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# Signal Transducer and Activator of Transcription-1 Is Critical for Apoptosis in Macrophages Subjected to Endoplasmic Reticulum Stress In Vitro and in Advanced Atherosclerotic Lesions In Vivo

Wah-Seng Lim, PhD; Jenelle M. Timmins, PhD; Tracie A. Seimon, PhD; Anthony Sadler, PhD; Frank D. Kolodgie, PhD; Renu Virmani, MD; Ira Tabas, MD, PhD

**Background**—Macrophage apoptosis is a critical process in the formation of necrotic cores in vulnerable atherosclerotic plaques. In vitro and in vivo data suggest that macrophage apoptosis in advanced atheromata may be triggered by a combination of endoplasmic reticulum stress and engagement of the type A scavenger receptor, which together induce death through a rise in cytosolic calcium and activation of toll-like receptor-4.

**Methods and Results**—Using both primary peritoneal macrophages and studies in advanced atheromata in vivo, we introduce signal transducer and activator of transcription-1 (STAT1) as a critical and necessary component of endoplasmic reticulum stress/type A scavenger receptor–induced macrophage apoptosis. We show that STAT1 is serine phosphorylated in macrophages subjected to type A scavenger receptor ligands and endoplasmic reticulum stress in a manner requiring cytosolic calcium, calcium/calmodulin-dependent protein kinase II, and toll-like receptor-4. Remarkably, apoptosis was inhibited by  $\approx 80\%$  to  $90\%$  ( $P < 0.05$ ) by STAT1 deficiency or calcium/calmodulin-dependent protein kinase II inhibition. In vivo, nuclear Ser-P-STAT1 was found in macrophage-rich regions of advanced murine and human atheromata. Most important, macrophage apoptosis was decreased by  $61\%$  ( $P = 0.034$ ) and plaque necrosis by  $34\%$  ( $P = 0.02$ ) in the plaques of fat-fed low density lipoprotein receptor null *Ldlr*<sup>-/-</sup> mice transplanted with *Stat1*<sup>-/-</sup> bone marrow.

**Conclusions**—STAT1 is critical for endoplasmic reticulum stress/type A scavenger receptor–induced apoptosis in primary tissue macrophages and in macrophage apoptosis in advanced atheromata. These findings suggest a potentially important role for STAT1-mediated macrophage apoptosis in atherosclerotic plaque progression. (*Circulation*. 2008; 117:940-951.)

**Key Words:** apoptosis ■ atherosclerosis ■ cholesterol ■ macrophage ■ plaque

In advanced atherosclerosis, death of macrophages in the setting of defective phagocytic clearance of apoptotic cells contributes to the development of plaque necrosis.<sup>1,2</sup> Plaque necrosis, in turn, is thought to promote plaque disruption and arterial thrombosis, which are the proximate causes of acute cardiovascular events.<sup>1-3</sup> Our laboratory established an important principle of advanced lesion macrophage death, namely involvement of the endoplasmic reticulum (ER) stress pathway known as the unfolded protein response (UPR).<sup>4,5</sup> Some laboratories have discovered important evidence that the UPR is activated in intimal cells, including macrophages, in advanced murine and human plaques.<sup>6-9</sup> In particular, Myoishi et al<sup>9</sup> recently showed a dramatic rise in UPR markers, including

the transcription factor CHOP (GADD153), and intimal cell apoptosis in autopsy specimens from humans with vulnerable and ruptured plaques but not stable lesions and in atherectomy specimens from humans with unstable angina but not stable angina. Although the UPR is primarily an ER repair pathway, a branch of the UPR involving the effector CHOP can trigger apoptosis when the cell senses that repair is no longer possible.<sup>4,10</sup> In terms of causation, we have shown that advanced lesion macrophage death and plaque necrosis are decreased in atherosclerotic apolipoprotein E–deficient (*ApoE*<sup>-/-</sup>) mice in the setting of ER stress prevention<sup>5</sup> or CHOP deficiency (Edward Thorp, PhD; Gang Li, PhD; George Kuriakose, MSc; David Ron, MD; and I.T., unpublished data, 2007).

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The online-only Data Supplement, consisting of Methods and a figure, can be found with this article at <http://circ.ahajournals.org/cgi/content/full/CIRCULATIONAHA.107.711275/DC1>.

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## Clinical Perspective p 951

Our work on the UPR began with a model of advanced lesional macrophage death that is present in advanced plaques, namely intracellular accumulation of lipoprotein-derived free cholesterol (FC).<sup>11</sup> FC enrichment of macrophages, like many ER stressors, activates the UPR through depletion of ER luminal calcium.<sup>12,13</sup> Since then, mechanistic studies have led to a broader concept of advanced lesional macrophage death beyond the FC model. These studies have shown that any combination of inducers of ER stress and ligands for the macrophage type A scavenger receptor (SRA), both of which are expressed prominently in advanced lesions, triggers macrophage apoptosis.<sup>14,15</sup> Macrophage SRA recognizes a number of lesional molecules and atherogenic lipoproteins, including those used to enrich macrophages with cholesterol in the FC model.<sup>16</sup> The SRA also is a pattern recognition receptor of the innate immune system, and endotoxin-free SRA ligands activate other pattern recognition receptors, notably toll-like receptor-4 (TLR4).<sup>15,17,18</sup> In this context, our studies have shown that SRA ligands trigger 2 critical proapoptotic events in ER-stressed macrophages: TLR4-mediated activation of a proapoptotic MyD88 pathway<sup>15</sup> and SRA-mediated suppression of a prosurvival TLR4-TRIF-interferon (IFN)- $\beta$  pathway.<sup>14,15</sup>

In this report, we show that apoptosis of ER-stressed macrophages also requires signal transducer and activator of transcription-1 (STAT1) and calcium/calmodulin-dependent protein kinase II (CaMKII) in a process involving cytosolic calcium and TLR4. Most important, we provide evidence that activated STAT1 is present in atheromata and that lesional macrophage apoptosis is suppressed in the setting of STAT1 deficiency.

