Tribbles Homolog 3 Promotes Foam Cell Formation Associated with Decreased Proinflammatory Cytokine Production in Macrophages: Evidence for Reciprocal Regulation of Cholesterol Uptake and Inflammation

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Abstract

Background: Insulin resistance is central in the pathophysiology of cardiometabolic disease; however, common mechanisms that explain the parallel development of both type 2 diabetes and atherosclerosis have not been elucidated. We have previously shown that tribbles homolog 3 (TRB3) can exert a chronic pathophysiological role in promoting insulin resistance and also has an acute physiological role to alternatively regulate glucose uptake in fat and muscle during short-term fasting and nutrient excess. Since TRB3 is expressed in human atherosclerotic plaques, we explored its role in foam cell formation to assess its potential contribution to atherogenesis.

Methods: We have used human THP-1 monocytes, which transition to lipid-laden macrophage foam cells when exposed to oxidized low-density lipoprotein (ox-LDL).

Results: We first observed that TRB3 was upregulated by more than twofold ($P < 0.01$) within 24 hr of treatment with ox-LDL. To determine whether TRB3 actively participated in foam cell formation, we overexpressed TRB3 in THP-1 monocytes and found that this led to a 1.5-fold increase in cholesterol accumulation after 48 hr ($P < 0.01$), compared with controls. At the same time, TRB3 overexpression suppressed inflammation in macrophages as evidenced by reduced expression and secretion of tumor necrosis factor alpha (TNF- α) and interleukin-1 beta (IL-1 β) (both $P < 0.01$).

Conclusions: (1) TRB3 is upregulated in macrophages upon treatment with ox-LDL; (2) TRB3 promotes lipid accumulation and suppresses cytokine expression; and (3) inflammation and foam cell formation can be reciprocally regulated, and TRB3 orients the macrophage to assume a more primary role for lipid accumulation while maintaining a secondary role as an inflammatory immune cell.

Introduction

METABOLIC SYNDROME IS A common metabolic disorder characterized by glucose intolerance, central obesity, dyslipidemia, and hypertension. Insulin resistance plays a central role in the pathogenesis of the metabolic syndrome and places individuals at an increased risk of both type 2 diabetes mellitus (T2DM) and cardiovascular disease.^{1–3} This spectrum of pathophysiology has been termed cardiometabolic disease, which we define as a process that begins early in life with relative insulin resistance progressing to clinically identifiable states of high risk, namely prediabetes

and metabolic syndrome, and culminating in T2DM, cardiovascular disease events, or both in single patients. We have previously shown that tribbles homolog 3 gene (TRB3) promotes insulin resistance in insulin target cells, is induced by glucose, and can mediate glucose-induced insulin resistance (*i.e.*, “glucose toxicity”) in diabetes.⁴ While these studies demonstrate a role for TRB3 in the metabolic component of cardiometabolic disease, we hypothesize that TRB3 also participates in atherogenesis, the vascular component of the disease.

The tribbles gene was initially discovered in *Drosophila* as a regulator of germ cell development.⁵ It can slow the

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progression of the G2 stage of the cell cycle and regulate DNA damage repair through ubiquitination.^{5,6} Mammals express three homologs of tribbles; TRB1, TRB2, and TRB3. All contain a consensus serine/threonine kinase catalytic core, but lack the key residues for catalytic phosphorylation, and are referred to as pseudokinases.^{4,7,8} Although the function of tribbles in humans is not fully understood, studies have demonstrated that TRB3 mediates a number of cellular functions. In particular, TRB3 has the ability to inhibit insulin signaling in multiple cells and tissues by binding to and inhibiting phosphorylation of Akt.⁹ Through its action on Akt and perhaps other phosphorylated signaling molecules, it has also been found to regulate nitric oxide synthesis, endoplasmic reticulum stress-induced apoptosis, cell differentiation, and glucose transport.⁸

