**Advanced Glycation End-Product of Low Density Lipoprotein Activates the Toll-Like 4 Receptor Pathway Implications for Diabetic Atherosclerosis**

Conrad P. Hodgkinson, Ross C. Laxton, Kunal Patel, Shu Ye

**Objective**—Diabetes is a major risk factor for coronary heart disease. Accumulation of advanced glycation end-products (AGEs) attributable to hyperglycemia in diabetics promotes the development of atherosclerosis. However, the underlying mechanisms remain unclear.

**Methods and Results**—The advanced glycation end-product of low-density-lipoprotein (AGE-LDL) induced proinflammatory cytokine production in human coronary artery endothelial cells and human- and mouse-macrophages. AGE-LDL stimulated cytokine synthesis was markedly reduced in mouse macrophages with a TLR4 loss-of-function mutation. Coimmunoprecipitation experiments indicated AGE-LDL interacts with TLR4, RAGE, and CD36. Incubation of cultured macrophages with TLR4, RAGE, or CD36 antibodies inhibited AGE-LDL stimulation of tumor necrosis factor (TNF)α production. A competitive binding inhibitor of TLR4 blocked AGE-LDL binding to the receptor. After transfection of a HEK293 cell system with wild-type TLR4, AGE-LDL activated a signaling pathway including p38α, JNK, and ERK1 kinases and AP1, Elk1, and NF-κB transcription factors; the net result being increased cytokine production. These effects were absent when cells were transfected with empty plasmid. Two common polymorphisms in TLR4, D299G and T399I, reduced the response of TLR4 to lipopolysaccharide (LPS) but had no effect on AGE-LDL signaling.

**Conclusions**—These results indicate that AGE-LDL activates a TLR4-mediated signaling pathway, thus inducing proinflammatory cytokine production. This mechanism may partly explain the increased risk of atherosclerosis observed in diabetics. (*Arterioscler Thromb Vasc Biol. 2008;28:2275-2281.)*

**Key Words:** diabetes ■ atherosclerosis ■ advanced glycation end-products ■ toll-like receptors

Diabetes have a 2- to 4-fold higher risk of developing atherosclerosis than nondiabetics.1 Hyperglycemia in diabetes induces nonenzymatic glycation of proteins and lipids, leading to the accumulation of advanced glycation end-products (AGEs). Studies have shown that AGEs are atherogenic. Inhibition of AGE, blocking either their formation or their interaction with cells, can suppress diabetic atherosclerosis.2-5 On the other hand, diets enriched in AGEs promote the development of atherosclerotic lesions.4 The mechanism whereby AGEs promote atherosclerosis, however, remains poorly understood. AGE-binding receptors have been characterized, including scavenger receptors types I and II, the receptor for advanced glycation endproducts (RAGE), oligosaccharyl transferase-48 (AGE-R1), 80K-H phosphoprotein (AGE-R2), and galectin-3 (AGE-R3). These AGE receptors are typically found in monocytes, macrophages, and endothelial cells.6

The advanced glycation end-product of low density lipoprotein (AGE-LDL) has been implicated in diverse processes: triggering apoptosis, reducing nitric oxide synthesis, modulating vascular cell adhesion molecule (VCAM)-1 and macrophage scavenger receptor gene expression, as well as impairing vascular reactivity.6,7 In addition, AGE-LDL levels have been found to be elevated in diabetics,8,9 and there is a positive correlation between AGE-LDL levels and risk of atherosclerosis and coronary heart disease.9 RAGE has been shown to be activated by AGE-LDL, but whether it is the sole AGE-LDL receptor is unknown.10

Toll-like receptor-4 (TLR4) is the primary receptor for LPS present in Gram-negative bacteria and plays a crucial role in innate immunity.11 It also recognizes endogenous ligands, such as oxidized LDL (oxLDL),12 fibroactin,13 and heat shock proteins,14 which are involved in the pathogenesis of atherosclerosis.15 Key cells in atherosclerotic lesions, including macrophages, endothelial cells, and smooth muscle cells, express TLR4.16 Activation of TLR4 on vascular endothelial cells by oxLDL induces proinflammatory cytokine synthesis,12 and TLR4 knockout reduces atherosclerosis in apoE-deficient mice.17

In this study we show that AGE-LDL activates TLR4, triggering TLR4-dependent signaling pathways and inducing
proinflammatory cytokine production. These findings are pertinent to the understanding of how AGE-LDL can promote atherosclerosis in diabetics.

