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Enhanced Atherosclerosis in TIPE2-Deficient Mice Is Associated with Increased Macrophage Responses to Oxidized Low-Density Lipoprotein

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Atherosclerosis has been widely recognized as an inflammatory disease of the arterial wall in which macrophages play a major role. Yet, how macrophage-mediated pathology is regulated during atherosclerosis is poorly understood. TNF-α-induced protein 8-like 2 (TIPE2, also known as TNFAIP8L2) is highly expressed in resting macrophages and can negatively regulate inflammation through inhibiting immune receptor signaling. We report in this article that TIPE2 plays a crucial atheroprotective role likely by regulating macrophage responses to oxidized-low-density-lipoprotein (ox-LDL). TIPE2-deficient macrophages treated with ox-LDL produced more oxidative stress and proinflammatory cytokines, and exhibited heightened activation of the JNK, NF-κB, and p38 signaling pathways. As a consequence, TIPE2 deficiency in bone marrow–derived cells exacerbated atherosclerosis development in Ldlr−/− mice fed a high-fat diet. Importantly, ox-LDL markedly downregulated TIPE2 mRNA and protein levels in macrophages, suggesting that ox-LDL mediates atherosclerosis by TIPE2 inhibition. These results indicate that TIPE2 is a new inhibitor of atherosclerosis and a potential drug target for treating the disease. The Journal of Immunology, 2013, 191: 000–000.

Recent studies have shown that atherosclerosis is a non-resolving inflammatory disease in which immune mechanisms interact with metabolic risk factors to initiate, propagate, and activate lesions in the intima of medium- and large-sized arteries (1, 2). Bloodborne inflammatory and immune cells play an important role during atherosclerosis. Monocytes migrate into the vessel wall from blood, differentiate into macrophages, and transform into foam cells by taking up oxidized-low-density lipoprotein (ox-LDL) (3, 4). Activated macrophages produce inflammatory cytokines, proteases, and cytotoxic oxygen and nitrogen radical molecules to promote atherosclerosis (5, 6). Functional abnormalities of macrophages led to reduced atherosclerosis in ApoE−/− mice (7). Hence atherogenesis is an inflammatory process in which the macrophage is the major player.

TNF-α–induced protein 8–like 2 (TIPE2, also known as TNFAIP8L2) is a newly identified protein of the TNFAIP8 family (also known as TIPE) (8, 9). This family consists of four members, TNFAIP8 (TIPE), TIPE1 (TNFAIP8L1), TIPE2, and TIPE3 (TNFAIP8L3), which share a homologous sequence, forming a unique structure with unknown functions (10). Early studies show that TIPE2 plays an essential role in the maintenance of immune homeostasis (8). TIPE2 can regulate both inflammation and carcinogenesis (8, 11) Interestingly, it is found highly expressed in macrophages and serves as a negative regulator of innate immunity through inhibiting TLR signaling (8, 12, 13). Its germine deletion leads to fatal inflammatory diseases, and abnormal expression of TIPE2 in humans was found to be associated with hepatitis B, diabetic nephropathy, and experimental stroke (8, 14–16). However, it remains unknown whether TIPE2 plays a role in atherosclerosis. We hypothesize that TIPE2 expressed in macrophages plays atheroprotective roles by negatively regulating inflammation. A number of in vivo and in vitro experiments were designed and performed in this study to test this hypothesis.

Materials and Methods

Animals and bone marrow transplantation

All animal experiments were performed according to the guidelines of the Animal Management Rules of the Chinese Ministry of Health (document no. 55, 2001) and were approved by the AnimalEthical Committee of Shandong University. All mice were housed in the Animal Facilities of Shandong University School of Medicine, under pathogen-free conditions throughout the experiment.

