SUPPLEMENTAL METHODS (Lim *et al*.)

Materials

Tissue culture media, cell culture reagents, and heat-inactivated FBS (GIBCO BRL) were purchased from Invitrogen. The acyl-coenzyme A-cholesterol acyltransferase (ACAT) inhibitor 58035 (3-[decyldimethylsilyl]-N-[2-(4-methylphenyl)-1-phenylethyl]propanamide (1) was from J. Heider, formally of Sandoz (East Hanover, NJ). A 10-mg/ml stock was made in DMSO and used at a concentration of 10 μg/ml in all experiments. Recombinant mouse IFNγ was from the PBL Biomedical Laboratories. The Vybrant Annexin V/Propidium Iodide Apoptosis Assay kit #2 was from Molecular Probes. LDL (d, 1.020-1.063 g/ml) from fresh human plasma was isolated by ultracentrifugation (2). Acetyl-LDL was prepared from LDL by reaction with acetic anhydride, as described previously (3), and used at a concentration of 50 μg/ml in all experiments. All other chemical reagents, including tunicamycin, thapsigargin, concanavalin A, and fucoidan, were purchased from Sigma-Aldrich.

Endotoxin Testing

Reagents used in this study were tested for endotoxin contamination by using the Limulus Amebocyte Lysate (LAL) kit (Cambrex, Walkersville, MD) and found to have <0.06 EU/ml endotoxin at working dilutions.

Eliciting and Culturing Mouse Peritoneal Macrophages

Macrophages were obtained from 8–10-wk-old female C57BL/6J mice (Jackson Laboratory); *Stat1*-/- mice on a C57BL/6J background (4); or *Tlr4*^{del} mice on a C57BL/10ScNJ background and wild-type C57BL/10ScSnJ background (Jackson Laboratory). The macrophages were harvested either three days after intraperitoneal (i.p.) injection of concanavalin A (5) or after immunization with methyl-BSA (6). For the latter method, 2 μg/ml methyl-BSA in 0.9% saline was emulsified in an equal volume of complete Freund's adjuvant (CFA) (Difco). Mice were immunized intradermally with 100 μl of the emulsion. After 14 days, the immunization protocol was repeated, except incomplete Freund's adjuvant was used instead of CFA. Seven days later, the mice were injected i.p. with 0.5 ml PBS containing 100 μg methyl-BSA and then the macrophages were harvested 4 days after that by peritoneal lavage. All macrophages were

cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS and 20% L-cell conditioned medium for 48-72 h, at which point they were typically at ~90% confluency.

Immunoblot Analysis

Cell lysates were prepared by homogenization in 1X sample buffer containing 2% SDS, 62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 50 mM DTT, and 0.01% bromphenol blue, and boiled at 100°C for 5 min. Cytosolic and nuclear extracts were isolated using the Nuclear Extraction Kit (Panomics) according to the manufacturer's protocol. Cell extracts were electrophoresed on 4–20% gradient SDS-PAGE gels (Invitrogen) and transferred to 0.45-μm nitrocellulose membranes. The membranes were blocked in Tris-buffered saline, 0.1% Tween 20 (TBST), containing 5% (w/v) nonfat milk at room temperature for 1 h and then incubated with primary antibodies diluted in TBST containing 5% (w/v) nonfat milk or 5% BSA (w/v) at 4 °C overnight. This incubation was followed by incubation with HRP-conjugated secondary antibodies at room temperature for 1 h. Proteins were detected by SuperSignal West Pico-enhanced chemiluminescent solution (Pierce Chemical Co.). When required, membranes were stripped with Restore Western Blot Stripping Buffer (Pierce Chemical Co.) for 15 min at room temperature and reprobed for β-actin (loading control) or other proteins. Antibodies against total STAT1, GADD 153 (CHOP), and α-tubulin were purchased from Santa Cruz Biotechnology, Inc. Antibodies against phospho-Thr286/287 CaMKII, CaMKII, phospho-Ser727 STAT1, and phospho-Tyr701 STAT1 were purchased from Cell Signaling Technology. Anti-nucleophosmin antibody was purchased from Zymed, and anti-β-actin mouse monoclonal antibody was from Chemicon International. The HRP-conjugated donkey anti–mouse and donkey anti–rabbit IgG secondary antibodies were purchased from Jackson ImmunoResearch Laboratories.

Plasma Cholesterol Assays

Total plasma cholesterol was determined using an enzymatic kits from Wako Chemicals GmbH. Plasma high-density lipoprotein (HDL) cholesterol was determined after dextran sulfate-Mg^{2+} precipitation of apoB-containing lipoproteins. Lipoprotein-cholesterol profiles were determined by FPLC gel-filtration fractionation consisting of two Superose 6 columns connected in series (Amersham Pharmacia).