**Methods**

AGE-LDL

AGE-LDL was made according to established protocols. LDL (200 µg/mL) was incubated, under Argon, with 0.15 mol/L EDTA pH8 and 50 mmol/L glucose in the presence of penicillin-streptomycin at 37°C for 14 days. Control LDL preparations were made under the same conditions but without glucose. AGE-LDL and LDL preparations were purified before use by dialysis against PBS. Agarose gel electrophoresis was used to assess any alterations in negative charge indicative of oxidation. Conjugated diene formation was determined by absorbance at 230 nm to measure lipid peroxidation. Protein modification was evaluated by measuring pentosidine formation spectrofluorometrically (excitation 335 nm, emission 385 nm).

Oxidized LDL was obtained commercially (Intracel, Autogen Bioclear). BSA and AGE-BSA were from Sigma.

**Cell Culture**

Human CD14+ monocytes were purchased from Cambrex. Human coronary artery endothelial cells were purchased from PromoCell (UK). Mouse TLR4/CRL-2751) and TLR4/CRL-2458) macrophages were purchased from ATCC (LGC Promochem). The cell-line HEK293-CD14-MD2 was purchased from InvivoGen. Human monocytes, mouse TLR4 macrophages and HEK293-CD14-MD2 cells were cultured in DMEM containing glucose (4.5g/L) and fetal bovine serum (10% v/v). Hygromycin (50 µg/mL) was added to the medium for culturing HEK293-CD14-MD2 cells to maintain expression of CD14 and MD2. Proprietary medium for the coronary artery endothelial cells was supplied by Promocell. Mouse TLR4 macrophages were maintained in DMEM supplemented with fetal bovine serum (10% v/v) and LADMAC conditioned media (20% v/v) as per ATCC guidelines. For experiments coronary artery endothelial cells and mouse TLR4 macrophages were washed with DMEM and then maintained in DMEM with fetal bovine serum (10% v/v). This had no effect on cell viability. All cell types were cultured at 37°C in a 5% CO₂ humidified atmosphere.

**Plasmid Constructs**

Full length wild-type TLR4 (Origene, Cambridge Bioscience) was subcloned into the plasmid vector pcDNA4-His-Max-C (Invitrogen). pGL3-basic-MMP1 plasmid has been described previously. The IL8 promoter (2kb) was generated by polymerase chain reaction (PCR) using genomic DNA as template and cloned into pGL3-basic (Promega). Transfection-grade DNA was prepared using Endo-free maxi kits (Qiagen).

**Transfection**

Exponentially growing HEK293-CD14-MD2 cells (2×10⁶) were transiently transfected with the plasmids as described in the figure legends, using GeneJuice transfection reagent according to the manufacturer’s instructions (Novagen, Nottingham).

**Coimmunoprecipitation**

Extracts from mouse macrophages were prepared in lysis buffer (50 mmol/L Tris–pH 7.5, 150 mmol/L NaCl, 1% v/v Sigma Protease and Phosphatase Inhibitor cocktails) and diluted to give a 500 µL final volume (500 µg). A polyclonal AGE antibody (5 µL; BD Biosciences) and Protein G:protein A beads (50:50, 20 µL; Sigma) were added. After 5-hour continuous gentle agitation at 4°C, the beads were collected by pulse spin and then washed three times in lysis buffer, after which they were resuspended in PBS.

**Immunoblotting**

Cell extracts (in 62.5 mmol/L Tris pH7.8 and 1% v/v SDS) were resolved by SDS/PAGE and transferred onto polyvinylidene fluoride (PVDF). Membranes were probed with primary antibody followed by incubation with horseradish peroxidase-conjugated secondary antibody according to manufacturer’s instructions. Blots were developed with the ECL system according to the manufacturer’s instructions (Amersham Biosciences). Primary antibodies were supplied from Cell Signaling Technology (New England Biolabs) or Abcam (Cambridge).