## Methods

See the online-only Data Supplement for expanded Methods.

### Assay of Macrophage Apoptosis

Midstage and late-stage apoptosis in peritoneal macrophages was assayed by annexin V and propidium iodine staining, respectively, with the Vybrant Apoptosis Assay Kit No. 2 (Molecular Probes, Carlsbad, Calif). At the end of incubation, the macrophages were gently washed once with PBS and incubated for 15 minutes at room temperature with 120  $\mu$ L annexin-binding buffer (25 mmol/L HEPES, 140 mmol/L NaCl, 1 mmol/L EDTA, pH 7.4, 0.1% BSA) containing 10  $\mu$ L Alexa Fluor 488-conjugated annexin V and 1  $\mu$ L of 100- $\mu$ g/mL propidium iodine. The staining mixture was then removed and replaced with 120  $\mu$ L annexin-binding buffer. The cells were viewed immediately at room temperature with an Olympus IX-70 inverted fluorescent microscope equipped with filters appropriate for fluorescein and rhodamine, and images were obtained with a Cool Snap charge-coupled device camera (RS Photometrics, Tucson, Ariz) equipped with imaging software from Roper Scientific (Trenton, NJ). Three fields of cells ( $\approx$ 650 cells per field) were photographed for each condition, and the number of annexin V/propidium iodine-positive cells in each field was counted and expressed as a percent of the total number of cells.

### Bone Marrow Transplantation

Ten-week-old female low-density lipoprotein receptor null *Ldlr*<sup>-/-</sup> mice were lethally irradiated with 10 Gy from a cesium  $\gamma$  source 4 to 6 hours before transplantation. Bone marrow cells were collected from the femurs and tibias of donor *Stat1*<sup>-/-</sup> or *Stat1*<sup>+/+</sup> mice by flushing with sterile medium (RPMI 1640, 2% FBS, 10 U/mL

heparin, 50 U/mL penicillin, 50  $\mu$ g/mL streptomycin). The bone marrow cells were washed extensively and resuspended in RPMI medium containing 20 mmol/L HEPES, 50 U/mL penicillin, and 50  $\mu$ g/mL streptomycin. Each recipient mouse was injected with  $5 \times 10^6$  bone marrow cells through the tail vein. The mice were given acidified water containing 100 mg neomycin and 10 mg polymyxin B sulfate 1 week before and 2 weeks after transplantation. Six weeks after transplantation, the mice were fed a "Western-type" diet (21% anhydrous milk fat and 0.15% cholesterol, TD88137, Harlan-Teklad) for 10 or 12 weeks.

### Atherosclerotic Lesion Analysis

On the day of the analysis, food was removed from the cages in the morning, and the mice were fasted for 8 hours. The animals were then anesthetized with isoflurane, and blood was withdrawn by cardiac puncture. The heart was then perfused with PBS, and the heart and proximal aorta were harvested. The heart and aorta were perfused ex vivo with PBS and then transferred to 10% buffered formalin, processed, and embedded in paraffin. Starting from the atrial leaflets, serial sections (6  $\mu$ m thick) were prepared so that every eighth section was stained with Harris hematoxylin and eosin. Atherosclerotic lesions in 6 sections were analyzed in a blinded fashion with a Nikon Labophot-2 microscope (Nikon Instruments Inc, Melville, NY) equipped with a Sony CCD-Iris/RGB color videocamera (Sony Electronics Inc, San Diego, Calif) attached to a computerized imaging system using IMAGE-PRO PLUS 3.0 software (Media Cybernetics, Bethesda, Md). Aortic lesion area was quantified by averaging the lesion areas of the 6 sections. Necrotic areas were defined as those regions of the lesions that lacked nuclei and cytoplasm.

### In Situ TdT-Mediated dUTP Nick-End Labeling Assays

Apoptotic cells in the intima of atherosclerotic lesions were detected by the TdT-mediated dUTP nick-end labeling (TUNEL) technique using the TMR red in situ cell death detection kit (Roche Diagnostics, Indianapolis, Ind) and the stringent method of Kockx.<sup>19</sup> Sections of proximal aorta were deparaffinized, rehydrated, and treated with 2  $\mu$ g/mL proteinase K (Roche) for 30 minutes at 37°C in a humidified chamber. The treated sections were incubated in TdT reaction mixture containing TMR red dUTP for 1 hour at 37°C in a humidified chamber. After washing, genomic DNA was stained with DAPI for 5 minutes at room temperature; the slides then were mounted with coverslips. TUNEL staining was analyzed with an Olympus IX-70 inverted fluorescent microscope equipped with a Cool Snap charge-coupled device camera and imaging software (Roper Scientific). Fluorescent images were captured and analyzed with image Photoshop analysis software (Adobe Systems, San Jose, Calif).

