Supplemental Methods

**Synthetic ApoB100 peptides**

A library of synthetic peptides of human ApoB100 was used. Briefly, 302 peptides that encompassed the entire sequence of human ApoB100 protein were synthesized (K.J. Ross Petersen AS, Denmark) and numbered consecutively from P1 to P302, starting from the N-terminus. Each peptide was 20 amino acids long, in each of which the first 5 amino acids overlapped with the C-terminal sequence of the previous peptide and the last 5 amino acids overlapped with the N-terminal sequence of the next peptide. A stock solution of the peptides was prepared with 10% EtOH in aqueous NaCl (9 mg/ml) or PBS. No thiobarbituric acid reactive substances (TBARS) were detected in the preparations. In addition, 3 truncated fragments of P216 (ApoBDS-1), referred to as P216a, P216b, and P216c, were synthesized and purified identically as peptide ApoBDS-1 (Ross Petersen AS, Denmark).

Endotoxin levels in the synthetic peptides never exceeded 50 pg/mg of peptide, as determined by Limulus amoebocyte lysate detection assay (Associates of Cape Cod, Inc., Woods Hole, MA, USA).

**Isolation and culture of human PBMCs**

Peripheral blood from healthy volunteers was obtained from the Blood Central of Karolinska University Hospital, Stockholm, Sweden. Peripheral blood mononuclear cells
(PBMCs) were isolated using Lymphoprep™ gradient medium (density 1.077 g/ml; Axis-Shield, Oslo, Norway) according to the manufacturer’s instructions. The cells were cultured in RPMI that was supplemented with 10% FCS. After 24 h in culture, \(2.5 \times 10^5\) PBMCs/well in RPMI with 1% FCS were incubated with the indicated concentrations of ApoBDS-1 for 24 h at 37°C, 5% CO\(_2\).

**Macrophage differentiation in vitro**

PBMCs were cultured in RPMI that was supplemented with 10% FCS in a dish at 1.5 \(\times\) 10\(^5\) cells/cm\(^2\). After a 1-h adherence step, floating cells were discarded. Adherent monocytes were used to generate macrophages, as described \(^2\). Briefly, monocytes were cultured for 7 days in medium that contained 60% AIM V\(^®\) (Gibco Invitrogen, Carlsbad, CA, USA) and 30% Iscove's Modified Dulbecco's Medium (IMDM, Gibco Invitrogen, Carlsbad, CA, USA) and was supplemented with 10% inactivated AB+ serum on dishes. The media was changed after 3 days. After differentiation, the cells were scraped, resuspended in RPMI that was supplemented with 10% FCS, and allowed to rest. After 24 h of rest, 2.5 \(\times\) 10\(^5\) macrophages/ml were plated in RPMI with 1% FCS and incubated with 25 µg/ml ApoB-DS1 for 24 h at 37°C, 5% CO\(_2\).

**Stable TLR4 transfectants**

HEK-293/TLR4 cells (stably transfected with human TLR4A, MD-2, and CD14, Invivogen, San Diego, CA, USA) were grown in DMEM with 10% FCS, HygroGold™ (50 µg/ml), Normocin™ (100 µg/ml), and blasticidin (10 µg/ml) and seeded at 1 \(\times\) 10\(^4\) cells per well. Cells were stimulated with LPS (100 ng/ml) or ApoBDS-1 (50 µg/ml) and incubated
overnight at 37°C, 5% CO₂. IL-8 concentrations in the supernatants were measured by ELISA.

**Cytokine and chemokine measurements**

TNF-α, IL-1β, IL-6, IL-10, IL-12p40, IFN-γ, IP-10, MIG, IL-8, CCL2 (MCP-1), and CCL5 (RANTES) in the supernatants of PBMC and plaque tissue cultures were measured by Cytometric Bead Array (CBA®, BD Bioscience, CA, USA) or ELISA (R&D Systems, Abingdon, United Kingdom), following the manufacturer’s protocol. Prostaglandin E2 (PGE2) in the supernatants of PBMC and plaque tissue cultures was measured using the PGE2 Express EIA Kit (Cayman Europe, Tallin, Estonia) per the manufacturer's instructions.

**RNA analysis**

RNA extraction and reverse-transcription were performed as described 3. Quantitative real time-PCR analysis was performed on an ABI Prism 7900 HT Sequence Detector (Applied Biosystems, Foster City, CA, USA) using Assay-on-Demand primers and probes (Applied Biosystems, Foster City, CA, USA). mRNA levels were normalized to the housekeeping genes β-2 macroglobulin and GAPDH. Relative expression was determined by the 2^ΔΔCT method 4.

**Analysis of calcium flux**

PBMCs were resuspended at 1 x 10⁶ cells/ml in Hanks’ balanced saline solution (HBSS), containing calcium, 1% (v/v) FCS, 5 μM Fluo-4 AM (Molecular Probes, Invitrogen, Carlsbad, CA, USA), and 2.5 mM probenecid (Molecular Probes, Invitrogen,
Carlsbad, CA, USA). After a 30 min pre-incubation at 37°C, the cells were washed with calcium-free HBSS and incubated with 25 µg/mL ApoBDS-1 or 2.5 µM calcium ionophore as a positive control. Increased calcium flux was recorded within 240 seconds, as indicated in the figures. Samples were excited at 485 nm, and emission spectra were recorded at 535 nm by fluorescence microscopy or on a 96-well plate fluorescence reader (Fluoroskan Ascent FL, Thermo Scientific). Data are presented as fold-increase compared with baseline readings from cells in medium alone.

