletion of Macrophage Vitamin D Receptor Promotes Insulin Resistance and Monocyte Cholesterol Transport to Accelerate Atherosclerosis in Mice

Graphical Abstract

Highlights

- Myeloid VDR deletion induces hepatic macrophage deposition and gluconeogenesis
- Myeloid VDR deletion enables M2 monocytes to transport cholesterol into plaques
- Impaired VDR-SERCA2b interaction results in macrophage ER stress and foam cells
- Bone marrow transplant of VDR into KODMAC mice rescues the metabolic phenotype

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In Brief

Identifying environmental conditions affecting chronic inflammation is essential to preventing cardiometabolic disorders. Oh et al. demonstrate that myeloid-specific Vdr deletion is sufficient to induce insulin resistance and accelerate atherosclerosis in mice. Furthermore, M2 monocyte transport of cholesterol into the vessel wall is described as a low-density lipoprotein (LDL)-independent pathway for atherosclerosis.
Deletion of Macrophage Vitamin D Receptor Promotes Insulin Resistance and Monocyte Cholesterol Transport to Accelerate Atherosclerosis in Mice

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SUMMARY

Intense effort has been devoted to understanding predisposition to chronic systemic inflammation because it contributes to cardiometabolic disease. We demonstrate that deletion of the macrophage vitamin D receptor (VDR) in mice (KODMAC) is sufficient to induce insulin resistance by promoting M2 macrophage accumulation in the liver as well as increasing cytokine secretion and hepatic glucose production. Moreover, VDR deletion increases atherosclerosis by enabling lipid-laden M2 monocytes to adhere, migrate, and carry cholesterol into the atherosclerotic plaque and by increasing macrophage cholesterol uptake and esterification. Increased foam cell formation results from lack of VDR-SERCA2b interaction, causing SERCA dysfunction, activation of ER stress-CaMKII-JNKp-PPARγ signaling, and induction of the scavenger receptors CD36 and SR-A1. Bone marrow transplant of VDR-expressing cells into KODMAC mice improved insulin sensitivity, suppressed atherosclerosis, and decreased foam cell formation. The immunomodulatory effects of vitamin D in macrophages are thus critical in diet-induced insulin resistance and atherosclerosis in mice.

INTRODUCTION

The combination of type 2 diabetes (T2DM) and cardiovascular disease (CVD) is the most common cause of morbidity and mortality in Western populations. Despite the known link between chronic inflammation, insulin resistance, and accelerated vascular disease, little is known about the mechanisms causing this immune activation. Environmental conditions that regulate macrophage differentiation and infiltration within metabolically active tissues play a role in disease progression (Olefsky and Glass, 2010). In high-fat-feeding-induced obese mice, classical M1 macrophage infiltration into adipose and liver generates pro-inflammatory cytokines and reactive oxygen intermediates that accelerate additional immune cell recruitment and promote insulin resistance (Lumeng et al., 2008). In early atherosclerotic plaque formation, retention of cholesterol in the subendothelial space triggers monocyte recruitment (Hansson and Hermansson, 2011). Both M1 and M2 macrophage phenotypes interchange dynamically depending on the plaque environment (Bouhlel et al., 2007). M1 macrophages, stimulated by the T-helper 1 cytokine, interferon γ, or by cholesterol crystals, are present early in the atherosclerotic plaque, promoting more inflammation, but they have lower scavenger receptor expression and cholesterol deposition and migrate more easily out of the plaque (Mantovani et al., 2009; Murray et al., 2014). In contrast, T-helper 2 cytokines or activation of lipid-sensing nuclear receptors by polyunsaturated fatty acids promotes M2 macrophage differentiation. This heterogeneous M2 macrophage subtype displays a spectrum of inflammatory responses (both pro- and anti-inflammatory) but is consistently characterized by expression of interleukin-10 (IL-10), arginase 1, and mannose receptor (MR) that contribute to tissue repair. Interestingly, these cells have increased scavenger receptor expression facilitating foam cell formation, suggesting that modified lipid deposition and M2 macrophage differentiation perpetuate one another within the atherosclerosis plaque (Chinetti-Gbaguidi et al., 2011; Oh et al., 2012). Therefore, understanding the environmental conditions that govern monocyte recruitment and subsequent macrophage immune programming and cholesterol deposition is key to the development of novel therapeutic strategies.

ER stress is an important cellular adaptation linking immune responses to obesity, insulin resistance, and atherosclerosis (Hotamisligil, 2010). Stimulation of ER stress induces responses to improve protein folding, but persistent stress triggers further inflammation through NF-κB and c-Jun N-terminal kinase (JNK) activation, increasing foam cell formation and inducing plaque necrosis in advanced atherosclerotic lesions.
Vitamin D receptor (VDR) is present in almost all cells of the immune system, and vitamin D deficiency is widely prevalent and has emerged as a potential contributor to the pathophysiology of type 2 diabetes mellitus (T2DM) and cardiovascular disease (CVD) (Holick, 2007; Norman and Powell, 2014; Veldman et al., 2000). Active vitamin D (1,25(OH)2D3) reduces adipocyte monocyte recruitment and inflammatory responses, improving adipocyte glucose uptake (Gao et al., 2013). In contrast, diet-induced vitamin D deficiency in mice results in hypertension and accelerated atherosclerosis due to increased plaque ER stress (Weng et al., 2013). Similarly, total body VDR knockout increases blood pressure and accelerates atherosclerosis, possibly by local activation of the renin angiotensin system in macrophages (Szeto et al., 2012). In vitamin-D-deficient diabetic patients, suppression of monocyte/macrophage ER stress by activating VDR decreases adhesion to endothelial cells and migration (Riek et al., 2012, 2013, 2014). Furthermore, 1,25(OH)2D3 suppresses macrophage cholesterol uptake and foam cell formation through downregulation of ER stress and subsequent reduction of JNK phosphorylation, peroxisome-proliferator-activated receptor gamma (PPARγ) expression, and scavenger receptor CD36 and SR-A1 expression. Together, these data suggest that regulation of ER stress by vitamin D exerts a pivotal role in macrophage cholesterol deposition and monocyte infiltration to critical metabolic tissues. This study was designed to determine the in vivo effects of VDR deletion on macrophage ER stress, chronic inflammation, insulin resistance, and atherosclerosis.