### Statistical Analysis

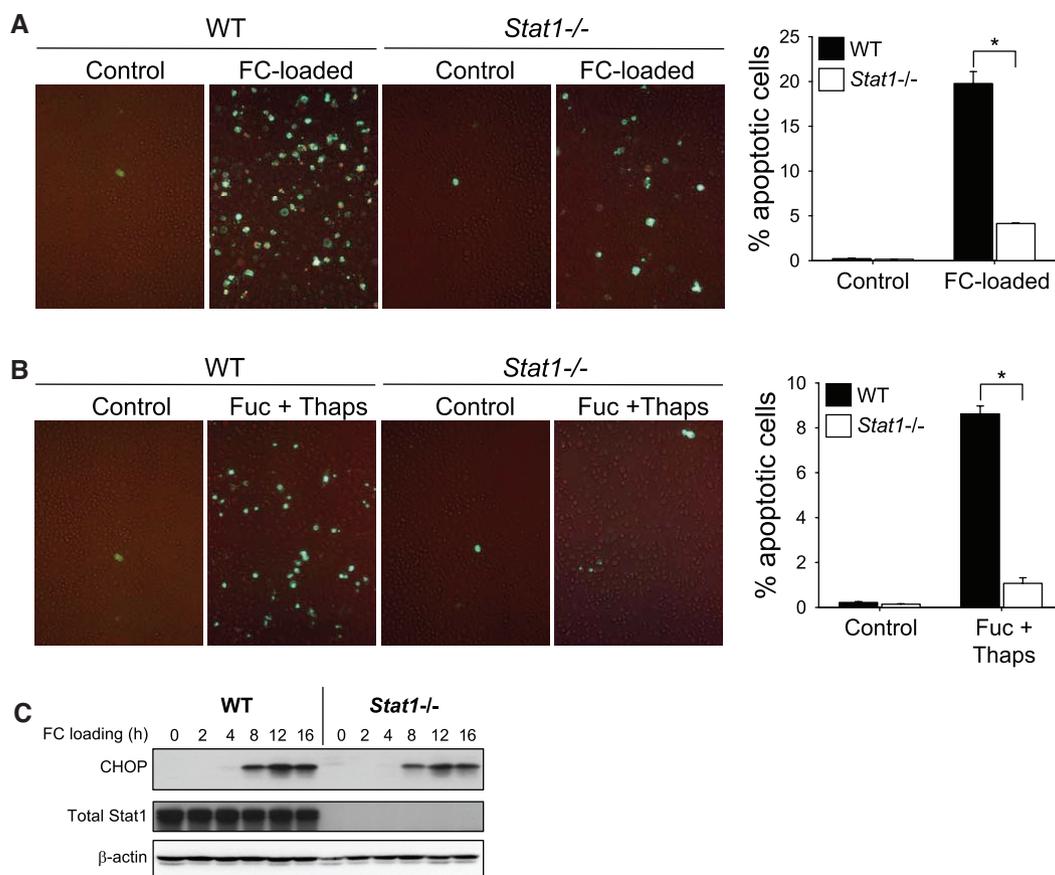
Data are presented as mean  $\pm$  SEM. Absent error bars in the bar graphs signify SEM values smaller than the graphic symbols. The significance of paired data was determined by Student *t* test. Data with  $>2$  groups or  $\geq 2$  independent variables were analyzed with ANOVA, followed by the Bonferroni post hoc test. Significance is indicated by an asterisk in the figures with an explanation in the figure legends; nonsignificance is indicated by NS in the figures.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

## Results

### SRA-Induced Apoptosis in ER-Stressed Macrophages Requires STAT1 and Is Preceded by Serine Phosphorylation of STAT1

During the course of another study investigating an ER stress response mediator called interferon-inducible, double-

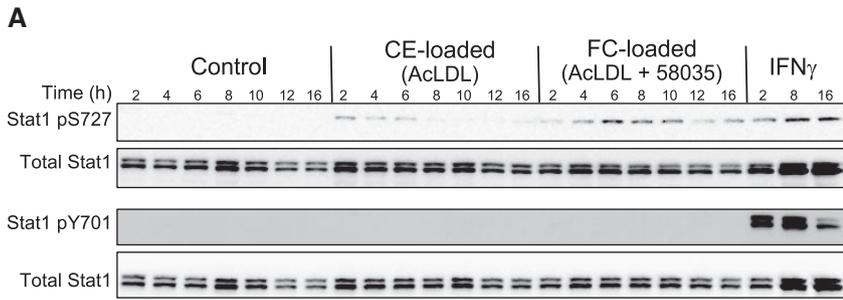


**Figure 1.** SRA/ER stress-induced macrophage apoptosis requires STAT1. A and B, Peritoneal macrophages from WT or *Stat1*<sup>-/-</sup> mice were incubated for 17 hours with medium alone (Control) or medium containing acetyl-low-density lipoprotein (LDL) plus the ACAT inhibitor 58035 (FC-loaded) or for 21 hours with medium alone (Control) or medium containing 50  $\mu$ g/mL fucoidan and 0.5  $\mu$ mol/L thapsigargin (Fuc+Thaps). Midstage and late-stage apoptosis was assessed by staining with Alexa Fluor 488-conjugated annexin V (green) and propidium iodide (orange), respectively. Representative merged fluorescence and bright-field images and quantitative data from 3 fields of cells for each condition are shown. C, Lysates from WT and *Stat1*<sup>-/-</sup> macrophages were FC loaded for the indicated times and subjected to immunoblot analysis to detect CHOP, total STAT1, and  $\beta$ -actin. \* $P=0.001$  by Bonferroni after ANOVA.

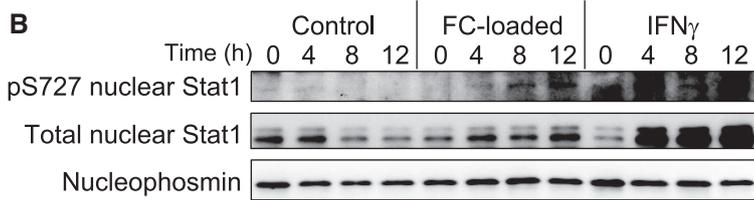
stranded RNA-regulated eIF-2 $\alpha$  protein kinase (PKR), we conceived the hypothesis that STAT1, the activity of which is modulated by PKR,<sup>20</sup> may play a role in ER stress-induced macrophage apoptosis. To test this idea, we compared SRA/ER stress-induced apoptosis in peritoneal macrophages from wild-type (WT) versus *Stat1*<sup>-/-</sup> mice. Confirming our previous work, both intracellular FC enrichment with an SRA-interacting lipoprotein and treatment with the SRA ligand fucoidan plus the UPR activator thapsigargin triggered apoptosis, as indicated by an increase in annexin V staining (Figure 1A and 1B, WT). In contrast, *Stat1*<sup>-/-</sup> macrophages were markedly protected from apoptosis by both inducers (80% to 90% inhibition of apoptosis;  $P<0.05$ ), indicating an essential role of STAT1 in this model of macrophage apoptosis (Figure 1A and 1B, *Stat1*<sup>-/-</sup>). The decrease in apoptosis in *Stat1*<sup>-/-</sup> macrophages could not be explained by a decrease in either SRA (not shown) or CHOP induction (Figure 1C).