Two weeks before and 3 wk after bone marrow transplantation, Ldlr−/− female mice received acidified water containing neomycin (100 mg/l) and
polymyxin B sulfate (60,000 U/l) in filter-top cages. One day before transplantation, Ldlr\textsuperscript{−/−} (age 11 wk, n = 16; purchased from Model Animal Research Center of Nanjing University, Nanjing, China) mice were subjected to lethal irradiation with 1000 rad (10 Gy) to eliminate bone marrow stem cells. Donor bone marrow from 11-wk-old TIPE2\textsuperscript{−/−} mice (n = 6, as previously described [8, 11, 13]) in C57BL/6J background or age- and sex-matched wild type (WT; C57BL/6J background; n = 6) was prepared by flushing the femur and tibia as previously described (17). For transplantation, recipient mice were injected with 5 \times 10\textsuperscript{5} bone marrow cells through the tail vein. Eight of these animals were transplanted with WT bone marrow and the other eight with TIPE2\textsuperscript{−/−} bone marrow. After 4 wk recovery on a sterilized regular chow, the mice were switched to a high-fat diet for 8 wk to induce atherosclerotic lesion development. A high-fat diet contained 15% fat and 0.25% cholesterol, whereas a chow diet had 5% fat and no added cholesterol. Twelve weeks after bone marrow transplantation, blood was collected after overnight fasting to determine plasma cholesterol and triglyceride concentrations using commercial kits (Roche Diagnostics, Indianapolis, IN). Peripheral blood cell counts were analyzed by Drew Hemavet 950FS (Drew Scientific, Oxford, U.K.) after 6 wk of high-fat feeding.

Atherosclerotic lesion analysis

Animals were anesthetized by i.p. pentobarbital injection and exsanguinated by femoral artery transection. In situ perfusion fixation through the left ventricle was performed by normal saline instillation for 15 min, followed by infusion of 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) for 30 min. The heart and aortas were dissected and fixed in 4% paraformaldehyde (Acros Organics, Geel, Belgium) for 48 h. The aortic root was cut into 2-mm-wide sections, which were fixed in 4% paraformaldehyde for 24 h before cryopreservation. The aortic sections were stained with Masson’s trichrome for evaluation of collagen contents. The mouse heart was isolated and embedded in OCT compound (Sakura Finetek USA, Torrance, CA) and frozen, to quantify atherosclerosis in the aortic root. Subsequently, sequential sections were cut at 6-μm thickness every 50 μm along the aortic root. Atherosclerotic lesions were quantified by taking an average of three cross sections from each aorta spaced 50 μm apart using Image Pro plus 6.0 software (Media Cybernetics).

Immunohistochemistry and immunofluorescence

Immunohistochemistry and immunofluorescence was performed as previously described (12). The primary Abs were anti-monocyte/macrophage Ab (MOMA-2; 1:100 dilution; Serotec), anti-CD3 (1:100 dilution; Abcam), and anti-rat IgG (H+L; Alexa Fluor 488 Conjugate, green, CST, 1:1000 dilution). Primers specific for the LDLr message were designed previously (18). The primary Abs were anti-monocyte/macrophage Ab (MOMA-2) (1:100 dilution; Serotec), anti-CD3 (1:100 dilution; Abcam), and anti-rat IgG (H+L; Alexa Fluor 488 Conjugate, green, CST, 1:1000 dilution). The process is shown in Supplemental Fig. 1. Twelve weeks after bone marrow transplantation, blood was collected after overnight fasting to determine plasma cholesterol and triglyceride concentrations using commercial kits (Roche Diagnostics, Indianapolis, IN). Peripheral blood cell counts were analyzed by Drew Hemavet 950FS (Drew Scientific, Oxford, U.K.) after 6 wk of high-fat feeding.

Cell culture

For isolation of elicited peritoneal macrophages, age- and sex-matched WT and TIPE2\textsuperscript{−/−} mice were injected i.p. with 1.0 ml of 3% sterile thiglycollate broth (Sigma-Aldrich). Four days after the injection, cells were harvested by i.p. lavage with ice-cold PBS. Then cells were seeded in DMEM medium (Life Technologies-BRL, Carlsbad, CA) with 10% FBS on 12-cell plates (Corning, Corning, NY) at 5 \times 10\textsuperscript{3} cells/well for RNA extraction or in 6-well plates (Corning) at 1 \times 10\textsuperscript{4} cells/well for flow cytometry or Western blot experiments.

Quantitative real-time PCR

Total RNA of cells or tissues was prepared using the TRizol reagent (Invitrogen). Quantitative PCR (qPCR) for genes of interest was performed using iQ5 SYBR Green iCycler iQ5 (BioRad, Hercules, CA). cDNA levels were determined using a standard curve and normalized to β-actin. Amplification conditions were: 95°C for 3 min, 95°C for 15 s, 55°C for 15 s, and 72°C for 40 cycles. Each sample was run in triplicate. The relative changes in gene expression were analyzed by the 2\textsuperscript{-ΔΔct} method, and a melting-curve analysis was performed to ensure the specificity of the products. The primers used were shown in Supplemental Table I and synthesized by Invitorgen.