**Detection of MAPK phosphorylation**

To examine MAPK responses, THP-1 cells were stimulated with 25 µg/mL ApoBDS-1 for the indicated times. Next, the cells were washed with cold PBS, and lysates were generated by homogenizing the cells in RIPA lysis buffer (1% Nonidet® P40, 50 mM Tris, pH 8.0, 150 mM NaCl, deoxycholate 0.5%, SDS 0.1%) supplemented with protease inhibitor cocktail (Complete™, Roche, Basel, Switzerland) and phosphatase inhibitors (10 mM sodium fluoride and 1 mM sodium orthovanadate, Sigma-Aldrich, St. Louis, MO, USA).

Equal amounts of proteins (30 µg) were separated by SDS-PAGE (4.0% - 15%, Bio-Rad, Richmond, CA, USA) and transferred to a PVDF membrane (GE Healthcare, Uppsala, Sweden). After being blocked with 5% nonfat dry milk, phosphorylated and total proteins were detected using monoclonal antibodies to ERK1/2, phospho-ERK1/2, p38, phospho-p38, JNK, and phospho-JNK (Cell Signaling Technology, Beverly, MA, USA). The blots were incubated with HRP-conjugated anti-rabbit IgG (Dako, Glostrup, Denmark) and developed by ECL (GE Healthcare, Uppsala, Sweden).
**Blocking of MAPK signaling pathways**

For pharmacological inhibition of ERK1/2, p38-MAPK, and JNK, PD98059 (10 μM), SB203580 (10 μM), and SP600125 (40 μM) (all purchased from Calbiochem, Darmstadt, Germany) were added 30 min before the PBMCs were stimulated with peptides. The inhibitors were dissolved in 100% dimethylsulphoxide (DMSO); the final concentration of DMSO in the cultures was < 0.1%. Unstimulated cell cultures that were incubated with 0.1% DMSO were included as a control and did not experience any biological effects (data not shown).

**Anti-ApoBDS-1 specificity analysis.**

Specificity of anti-ApoBDS-1 rabbit polyclonal IgG antibody against ApoBDS-1 was evaluated by ELISA. Briefly, 50 µL of the different antigens (1 µg/ml in Carbonate/Bicarbonate pH 9.5) was added to 96-well ELISA plates and incubated overnight at 4°C. Coated plates were washed with PBS and blocked with 1% gelatin (Gibco Invitrogen, Carlsbad, CA, USA) in PBS for 1 hr at room temperature. Next, plates were washed and incubated for 2 additional hours with anti-ApoBDS-1 antibody diluted 1:1000 in Tris-buffered saline (TBS)/gelatin 0.1%. After washing, total IgG levels were measured using enzyme-conjugated goat anti-rabbit antibodies (Dako, Glostrup, Denmark). The plates were washed, and colorimetric reactions were developed using TMB (3,3′,5,5′-Tetramethylbenzidine, BD Biosciences, Franklin Lakes, NJ, USA). The absorbance was measured using a microplate reader (VersaMax, Molecular Devices, Sunnyvale, CA, USA).
Supplemental figure II

\[ y = 0.4026x^2 - 46.434x + 1344.5 \]

\[ R^2 = 0.9869 \]
Supplemental figure III

IL-8 (ng/mL)

control  ApoBDS-1  48 min  49 min  50 min  51 min  52 min  53 min  54 min
Supplemental figure IV

[Graph showing IL-8 levels with different treatments: ApoBDS-1, P216a, P216b, P216c, and LPS. The graph indicates various concentrations of IL-8 (pg/mL) in response to these treatments.]
Supplemental figure legends

Supplemental figure I. Anti-ApoBDS-1 antibody specificity analysis.
Specific reactivity of anti-ApoBDS-1 rabbit polyclonal IgG antibody to the ApoB100 peptides, ApoB100 protein, native LDL, and oxLDL was evaluated by ELISA. Values are expressed as mean ± SEM of the OD at 450 nm.

Supplemental figure II. Molecular weight calibration of Superose 6/Discovery BIO GFC-500.
Molecular weight calibration of the coupled columns Superose 6 and Discovery BIO GFC-500 was performed using LMW and HMW protein cocktails from GE Healthcare, Uppsala, Sweden. Samples fractionated at a flow rate of 0.4 mL/min, using Ammonium bicarbonate buffer, pH 7.4. Thyroglobulin 669 kDa; Ferritin 440 kDa; Catalase 232 kDa; Lactate dehydrogenase 140 kDa; Albumin 66 kDa; Carbonic Anhydrase 29 kDa; Ribonuclease A 13.7 kDa; Aprotinin 6.5 kDa. X axis, retention time; Y axis, molecular weight (kDa).

Supplemental figure III. Assessment of IL-8 production in response to low molecular weight ApoBDS-1 positive fractions from plaque homogenates.
2 x 10^5 PBMCs were stimulated with low molecular weight fractions (10 to 42 kDa) from 2 different plaque homogenates. ApoBDS-1 (50 µg/ml) was used as positive control (black bar), and medium alone as negative control (grey bar). IL-8 was measured after 24h. Data are means ± SEM of IL-8 levels of 4 experiments. *) P<0.05; **) P<0.01 for samples vs negative control.
Supplemental figure IV. Blockade of ApoBDS-1 induced IL-8 release from PBMCs by truncated ApoBDS-1 peptides.

$2 \times 10^5$ PBMCs were stimulated with ApoBDS-1 (25 µg/ml) or in combination with 5-fold molar excess of truncated ApoBDS-1 peptides P216a, P216b, or P216c. LPS (100 ng/ml) was used as positive control, and medium as negative control. IL-8 was measured after 24 h incubation. Truncated peptides are able to specifically block ApoBDS-1 induced IL-8 release. Data are presented as means ± SEM of IL-8 levels. *) $P<0.05$. The data are representative of 2 experiments.
Supplemental References