**ELISA**

Kinase ELISA kits were from RnD Systems (Abingdon). Transcription factor ELISA kits were from StressGen (NFκB; Cambridge Bioscience), or Active Motif (Elk-1, AP-1). Interleukin (IL)-6 and TNFα ELISA kits from BioLegend (Cambridge Bioscience). All kits were used according to manufacturer’s instructions. For the transcription factors, nuclei extracts were used (manufacturer’s instructions).

**Luciferase Assays**

HEK293-CD14-MD2 cells were transfected with plasmid mix containing a pGL3 construct, pcDNA4-TLR4, and pRL-TK (renilla luciferase gene to measure transfection efficiency) using GeneJuice transfection reagent (Novagen). Twenty-four hours after transfection, cells were lysed, and activities of firefly luciferase and renilla luciferase in the lysates were measured using a dual-luciferase assay kit (Promega). Gene promoter activity was determined according to the ratio of firefly luciferase activity to renilla luciferase activity.
Five independent experiments were performed. In each experiment, transfection and luciferase assays were carried out in duplicate for each construct.

**Endotoxin Measurement**

LPS levels in the cell culture media, transfection reagent, AGE-LDL preparation, AGE-BSA, LDL, plasmid DNA, and transfection reagent were measured using a Limulus Amebocyte Lysate (LAL) kit from Cambrex. LPS levels were below 5 pg/mL, significantly lower than the concentrations of LPS required to activate TLR4.21

**Results**

Absorbance at 234 nm, fluorescence and electrophoretic mobility are measures of lipid peroxidation, protein modification, and oxidation, respectively, and the results are shown in supplemental Figure I (available online at http://atvb.ahajournals.org). As expected, absorbance, fluorescence and electrophoretic mobility of oxidized LDL (oxLDL) was significantly greater than unmodified LDL. AGE-LDL, prepared by incubating LDL with glucose for 14 days under argon, had higher levels of pentosidine formation with no change in the absorbance at 234 nm or electrophoretic mobility when compared to control LDL preparations incubated in the absence of glucose. These features are indicative of LDL glycation.18 The fluorescent data and the known labeling of the commercially prepared AGE-BSA indicates that labeling of AGE-LDL was 6 mol of glucose per mol LDL. No increase in the absorbance at 234 nm or electrophoretic mobility indicated that the AGE-LDL preparation was neither oxidized nor glycoxidised.

The commercially obtained AGE-BSA had a lower amount of pentosidine compared to AGE-LDL, which may indicate a reduced level of glycation.

Initial experiments were conducted with human coronary artery endothelial cells and macrophages as they both have important roles in atherogenesis. AGE-LDL induced IL6 production in both of these cell types (Figure 1, third bar versus first bar), whereas unmodified LDL had no effect in coronary artery endothelial cells and a nonsignificant increase in macrophages (Figure 1b, fourth bar), suggesting that only AGE modified LDL, but not native LDL, could induce cytokine expression in these cells.

AGE-BSA (AGE-Bovine Serum Albumin), a potent RAGE ligand,2 activated IL6 synthesis in both cell-types (Figure 1, second bar). Treating human macrophages and endothelial cells with the potent TLR4 ligand LPS induced IL6 synthesis (Figure 1b, fifth bar). These results suggest that the human coronary artery endothelial cells and macrophages contained RAGE and TLR4.

Because TLR4 recognizes oxLDL,12 we investigated whether TLR4 mediated the effect of AGE-LDL on cytokine production. To address this question mouse macrophages with either a functional TLR4 or a TLR4 loss-of-function mutation were used. As expected, LPS increased IL6 and TNFα synthesis by macrophages with functional TLR4; this effect was completely abolished in macrophages with the TLR4 mutation (supplemental Figure II; Figure 2 TLR4+/+ versus TLR4−/−). AGE-LDL increased the synthesis of these cytokines in macrophages with functional TLR4; this effect was reduced ≈50% in mouse macrophages with the TLR4 loss-of-function mutation (supplemental Figure II; Figure 2). The effect of AGE-LDL on cytokine synthesis was therefore partly mediated by TLR4. To verify the result macrophages were treated with AGE-LDL that had been heated at 95°C for 5 minutes (b-AGE-LDL). This would cause AGE-LDL deg-
radiation but leave the ability of LPS to stimulate TLR4 unaffected. The heat-treated b-AGE-LDL did not increase cytokine synthesis compared with unstimulated cells (supplemental Figure II; Figure 2), indicating that the stimulatory effect of AGE-LDL on cytokine synthesis was not caused by LPS contamination. Unmodified LDL did not have an effect on the synthesis of either cytokine in mouse macrophages (supplemental Figure II; Figure 2).