Immunohistochemistry of Murine and Human Atherosclerotic Lesions

Ldlr-/- mice on the C57BL/6 background were fed a "Western-type" diet (21% anhydrous milk fat and 0.15% cholesterol from Harlan-Teklad; TD88137) for 10 weeks. After anesthetization, the hearts of the mice were perfused with PBS. The hearts and proximal aortae were harvested, perfused *ex vivo* with PBS, embedded in OCT compound, snap frozen in an ethanol-dry ice bath, and stored at −70°C. Sections of proximal aortae (6-μm thick) were prepared at −20°C by using a Microm Microtome Cryostat HM 505 E and placed on poly-L-lysine-coated slides (Fisher Scientific). The sections were then fixed in ice-cold acetone for 10 min, air dried for 10 min, and stored at −70°C until use. All of the following procedures were conducted at room temperature. To detect phospho-S727 STAT1, the frozen sections were washed 3 times in PBS for 2 min, blocked for 1 h with 10% normal donkey serum (Jackson ImmunoResearch), and incubated for 1 h with 2.7 μg/ml rabbit anti-phospho-S727 STAT1 (Cell Signaling #9177) or, as a negative control, 2.7 μg/ml nonimmune rabbit IgG (Jackson ImmunoResearch). After washing 3 times in PBS, the sections were incubated for 30 min with biotinylated donkey anti-rabbit IgG (Jackson ImmunoResearch). Finally, the sections were incubated with streptavidin-horseradish peroxidase and the DAB chromogen (both from BD Pharmingen), mounted in Permount, and viewed with an Olympus IX 70 inverted microscope using a ×40 objective. Parallel sections were stained with anti-Mac-3 (BD Pharmingen), anti-α-actin (Abcam), and anti-PECAM-1 (Santa Cruz Biotechnology) antibodies to label macrophages, smooth muscle cells, and endothelial cells, respectively. To stain macrophages in parafinized sections used for *in-situ* TUNEL analysis, sections were deparaffinized, rehydrated, blocked for 1 h with 10% normal donkey serum (Jackson ImmunoResearch), and incubated overnight with a rabbit anti-mouse macrophage antibody (AIA31240, Accurate Chemical & Scientific Corp.). The subsequent steps were identical to that of staining frozen sections described above. To visualize foam cells, frozen sections were fixed with buffered formalin and stained with Oil Red O for neutral lipid and Harris hematoxylin for nuclei.

For the human coronary artery study, the heart of a patient who had died suddenly of coronary causes was obtained as described previously (7). Coronary segments (3-4 mm in length) were frozen, cryosectioned, and stored at –80°C until use. For immunostaining, the sections were thawed, fixed in cold acetone (-20^{o}C), air-dried, and stained with primary antibodies against phosphorylated STAT1 (Millipore, Lake Placid, NY, Catalog # 07-714,

dilution 1:300) or the macrophage marker CD68 (Dako, Carpinteria, CA, Catalog #M0814, dilution 1:600) for 1 hour. Primary antibodies were labeled using an EnVision^{+} System, peroxidase kit (Catalog # K4009) for 30 min. Negative control staining for STAT1 was performed with non-immune IgG (Dako, catalog # X0903) at a similar protein concentration. Finally the signal was visualized by a 3-amino-9-ethylcarbozole substrate-chromogen system producing a rose red color; the sections were counterstained with Gill hematoxylin. For co-immunostaining with anti-Ser-P-STAT1 and anti-CD68, anti-Ser-P-STAT1 was first visualized using diaminobenzidine (DAB) tinted with nickel chloride (black color). The sections were then immunostained for CD68 and visualized using an alkaline phosphatase ABC kit (Vector, Burlingame, CA) with Vector red; sections were then counterstained with methyl green.

Apoptosis was identified by TUNEL (terminal deoxynucleotidyl transferase [TdT]-mediated dUTP nick-end labeling) using TdT from VasoTACS, Trevigen, Gaithersburg MD. Human tonsil served as a positive control. The reaction produce was visualized with DAB tinted with nickel chloride. The section was then stained with CD68 at 1:600 for 1 hour and developed with alkaline phosphatase red substrate kit (Vector, Burlingame, CA). The sections were then counterstained with methylgreen.