RESULTS

Efficiency of Macrophage VDR Deletion

Animals with specific inactivation of VDR in myeloid cells (knockout of vitamin D receptor in macrophages; KODMAC) were obtained by crossing VdrloxP/loxP mice (Masuyama et al., 2006) with transgenic mice expressing Cre recombinase under the control of the lysosomal M promoter (Lyz2Cre+/−) in Ldr−/− or Apoe−/− backgrounds, models of diet-induced insulin resistance and atherosclerosis, to produce KODMAC-L and KODMAC-E mice, respectively (Figure 1A). All animals were fed chow diet for 6 weeks after weaning, then high-fat diet (HFD) for an additional 8 weeks in Apoe−/− and 10 weeks in Ldr−/− mice unless otherwise specified.

The efficiency of macrophage-specific Vdr inactivation in KODMAC-L was demonstrated with Vdr mRNA levels in bone marrow-derived macrophages (BMDM), peripheral blood monocytes, and peritoneal macrophages and VDR protein levels in multiple tissues (Figures 1B and 1C). Similar results were found for KODMAC-E (data not shown). Incubation of KODMAC-L peritoneal macrophages with 1,25(OH)2D3 confirmed suppressed expression of the VDR target gene Cyp24a1 (Figure 1D). KODMAC-L mice had similar body weight and composition compared to control littermates (control-L) (Figures 1E–1G). Cholesterol and triglyceride levels were also similar before and after HFD (Figures 1H and 1I). In contrast, fasting glucose was higher in KODMAC-L before and after HFD (Figure 1J). Similar results were found in KODMAC-E mice in comparison to littermates (control-E) (Figures S1A–S1D).

Deletion of Macrophage Vdr Induces Insulin Resistance

To characterize the altered glucose metabolism in KODMAC-L, we performed glucose tolerance tests (GTTs) and insulin tolerance tests (ITTs) after 6 weeks on chow and after an additional 10 weeks of HFD. KODMAC-L developed glucose intolerance before and after HFD with no compensatory increase in insulin secretion (Figures 2A and S2A). In addition, KODMAC-L mice were more resistant to glucose lowering by exogenous insulin, suggesting insulin resistance (Figures 2B and S2B). KODMAC-E also showed insulin resistance before and after 8 weeks on HFD (Figures S2C–S2F). To clarify the primary site of insulin resistance in KODMAC-L, we performed hyperinsulinemic-euglycemic clamps in mice before HFD. KODMAC-L had significantly reduced glucose infusion rate and increased hepatic glucose production, with no difference in disposal rate, consistent with increased hepatic gluconeogenesis (Figures 2C–2E). mRNA expression of two key gluconeogenic enzymes, phosphoenolpyruvate carboxykinase (Pck1) and glucose 6-phosphatase (G6pc), was higher in KODMAC-L hepatocytes (Figure 2F), and hepatic AKT phosphorylation was diminished in KODMAC-L mice after inferior vena cava insulin injection, confirming hepatic insulin resistance (Figure 2G). To determine whether KODMAC-L macrophages directly modulate hepatic glucose production, we co-cultured primary hepatocytes from control-L mice with peritoneal macrophages from KODMAC-L or control-L and found increased hepatocyte expression of Pck1 and G6pc after incubation with KODMAC-L macrophages. Moreover, culture of primary control-L hepatocytes with KODMAC-L macrophage media recapitulated the induction of gluconeogenic enzymes (Figure 2H), implying a macrophage secretory factor affecting hepatic gluconeogenesis. Media from the KODMAC-L culture had a higher concentration of tumor necrosis factor α (TNF-α), IL-1β, and IL-6, all cytokines reported to induce hepatic gluconeogenesis (Figure 2I). Liver histologic analysis showed increased macrophage accumulation and cholesterol deposition in KODMAC-L mice (Figures 2J–2L), suggesting increased cytokine secretion from hepatic macrophage infiltration induces gluconeogenic enzymes. We next explored the macrophage subtype in KODMAC-L livers. Interestingly, KODMAC-L liver macrophages showed an M2 pattern of membrane markers by flow cytometry with a consistent M2 gene signature (Figures 2M, S2G, and S2H). To clarify whether the M2 macrophage subtype differentiation was liver specific, we analyzed BMDM and peritoneal macrophages from KODMAC-L mice, with all cells demonstrating an M2 membrane and mRNA expression pattern (Figures 2N, S2O, and S2I–S2L), suggesting that macrophage Vdr deletion increased M2 macrophages and cytokine activation of hepatic insulin resistance.

Deletion of Macrophage Vdr Increases Foam Cell Formation and Accelerates Atherosclerosis

Macrophage infiltration and lipid accumulation in the vessel wall are critical in atherosclerosis initiation and progression. In type 2 diabetics, active vitamin D promotes macrophage differentiation in vitro and suppresses cytokine secretion (Giulietti et al., 2007).
To determine whether the immunomodulatory effects of macrophage VDR signaling play a role in atherosclerosis, we measured atherosclerotic lesions in KODMAC mice after HFD. KODMAC-L and -E had ~3-fold and ~2-fold greater median plaque area, respectively, in all aortic regions compared to control-L and control-E (Figures 3A and S3A) and to Cre+/− Ldlr−/− littermates (data not shown). Moreover, KODMAC-L and KODMAC-E mice on prolonged chow diet only, without exposure to HFD, had 4- to 10-fold increased plaque area across all aortic regions (Figures S3B and S3C), suggesting that defective macrophage VDR signaling is critical to atherosclerosis progression.