STAT1 is activated by phosphorylation of Y701 or S727.<sup>21</sup> Y701 phosphorylation is essential for STAT1 dimerization, nuclear translocation, and DNA binding.<sup>21</sup> S727 phosphorylation enhances the transcriptional activity of tyrosine-phos-

phorylated STAT1 or, in some cases, has been reported to participate in signaling in the absence of Y701 phosphorylation.<sup>21–23</sup> As shown in Figure 2A, FC loading of macrophages induced serine, whereas tyrosine phosphorylation was not detected, and total STAT1 was not increased. In contrast, very little serine phosphorylation was seen in nonloaded or cholesteryl ester-loaded macrophages, which show no or very little evidence of ER stress.<sup>4</sup> As expected, IFN $\gamma$  induced highly detectable levels of tyrosine phosphorylation and serine phosphorylation of STAT1.<sup>21</sup> Previous work has suggested that nuclear Ser-P-STAT1 can occur through serine phosphorylation of a constitutive pool of nuclear STAT1.<sup>23</sup> We detected STAT1 in nuclear fractions isolated from untreated macrophages, and Ser-P-STAT1 was increased with FC loading. Although total nuclear STAT1 was modestly increased after FC loading, this increase was much less than that seen with IFN $\gamma$ , which is known to induce STAT1 nuclear translocation<sup>21</sup> (Figure 2B). These data suggest that at least a portion of FC-induced Ser-P-STAT1 occurs through phosphorylation of constitutively nuclear STAT1. It also is possible that at least a portion of the STAT1 was tyrosine phosphorylated but below the limits of detection of our immunoblot assay.

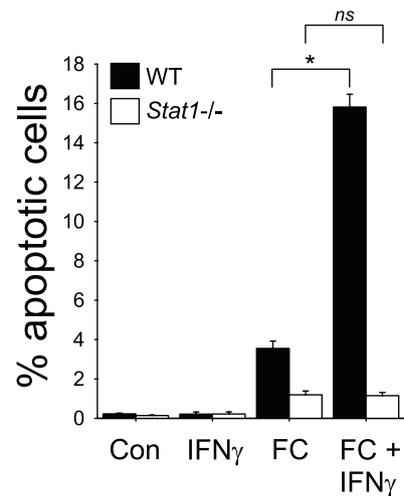
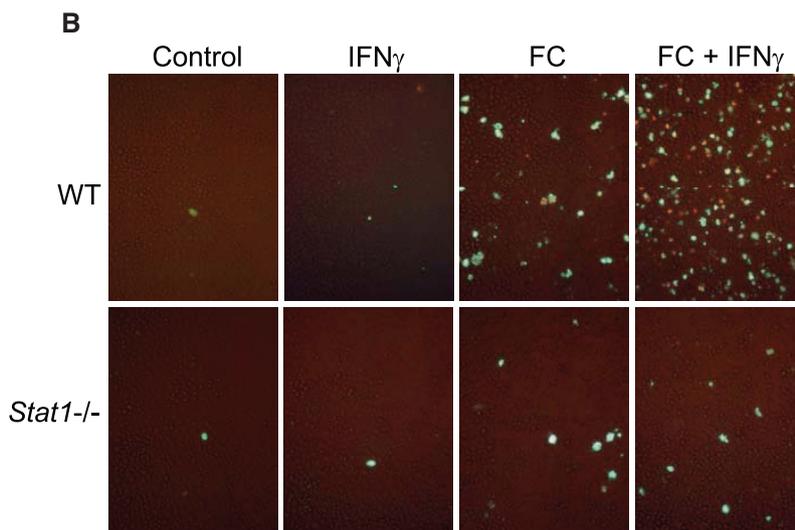
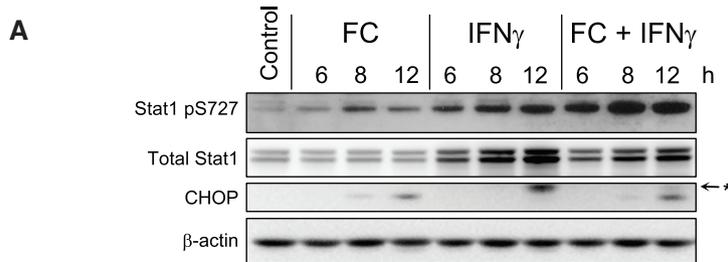


**Figure 2.** FC loading induces serine but not tyrosine phosphorylation of STAT1. A, Macrophages were incubated for the indicated times with medium alone (Control) or medium containing acetyl-LDL (CE-loaded), acetyl-LDL plus 58035 (FC-loaded), or 100 U/mL IFN $\gamma$ . Whole-cell lysates were then prepared and subjected to immunoblot analysis to detect phospho-S727 STAT1 (Stat1 pS727), phospho-Y701 STAT1 (Stat1 pY701), and total STAT1. B, Nuclear fractions from control, FC-loaded, and IFN $\gamma$ -treated macrophages were subjected to immunoblot analysis to detect STAT1 pS727, total STAT1, and the nuclear marker nucleophosmin.