Figure 3. AGE-LDL receptor binding experiments. a, AGE compounds were immunoprecipitated with an AGE antibody from TLR4 wild-type mouse macrophage cell lysates. Proteins in the immunocomplex were immunoblotted with TLR4, RAGE, or CD36 antibodies. b, Native- or AGE-LDL were immunoprecipitated from TLR4 wild-type mouse macrophage cell lysates. Proteins in the immunocomplex were immunoblotted with a LDL-receptor antibody. n=2.

Figure 4. AGE-LDL activates kinases through TLR4. HEK293-MD2-CD14, transfected with empty vector or TLR4 receptor, were incubated with AGE-LDL for the specified times. Total cell extracts were assayed for phospho-p38α, phospho-JNK [pan], phospho-ERK1, and phospho-Akt S473 [pan]. t tests were performed against the unstimulated-no-TLR4 control at zero hours. probability values <0.05 are asterisked. n=4.
AGE-BSA stimulated IL6 and TNFα synthesis; the effect being similar between mouse macrophages with functional TLR4 and those possessing the TLR4 mutation (supplemental Figure II; Figure 2), suggesting that the effect of AGE-BSA was not mediated by TLR4.

To test whether AGE-LDL could interact with TLR4, wild-type mouse macrophages, treated with AGE-LDL, were lysed and lysates incubated with an AGE antibody. The immunoprecipitates were then probed for TLR4. As shown in Figure 3a precipitation of AGE resulted in the coimmunoprecipitation of TLR4; indicating an association between TLR4 and AGE-LDL. No coimmunoprecipitation of TLR4 was observed in the absence of the AGE antibody, nor with extracts of macrophages exposed to LDL or AGE-BSA.

Both RAGE and CD36, two known AGE receptors, were also present in AGE immunoprecipitates from AGE-LDL treated macrophage extracts (Figure 3a). As expected, AGE-BSA was also found to bind to RAGE and CD36, whereas unmodified LDL did not (Figure 3a). No binding between AGELDL and the LDL-receptor could be observed however (Figure 3b).

In a binding assay a strong signal was observed when plasma membranes from wild-type macrophages cultured in the presence of AGE-LDL were immunoblotted with an AGE antibody. 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphorylcholine inhibits TLR4 signaling by preventing LPS from binding to the TLR4 complex. Plasma membranes from macrophages treated with AGE-LDL and 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphorylcholine had no AGE signal (supplemental Figure IIIa). This is further evidence of binding between TLR4 and AGE-LDL.

AGE-LDL did not stimulate TNFα synthesis in wild-type macrophages cocultured with AGE, TLR4, RAGE, or CD36 antibodies whereas the LDL-receptor antibody had no effect (supplemental Figure IIIb), further verifying the association between AGE-LDL and TLR4.