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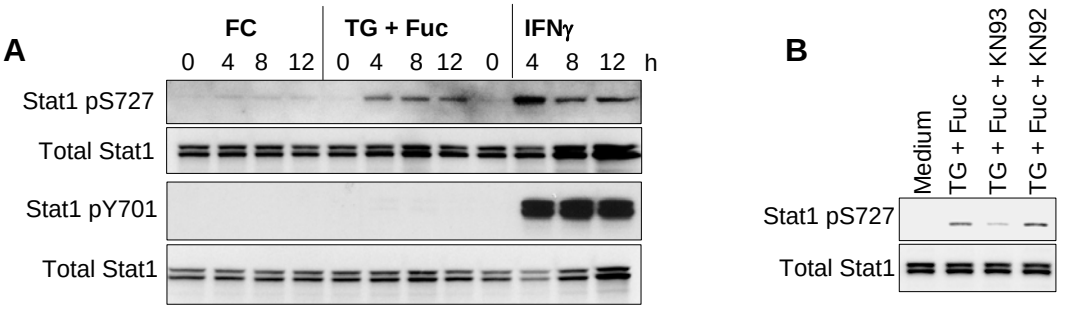
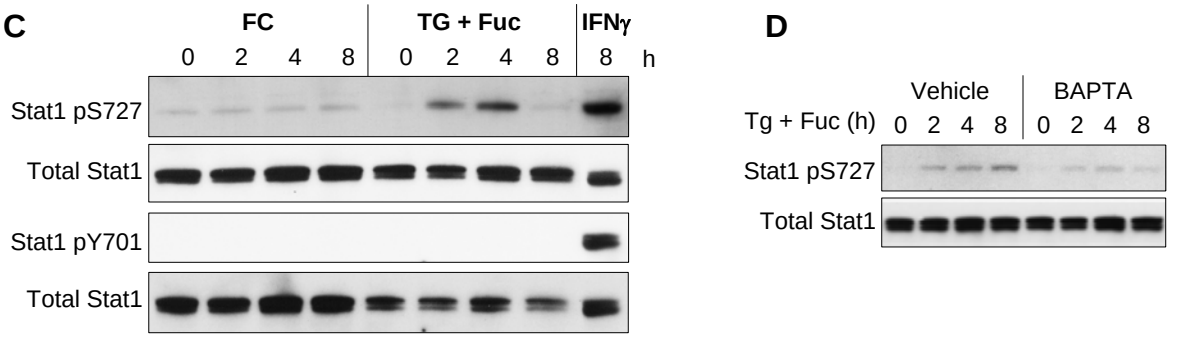
Mouse bone marrow-derived macrophages



Human monocyte-derived macrophages



Mouse bone marrow-derived macrophages (mBMDM) were collected as described in Methods and cultured for 7 days to permit differentiation into mature macrophages. **(*A*)** mBMDM were incubated with acetyl-LDL plus the ACAT inhibitor 58035 (*FC-loaded*), 50 μg/ml fucoidan and 0.5 μM thapsigargin (*TG+Fuc*), or 100 U/ml IFNγ for the indicated times. **(*B*)** mBMDM were incubated with 50 μg/ml fucoidan and 0.5 μM thapsigargin (*TG+Fuc*) plus 50μM KN93 or 50μM KN92 for 2 h.

Human monocyte-derived macrophages (hMDM) were isolated from normal human buffy coats as previously described (Bottalico *et al*. [1993] *J Biol Chem* 268: 8569-8573) and cultured in the presence of 1 ng/ml GM-CSF for 10 days. **(*C*)** hMDM were incubated with acetyl-LDL plus the ACAT inhibitor 58035 (*FC-loaded*), 50 μg/ml fucoidan and 0.5 μM thapsigargin (*TG+Fuc*), or 100 U/ml IFNγ for the indicated times. **(*D*)** hMDM were incubated with 50 μg/ml fucoidan and 0.5 μM thapsigargin (*TG+Fuc*) plus 10 μg/ml BAPTA or vehicle control for the indicated times. Whole-cell lysates were then subjected to immunoblot analysis to detect phospho-S727 STAT1 (*Stat1 pS727*), phospho-Y701 STAT1 (*Stat1 pY701*), and total STAT1.

CLINICAL PERSPECTIVE

Lim et al.

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In industrialized societies, virtually all young adults have atherosclerosis. Most of these lesions are asymptomatic and will remain so for the rest of the person's life. However, a small percentage will progress to a dangerous stage involving plaque breakdown, acute lumenal thrombosis, and acute vascular events, like myocardial infarction and sudden cardiac death. Thus, a major goal is to elucidate the cellular-molecular events involved in benign-to-vulnerable plaque progression. A key feature of vulnerable plaques are "necrotic cores," which likely promote plaque breakdown and acute thrombosis. Necrotic cores are "graveyards of dead macrophages," a prominent cell type in atherosclerosis. Lim *et al*. used a cell-culture model of macrophage death to explore death-promoting molecules that may be relevant to advanced atherosclerosis. These experiments revealed an important role for a calcium-signaling pathway involving a two molecules called CaMKII and STAT-1. The authors showed that both mouse and humans advanced atheromata have activated STAT-1. Most importantly, when macrophages were made deficient in STAT-1 in a mouse model of advanced atherosclerosis, macrophage death and plaque necrosis were diminished. Two important caveats of this study need to be mentioned. First, the processes of macrophage death and plaque necrosis are complex, and so the CaMKII-STAT1 pathway represents only one piece of the puzzle. Second, the mouse is a poor model of plaque disruption and acute thrombosis. Thus, father studies will be needed to explore other pathways involved in advanced lesional macrophage death, and improved mouse models will be required to prove the hypothesis that macrophage death and plaque necrosis promote plaque disruption and acute thrombosis. Nonetheless, the Lim *et al*. study provides important new molecular-cellular information related to the progression of advanced atherosclerotic lesions—information that some day may be translated into therapy designed to block benign-to-vulnerable plaque transformation.