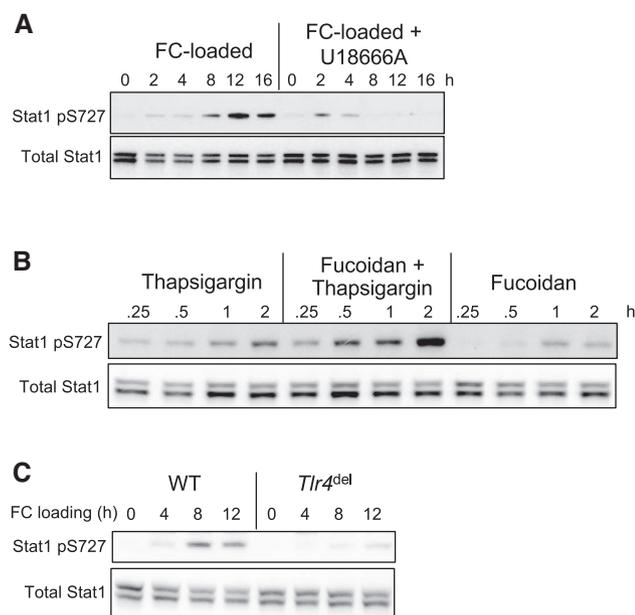


The ability of IFN $\gamma$  to stimulate both serine and tyrosine phosphorylation of STAT1, the presence of IFN $\gamma$  in atherosclerotic lesions, and recent evidence that IFN $\gamma$  promotes advanced plaque progression<sup>24,25</sup> led us to ex-

plore the effect of the combination of FC loading and IFN $\gamma$  treatment on Ser-P-STAT1 and apoptosis. FC-loaded macrophages treated with IFN $\gamma$  showed an increase in Ser-P-STAT1 that was greater than either condition alone (Figure



**Figure 3.** IFN $\gamma$  enhances FC-induced STAT1 serine phosphorylation and STAT1-dependent FC-induced apoptosis. A, Macrophages were incubated for the times indicated with medium alone (Control) or medium containing acetyl-LDL plus 58035 (FC), 100 U/mL IFN $\gamma$ , or acetyl-LDL, 58035, and IFN $\gamma$  (FC+IFN $\gamma$ ). Whole-cell lysates were then prepared and subjected to immunoblot analysis to detect STAT1 pS727, total STAT1, CHOP, and  $\beta$ -actin. In the CHOP blot, a nonspecific band is indicated by the asterisk. B, Macrophages from WT or *Stat1*<sup>-/-</sup> mice were incubated for 13 hours with medium alone (Control) or medium containing 100 U/mL IFN $\gamma$ , acetyl-LDL plus 58035 (FC), or acetyl-low-density lipoprotein, 58035, and IFN $\gamma$  (FC+IFN $\gamma$ ). Apoptosis was assayed and quantified as in Figure 1. \* $P=0.01$  for FC and  $P=0.001$  for FC+IFN $\gamma$  by Bonferroni after ANOVA.



**Figure 4.** STAT1 serine phosphorylation in SRA-engaged, ER-stressed macrophages is amplified by ER stress and requires TLR4 activation. Whole-cell lysates were subjected to immunoblot analysis to detect STAT1 pS727 and total STAT1 under the following conditions. A, Macrophages were incubated for the times indicated with acetyl-LDL and 58035 (FC-loaded) or acetyl-LDL, 58035, and 1  $\mu\text{mol/L}$  U18666A (FC-loaded+U18666A). B, Macrophages were incubated for the times indicated with 0.5  $\mu\text{mol/L}$  thapsigargin, 50  $\mu\text{g/mL}$  fucoidan plus thapsigargin, or fucoidan alone. C, Macrophages from WT or *Tlr4*<sup>del</sup> mice were incubated for the times indicated under FC-loading conditions.

3A). Note that IFN $\gamma$  alone did not induce CHOP, nor did it further increase CHOP in the setting of FC loading. Most important, under conditions in which IFN $\gamma$  alone induced no apoptosis, IFN $\gamma$  treatment led to a >5-fold enhancement of FC-induced apoptosis (Figure 3B, WT). This effect of IFN $\gamma$  required STAT1 because it was inhibited by 93% ( $P<0.05$ ) in *Stat1*<sup>-/-</sup> macrophages (Figure 3B, *Stat1*<sup>-/-</sup>). Thus, in atheromata, where macrophages are likely exposed to the combination of SRA ligands, ER stressors, and IFN $\gamma$ , the role of STAT1 in macrophage apoptosis may be particularly important (below).

### Cytosolic Calcium, TLR4, and CaMKII Activation Is Required for Stat1 Serine Phosphorylation and Apoptosis in FC-Loaded Macrophages

Three kinases that are able to catalyze serine phosphorylation of STAT1 are p38, extracellular signal-regulated kinase (ERK), and protein kinase C- $\delta$ .<sup>23</sup> However, using a combination of gene targeting and chemical inhibitors, we found that inhibiting these kinases did not abrogate FC-induced serine phosphorylation of STAT1 (data not shown). In the face of these negative data, we next asked whether 2 critical components of the multihit model, ER stress and TLR4 signaling, were necessary for STAT1 serine phosphorylation. The data in Figure 4A and 4B show that blocking FC-induced ER stress by the cholesterol trafficking inhibitor U18666A<sup>4</sup> or omitting thapsigargin from the fucoidan-plus-thapsigargin model markedly suppressed Ser-P-STAT1. In addition, FC-