To characterize TLR4 signaling events activated by AGE-LDL we used HEK293-MD2-CD14 cells which stably express cofactors necessary for TLR4 function (MD2, CD14) but lack TLR4 itself. The HEK293-MD2-CD14 cells possess a functional TLR4 complex only on transfection with a TLR4 expression construct. In HEK293-MD2-CD14 cells transfected with the TLR4 expressing plasmid, AGE-LDL activated MAPKs as indicated by increases in phosphorylation of p38, JNK, and ERK1. Maximal phosphorylation occurred at 15 to 30 minutes after AGE-LDL treatment (Figure 4). In contrast, in HEK293-MD2-CD14 cells transfected with empty plasmid, there was no phosphorylation of p38, JNK, and ERK1 in response to AGE-LDL treatment (Figure 4). This suggests that AGE-LDL triggered p38, JNK, and ERK1 activation via TLR4. Activation of TLR4 by LPS has been shown to induce phosphorylation of Akt/PKB. However, AGE-LDL stimulation had no effect on the phosphorylation of this kinase (Figure 4). Expression of TLR4 in the cells transfected with the TLR4 expressing plasmid, but not in the untransfected cells, was verified by immunoblotting (supplemental Figure IV). The phosphorylation of p38, JNK, and ERK1 in response to AGE-LDL stimulation of TLR4 was not attributable to changes in the expression levels of these kinases (supplemental Figure V). Neither AGE-BSA nor native LDL induced p38 activation (supplemental Figure VI), suggesting that the effect of AGE-LDL on TLR4 activation cannot be attributed to advanced glycation in general or LDL itself.

Transcription factors AP1 and Elk1, known to be downstream of the MAPK pathway, were activated in AGE-LDL–treated HEK293-MD2-CD14 cells transfected with the TLR4 expressing plasmid but not in cells transfected with the empty vector. In addition, in HEK293-MD2-CD14 cells transfected with the TLR4 expressing plasmid but not in cells transfected with the empty vector, AGE-LDL induced activation of NFκB, which occurs independently of MAPKs via IKKβ (Figure 5).

To compare the effects of AGE-LDL and LPS on TLR4 activation, we treated TLR4 transfected HEK293-MD2-

Figure 5. AGE-LDL activates transcription factors via TLR4. HEK293-MD2-CD14, transfected with empty vector or the TLR4 receptor, were incubated with AGE-LDL. Nuclear extracts were assayed for p-Jun, Elk-1, or NFκB. t tests were performed against the unstimulated-no-TLR4 control at zero hours. Differences with a probability value <0.05 are asterisked. n=4 to 6.
CD14 cells with either LPS (5 ng/mL) or AGE-LDL (8 μg/mL) and measured activation of NFκB and p38α. LPS stimulation increased active NFκB 40-fold, substantially greater than that of AGE-LDL. Similarly, LPS activation of p38α activation was stronger than that caused by AGE-LDL (supplemental Figure VII).

We used the HEK293-MD2-CD14 to test whether AGE-LDL stimulated cytokine synthesis via TLR4. The experiments showed that in HEK293-MD2-CD14 cells transfected with the TLR4 expressing plasmid, but not in cells transfected with the empty vector, AGE-LDL treatment induced IL6 and TNFα production (Figure 6), suggesting that AGE-LDL induced cytokine synthesis requires TLR4. AGE-LDL also activated other proinflammatory markers through TLR4. HEK293-MD2-CD14 cells were transfected with the TLR4 expressing plasmid and a luciferase reporter gene construct under the control of the IL-8 or matrix metalloproteinase (MMP)-1 gene promoter. Treating the cells with AGE-LDL increased the activity of the IL-8 and MMP-1 gene promoters approximately 1.5- and 2-fold respectively (supplemental Figure VIIIa).

Two single nucleotide polymorphisms in the coding region of TLR4 gene, resulting in amino acid substitutions (D299G and T399I), reduce TLR4 activation by LPS. We also found that LPS induced IL6 and TNFα production was reduced in HEK293-MD2-CD14 cells expressing the TLR4 299G-399I variant compared to cells expressing wild-type TLR4 (supplemental Figure VIIIb right panels). However, AGE-LDL stimulation of IL6 and TNFα production was unaffected by the D299G and T399I substitutions (supplemental Figure VIIIb left panels).

**Discussion**

The results of our study indicated that AGE-LDL induces proinflammatory cytokines in part by TLR4 (supplemental Figure X). AGE-LDL–induced cytokine production was markedly reduced in TLR4 knock-out macrophages when compared to wild-type cells. This result was strengthened by several assays which indicated that AGE-LDL interacted with TLR4. Furthermore, AGE-LDL activated a signaling cascade in HEK293-MD2-CD14 cells only when the cells had been transfected with a TLR4 expression plasmid (supplemental Figure IX).