Next, we explored whether alterations in KODMAC-L macrophage cholesterol metabolism contribute to increased atherosclerosis. Peritoneal macrophages from KODMAC-L mice after 10 weeks of HFD had increased foam cell formation by oil-red-O staining and higher free and total cholesterol content (Figures 3B–3D). Foam cell formation was mediated by increasing oxidized low-density lipoprotein (oxLDL) cholesterol binding and uptake and cholesterol ester formation with no differences in cholesterol efflux (Figures 3E–3H). To determine whether the M1/M2 macrophage profile induced in KODMAC-L correlates with the deposition of atherosclerotic plaque foam cells, we examined frozen sections of the aortic root from KODMAC-L mice after 10 weeks on HFD. Immunofluorescent staining revealed increased vessel wall infiltration with predominantly M2 macrophages that co-localized with fat staining by adipose differentiation-related protein (ADRP) compared to control-L, consistent with our previous observation that M2 macrophages induced by vitamin D deficiency accumulate more cholesterol (Figures 3I–3K) (Weng et al., 2013).

CaMKII Links ER Stress to Increased Cholesterol Uptake in KODMAC-L Macrophages

In diabetic patients, ER stress activation induced by vitamin D deficiency triggers M2 macrophage differentiation and foam cell formation by increasing scavenger-receptor-mediated cholesterol uptake (Oh et al., 2009; Riek et al., 2013). Therefore, we explored whether the effects of vitamin D on foam cell formation are ER stress-VDR-dependent in vivo. Consistent with our previous data, peritoneal macrophages from KODMAC-L mice after 6 weeks of chow showed upregulation of ER stress, JNK phosphorylation, PPARγ, and scavenger receptors CD36 and SRA-1, regardless of vitamin D conditions (Figure 4A). KODMAC-L macrophages also demonstrated increased acetylated LDL cholesterol binding and uptake (Figures S4A and S4B). We then confirmed that the effects of Vdr deletion on macrophage subtype are ER-stress-dependent. Incubation of KODMAC-L macrophages with an ER stress inhibitor, 4-phenylbutyrate (PBA), shifted them toward an M1 expression pattern similar to that of control-L cells (Figures S4C and S4D). In previous studies in cholesterol-loaded macrophages, increased cytosolic calcium resulting from ER stress activates calmodulin-dependent protein kinase II (CaMKII), linking ER stress to JNK-mediated macrophage apoptosis (Timmins et al., 2009).

We studied type 2 diabetic human macrophages cultured in vitamin-D-deficient or 1,25(OH)2D3-supplemented conditions and chow-fed KODMAC-L or control-L peritoneal macrophages to explore whether CaMKII activation links impaired vitamin D signaling to macrophage cholesterol uptake. Both KODMAC-L and vitamin-D-deficient human macrophages had increased CaMKII phosphorylation compared to control-L or 1,25(OH)2D3-supplemented human macrophages, respectively, but no differences in total CaMKII expression (Figure 4B). To determine the effects of CaMKII inhibition on macrophage cholesterol uptake, we used KN-93, a CaMKII inhibitory agent, in KODMAC-L and control-L peritoneal macrophages under vitamin-D-deficient or 1,25(OH)2D3-supplemented conditions. Vitamin-D-deficient control-L macrophages and KODMAC-L macrophages in both vitamin D conditions all had significantly higher oxLDL cholesterol uptake compared to 1,25(OH)2D3-supplemented control-L macrophages, but CaMKII inhibition abolished this increased cholesterol uptake in all circumstances (Figure 4C), suggesting that CaMKII activation is key in macrophage cholesterol uptake induced by defective vitamin D signaling. Moreover, inhibiting CaMKIIp in KODMAC-L or control-L macrophages under vitamin-D-deficient conditions suppressed JNKp, PPARγ, CD36, and ER stress (Figure 4D), suggesting that activation of CaMKII links vitamin-D-deficiency-induced ER stress to JNK-mediated macrophage cholesterol uptake.

VDR Signaling Facilitates SERCA2b Function and Suppresses ER Stress

Sarc endothelial reticulum calcium ATPase 2b (SERCA2b) is a critical enzyme maintaining high ER calcium levels to optimize protein production and folding. Induction of macrophage ER stress by the SERCA inhibitor thapsigargin in control-L macrophages shifts macrophages toward an M2 expression pattern similar to that of KODMAC-L (Figures S4E and S4F), implying similar effects of Vdr deletion and SERCA dysfunction. To clarify whether Vdr deletion induces ER stress by changing SERCA2b expression or function, we again studied peritoneal macrophages from KODMAC-L and control-L mice after 6 weeks of chow and human type 2 diabetic macrophages cultured in...
Figure 2. Deletion of Macrophage Vdr Induced Insulin Resistance

KODMAC-L and control-L mice after 6 weeks of chow were assessed for glucose metabolism and insulin resistance after overnight fast.

(A and B) Glucose tolerance test (A) with 30-min plasma insulin levels (inset) (n = 35 per group) and (B) insulin tolerance test (B) (n = 16 per group).

(C–E) From hyperinsulinemic euglycemic clamps, (C) glucose infusion rate, (D) hepatic glucose production after insulin stimulation, and (E) glucose disposal rate (n = 7–8 per group).

(F) qRT-PCR analysis of relative Pck1 and G6pc mRNA expression from hepatocytes, normalized to Mrpl32 (n = 6 per group).