TRBs have also been implicated in atherosclerosis. They were found to be expressed in unstable regions of human carotid plaques, and the silencing of TRB3 suppressed atherosclerosis and stabilized plaques in diabetic ApoE^{-/-}/LDL receptor^{-/-} mice.^{10,11} There are many events that lead to the formation of an atherosclerotic lesion, but the initial lesion is the formation of macrophage foam cell. These are cells that coalesce to produce fatty streaks in the endothelium of large arteries.¹² Recruitment of monocytes to areas of endothelial dysfunction, followed by their migration into the endothelial space and the subsequent differentiation into macrophages, sets the stage for foam cell formation. These cells contribute to inflammation in the vascular wall and protect vessels by removing cytotoxic oxidized low-density lipoprotein (ox-LDL) particles. Therefore, we investigated whether TRB3 could be induced by ox-LDL and help modulate foam cell formation. We surmised that the signaling of ox-LDL through Toll-like receptor 4 (TLR4) was involved in the induction of TRB3 and used lipopolysaccharide (LPS) stimulation to further test this theory.¹³ We were surprised to observe that TRB3 accelerated cholesterol accumulation and foam cell formation, but had an opposing effect to suppress the inflammatory status of these cells, possibly through its regulation of fatty acid binding protein 4 (FABP4). Our laboratory has previously shown that ox-LDL is responsible for an increase in transcription of FABP4 and subsequent overexpression of this gene increased cholesterol ester accumulation in macrophage foam cells.¹⁴ To further examine the phenomenon of reciprocal regulation of inflammation and cholesterol accumulation, we disrupted the functional status of the inflammasome and observed similar findings. The data suggest that macrophages can be alternatively programmed for cholesterol uptake versus inflammation early in the process of atherogenesis.

Materials and Methods

Culture of human monocytic cells (THP-1)

THP-1 cells, derived from an acute monocytic leukemia patient, were obtained from ATCC. They were cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented by fetal bovine serum to 10%, 2-mercaptoethanol to a final concentration of 0.05 mM, 100 U/mL of penicillin, and 100 mg/mL of streptomycin in a 5% CO₂ atmosphere at 37°C. The monocytes were differentiated into macrophages by treatment with phorbol 12-myristate 13-acetate (PMA) at a concentration of 100 ng/mL for 24 hr. The adherent macrophages were then washed with phosphate-buffered

saline (PBS) thrice and allowed to rest in the aforementioned complete IMDM for 24 hr before experiments. Foam cell formation was achieved by treatment of the rested macrophages with 100 µg/mL of ox-LDL for 24, 48, and 72 hr. NLRP3-deficient THP-1 cells were ordered from InvivoGen (thp-dlnp). They were cultivated, differentiated, and treated in the same media conditions as our native wild-type (WT) and TRB3 overexpressing (OE) macrophages.

LDL oxidation

Sterile human LDL was purchased from INTRAcEL. The LDL was dialyzed against a solution of 0.01 M Tris and 0.15 M NaCl at 4°C overnight. Copper chloride (CuCl₂) was then added at a concentration of 10 µM overnight in the Tris/NaCl solution to oxidize the LDL. Finally, to stop the oxidation, the LDL was placed back in the Tris/NaCl solution (this time with PBS) and the buffer was replaced thrice over 24 hr. The Bio-Rad DC protein assay was then used to determine the concentration of the ox-LDL.

Lentiviral-induced THP-1 cells

Full-length coding sequence of Trb3 with cMyc tag at 3' end was cloned into the lentiviral vector for overexpression. A control vector with enhanced green fluorescent protein (GFP) cloned into the same vector backbone was also generated. To generate the lentiviral construct, 7.5 µg of OE plasmid was cotransfected with 5 µg vesicular stomatitis virus-G protein and 7.5 µg pGag/Pol packaging plasmids into HEK293T cells in 10-cm plates. Using the calcium precipitation method, viral particles were harvested from the supernatant 48 hr after transfection. THP-1 cells were then transduced with virus at multiplicity of infection ~50 with 8 µg/mL Polybrene (Sigma-Aldrich) for 24 hr. Stable cell lines OE TRB3 were generated by puromycin selection for 2 weeks.