In mouse macrophages with the TLR4 loss-of-function mutation the effect of AGE-LDL on cytokine production was reduced but not completely absent, suggesting that AGE-LDL induces cytokine synthesis by activating several receptors. Indeed, coimmunoprecipitation assays indicated that AGE-LDL associated with RAGE and CD36, two receptors which promote cytokine synthesis.

The effects of AGE-LDL observed in our experiments were not attributable to LPS contamination of reagents. Limulus amebocyte lysate assays determined LPS concentrations were below 5 pg/mL in all reagents, significantly lower than the concentrations (>50 pg/mL) of LPS required to activate TLR4.21 Furthermore AGE-LDL, heated at 95°C for 5 minutes so as to cause AGE-LDL degradation but leaving the ability of LPS to activate TLR4 unaffected, abolished AGE-LDL–stimulated cytokine production. Additionally, the TLR4 D299G and T399I substitutions, which significantly reduced the ability of LPS to activate the receptor and so produce cytokines, had no effect on AGE-LDL stimulation of cytokine production through TLR4. Finally, LPS activates Akt/PKB through TLR4, an observation not observed with AGE-LDL stimulation of the receptor.

LDL incubated with glucose produces AGE moieties attached to both the lipid and apoprotein components, and these have also been detected in LDL from diabetic individuals.8 Epitopes close to the LDL receptor binding site of apolipoprotein-B have been shown to be modified by glycation, which may prevent binding to the LDL-receptor.25 The lipid component of LPS is sufficient to activate TLR4,23 and a partially oxidized form of LDL has been shown to activate the receptor TLR4.12 Taken together with our finding that AGE-LDL activates TLR4, it suggests that modified LDL mimics the structure of the lipid component of LPS. Furthermore, results of our study indicate that TLR4 activation by AGE-LDL was not brought about by the LDL itself or its lipid component.
glycation products in general, as we found that neither native LDL nor AGE-BSA activated TLR4.

Serum concentrations of glycated LDL in diabetics are around 10 to 35 μg/mL.9 This is higher than the concentration of AGE-LDL, 8 μg/mL, used in our experiments, indicating that the concentration in our experiments can be physiologically relevant.

Diabetics have a higher risk of developing atherosclerosis than nondiabetics,1 yet the underlying mechanisms remain incompletely understood. Substantial evidence indicates that the association between diabetes and atherosclerosis arises from the elevated levels of AGEs generated as a result of hyperglycemia, and AGE-LDL is likely to be important. Despite good glycemic control, AGE-LDL levels remain elevated in diabetics.4,8,9 Furthermore, AGE-LDL levels directly correlate with the risk of myocardial infarction irrespective of an individual’s glycemic status.9 Reduced LDL clearance, endothelial dysfunction and apoptosis,26 and enhanced platelet aggregation27 have all been proposed as explanations as to how AGE-LDL promotes atherosclerosis.

Our finding that AGE-LDL activates TLR4 and induces proinflammatory cytokine production in macrophages and endothelial cells provides another mechanism by which AGE-LDL promotes atherosclerosis. Recent studies have indicated that TLR4 has a role in the inflammatory processes of atherosclerosis, though it is unclear as to what triggers TLR4 activation during atherogenesis.28 The TLR4 ligands LPS, oxLDL, and heat shock protein 60, are all associated with an increased risk of atherosclerosis.12,14 Our study implicates AGE-LDL as another ligand that can trigger TLR4 activation during atherogenesis.

In summary, the results of our study indicate that AGE-LDL can activate TLR4 leading to increased cytokine production. This finding provides an understanding of the mechanisms underlying the increased risk of coronary heart disease in diabetics and aids identification of therapeutic targets.

Sources of Funding

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Disclosures

None.

References

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Figure 1

Macrophage

Coronary Artery Endothelial Cell

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* indicates statistical significance.
Figure 3

(a) AGE Antibody IP

TLR4 Immunoblot  
RAGE Immunoblot  
CD36 Immunoblot  

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(b) LDL Antibody IP

LDL-Rc Immunoblot  

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Supplementary Figures

Supplemental Figure I. Characterisation of LDL. The absorbance (234nm), fluorescence (excitation 360nm, emission 430nm) and electrophoretic mobility of LDL, AGE-LDL, Oxidized-LDL, BSA, and AGE-BSA are shown. Absorbance and fluorescence data are expressed as a ratio to LDL or BSA. Data are means ± S.E.M. of four preparations.