Preparation of ox-LDL

Ox-LDL was obtained from Peking Union Medical University (Beijing, China), as previously described (21). Ox-LDL preparations were tested for possible endotoxin contamination using a Limulus amebocyte lysate kit (Cambrex, Walkersville, MD). The endotoxin concentration of the ox-LDL preparations >0.1 endotoxin unit/ml protein was discarded. The ox-LDL was stored at 4°C and used within 2 wk after receipt.

Flow cytometry

Peritoneal macrophages were stimulated with 50 μg/ml ox-LDL (Peking Union Medical University, Beijing, China) for 1 h and then incubated with 10 μmol/l 2′, 7′-dichlorodihydrofluorescein diacetate (DCF-DA; Sigma-Aldrich) or 10 μmol/l 4-amino-5-methylamino-2′, 7′-dichlorofluorescein diacetate (Molecular Probes, Eugene, OR) for 30 min. Flow cytometry was performed using the Beckman Coulter Cytomics FC 500 (Beckman Coulter, Fullerton, CA). A total of 10,000 stained cells was analyzed.

Analysis of NO production

Primary peritoneal macrophages incubated with DMEM medium containing 10% FBS overnight before stimulation were plated at 3 \times 10\textsuperscript{5} cells/well in a 24-well culture plate (Corning). After the cells were treated with 50 μg/ml ox-LDL for 24 h, culture supernatant was collected. NO production in the medium sample was measured by the Griess assay. In brief, cell supernatants (50 μl) were mixed with 50 μl Griess reagent (Sigma-Aldrich). After 15 min, nitrite concentrations were determined by the measurement of the OD at 540 nm. The cells were collected to determine the levels of mRNA and protein of inducible NO synthase (iNOS) by qPCR and Western blot, respectively. The experiments were conducted in triplicates and repeated at least three times.

FIGURE 1. TIPE2 deficiency in hematopoietic cells accelerates atherosclerosis in Ldlr\textsuperscript{−/−} mice. (A) Representative images of Oil Red O−stained descending aortas of mice (en face assay). (B) Quantitative analysis of en face lesion area. (C) Representative images of Oil Red O−stained sections of the aortic sinus. (D) Quantitative analysis of the cross-sectional lesion area of the aortic root in Ldlr\textsuperscript{−/−} recipients. Original magnification ×100. Each circle indicates an individual mouse. Data are shown as mean ± SE (n = 8). *p < 0.05 versus WT controls.
Western blot

Cells were lysed and the supernatant was collected to determine the protein concentration. Fifty micrograms of proteins was separated by 12% SDS-PAGE and transferred to polyvinylidene difluoride membrane (Millipore, Billerica, MA). After being blocked with 5% BSA in TBST, the membranes were incubated with primary Abs: anti-TIPE2 (1:2), anti-iNOS, anti-ERK and p-ERK, anti-p38 and p-p38 (1:1000; Cell Signaling Technology, Beverly, MA), anti-JNK and p-JNK, anti-IκB and p-IκB (1:500; Cell Signaling Technology), and anti–β-actin (1:1000; Santa Cruz, CA). Then immunoblotting was conducted with secondary Abs (goat anti-rabbit IgG or goat anti-mouse IgG) conjugated with peroxidase. After washing, bound

FIGURE 2. Macrophages, collagen, and necrosis in atherosclerotic lesions from WT- or TIPE2<sup>−/−</sup>-transplanted Ldlr<sup>−/−</sup> mice. (A and B) Collagen and necrosis in the aortic lesions were detected by Masson’s trichrome staining and H&E staining, respectively, and quantified, as described in (B). (C and D) Macrophages and T cells in the aortic lesions were stained by immunohistochemistry using Abs against MOMA-2 and CD3 (left panel). Quantitative analysis of macrophages from WT- or TIPE2<sup>−/−</sup>-transplanted mice. Percentages of the MOMA-2+ and CD3+ areas to the total lesion area were determined by microscopy with 100× magnification (right panel), using three sections per animal and averaged for each group. Scale bars, 100 μm. (E) The genotypes of TIPE2 were confirmed with PCR. Mice transplanted with WT bone marrow cells had a 300-bp PCR product, whereas mice transplanted with TIPE2<sup>−/−</sup> bone marrow cells had no such a product. (F) Serum concentrations of IL-6 and MCP-1 in Ldlr<sup>−/−</sup> recipients determined by ELISA. (G) Quantitative real-time PCR analysis of total RNA extracted from aortic tissue of recipient Ldlr<sup>−/−</sup> mice was performed for IL-1β, IL-6, IL-12p40, iNOS, KC, and MCP-1. Shown is the gene expression normalized to β-actin expression. Data are shown as mean ± SEM (n = 3). (H) Plasma total cholesterol, triglycerides, LDL, and high-density lipoprotein showed no significant differences between Ldlr<sup>−/−</sup> mice that received TIPE2<sup>−/−</sup> and WT bone marrow. Data are shown as mean ± SE (n = 8). *p < 0.05, **p < 0.01 versus WT controls.
peroxidase activity was detected by the ECL detection system (ECL, F-ChemiBlsu.6pro, DNR, Tel Aviv, Israel) using the SuperSignal West Pico kit (Pierce).