(G) Western blot analysis of phosphorylated AKT from hepatocytes (n = 3 per group).

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vitamin-D-deficient or 1,25(OH)2D3-supplemented conditions. We found no differences in SERCA2b protein expression among all groups (Figure 4B). However, SERCA2b activity in KODMAC-L and vitamin D-deficient human macrophages was decreased by at least 20% compared to control-L and 1,25(OH)2D3-supplemented macrophages, respectively, suggesting that VDR activates SERCA2b to maintain normal ER functionality (Figures 4E and 4F). To confirm the effects of deletion of macrophage VDR on SERCA2b function in diabetic patients, we infected human macrophages cultured in 1,25(OH)2D3-supplemented media with lentivirus containing either small interfering RNA (siRNA) VDR hairpins or control siRNA. VDR-siRNA-infected macrophages showed significantly lower SERCA2b activity compared to control-siRNA-infected macrophages (Figure 4G). Finally, we immunoprecipitated KODMAC-L and control-L peritoneal macrophage lysates with VDR antibody and blotted for SERCA2b, identifying expression of VDR-SERCA2b interaction (Figure 4H). Co-localization of SERCA2b with VDR by immunofluorescence in control-L peritoneal macrophages confirmed a mechanism of VDR interaction with SERCA2b to optimize ER function (Figures 4I and 4J).

Deletion of VDR Signaling Enables Monocyte Cholesterol Transport into the Atherosclerotic Plaque

Monocyte adhesion to the endothelium and migration into the subendothelial space are critical to atherosclerotic plaque macrophage deposition and foam cell formation (Gautier et al., 2009). However, recent studies suggest that non-classical monocytes can become cholesterol laden in the circulation and may be a potential source of vascular cholesterol deposition in addition to differentiated plaque macrophages (Mosig et al., 2009; Wu et al., 2009). We evaluated cholesterol content in KODMAC-L circulating monocytes after 3 weeks of HFD, demonstrating higher cholesterol content and oxLDL uptake (Figures 5A and 5B). Moreover, KODMAC-L monocytes demonstrated higher adhesion to fibronectin and increased migration in response to monocyte chemoattractant protein 1 (MCP-1) and, accordingly, increased mRNA expression of adhesion membrane receptor β1 integrin and MCP-1 receptor (CCR2) (Figures 5C, 5D, S5A, and S5B), indicating that VDR deletion induces a pro-atherogenic phenotype that might enable monocytes to carry cholesterol into the atherosclerotic plaque. To study this potential mechanism directly, we incubated KODMAC-L or control-L monocytes from mice after 6 weeks of HFD with labeled DiI-oxLDL cholesterol, and infused them daily for 3 days into high-fat-fed LDLR<sup>−/−</sup> mice. Two-photon microscopy from explanted aortas showed localization of DiI-oxLDL inside the vascular wall in the subendothelial space, implying accumulation of lipid-laden monocytes (Figure 5G), confirming an LDL-independent pathway for vascular cholesterol deposition.

To characterize the circulating monocytes associated with cholesterol deposition in KODMAC-L, we used flow cytometry to evaluate traditional murine monocyte markers. Monocytes from KODMAC-L mice after 3 weeks of HFD had significantly more Ly6<sup>clo</sup> cells and fewer Ly6<sup>c+</sup> cells compared to control-L cells. However, there was no difference in Ly6<sup>c+</sup> cells between the groups. In addition, there were significantly more cells positive for CCR2 in KODMAC-L, and this was driven by the shift toward Ly6<sup>clo</sup> cells (Figure 5H), suggesting that deletion of VDR induces a non-classical subtype of monocytes that express membrane markers facilitating vascular invasion. In our previous work, M1/M2 macrophage markers assessed in diabetic mice correlate better with adhesion and migration than the typical markers used to classify human monocytes (CD14/CD16). Therefore, we tested whether the M1/M2 expression phenotype of circulating monocytes would reflect the early plaque macrophage subtype. KODMAC-L monocytes expressed predominantly M2 membrane markers with mRNA expression typical of M2 polarization (Figures 5I, 5S, and 5D). Frozen sections of early atherosclerotic plaques in the aortic root from KODMAC-L mice after 3 weeks on HFD also showed a predominance of M2 macrophages with more ADRP co-localization compared to M1 macrophages (Figures 5J–5L), similar to KODMAC-L after HFD. Therefore, deletion of monocyte VDR promotes a pro-atherogenic M2 monocyte phenotype, resulting in M2-predominant plaque macrophages.
**Figure 3. Deletion of Macrophage Vdr Accelerated Atherosclerosis**

KODMAC-L and control-L mice were assessed for atherosclerosis and macrophage cholesterol metabolism after HFD. (A) Atherosclerotic plaque area of pinned aortas (n = 16 per group). Lines indicate median values. (B–H) In peritoneal macrophages, (B) representative image of oil red O staining (scale bar represents 10 μm), (C) free and (D) total cholesterol content (n = 6 each group), (E) cholesterol binding and (F) cholesterol uptake after stimulation with DiI-oxLDL for 6 hr, (G) cholesteryl ester formation after stimulation with oxLDL and 3H oleic acid for 6 hr, and (H) cholesterol efflux after incubation with 3H cholesterol for 24 hr and stimulation with HDL or apolipoprotein AI for 6 hr (n = 6 per group). *p < 0.05 versus control-L for (A)–(H).

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with increased cholesterol deposition that persist throughout plaque progression.