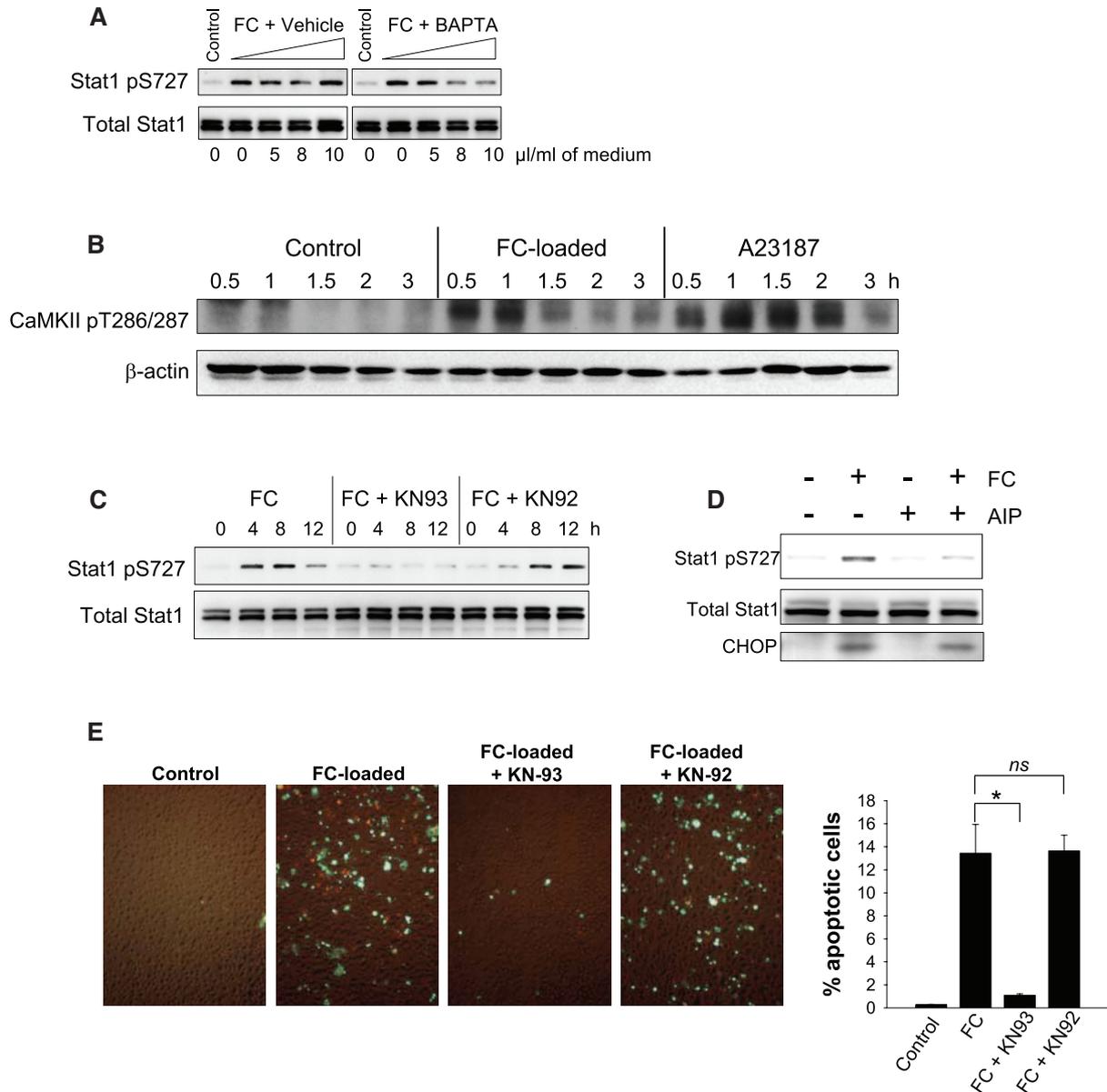
induced serine phosphorylation of STAT1 was almost completely prevented in TLR4-deficient macrophages (Figure 4C). Note that all of these manipulations also block macrophage apoptosis.<sup>4,15</sup>

Both ER stress and TLR4 signaling can affect cellular calcium metabolism (see Discussion).<sup>12,13,15,26,27</sup> Moreover, we recently showed that buffering cytosolic calcium with 1,2-bis[2-aminophenoxy]ethane-N,N,N',N'-tetraacetic acid tetrakis [acetoxymethyl ester] (BAPTA-AM) markedly inhibited both FC-induced and thapsigargin/fucoidan-induced apoptosis.<sup>15</sup> To test the role of cytosolic calcium in STAT1 serine phosphorylation, we incubated FC-loaded macrophages with increasing concentrations of BAPTA-AM or equivalent volumes of vehicle control. As shown in Figure 5A, BAPTA-AM suppressed FC-induced serine phosphorylation of STAT1 in a dose-dependent manner.

One mechanism by which cytosolic calcium might participate in STAT1 serine phosphorylation is by activating CaMKII, which may directly phosphorylate STAT1<sup>28</sup> and/or lead to its phosphorylation by enhancing TLR4 signaling (see Discussion).<sup>15,29,30</sup> As shown in Figure 5B, FC loading led to a rapid and marked enhancement of CaMKII threonine phosphorylation, which is a marker of its activation. At the 30- and 60-minute time points, the degree of activation was similar to that of the calcium ionophore A23187, a known potent activator of CaMKII. Similar results were found with fucoidan plus thapsigargin (data not shown). Note that the time course of CaMKII activation by FC loading or by thapsigargin plus fucoidan precedes the onset of STAT1 serine phosphorylation in these cells. To show a functional role for CaMKII activation in both STAT1 serine phosphorylation and apoptosis in FC-loaded macrophages, we used 2 structurally diverse CaMKII inhibitors. The data in Figure 5C and 5D show that the chemical CaMKII inhibitor KN93,<sup>31</sup> but not the inactive homologue KN92, and the peptide CaMKII inhibitor AIP<sup>32</sup> markedly suppressed FC-induced STAT1 serine phosphorylation. Most important, KN93 but not KN92 suppressed FC-induced apoptosis by 92% ( $P<0.05$ ) (Figure 5E). Note that neither KN93 nor AIP decreased the uptake or ER trafficking of lipoprotein-derived FC or the induction of CHOP (data not shown and Figure 5D). In summary, these data indicate that cytosolic calcium and CaMKII are essential for STAT1 serine phosphorylation and apoptosis in the SRA-ER stress model. We also conducted experiments on 2 additional macrophage models, namely mouse bone marrow-derived and human peripheral blood-derived macrophages. In both of these cell types, the SRA-ER stress model exclusively induced STAT1 serine phosphorylation via a pathway mediated by cytosolic calcium and CaMKII (see the Figure in the online-only Data Supplement), suggesting the universality of this signaling pathway among macrophages.