Cholesterol accumulation

After treatment with ox-LDL for the indicated time periods, the media were removed and the cells were washed thrice with PBS. The cells were then collected using 300 µL/well Cell-lytic M Cell Lysis (Sigma-Aldrich) reagent fortified with complete EDTA-free protease inhibitor cocktail (Sigma-Aldrich) and PhosSTOP phosphatase inhibitor cocktail (Sigma-Aldrich). The cells were disrupted by a sonic dismembrator. Protein concentration was then measured using the Bio-Rad DC protein assay to determine a baseline measure of the cellular samples, and cholesterol accumulation was measured using the Wako Cholesterol E Kit.

RNA preparation and quantitative polymerase chain reaction

After treatment, media were removed and the cells were washed thrice with PBS. The cell samples were removed using the Buffer RLT, which is the lysis buffer provided by the Qiagen RNeasy Mini Kit. A cell sonicator was used to disrupt the cellular membranes, and the rest of the RNA isolation was carried out using the RNeasy Mini Kit (Qiagen), as described by the manufacturer. RNA concentration and integrity were assessed using the Synergy 2 Multimode Microplate reader (BioTek).

The isolated RNA was then reverse transcribed into cDNA using the SuperScript VILO cDNA Synthesis Kit (Qiagen) using the Veriti 96-Well Fast Thermal Cycler (Applied Biosystems). The cDNA concentration and integrity were also assessed using the BioTek plate reader. mRNA levels assessed by quantitative real-time polymerase chain reaction (RT-PCR) were normalized to GAPDH and then expressed as arbitrary mRNA units.

Western blotting

Cultured macrophages were lysed in a buffer (Sigma-Aldrich) supplemented with complete protease inhibitor cocktail (Roche). Antibodies to TRB3 were used to detect the presence of TRB3 in the cell lysates.

Statistics

All data are given as mean $\pm$ standard deviation, unless otherwise indicated. Comparisons between means were performed using a two-tailed Student's *t*-test. Comparisons among more than two variables were calculated with ANOVA following the *post hoc* Tukey test, if appropriate. Differences were accepted as significant at $P < 0.05$.

Results

TRB3 expression is upregulated by ox-LDL

To determine whether TRB3 could be playing a role in foam cell formation, we first assessed whether the TRB3 mRNA gene expression was regulated in macrophages exposed to ox-LDL. THP-1 macrophages were treated with 100 μ g/mL of ox-LDL, and the expression of TRB3 was measured. As shown in Fig. 1, TRB3 expression was induced by ox-LDL with progressive increases in TRB3 mRNA levels over baseline quantified as 2.3 ± 0.3 -fold at 24 hr in THP-1 cells (Fig. 1A) and 3.0 ± 0.5 in Raw 264.7 cells (Fig. 1B). We confirmed our data by measuring protein levels of TRB3 in Raw 264.7 cells treated with ox-LDL (Fig. 1C).

TRB3 overexpression increases cholesterol accumulation in THP-1 macrophages

We then studied whether TRB3 actively participated in foam cell formation by directly enhancing lipid accumulation in THP-1 cells. We transfected WT THP-1 cells with a lentiviral expression vector containing a full-length TRB3 sequence with a cMyc tag at the 3' end. As shown in Fig. 2, stable transfectants exhibited a >60-fold increase in TRB3 mRNA compared to WT macrophages and to control cells transfected with a lentivirus construct expressing GFP. Treatment with ox-LDL for 24 hr produced significant increases in TRB3 mRNA in both controls and in TRB3 OE cell lines. To assess effects of TRB3 expression on lipid accumulation, we treated WT macrophages, controls expressing GFP, and TRB3 OE macrophages with ox-LDL for 48 hr. When compared with the control cell lines, we observed a significantly greater uptake in the cellular content of cholesterol in the TRB3 OE macrophages and a significantly greater fold increase in cholesterol accumulation in response to ox-LDL (1.6 ± 0.5 - vs. 1.9 ± 0.3 - vs. 2.5 ± 0.4 -fold in the WT, GFP expressing, and TRB3 OE cells, respectively; $P < 0.05$ comparing TRB3 OE to WT and GFP; $P =$ not significant WT vs. GFP) as shown in Fig. 3.