**Cardiometabolic Abnormalities from Vdr Deletion Are Transplantable**

To determine whether introduction of normal macrophage VDR signaling by bone marrow (BM) transplant could prevent metabolic dysregulation and atherosclerosis in KODMAC-L, we transplanted previously irradiated KODMAC-L mice with BM from KODMAC-L (KOD/KOD) or control-L (Con/KOD) animals. After 6 weeks of chow diet, Con/KOD mice had decreased fasting glucose, improved insulin sensitivity with decreased expression of hepatic gluconeogenic enzymes, and liver macrophages shifted toward an M1-predominant phenotype (Figures 6A–6D). After HFD, Con/KOD mice also had increased insulin sensitivity (Figures S6A–S6C) and decreased atherosclerotic area in the proximal and thoracic aorta compared to KOD/KOD (Figure 6E). Moreover, peritoneal macrophages had decreased cholesterol uptake and cholesteryl ester formation with no differences in cholesterol efflux (Figures 6F–6H). Conversely, we transplanted control-L mice with BM from KODMAC-L (KOD/Con) or control-L (Con/Con) animals to induce the metabolic phenotype. As expected, KOD/Con mice demonstrated cardiometabolic dysregulation compared to Con/Con, with increased glucose levels, insulin resistance, atherosclerosis, M2 macrophages, and foam cell formation with greater cholesterol uptake (Figures 6I–6P and S6D–S6F). There was no differential effect of BM transplantation on fasting cholesterol or triglycerides (Figures S7G–S7J). These data confirm the fundamental role of macrophage VDR signaling in chronic inflammation, affecting metabolic function and atherosclerosis progression.

**DISCUSSION**

Little is known about environmental immunomodulators affecting the chronic inflammation in metabolic tissues that contribute to vascular disease. In this work, we demonstrate that Vdr deletion in a single cell lineage in KODMAC mice is sufficient to induce insulin resistance and accelerate atherosclerosis. KODMAC-L mice had accelerated M2 macrophage infiltration and cholesterol deposition in the liver, increasing cytokine secretion and hepatic glucose production. Moreover, KODMAC-L animals were predisposed to plaque cholesterol deposition via both M2 monocyte transport of cholesterol into the vessel wall and cholesterol uptake by M2 plaque macrophages. Increased cholesterol deposition precipitated by IL-4-induced M2 macrophages, CD36 signaling activation by oxLDL results in increased cholesterol esterification and cholesteryl ester formation, increased cholesterol efflux, and increased atherosclerosis (Chinetti-Gbaguidi et al., 2011). In contrast, BM transplant from mice deficient for Ppara, a transcription factor that facilitates IL-4-induced M2 macrophage differentiation, causes hepatic infiltration and increased gluconeogenesis (Odegard et al., 2008). In contrast, we found that deletion of macrophage Vdr favors hepatic accumulation of M2 cells that stimulate gluconeogenesis. The transplantability of this phenotype confirms that VDR signaling links macrophage subtype to hepatic gluconeogenesis. These contrasting findings imply that the M1–M2 paradigm to describe the inflammatory profile of macrophage subtypes as they contribute to hepatic insulin resistance may be oversimplified.

The recruitment of circulating monocytes into the vessel wall is critical in the pathophysiology of vascular complications (Hansson and Hermansson, 2011). Recent studies in patients with familial hypercholesterolemia and in mouse models of atherosclerosis show that non-classical monocytes can become cholesterol laden in the circulation, suggesting they may carry cholesterol into the vessel wall (Mosig et al., 2009; Wu et al., 2009). We found that VDR signaling regulates atherosclerosis initiation by modifying circulating monocyte cholesterol transport and plaque macrophage phenotype. Monocyte Vdr deletion induced a cholesterol-laden M2 phenotype with increased adhesion to and migration into the vessel wall, facilitating an LDL-independent pathway for cholesterol transport into the early atherosclerotic plaque.

Macrophages are key cells in atherosclerosis progression. The cellular microenvironment directs macrophages into different subtypes with distinct cholesterol metabolism phenotypes. In IL-4-induced M2 macrophages, CD36 signaling activation by long-chain free fatty acids generates small lipid droplets and increased β-oxidation (Huang et al., 2014). In contrast, CD36 signaling activation by oxLDL results in increased cholesterol deposition and nuclear sequestration of AMPK, diminishing β-oxidation (Oh et al., 2009; Samovski et al., 2015). In vivo, the location of M2 macrophages in atherosclerotic plaque affects cholesterol metabolism. Increased IL-4 overlying the lipid core induces M2 macrophages (CD68* and MR*) with increased cholesterol ester formation, reduced cholesterol efflux, and small lipid droplets (Chinetti-Gbaguidi et al., 2011). In contrast,
Figure 4. Deletion of Macrophage Vdr Increased ER Stress and Accelerated Cholesterol Uptake by Activation of the CaMKII Signaling Pathway

Peritoneal macrophages from KODMAC-L or control-L before HFD and/or human monocyte-derived macrophages from type 2 diabetics were cultured for 5 days in vitamin-D-deficient or 1,25(OH)2D3-supplemented conditions.

(A) Western blot of ER stress and cholesterol signaling proteins.

(B) Heat map of ER stress and cholesterol signaling proteins.

(C) Bar graph of Di-ox-DL Cholesterol Uptake (ng/mg cellular protein).

(D) Western blot of ER stress and cholesterol signaling proteins.

(E) Graph of SERCA-Dependent ER Calcium Influx (nmol/min/mg cellular protein).

(F) Graph of SERCA-Dependent ER Calcium Influx (nmol/min/mg cellular protein).

(G) Graph of SERCA-Dependent ER Calcium Influx (nmol/min/mg cellular protein).

(H) Graph of SERCA-Dependent ER Calcium Influx (nmol/min/mg cellular protein).

(I) Immunofluorescence images of VDR, SERCA2b, and VDR/SERCA2b.

(J) Diagram of CaMKII signaling pathway.