### STAT1 Is Serine Phosphorylated in Murine and Human Atherosclerotic Lesions, and STAT1 Plays a Role in Advanced Lesional Macrophage Apoptosis and Plaque Necrosis in Female *Ldlr*<sup>-/-</sup> Mice

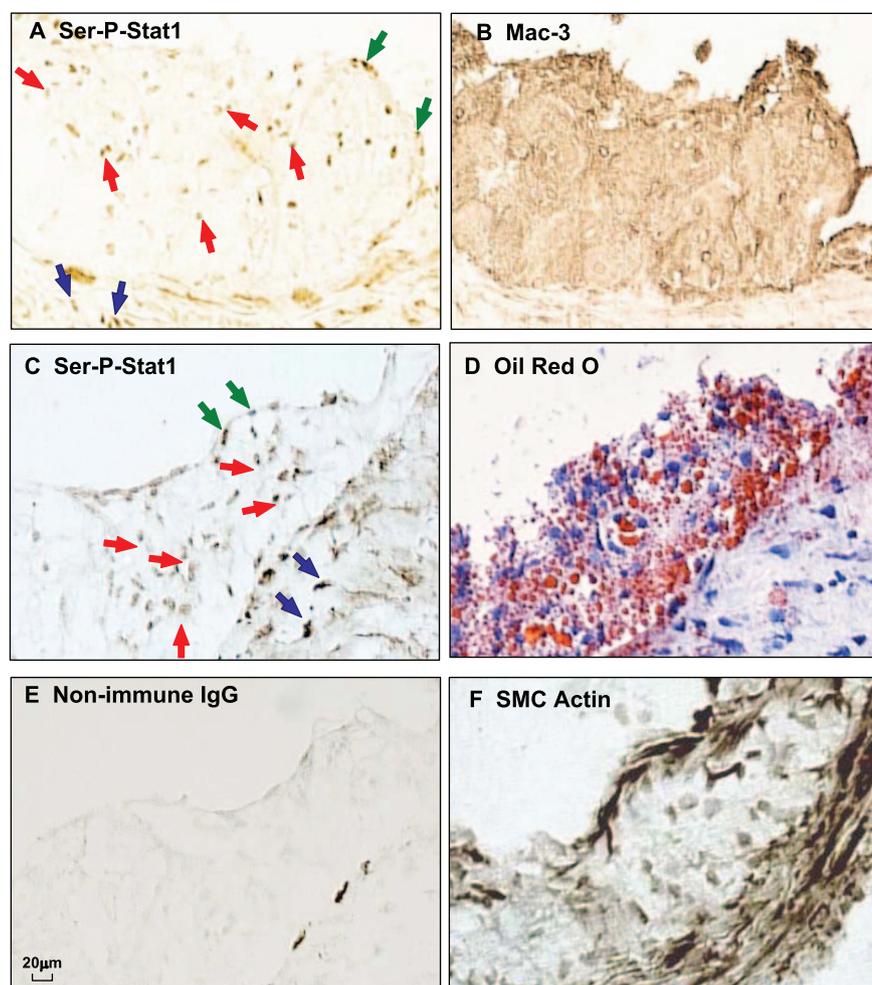
To provide evidence for the relevance of Ser-P-STAT1 in atherosclerosis, we first used immunohistochemistry to



**Figure 5.** Cytosolic calcium and CaMKII activation is required for FC-induced STAT1 serine phosphorylation, and inhibition of CaMKII blocks FC-induced apoptosis. A through D, Whole-cell lysates were subjected to immunoblot analysis to detect STAT1 pS727, total STAT1, CaMKII pT286/287, β-actin, or CHOP, as indicated in the individual blots, under the following conditions. A, Macrophages were incubated for 8 hours in medium alone (Control); medium containing acetyl-LDL and 58035 plus vehicle control (FC+Vehicle); or medium containing acetyl-LDL, 58035, and increasing concentrations of BAPTA (FC+BAPTA). The indicated microliters of vehicle or BAPTA-AM stock solution (1 mg/mL) were added per 1 mL medium. B, Macrophages were incubated for the times indicated with medium alone (Control) or medium containing acetyl-LDL and 58035 (FC-loaded) or 2 μg/mL A23187. C, Macrophages were incubated for the times indicated with acetyl-LDL and 58035 (FC) or acetyl-LDL and 58035 plus either 10 μmol/L KN93 or 10 μmol/L KN92 (FC+KN93 or FC+KN92). D, Macrophages were incubated for 8 hours with acetyl-LDL and 58035 (FC), 10 μmol/L AIP, or acetyl-LDL, 58035, and AIP. E, Macrophages were incubated for 24 hours in medium alone (Control) or medium containing acetyl-LDL and 58035 (FC-loaded) or acetyl-LDL and 58035 plus either 10 μmol/L KN93 or 10 μmol/L KN92 (FC-loaded+KN93 or FC-loaded+KN92). Apoptosis was assayed and quantified as in Figure 1. For all experiments involving KN93, KN92, or AIP, the macrophages were pretreated for 1 hour with medium alone or medium containing these inhibitors before FC loading. \**P*<0.01 by Bonferroni after ANOVA.

assess expression of Ser-P-STAT1 in murine and human atheromata (Figures 6 and 7). In mouse lesions, Ser-P-STAT1 was present in numerous macrophage foam cells, as assessed by staining adjacent sections with anti-Mac-3 antibody (Figure 6A and 6B) and Oil Red O (Figure 6D). As illustrated by these images, Ser-P-STAT1 staining also

was observed in the endothelial cells lining the lumen, which was PECAM-1 positive (not shown), and in smooth muscle cells in the media, which were α-actin positive (Figure 6F). In human lesions, staining of Ser-P-STAT1 was found in the advanced stages called pathological intimal thickening and fibroatheroma (Figure 7B and 7C)