FIG. 1. TRB3 expression is up-regulated by ox-LDL. Quantitative real time PCR was done on the cellular samples at the time points shown. mRNA expression was normalized to GAPDH. (A) THP-1 macrophages were rested for 24 hours and incubated in the presence of ox-LDL (100 μ g/mL) to confirm that TRB3 expression is increased in macrophages. (B) Raw 264.7 macrophages were also incubated in the presence of ox-LDL (100 μ g/mL) for 24 hr to confirm that ox-LDL increased TRB3 expression in macrophages. * $P < 0.001$ compared to baseline. (C) A western blot analysis was performed on Raw 264.7 macrophages using a TRB3 antibody. After 48 hr of incubation with ox-LDL, there was a significant increase in the TRB3 protein expression. * $P < 0.05$. ox-LDL, oxidized low-density lipoprotein; TRB3, tribbles homolog 3.

FIG. 2. Lentiviral vector-mediated expression of TRB3 in THP-1 (WT) cells. TRB3 expression levels were assessed in both WT control cells (*open bars*) and in stable TRB3 OE cells (*closed bars*) in the absence and presence of 100 μ g/mL of ox-LDL for 24 hr. * $P < .001$ compared with WT control values. OE, overexpressing; WT, wild type.

Ox-LD-induced secretion of MCP-1, TNF- α , and IL-1 β is TRB3 dependent

Ox-LDL is known to induce inflammation, which is involved in the pathophysiology of atherogenesis in the vascular wall. Therefore, we tested for effects of TRB3 overexpression on production of proinflammatory cytokines in response to ox-LDL. WT THP-1 and TRB3 OE macrophages were treated with ox-LDL for 24 hr, and cytokine secretion in the media was measured. As shown in Fig. 4, compared with WT cells, TRB3

FIG. 3. TRB3 overexpression augments lipid accumulation in response to ox-LDL in THP-1 macrophages. WT (human THP-1), GFP expressing, and TRB3 OE macrophages were treated with 100 μ g/mL of ox-LDL for 48 hr. The fold change represents the cholesterol accumulation difference between untreated macrophages for 48 hr and the ox-LDL-treated macrophages for 48 hr, normalized to grams of protein. There was no significant change in the lipid accumulation between WT and GFP controls. The TRB3 overexpression cells exhibited a significant increase in the amount of lipid accumulation; * $P < .05$ compared with WT and GFP. GFP, green fluorescent protein.

overexpression led to marked reductions in tumor necrosis factor alpha (TNF- α) secretion (10.5 ± 5.6 vs. 0.8 ± 1.9 pg/100 μ L media/mg protein) in Fig. 4A, interleukin-1 beta (IL-1 β) secretion (55.7 ± 5.7 vs. 7.3 ± 3.7) in Fig. 4B, and monocyte chemoattractant protein 1 (MCP-1; 1977.9 ± 106.6 vs. 1121.6 ± 87.7) in Fig. 4C, in response to ox-LDL (Fig. 4A, B, $P < 0.001$). TNF- α (Fig. 4D), IL-1 β (Fig. 4E), and MCP-1 (Fig. 4F) mRNA decreased in TRB3 OE cells ($P < 0.05$).

LPS-induced secretion of TNF- α through TLR4 is TRB3 dependent

It has been shown that ox-LDL can signal the inflammatory response, in part, through TLR4.¹³ In fact, we have observed that the TRB3 expression in response to ox-LDL was severely blunted in isolated peritoneal macrophages from TLR4^{-/-} mice compared with those from WT mice (unpublished data; not shown).¹⁵ We, therefore, examined whether TRB3 overexpression blunted cytokine production in response to LPS, a direct stimulator of TLR4. We first observed in Fig. 5A that treatment with LPS induced TRB3 mRNA expression in both WT and TRB3 overexpression cell lines, similar to our findings for ox-LDL (compare with Fig. 2). Moreover, TRB3 OE macrophages secreted significantly less TNF- α into the media in response to LPS compared with WT cells (207.7 ± 115.1 vs. 474.0 ± 179.1 pg/100 μ L media/mg protein $P < 0.05$), as demonstrated in Fig. 5B.

mRNA of proatherosclerotic gene FABP4 is increased TRB3 overexpression genes

We then examined the impact of TRB3 overexpression on a gene that we have previously shown to mediate an increase in lipid accumulation and foam cell formation, namely FABP4.⁴ Figure 6 shows FABP4 mRNA levels in WT and TRB3 OE macrophages treated in the absence and presence of ox-LDL for 24 hr. TRB3 OE cells displayed significantly greater induction of FABP4 in response to ox-LDL (8.3 ± 0.1 vs. 19.0 ± 0.5 relative units; $P < 0.001$).