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in areas of previous intraplaque hemorrhage, M2 macrophages (MR+ and CD163+) produce IL-10 and have increased cholesterol efflux and lower cholesterol content (Finn et al., 2012), highlighting the effect of the microenvironment on the interplay between macrophage subtype and functionality. In our study, KODMAC-L mice had increased atherosclerosis with a persistent predominance of cholesterol-laden M2 cells as plaques progressed. Similar results of increased atherosclerosis have been demonstrated in Vdr total knockout mice; these animals also have increased ex vivo macrophage foam cell formation in response to modified LDL cholesterol (Szeto et al., 2012). Our model shows that deletion of VDR is sufficient to program a sustained, pro-atherogenic monocyte/macrophage phenotype, confirmed by the transplantability of the cardiometabolic outcomes.

Chronic excess of cytokines, glucose, and lipids triggers ER stress, provoking inflammatory signaling, macrophage cholesterol uptake, or macrophage death, key mechanisms in insulin resistance and atherosclerosis (Hummasti and Hotamisligil, 2010; Oh et al., 2012; Tabas and Ron, 2011). Vitamin D acts as an ER stress reliever that suppresses pro-atherogenic macrophage properties and decreases atherosclerosis. This work demonstrates that the macrophage VDR interacts with SERCA2b to improve its function, identifying a mechanism by which vitamin D decreases ER stress. Macrophage accumulation of modified lipids impairs SERCA2b expression, increasing cytosolic calcium activation of the CaMKII-JNK pathway to cause macrophage apoptosis and plaque instability (Liang et al., 2012; Timmins et al., 2009). Similarly, we found that macrophage VDR signaling activation diminished CaMKII-JNK activation and suppressed cholesterol accumulation to break the cycle of ER stress activation.

We recognize that the KODMAC model of Vdr deletion in monocyte/macrophages is an extreme model of vitamin D deficiency and may not directly represent animal and human physiology. However, in animal models of insulin resistance and in patients with T2DM, adequate dietary vitamin D also promotes a monocyte/macrophage phenotype with lower adhesion, migration, and foam cell formation, as well as decreasing atherosclerosis (Oh et al., 2009; Riek et al., 2012; Weng et al., 2013). It is also important to acknowledge that these metabolic changes induced by macrophage-specific Vdr deletion might represent just a component of the myriad of cardiometabolic effects linked to vitamin D deficiency in humans, such as hypertension, thrombosis, and systemic inflammation. However, the KODMAC model highlights that VDR-dependent signaling in a single cell lineage is a critical pathway integrating inflammation, insulin resistance, and atherosclerosis.

**EXPERIMENTAL PROCEDURES**

Additional details and references are provided in the Supplemental Experimental Procedures.

**Experimental Models**

Vdrfl/fl mice were backcrossed into C57BL/6 for ten generations (Masuyama et al., 2006). Inactivation of VDR in myeloid cells (KODMAC) was generated by crossing Vdrfl/fl mice with transgenic mice expressing Cre recombinase under the control of the lyzosomal M promoter (Lyz2Cre+) in Ldr−/− (KODMAC-L) or Apeo−/− (KODMAC-E) backgrounds. Littermate mice Lyz2Cre+ Vdrfl/fl Ldr−/− (control-L) and Lyz2Cre+ Vdrfl/fl Apeo−/− (control-E) were used as controls. Mice received normal chow diet (Harlan TD87095) for 6 weeks after weaning, then vitamin D-sufficient HFD for 8 weeks in the Apeo−/− and for 10 weeks in the Ldr−/− background (Harlan TD88137). Additional mice were maintained on chow for 1 year for atherosclerosis assessment. For BM transplants, lethally irradiated 8-week-old chow-fed KODMAC-L or control-L recipient mice were reconstituted with ~5 x 10^7 BM cells from 8-week-old chow-fed KODMAC-L or control-L mice via injection. Recipient mice were given 6 weeks of chow, then 10 weeks of HFD. All experiments included male and female animals and were approved by the Washington University Animal Studies Committee. Adult subjects with T2DM were voluntarily recruited and provided with written informed consent, approved by the Human Research Protection Office of Washington University School of Medicine.

**Isolation of Murine Peritoneal, Hepatic, and BMDMs and Peripheral Monocytes**

Peritoneal macrophages were collected immediately following injection of PBS into the peritoneum and cultured for 3 hr in vitamin-D-deficient media (deficient in both 25(OH)D and 1,25(OH)2D; obtained by DMEM plus 10% charcoal/dextran-treated FBS) with or without 1.25(OH)2D3 (Sigma-Aldrich, 10−8 M) prior to assessment. Cells were subjected to CD11b microbeads (Miltenyi Biotec) prior to flow cytometry. For hepatic macrophage isolation, livers were resected, minced, treated with collagenase, and filtered (70-μm cell strainer, BD Bioscience) to remove hepatocytes. Hepatic macrophages were selected for CD11b (present in macrophages, but not Kupffer cells) with microbeads. For BMDMs, BM was flushed from femurs and tibias and subjected to erythrocyte lysis, then CD11b microbeads prior to 5 days of culture. Cytosolic calcium activation of the CaMKII-JNK pathway to cause macrophage apoptosis and plaque instability (Liang et al., 2012; Timmins et al., 2009). Similarly, we found that macrophage VDR signaling activation diminished CaMKII-JNK activation and suppressed cholesterol accumulation to break the cycle of ER stress activation.

We recognize that the KODMAC model of Vdr deletion in monocyte/macrophages is an extreme model of vitamin D deficiency and may not directly represent animal and human physiology. However, in animal models of insulin resistance and in patients with T2DM, adequate dietary vitamin D also promotes a monocyte/macrophage phenotype with lower adhesion, migration, and foam cell formation, as well as decreasing atherosclerosis (Oh et al., 2009; Riek et al., 2012; Weng et al., 2013). It is also important to acknowledge that these metabolic changes induced by macrophage-specific Vdr deletion might represent just a component of the myriad of cardiometabolic effects linked to vitamin D deficiency in humans, such as hypertension, thrombosis, and systemic inflammation. However, the KODMAC model highlights that VDR-dependent signaling in a single cell lineage is a critical pathway integrating inflammation, insulin resistance, and atherosclerosis.