**ELISA assay**

The concentrations of IL-6 and MCP-1 in sera or in culture supernatants were determined using commercial ELISA kits for IL-6 and MCP-1 (eBioscience, San Diego, CA), according to the manufacturer’s instructions.

**Statistical analysis**

All analyses were performed using SPSS 16.0 (SPSS, Chicago, IL). Data were expressed as mean ± SE. An independent-samples two-tailed Student t test was used to compare continuous data for between-group differences, and comparisons among multiple groups involved the use of ANOVA with the least significant difference post hoc test. For analysis of plaque morphology and areas of immunohistochemical or immunofluorescent positive staining, nonparametric Mann–Whitney U test was used for group comparison. Each experiment was conducted at least three times. The p values < 0.05 were considered statistically significant.

**Results**

**TIPE2 deficiency in hematopoietic cells accelerates atherosclerosis**

To study the role of TIPE2 in atherogenesis, bone marrow transplantation was performed in atherosclerosis-prone female Ldlr−/− mice using TIPE2−/− donor cells. The en face area of the aortic atherosclerotic lesions was significantly increased in Ldlr−/− (5.69 ± 1.02 versus 3.03 ± 0.41%) mice transplanted with TIPE2−/− bone marrow cells compared with the mice transplanted with control cells (Fig. 1A, 1B). Similarly, the cross-sectional area of the atherosclerotic lesions in the aortic root was also increased by an average of 44% in Ldlr−/− mice transplanted with TIPE2−/− bone marrow cells relative to the mice transplanted with control cells (Fig. 1C, 1D). These differences were significant (p < 0.05; Fig. 1B, 1D). Thus, TIPE2 deficiency in bone marrow–derived cells resulted in an accelerated atherosclerotic lesion formation.

**TIPE2 deficiency in hematopoietic cells affects the amount of macrophages of atherosclerotic lesions**

To further explore the characteristics of atherosclerosis, we next analyzed the plaque morphology and composition. The necrois-positive areas that were nuclear, fibrotic, and eosin-negative were equal between TIPE2−/− cell– and WT cell–transplanted mice (Fig. 2A). To quantify the extent of fibrosis, collagen in lesions was stained with Masson’s trichrome, but no difference in the extent of fibrosis was found between the two groups (Fig. 2B). Interestingly, quantitative morphometry of the cross-sectional area of the aortic lesions by immunohistochemistry showed a significant increase in the amount of macrophages per lesion in TIPE2−/− cell–transplanted mice in comparison with the WT cell–transplanted mice (p < 0.05; Fig. 2C). However, the number of CD3+ T cells in lesions of TIPE2−/− chimeras was not significantly increased compared with lesions from WT controls; although there appeared to be a trend for an increment, the difference was not statistically significant (Fig. 2D). These data suggest that TIPE2 expression in bone marrow–derived cells affect the inflammatory component of atherosclerotic lesions in a significant manner.

**TIPE2 deficiency in hematopoietic cells does not affect chimerism, blood cell counts, and plasma lipid levels but increases proinflammatory cytokine expression**

After 8 wk of high-fat diet feeding, repopulation of the bone marrow cells in the recipients was confirmed by detection of TIPE2 gene by PCR using genomic DNA from peripheral blood cells (18). TIPE2 mRNA was detected in the cells from the control group, but not in those from the TIPE2−/− group (Fig. 2E). The levels of repopulation were also confirmed for the Ldlr genes of donor origin by PCR, because both sets of donor bone marrow cells were Ldlr+, consistently, the WT Ldlr gene was detected in the genomic DNA extracted from the peripheral whole blood cells of all mice from both groups. On the contrary, the mutant Ldlr gene in both groups was below the detectable level after 30 PCR cycles (data not shown). These results indicated a complete reconstitution of the recipient hematopoietic cells by the donor bone marrow cells.