Cell viability of TRB3 OE expression cells is similar when treated with ox-LDL

Previous reports have shown that overexpression of TRB3 in macrophages can inhibit viability.⁷ To exclude the possibility that TRB3 effects were due to differential impact on macrophage viability, we performed the yellow tetrazolium [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] MTT assay and found that there were no significant differences in the viability of macrophages comparing WT cells, cells expressing GFP, and cells OE TRB3 treated with or without ox-LDL (Fig. 7).

Opposite regulation of cholesterol accumulation and inflammation during foam cell formation

The aforementioned data indicated that TRB3 overexpression enhanced macrophage lipid accumulation and foam cell formation, while at the same time suppressing inflammatory responses, in response to ox-LDL. The implication is that macrophages can be regulated to favor lipid accumulation at the expense of inflammation and vice versa. To further test this reciprocal relationship, we diminished the inflammatory potential of macrophages by knock down

FIG. 4. TRB3 suppresses inflammatory responses. THP-1 WT cells were treated with 100 μ g/mL of ox-LDL for 24 hr. The media were collected, and a subsequent ELISA was run on samples of the media to determine the cytokine secretion of the different cell lines. Levels of the corresponding mRNA were also measured. (A) TNF- α secretion was decreased in the TRB3 OE cell lines. (B) IL-1 β secretion was decreased in the TRB3 OE cell lines. (C) MCP-1 secretion was decreased in the TRB3 OE cell lines. (D) TNF- α mRNA was lower in TRB3 OE cell lines. (E) IL-1 β mRNA was lower in TRB3 OE cell lines. (F) MCP-1 mRNA was lower in TRB3 OE cell lines. * P < 0.001 in each panel comparing TRB3 OE and WT. IL-1 β , interleukin-1 beta; MCP-1, monocyte chemoattractant protein 1; TNF- α , tumor necrosis factor alpha.

of the critical inflammasome protein, NLRP3, and observed effects on cholesterol accumulation and cytokine production in response to ox-LDL and LPS. As demonstrated in Fig. 8B, NLRP3 knockdown cells accumulated greater amounts of cholesterol than control cells following ox-LDL treat-

ment, although TRB3 mRNA levels remained relatively similar in the two cell lines (Fig. 8A). At the same time, the induction of IL-1 β protein by LPS was greatly diminished in the NLRP3 knockdown cells over a time course study as indicated in Fig. 8B. The data confirm the concept that

FIG. 5. TRB3 expression in response to LPS. (A) TRB3 mRNA levels are induced by LPS in WT cells (*open bars*) and increased above baseline in TRB3 OE cells (*closed bars*). Cells were treated with 1 μ g/mL of LPS for 24 hr. (B) TNF- α secretion was suppressed in response to LPS. * $P < 0.01$ comparing OE values to control values. LPS, lipopolysaccharide.

manipulations, which suppress inflammatory capabilities, can augment the macrophage's capacity to accumulate lipid in the transition to foam cells.

Discussion

Two sets of observations provided rationale for the current study. First, a potential role for TRB3 in atherogenesis was established by studies demonstrating that TRB3 was expressed in unstable regions of human carotid plaques¹⁰ and that silencing of TRB3 suppressed atherosclerosis and stabilized plaques in diabetic ApoE^{-/-}/LDL receptor^{-/-} mice.¹¹ Second, we have shown that TRB3 induces insulin resistance in cultured muscle and adipose cells and mediates glucose-induced insulin resistance in diabetes.⁸ Thus, we hypothesized that TRB3 could participate in both the metabolic and vascular components of the cardiometabolic disease. In this study, we investigated the role of TRB3 in foam cell formation in macrophages. We showed that one of the principal proponents of atherosclerosis progression, ox-LDL, can induce TRB3 expression in macrophages. We then examined whether TRB3 actively modulated cholesterol accumulation and foam cell formation in macrophages and found that TRB3

did indeed promote lipid accumulation. One underlying mechanism was that TRB3 induced expression of FABP4, which we have previously shown to be sufficient to augment lipid uptake and foam cell formation in macrophages.¹⁴