**EXPERIMENTAL PROCEDURES**

Additional details and references are provided in the Supplemental Experimental Procedures.

**Experimental Models**

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**Isolation of Human Monocyte-Derived Macrophages**

Peripheral monocytes were isolated by standard Ficoll isolation techniques and selected by CD14 microbeads (Miltenyi Biotec) with CD14+ cell
Figure 5. Deletion of Macrophage Vdr Increases Lipid-Laden M2 Cells in the Atherosclerotic Plaque

(A–D) Peripheral blood monocytes from KODMAC-L and control-L mice after 3 weeks of HFD were assessed for (A) total cellular cholesterol (n = 6 per group), (B) cholesterol uptake after DiI-oxLDL stimulation for 6 hr (n = 4 per group), (C) adhesion to fibronectin (n = 6 per group), and (D) transwell migration in response to MCP-1 stimulation (n = 12 per group).

(E and F) KODMAC-L and control-L monocytes were incubated with stably labeled cholesterol-d7 for 24 hr and transfused into 6-month-old Ldlr−/− mice on chow. Cholesterol-d7 ester enrichment in (E) monocytes (n = 5 per group) and (F) aortic arch relative to plasma (n = 3 per group).

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purity of 97% by flow cytometry (FACStar Plus). For monocyte-derived macrophages, monocytes were cultured with 100 ng/ml of M-CSF for 5 days in vitamin-D-deficient media with or without supplementation of 1,25(OH)2D3 (10^{-8} M). For ER stress suppression or induction, differentiated macrophages were cultured with PBA (10 mM) or thapsigargin (0.25 mM), respectively, for an additional 18 hr. For lentiviral infection, after 5 days of differentiation in 1,25(OH)2D3-supplemented conditions, macrophages were infected with lentivirus containing either Vdr-siRNA or control-siRNA, with assays performed 72 hr later.

**Metabolic Assessment**

Mice underwent metabolic characterization after 6 weeks of chow and after an additional 8–10 weeks of HFD (Apoe^{-/-} or Ldlr^{-/-} background, respectively). Body composition was performed by dual energy X-ray absorptiometry (PXImus, GE Corporation). Serum glucose, cholesterol, and triglycerides and insulin were measured after 6 hr of fasting using commercially available kits. For GTTs and ITTs, mice were fasted overnight prior to peritoneal injection with 10% D-glucose (1 g/kg) or insulin (0.75 U/kg, Humulin R). Hyperinsulinemic eu-glycemic clamps were performed in conscious mice after 6 hr of fasting with a clamped glucose level of 120 mg/dl. For hepatic insulin signaling, livers were isolated and snap frozen 2 min after inferior vena cava insulin injection in fasted mice and were then homogenized and immunoblotted for phospho-AKT (Ser473).

**Hepatocyte Isolation and Macrophage Co-culture**

Livers of anesthetized 8-week-old control-L mice were perfused with collagenase-containing media (Invitrogen) and resected. The dissociated hepatocytes were allowed to recover for 24 hr. Peritoneal KODMAC-L and control-L macrophages were cultured for 24 hr in 1,25(OH)2D3-supplemented media prior to co-culture onto hepatocyte cultures at a 1:2 macrophage:hepatocyte ratio for 18 hr. TNF-α (BD Biosciences) and IL-1β (R&D Biosystems) concentrations were measured by ELISA in co-culture media. For liver histology, sections of frozen livers were stained with H&E or with macrophage-specific antibody F4/80 (1:100, Santa Cruz Biotechnology). Nuclei were manually counted and cells were identified as positive or negative for F4/80 in ten fields at 10× magnification per animal.

**Flow Cytometry**

Monocytes and hepatic, BM-derived, and peritoneal macrophages were selected for CD11b with microbeads, then stained with antibodies directed against CCR7, CD86, Ly6C, and CD115 (eBioscience), CD163 (Bioss USA), and MR and CCR2 (R&D Systems). Monocytes and macrophages were gated for live cells, and monocytes were additionally gated for CD115+ for analysis.

**Hepatocyte Isolation and Macrophage Co-culture**

Livers of anesthetized 8-week-old control-L mice were perfused with collagenase-containing media (Invitrogen) and resected. The dissociated hepatocytes were allowed to recover for 24 hr. Peritoneal KODMAC-L and control-L macrophages were cultured for 24 hr in 1,25(OH)2D3-supplemented media prior to co-culture onto hepatocyte cultures at a 1:2 macrophage:hepatocyte ratio for 18 hr. TNF-α (BD Biosciences) and IL-1β (R&D Biosystems) concentrations were measured by ELISA in co-culture media. For liver histology, sections of frozen livers were stained with H&E or with macrophage-specific antibody F4/80 (1:100, Santa Cruz Biotechnology). Nuclei were manually counted and cells were identified as positive or negative for F4/80 in ten fields at 10× magnification per animal.

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**Monocyte Adhesion and Migration**

Monocytes were added to fibronectin-coated plates and incubated for 4 hr at 37°C before adhered cells were stained and quantified. Transwell migration assays were performed by addition of monocytes to the upper chamber with 8 hr of MCP-1 (100 ng/well) stimulation in the lower chamber. Cells migrated into the lower chamber were manually counted.