To examine the effects of bone marrow transplantation on blood cells and biochemistry, we determined relative blood cell counts and plasma lipid levels. All blood cell counts were normal and showed no significant difference between groups (Supplemental Table II). As shown in Fig. 2H, there were no significant differences in total plasma cholesterol, triglycerides, LDL, and high-density lipoprotein between Ldlr−/− mice receiving TIPE2−/− bone marrow cells and those receiving WT cells. In addition, no significant differences in body weights were observed between groups (data not shown). These results suggest that TIPE2 deficiency in hematopoietic cells does not affect lipoprotein metabolism and blood cell counts of Ldlr−/− recipient mice.

To determine the potential contribution of nonlipoprotein circulating factors, we examined inflammatory cytokines in the serum of transplanted Ldlr−/− mice. Serological testing showed signifi-
cantly higher levels of inflammatory cytokines such as IL-6 and MCP-1 in the blood of mice transplanted with TIPE2<sup>−/−</sup> bone marrow cells compared with mice transplanted with WT cells (p < 0.05 and p < 0.01, respectively; Fig. 2F). We also performed quantitative RT-PCR for inflammatory mediators using aortic roots isolated from TIPE2<sup>−/−</sup>→Ldlr<sup>−/−</sup> and WT→Ldlr<sup>−/−</sup> mice fed a high-fat diet for 8 wk. Consistent with the increased vascular lesion formation in TIPE2<sup>−/−</sup>→Ldlr<sup>−/−</sup> mice, the production of proinflammatory cytokines and chemokines, such as IL-1β, IL-6, IL-12p40, iNOS, keratinocyte chemoattractant (KC), and MCP-1, was indeed significantly enhanced in TIPE2<sup>−/−</sup>→Ldlr<sup>−/−</sup> mice compared with WT→Ldlr<sup>−/−</sup> mice (p < 0.05 or p < 0.01; Fig. 2G). These results are consistent with the histological data that showed increased accumulation of macrophages in the lesions of TIPE2<sup>−/−</sup>-chimeric mice, indicating an escalation of local inflammation. These results suggest that TIPE2 deficiency in hematopoietic cells increases the inflammatory cytokines in animals and may contribute to the progression of atherosclerosis.

**Ox-LDL downregulates TIPE2 expression in J774A.1 and peritoneal macrophages**

To assess the effects of ox-LDL on macrophage expression of TIPE2, J774 cells, a murine macrophage-like cell line, and peritoneal macrophages were treated with ox-LDL, a major atherogenic factor (22). As shown in Fig. 3, ox-LDL significantly downregulated the expression levels of TIPE2 mRNA and protein in murine macrophages. The downregulation of TIPE2 was detectable as early as 1 h and was sustained 48 h after ox-LDL treatment in J774 cells (p < 0.01; Fig. 3A). We also examined the effect of ox-LDL on TIPE2 expression in murine peritoneal macrophages. As expected, the levels of TIPE2 mRNA decreased significantly in both dose-dependent and time-dependent manners (all p < 0.01; Fig. 3B, 3C). Furthermore, we found that the expression levels of TIPE2 protein by Western blot decreased in WT peritoneal macrophages treated with 50 μg/ml ox-LDL (p < 0.01; Fig. 3D, 3E). These results indicate that ox-LDL can downregulate TIPE2 expression both in J774 cells and peritoneal macrophages, suggesting that TIPE2 may be involved in the regulation of ox-LDL-mediated signaling in macrophages.

**TIPE2 expression in macrophages regulates ox-LDL-induced reactive oxygen species generation**

To study possible functions of TIPE2 expression in macrophages, we isolated and cultured peritoneal macrophages from WT and TIPE2<sup>−/−</sup> mice, and first focused on cellular reactive oxygen species (ROS) generation. As shown in Fig. 4A and 4B, the absence of TIPE2 resulted in increased ROS generation detected by DCF fluorescence. Without any treatment, the level of ROS in TiPe2<sup>−/−</sup> macrophages tended to be higher than that in WT cells. After exposure to 50 μg/ml ox-LDL for 1 h, the level of ROS in TIPE2<sup>−/−</sup> macrophages was upregulated dramatically (p < 0.05). These results suggest that TIPE2 expression in macrophages affects the degree of ROS generation, particularly under conditions of increased oxidative stress.