In addition to the impact of TRB3 on lipid accumulation, we were surprised to find that the effect to accelerate foam cell formation was associated with a reduction in the production and secretion of proinflammatory cytokines. Specifically, TRB3 overexpression resulted in suppressed release of critical proinflammatory cytokines in the progression of atherosclerosis such as IL-1 β , MCP-1, and TNF- α in response to ox-LDL. To explore whether there was a reciprocal relationship between lipid accumulation and inflammation in macrophages, we disrupted the inflammasome by silencing NLRP3, a key constituent protein, and found that the resulting inhibition of inflammation led to an increase in lipid accumulation. Our results have led us to conclude that increased TRB3 expression alters a macrophage's primary function; that is, TRB3 increases a macrophage's capacity for lipid accumulation, while deemphasizing its function as an inflammatory cell.

Cardiovascular disease has been shown to be increased in patients with type 2 diabetes compared to those without diabetes. The current data suggest that TRB3 sits at the

FIG. 6. Effect of TRB3 on induction of the proatherogenic gene, FABP4, by ox-LDL. WT and TRB3 OE macrophages were treated with and without 100 μ g/mL for 24 hr, and then levels of FABP4 mRNA were measured. * $P < 0.001$ comparing ox-LDL-stimulated WT and TRB3 OE cells. FABP4, fatty acid binding protein 4.

FIG. 7. No effects of TRB3 on cell viability. WT, GFP vector, and TRB3 OE cells were treated for 24 hr with 100 μ g/mL of ox-LDL. The yellow tetrazolium [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] MTT assay was then used to check the viability of the cells. There was no significant difference among these cell lines whether treated with or without ox-LDL for 24 hr (all comparisons, $P = \text{NS}$). NS, not significant.

FIG. 8. Disruption of the inflammasome by silencing of NLRP3 and effects on cholesterol accumulation and inflammation. (A) Cholesterol accumulation by NLRP3^{-/-} THP-1 cells. WT THP-1 and NLRP3 knockdown (NLRP3^{-/-}) macrophages were treated with 100 μg/mL of ox-LDL for 48 hr, as described in our previous experiments. Cholesterol accumulation was significantly higher in the NLRP3^{-/-} cells compared to the WT THP-1 cells at the same time point. (B) WT and NLRP3^{-/-} macrophages were treated with 5 μg/mL of LPS, and IL-1β secretion was measured. (C) The silencing of NLRP3 suppressed the secretion of IL-1β in the NLRP3^{-/-} macrophages compared to WT. **P* < 0.01 in comparing all NLRP3^{-/-} with WT.

crossroads between type 2 diabetes and cardiovascular disease, because of its influence on many of the various pathways and processes involved in both insulin resistance and foam cell formation.

Multiple pathogenic factors have shown to lead to enhanced TRB3 expression in several tissues. High-glucose concentrations induce insulin resistance in muscle cells in a TRB3-dependent manner, and induction of TRB3 involves glucose metabolism through the hexosamine biosynthetic pathway.¹⁶ Increased TRB3 expression has been seen in the skeletal muscle of Zucker fatty rats and streptozotocin-treated diabetic rats in the adipose tissue of rats with metabolic syndrome induced by a high-fructose diet and in the liver of rats with nonalcoholic fatty liver disease.^{4,17,18} Hyperglycemia can lead to vascular complications. High-glucose concentrations can activate nuclear factor κB (NF-κB), which can increase the expression of various genes in multiple cells, including macrophages.^{19,20} Glucose also increases oxidative stress producing reactive oxygen species, such as the superoxide anion, which can cause LDL oxidation.²¹ We have shown that ox-LDL is a potent inducer of the expression on TRB3, adding to the list of metabolic pathogenic factors or nutrient sensors that are capable of inducing TRB3.