**Monocyte Cholesterol Transport**

The deuterium-labeled cholesterol-d7 tracer was added to 20% Intralipid, generating a cholesterol-d7-containing emulsion. Monocytes were incubated with the emulsion for 6 hr prior to infusion into recipient mice. Neutral lipids from monocytes; plasma at 6, 12, and 24 hr; and recipient aortas at 24 hr after infusion were extracted with chloroform:methanol (1:2 v/v), and each sample representing an image of 220 μm (x) by 240 μm (y) at 2 pixels/μm. Imaging was performed using a custom-built video-rate 2P microscope equipped with a Chameleon Vision II Ti:Sapphire laser.

**Cellular Cholesterol Metabolism**

Foam cell formation was assessed with fixation and staining with oil red O. For cellular total or free cholesterol, lipids were extracted with chloroform:methanol (1:2 v/v), dried, and reconstituted for enzymatic assays using commercial reagents. For cholesterol binding and uptake, cells were incubated for 6 hr with 10 μg/ml Dil-labeled oxidized LDL (DiL-oxLDL) at 4°C (binding) or at 37°C (binding and uptake), with uptake calculated as the difference. For cholesterol uptake assessments with the MacMII inhibitor KN93, macrophages were incubated with KN93 (10 μM) for 24 hr with Dil-oxLDL added for the final 6 hr. For cholesterol efflux, macrophages were incubated with oxoLDL (200 μg/ml) with 3H oleic acid (0.1 mM) for 6 hr prior to lipid extraction and radioactivity assessment. For cholesterol efflux, assays were incubated with oxoLDL (100 μg/ml) with 5 μCi of 3H cholesterol for 24 hr, then with apolipoprotein AI (25 μg/ml) or HDL (50 μg/ml) for 6 hr, after which supernatant and cells were assessed for radioactivity.

**SERCA2a Activity**

SERCA2a activity was assayed by isolating macrophage ER fractions with different centrifugation, incubating with radiolabeled CaCl2 for 15 min at 37°C, and measuring radioactivity.

**Immunofluorescence**

Peritoneal macrophages were fixed on slides with 4% formalin, blocked with 5% goat serum, and incubated with SERCA2a (1:250, Abcam) and VDR (1:200, Santa Cruz) overnight, then incubated with secondary antibody 488-conjugated goat anti-rabbit (green) and 532-conjugated goat anti-rabbit (red). Images were captured on a confocal microscope (Olympus Fv1000).

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Figure 6. Deletion of Macrophage Vdr Was Sufficient to Induce Insulin Resistance and Atherosclerosis
Irradiated KODMAC-L mice were transplanted with BM from KODMAC-L (KOD/KOD) or control-L (Con/KOD) animals to rescue the phenotype (A–H), and irradiated control mice were transplanted with BM from KODMAC-L (KOD/Con) or control-L (Con/Con) animals to induce the phenotype (I–P). All experiments except (E) and (M) were performed before HFD.

(A and I) Glucose tolerance test with 30-min plasma insulin levels (inset) and (B and J) insulin tolerance test (n = 7–11 per group).

(C and K) qRT-PCR analysis of relative Pck1 and G6pc mRNA expression from hepatocytes, normalized to Mrpl32 (n = 3 per group).

(D and L) Flow cytometry expression of membrane M1 and M2 markers in liver macrophages (n = 4 per group).

(E and M) Atherosclerotic plaque area of pinned aortas after HFD (n = 8–11 per group).

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Gene Expression
qRT-PCR was performed using the TaqMan or SYBR Green reagent kit, with relative expression levels of mRNAs calculated by the comparative threshold cycle method using L32 (Mrpl32) as an internal control.

Immunoblotting and Immunoprecipitation
Cellular proteins were immunoblotted for VDR (Santa Cruz), ER stress proteins (pPERK and PERK [Cell Signaling], CHOP [Santa Cruz]), proteins involved in cholesterol signaling (JNKp [Cell Signaling], PPARY, CD36, and SR-A1 [Santa Cruz]), SERCA2 (Abcam), and pCAMKII and CAMKII (Cell Signaling), and I-actin (Cell Signaling) was used as a loading control. For immunoprecipitation, macrophage lysates from control-L mice after 6 weeks of chow were incubated with monoclonal anti-VDR (Abcam), then blotted with anti-SERCA2 (Abcam).

Statistical Analysis
Experiments were carried out in duplicate or triplicate. Parametric data are expressed as the mean ± SEM and analyzed by t tests, paired or unpaired as appropriate, or by one-way ANOVA and Tukey’s post test for more than two groups. Non-parametric data (atherosclerosis) are presented as the median and analyzed using the Mann-Whitney test. Differences were considered statistically significant if p ≤ 0.05. Statistical analysis was carried out using GraphPad Prism.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.02.043.

AUTHOR CONTRIBUTIONS
All authors assisted in drafting and revising the work, approved the final version for publication, and agree to be accountable for all aspects of the work. Individual contributions are as follows: J.O. conceived and designed overall work and acquired data. A.E.R. conceived and designed overall work and analyzed and interpreted data. I.D. designed glucose metabolism experiments and acquired data. K.F. designed glucose metabolism and SERCA function experiments and acquired, analyzed, and interpreted data. J.S.S. and K.C. designed monocyte cholesterol transport experiments and acquired, analyzed, and interpreted data. C.B.-M. conceived and designed overall work and analyzed and interpreted data.

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REFERENCES


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(A and B) Cholesterol uptake after Dil-oxLDL stimulation (n = 6 per group). (C and D) Cholesterol ester formation after stimulation with oxLDL and 3H oleic acid (n = 4–6 per group). (E and F) Cholesterol efflux after incubation with 3H cholesterol and stimulation with HDL or apolipoprotein AI in peritoneal macrophages (n = 6 per group). *p < 0.05 versus KOD/KOD or Con/Con, respectively. Data are presented as mean ± SEM. See also Figure S6.