We next examined whether TIPE2 expression in macrophages affects oxidant or antioxidant gene expression. As shown in Fig. 4C, TIPE2-deficient macrophages expressed lower levels of antioxidant genes, such as heme oxygenase-1 (HO-1) and NAD(P)H:Quinone oxidoreductase 1 (NQO1). Once exposed to 50 μg/ml ox-LDL for 4 h, the mRNA levels of HO-1, NQO1, and extracellular superoxide dismutase (EC-SOD) in TIPE2-deficient macrophages were ∼2.5-, 2.3-, and 10-fold lower than that in WT controls, respectively (p < 0.001, p < 0.01, and p < 0.01, respectively). Expression levels of catalase mRNA was not different between the two groups of macrophages. Interestingly, the mRNA levels of NOX2 and p47phox, two key subunits of NAD phosphate oxidase (NAPDH oxidase family) (23, 24), were higher in TIPE2-deficient macrophages in response to ox-LDL treatment (p < 0.05 and p < 0.01, respectively; Fig. 4D). These results suggest that the accumulation of ROS in TIPE2-deficient macrophages is due to increased oxidant gene expression and decreased antioxidant gene expression.

**TIPE2 expression in macrophages promotes the expression of antioxidant pathway genes**

Why does TIPE2 deficiency lead to the significant loss of antioxidant enzyme gene expression? To address this question, we investigated upstream regulators of the antioxidant response. Quantitative real-time PCR was used to determine the expression...
of several key transcription factors, such as DJ-1, which regulates nuclear transport of Nrf-2 whose downstream target genes are important in the cellular antioxidant defense system (25), and the FOXO family, which regulates the expression of antioxidant enzyme genes (26). We found no difference in DJ-1 expression between the two groups of macrophages treated with ox-LDL (Fig. 5A). However, Nrf-2 expression was decreased in TIPE2-deficient cells, after exposure to 50 μg/ml ox-LDL for 4 h (p < 0.001; Fig. 5B). FOXO1 and FOXO4 expression were also significantly decreased in TIPE2-deficient cells, whereas no detectable differences in FOXO3a expression were noted between the two groups (p < 0.001 and p < 0.01, respectively; Fig. 5C–E). It is likely that attenuated activity of these two important antioxidant pathways contribute, at least in part, to the loss of the ability to mount an antioxidant response in TIPE2-deficient macrophages.

**TIPE2 deficiency affects inflammatory response genes**

To evaluate whether TIPE2 expression in macrophages has an impact on the inflammatory response against ox-LDL, we isolated peritoneal macrophages from WT and TIPE2−/− mice. We first compared the uptake of modified LDL. At two different time points (15 min and 2 h), we could not detect a difference between WT and TIPE2−/− macrophages in the uptake of either Dil-labeled acetylated LDL or ox-LDL (data not shown), suggesting that endocytosis of modified LDL was not affected by TIPE2 deficiency. In addition, the TIPE2−/− group showed no change in the number of ox-LDL–induced foam cells compared with WT macrophages as detected by Red Oil O staining (data not shown). These data indicate that TIPE2 is dispensable for endocytosis of modified LDL and foam cell formation. The mRNA and protein levels of iNOS in ox-LDL–stimulated macrophages were determined by qPCR and Western blot, respectively. Both mRNA and protein expression levels of iNOS were significantly higher in WT macrophages as detected by Red Oil O staining (data not shown). The number of doubly stained cells in ox-LDL–induced foam cells compared with WT macrophages alters the expression of several regulators of antioxidant enzyme genes. Peritoneal macrophages from WT and TIPE2−/− mice were stimulated with or without ox-LDL (50 μg/ml) for 4 h. The mRNA levels of DJ-1 (A), Nrf2 (B), FOXO1 (C), FOXO3 (D), and FOXO4 (E) were determined using quantitative real-time PCR. Data are shown as mean ± SE (n = 5). *p < 0.05, **p < 0.01, ***p < 0.001 versus WT controls.

Discussion

Atherosclerosis is widely appreciated as a chronic inflammatory disease of the vessel wall, characterized by the accumulation of lipid-laden macrophages and fibrous tissues in the large arteries (1, 27). In this study, we first found that TIPE2, a newly identified regulator of immune receptor signaling (8), is expressed in atherogenic macrophages and plays an important atheroprotective role.
role. The effects of TIPE2 in the pathogenesis of atherosclerosis were examined in Ldlr^{2/-} mice reconstituted with TIPE2^{2/-} macrophages or controls, and the effect appears to be mediated by several key atherogenic processes including intracellular oxidative stress, proinflammatory cytokine secretion, and foam cell signaling in response to ox-LDL stimulation.