Inflammation is a well-defined feature of atherosclerotic progression. Macrophages are known to release a myriad of cytokines in the vascular wall and contribute to lesion progression. MCP-1 is important in recruiting monocytes to atherosclerotic lesions in mice lacking apolipoprotein E or LDL receptor.^{22,23} In addition, ox-LDL promotes the secretion of IL-1β, considered to be a multifunctional cytokine in inflammation.²⁴ Previous studies have shown a role for IL-1β in the initiation and progression of atherosclerosis.^{25,26} TNF-α is yet another critical inflammatory cytokine released by modified lipoprotein-induced macrophages, and TNF-α production is augmented in macrophages that are part of carotid plaques.^{27,28} One of the auxiliary roles of macrophages is the removal of apoptotic cells and other harmful debris in the body, including oxidized forms of LDL. We have shown that increased TRB3 expression in response to ox-LDL can program the macrophage's primary function to favor lipid accumulation and diminish its role as an inflammatory cell.

It has been established that TLR4 is expressed in human atherosclerotic plaques, in addition to being upregulated on the macrophage cell surface in response to ox-LDL.²⁹ Recently, there has been an increase in the study of another class of pattern recognition receptors, NOD-like receptors (NLRs). One of the best characterized NLRs is known as the NLRP3 inflammasome, which is a large multimeric protein complex consisting of apoptosis-associated speck-like protein containing a CARD (ASC) and procaspase-1. Activation of NLRP3 leads to the secretion of IL-1β and IL-18.³⁰ For optimal activation of this inflammasome, a priming signal is needed, and TLR4 can often serve as this first priming signal in the NLRP3 cascade. In addition, NLRP3 inflammasomes secrete large amounts of IL-1β in response to cholesterol crystals. Previous studies have also shown that bone marrow from mice deficient in NLRP3 or ASC reduces atherosclerosis lesion size when introduced into mice susceptible to atherosclerosis.^{31,32} Although not yet confirmed, it is generally accepted that ox-LDL is an agonist for TLR4.²⁹ Using this information, we used LPS as a potent TLR4 ligand to study its effect on TRB3 expression. In other unpublished

experiments in macrophages, we have observed that LPS can induce TRB3 expression in a TLR4-dependent manner. In the current study, we show that cytokine secretion following LPS activation of TLR4 is depressed in the TRB3 OE cells, as was observed in response to ox-LDL. We surmised that there might be some interplay and overlap between the effects of TRB3 overexpression and NLRP3 on foam cell formation. In fact, silencing of NLRP3 incapacitated inflammatory responses concomitant with an increase in lipid accumulation.

In several previous studies, ox-LDL exerted a toxic effect and induced apoptosis in macrophages contributing to the formation of the lipid core.^{33–35} Shang et al. have also reported that TRB3 overexpression increased apoptosis.⁷ We have shown that stable TRB3 OE macrophages do not show any signs of increased apoptosis in macrophages and that the effects of TRB3 to augment foam cell formation and suppress secretion of cytokines cannot be attributed to any toxicity. It has been well characterized that apoptosis contributes to the development of vulnerable atherosclerotic plaques and lesion rupture, while the increased secretion of proinflammatory cytokines results in the cell death of endothelial and muscle cells in the lesion area.^{36–38} This could be explained by the fact that macrophage apoptosis is well regulated in early atherosclerotic lesions, while becoming less efficient and contributing more to the necrotic core of advanced lesions.³⁹ Short-term treatment (0–72 hr) with ox-LDL *in vitro* could be considered acute when compared to the chronic disease progression that evolves over several decades.

In conclusion, (1) TRB3 has the ability to alter the function of a macrophage by diminishing its status as a proinflammatory releasing cell and augmenting its capacity to accumulate lipid; (2) disruption of the inflammasome through NLRP3 silencing also promotes foam cell formation; and (3) inflammation and lipid accumulation can be reciprocally regulated suggesting that there may exist populations of macrophages in the vascular wall with variable capacities for inflammation and lipid uptake. In light of previous data demonstrating a role for TRB3 in insulin resistance, TRB3 could represent an important mechanistic link between insulin resistance and increased cardiovascular disease risk in cardiometabolic disease.

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Author Disclosure Statement

No competing financial interests exist.

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