Supplemental Figure II. Reduced AGE-LDL induction of cytokine synthesis in macrophages lacking the TLR4 gene. Wild-type (TLR4+/+) and TLR4 knock-out (TLR4 -/-) macrophages were incubated with either 8µg/ml AGE-LDL, 8µg/ml AGE-BSA, 8µg/ml LDL, 8µg/ml heat-denatured AGE-LDL (b-AGE-LDL), or 5ng/ml LPS. Cell culture medium was assayed at 0, 6 and 24 hours for TNFα and IL6 by ELISA. Results are expressed as the mean pg TNFα /mg total cell protein ± S.E.M. for four independent experiments. T-tests comparing AGE-BSA, AGE-LDL, LDL, b-AGE-LDL or LPS stimulus with un-stimulated control were performed on 24 hour cytokine expression values only. Differences with a p-value <0.05 are asterisked. T-tests comparing 24 hour cytokine expression values between the same stimulus in TLR4-/- and TLR4+/+ macrophages were performed with p-value <0.05 differences marked with a cross (†).

Supplemental Figure III. (a) Wild-type mouse macrophages were incubated with AGE-LDL in combination with the TLR4 binding inhibitor, 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphorylcholine, as appropriate. Plasma membrane extracts were immunoblotted with an AGE antibody. n=2. (b) Wild-type mouse macrophages were incubated with AGE-LDL and where appropriate AGE, TLR4, RAGE, CD36 or LDL-
receptor antibodies and TNFα levels in the culture medium measured after 16 hours. Results are expressed as mean pg TNFα/mg total cell protein ± S.E.M. for four independent experiments.

**Supplemental Figure IV.** Expression of TLR4 constructs in HEK293-MD2-CD14 cells. HEK293-MD2-CD14 cells were transfected with either the empty plasmid or the TLR4 plasmid. After 48h cell lysates were prepared and 20µg protein was subjected to immunoblotting with a TLR4 antibody.

**Supplemental Figure V.** AGE-LDL activates p38α, JNK and ERK but not Akt through TLR4. HEK293-MD2-CD14 transfected with either the empty vector (no TLR4) or the TLR4 receptor (TLR4) were incubated in the presence of 8µg/ml AGE-LDL for the specified times. Total cell extracts were assayed for total-p38α, total-JNK [pan], total-ERK1, and total-Akt1. Results are expressed as the mean pmol total-kinase amount/mg total cell protein ± S.E.M. for four independent experiments. T-tests comparing cells containing no TLR4 stimulated with AGE-LDL, cells containing TLR4, or cells containing TLR4 stimulated with AGE-LDL with the un-stimulated no TLR4 control at zero hours were performed. Differences with a p-value <0.05 are marked by an asterisk.

**Supplemental Figure VI.** Un-modified LDL does not activate TLR4. HEK293-MD2-CD14 transfected with the TLR4 plasmid were incubated in the presence of 8µg/ml AGE-LDL, 8µg/ml AGE-BSA or 8µg/ml LDL for 24h. Total cell extracts were assayed for either phospho-p38α (left panel) or total p38α (right panel). Results are expressed as
the mean pmol phospho- or total-p38/mg total protein ± S.E.M. for four independent experiments. T-tests comparing AGE-BSA, AGE-LDL, or LDL with the un-stimulated control were performed. Differences with a p-value <0.05 are marked by an asterisk.

**Supplemental Figure VII.** AGE-LDL is a weaker inducer of TLR4 than LPS. HEK293-MD2-CD14 transfected with either the empty vector (no TLR4) or the TLR4 plasmid (TLR4) were incubated in the presence or absence of 5ng/ml LPS or 8µg/ml AGE-LDL for 24h. Nuclei extracts were assayed for NFκB using ELISA, and results are expressed as the mean RLU ± S.E.M. for eight independent experiments. Total cell extracts were assayed for phospho-p38α and results are expressed as pmol/mg total protein ± S.E.M. for three independent experiments. T-tests comparing LPS or AGE-LDL stimulated TLR4 containing cells with un-stimulated cells without TLR4 at zero hours were performed. Differences with a p-value <0.05 are marked by an asterisk.