In this study, we first report that TIPE2 is a new atheroprotective protein. To study the roles of TIPE2 in atherogenesis, we performed bone marrow transplantation in atherosclerosis-prone mice (Ldlr^{2/-}), using TIPE2^{2/-} and control donor cells. We found that atherosclerotic lesions significantly increased in recipients with TIPE2^{2/-} bone marrow cells compared with controls. Interestingly, TIPE2 expression in bone marrow–derived cells significantly affects the amount of macrophages in atherosclerotic lesions. These data indicate that TIPE2 deficiency in bone marrow–derived cells resulted in an increased atherosclerotic lesion formation, indicating that macrophages may play an important role during atherogenesis.

Early studies showed that TIPE2 was highly expressed in macrophages (8, 12), but whether TIPE2 expression is regulated by ox-LDL in atherogenesis remains unclear. To address this

FIGURE 6. TIPE2 deficiency affects inflammatory response genes. (A–E) TIPE2 deficiency in macrophages upregulated iNOS generation upon ox-LDL stimulation. (A and B) Thioglycollate-elicited peritoneal macrophages from WT and TIPE2^{2/-} mice were treated with 50 μg/ml ox-LDL for the indicated time. The mRNA (A) and protein (B) levels of iNOS were determined by qPCR and Western blot, respectively. (C and D) Cells were cultured for 1 h in the absence or presence of 50 μg/ml ox-LDL, followed by 30 min of incubation with the NO indicator 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate. Intracellular NO was then detected by flow cytometry. The histogram plots showed the fluorescence intensity distributions (C), whereas the bar graphs showed mean fluorescence intensity (MFI) (D). (E) Cells were treated with or without ox-LDL (50 μg/ml) for 24 h. NO production in culture supernatants was determined by the Griess assay. Data are shown as mean ± SE (n = 4) of one representative experiment. (F) Cells were treated with or without ox-LDL (50 μg/ml) for 24 h. IL-6 and MCP-1 production in culture supernatants was determined by ELISA. Data are shown as mean ± SE (n = 4) of one representative experiment. (G and H) Thioglycolate-elicited peritoneal macrophages from WT and TIPE2^{2/-} mice were treated with 50 μg/ml ox-LDL for the indicated times. The mRNA levels of IL-1β, KC, and IL-12p40 were determined by RT-PCR (G) and qPCR (H), respectively. Data are shown as mean ± SE (n = 4) of one representative experiment. (I) Double-staining immunofluorescence microscopy with MCP-1–FITC Abs and MOMA-2–PE Abs specific for macrophages. The MCP-1+ staining cells were all positive for MOMA staining (with arrows). The number of doubly stained cells in TIPE2^{2/-} chimeras was significantly increased compared with the WT controls. *p < 0.05, **p < 0.01, ***p < 0.001 versus WT controls.
TIPE2 DEFICIENCY EXACERBATES ATHEROSCLEROSIS

FIGURE 7. TIPE2 deficiency increased IkB, JNK, and p38 phosphorylation in macrophages. Peritoneal macrophages from WT and TIPE2−/− mice (n = 4) were incubated with or without ox-LDL (50 μg/ml) for the indicated time. Total cell lysates were examined with Abs against total or phosphorylated IkBα, JNK1/2, p38, and ERK1/2 by Western blot. Significant increases in c-JNK and p38 phosphorylation in TIPE2-deficient macrophages were observed 5–60 min after ox-LDL stimulation, whereas increases in p-ERK phosphorylation were noted at 30 min. β-Actin served as a protein loading control.

issue, we examined TIPE2 expression levels in murine macrophage cell line J774.A.1 and peritoneal macrophages treated with ox-LDL in vitro. We found that ox-LDL significantly downregulated the expression levels of TIPE2 in J774.A.1 cells and peritoneal macrophages. These results indicate for the first time, to our knowledge, that TIPE2 may provide a novel target for the intervention of ox-LDL-induced effects during atherosclerosis.

Oxidation of LDL is a major pathogenic process in the development of atherosclerosis. A number of studies showed that ox-LDL has chemoattractant activity on monocytes, promotes their differentiation into macrophages, but inhibits their mobility and triggers the release of proinflammatory cytokines by binding to CD36 (28). Our data indicate that lack of TIPE2 expression in bone marrow-derived cells exacerbates the progression of atherosclerosis not by affecting lipoprotein metabolism, but by increasing the proinflammatory responses to ox-LDL stimulation.

The mechanisms underlying the atheroprotective effects of TIPE2 may involve several aspects. First, TIPE2 expression in macrophages affects the degree of ROS generation, particularly in conditions of increased oxidative stress. Increased generation of ROS may induce inflammatory responses and contribute to atherogenesis (29, 30). In the untreated condition, we found that TIPE2-deficient macrophages displayed increased DCF fluorescence levels compared with WT controls. This may be the result of increased baseline levels of oxidant gene expression and decreased levels of antioxidant enzymes in TIPE2-deficient macrophages. Upon treatment with ox-LDL, the DCF fluorescence levels were upregulated markedly in macrophages with TIPE2−/− genotype compared with WT controls, suggesting that TIPE2 expression in macrophages inhibits ROS generation. This result coincides with the notion that TIPE2 serves as a negative regulator of oxidative burst during infection (13). Recent studies suggest that HO-1, a rate-limiting enzyme in the catabolism of heme, plays an antiatherosclerotic role by reducing ferritin and seques-

tering Fe, which is an important catalyst for ROS formation (21, 31, 32). We found that TIPE2-deficient macrophages expressed lower levels of HO-1, NQO1, and EC-SOD, especially after exposure to ox-LDL. In contrast, the levels of NOX2 and p47phox, the important sources of ROS in macrophages (23, 24), were increased in response to ox-LDL stimulation in TIPE2-deficient macrophages. These data suggest that the accumulation of ROS in TIPE2-deficient macrophages is due to increased oxidant gene expression and decreased antioxidant enzyme expression. In addition, TIPE2 also affects upstream regulator, such as Nrf2, FOXO1, and FOXO4, of the antioxidant response. It appears that the mechanism behind the global drop in antioxidant gene expression is related to the decline in Nrf2, FOXO1, and FOXO4 expression, although the exact regulatory mechanisms remain to be elucidated.

The second mechanism involves modification of inflammatory responses by TIPE2. It was reported that TIPE2 knockout and knockdown cells produced more IL-6 and IL-12 upon stimulation with LPS (8). In this study, we used primary peritoneal macrophages from WT and TIPE2−/− mice to determine the ability of ox-LDL to induce cytokine expression. Deficiency of the TIPE2 gene in macrophages significantly accelerated ox-LDL-mediated activation of the inflammatory response genes, such as iNOS, IL-1β, KC, and IL12p40. iNOS, an enzyme induced in inflammation and able to generate O2− and peroxy nitrite (ONOO−), is a proinflammatory molecule whose expression is regulated by NF-κB. Studies demonstrated that genetic deficiency of iNOS in ApoE null mice fed a Western diet caused a decrease in atherosclerosis (33). In this article, we report that both the mRNA and protein expression levels of iNOS were increased in TIPE2−/− macrophages after ox-LDL stimulation, suggesting that TIPE2 plays an atheroprotective role possibly through holding back the generation of iNOS. Similarly, the mRNA expression levels of other cytokines, such as IL-1β, KC, and IL12p40, were increased in TIPE2−/− macrophages treated with ox-LDL. These results suggest that TIPE2 could alleviate atherosclerosis by negatively regulating cytokine production in macrophages.

The third mechanism may involve modification of intracellular signaling pathways by TIPE2. Previous studies showed that ox-LDL can activate MAPKs JNK1 and JNK2 through CD36 (34). Thus, in this study, we compared the ox-LDL–induced signals in TIPE2-deficient macrophages and WT controls. The results showed an increased phosphorylation of JNK1/2, p38, and IkB in TIPE2-deficient macrophages with ox-LDL treatment, suggesting that TIPE2 diminishes atherosclerosis by negatively regulating JNK1/2, NF-κB, and p38 pathways. The activation of the NF-κB pathway in macrophages leads to more severe atherosclerosis in mice, possibly by affecting the proinflammatory and anti-inflammatory balance that controls the development of atherosclerosis.

In conclusion, our data indicate that TIPE2 expressed in macrophages may play an atheroprotective role by negatively regulating ox-LDL–induced inflammatory responses. This finding may not only advance our understanding of the mechanisms of atherosclerosis, but also lead to the development of TIPE2-based strategies for treating the disease.

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