**Supplemental Figure VIII.** (a) HEK293-MD2-CD14 were transfected with a plasmid mix that contained either IL-8-promoter or MMP-1-promoter coupled to firefly luciferase, a *renilla* luciferase construct, and either empty vector (no TLR4) or the TLR4 plasmid (TLR4). Cells were incubated with 8µg/ml AGE-LDL for 20h after which firefly and *renilla* luciferase levels were measured. Results are expressed as the ratio between firefly and *renilla* luciferase and are the mean ratio ± S.E.M. for five independent experiments. T-tests comparing AGE-LDL treated cells with un-stimulated cells were performed. Differences with a p-value <0.05 are asterisked. (b) HEK293- MD2-CD14 cells were transfected with either the empty vector (cell), wild-type TLR4 (299D-399T),
or double mutation TLR4 (299G-399I) and incubated with 8µg/ml AGE-LDL or 5ng/ml LPS. After 24 hours the culture medium was assayed for IL6 or TNFα by ELISA. Results are expressed as mean pg cytokine/mg total cell protein ± S.E.M. for five independent experiments. T-tests comparing stimulus with un-stimulated cells were performed. Differences with a p-value <0.05 are asterisked. T-tests were also performed between wild-type TLR4 (299D-399T) and mutant TLR4 (299G-399I) with p-value <0.05 differences being marked with a cross (†).

Supplemental Figure IX. Human Macrophages were exposed to 8µg/ml AGE-LDL for the indicated times. (a) Total cell extracts were assayed for phospho-p38α or phospho-JNK and results are expressed as pmol phospho-kinase/mg total protein ± S.E.M. for three independent experiments. (b) Nuclei extracts were assayed for NFκB using ELISA, and results are expressed as the mean RLU ± S.E.M. for eight independent experiments. T-tests comparing AGE-LDL stimulated cells with un-stimulated cells were performed. Differences with a p-value <0.05 are marked by an asterisk.

Supplemental Figure X. AGE-LDL activates a TLR4 signalling pathway. (a) LPS activation of TLR4 induces NFκB which leads to cytokine synthesis. Two mutations in TLR4, 299G and 399I, render the receptor hyporesponsive to LPS. Activation of NFκB, and consequently cytokine synthesis, is reduced. However, AGE-LDL activation of NFκB by TLR4 is un-affected by these mutations. Cytokine synthesis is identical regardless of whether AGE-LDL stimulates the wild-type 299D-399T TLR4 or double-
mutant 299G-399I TLR4. (b) AGE-LDL activation of TLR4 activates a kinase and transcription factor cascade which leads to pro-inflammatory cytokine synthesis. AGE-LDL is also likely to promote pro-inflammatory cytokine production via binding to RAGE.
Supplemental Figure I

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Supplemental Figure II

TLR4 +/-

TLR4 +/-

No Stimulus
LPS
AGE-LDL
LDL
b-AGE-LDL
AGE-BSA

pgTNFα/mg protein

Time (hr)

pgTNFα/mg protein

Time (hr)

pgTNFα/mg protein

Time (hr)

pgTNFα/mg protein

Time (hr)
a) Plasma membrane bound AGE-LDL

Lane: 1. AGE-LDL
2. AGE-LDL + TLR4 Competitive Inhibitor

b) Antibody blocking experiments
Supplemental Figure IV

Lanes: 1. Empty plasmid
2. TLR4
Supplemental Figure VI

![Graphs showing p-p38α/mg protein and total-p38α/mg protein for different conditions: WT, WT + AGE-LDL, WT + AGE-BSA, and WT + LDL. The graphs indicate differences in protein levels across these conditions.](image-url)
Supplemental Figure VII

- LPS
- LPS
- AGE-LDL

![Graph showing RLU for nuclear NF-κB and p-p38α/mg protein for different conditions.](image-url)

- 0 hr
- 0.5 hr
Supplemental Figure VIII

(a)

IL-8

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MMP-1

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(b)

IL-6

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LPS

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