**Figure 6.** STAT1 is serine phosphorylated in atherosclerotic lesions from *Ldlr*<sup>-/-</sup> mice. Adjacent frozen sections of an aortic root lesion from an *Ldlr*<sup>-/-</sup> mice fed a Western-type diet for 12 weeks were immunostained with anti-Ser-P-STAT1 or anti-Mac3 (macrophages) (A and B) or anti-Ser-P-STAT1, oil red O, nonimmune immunoglobulin G (IgG), and  $\alpha$ -actin (C through F). Note examples of brown stain in the nuclei of the intimal cells (red arrows), endothelial cells (green arrows), and smooth muscle cells (SMC) in the media (blue arrows). The dark streaks at the intima-media interface in E represent nonspecific staining.

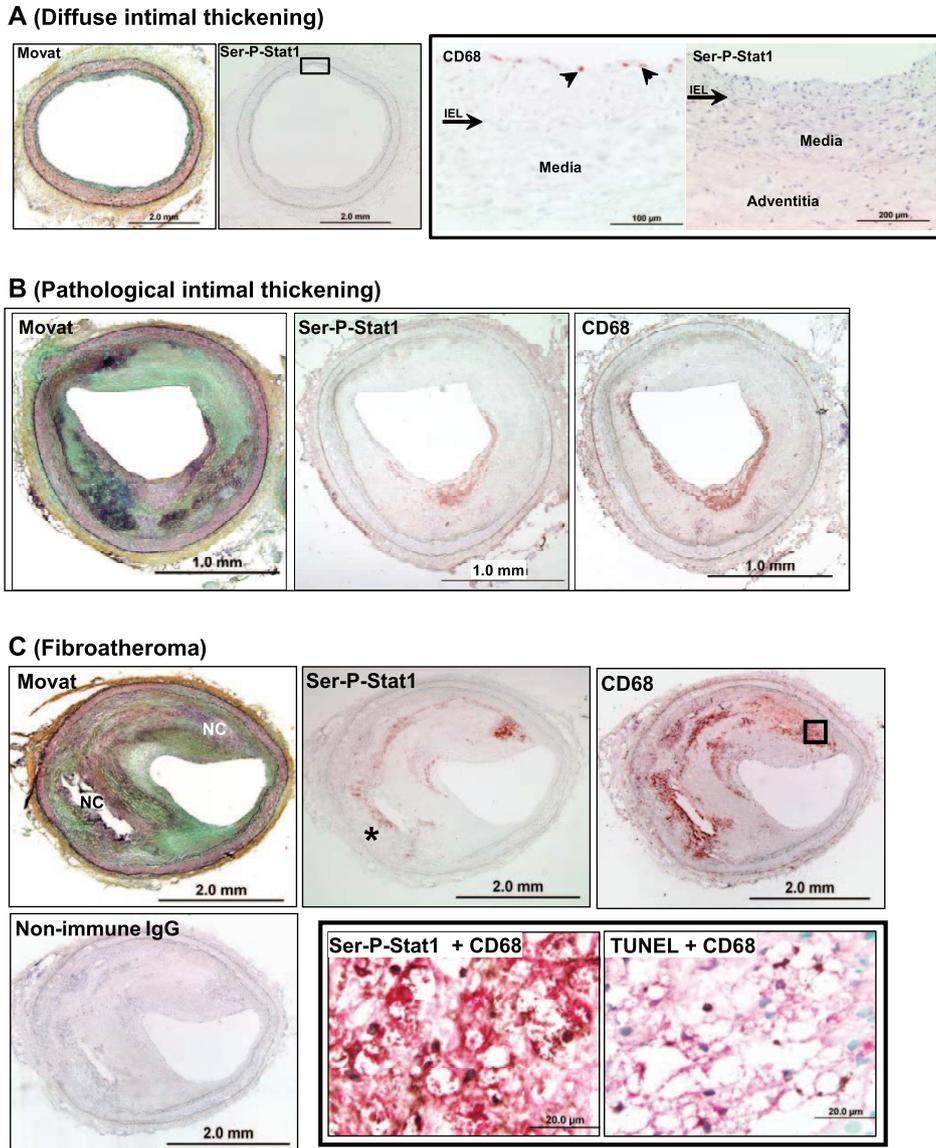
but not in the early stage of diffuse intimal thickening (Figure 7A). In the advanced lesions, most of the Ser-P-STAT1 colocalized with macrophages (Figure 7B and 7C). Note that Ser-P-STAT1 was found in the nuclei of these cells (Figure 7C, bottom middle) and in areas that were TUNEL positive, a marker of apoptosis (Figure 7C, bottom right). Of interest, some of the Ser-P-STAT1 in the most advanced fibroatheroma was found in macrophages surrounding necrotic areas (Figure 7C, top middle, asterisk).

To further investigate a causal link between STAT1 and lesional macrophage apoptosis, we compared advanced plaques of Western diet-fed *Ldlr*<sup>-/-</sup> mice reconstituted with either WT or *Stat1*<sup>-/-</sup> bone marrow. The mice were fed the Western diet for 10 or 12 weeks. Plasma lipoprotein cholesterol and body weight were similar between the 2 groups of mice (Figure 8A for 10-week protocol; data not shown for 12-week protocol). In the 10-week study, overall lesion areas were similar (Figure 8B and 8C). However, the number of TUNEL-positive cells in macrophage-rich regions was decreased by 61% ( $P=0.034$ ) in the *Stat1*<sup>-/-</sup>→*Ldlr*<sup>-/-</sup> lesions, and a trend toward decreased plaque necrosis existed that did not quite reach statistical significance ( $P=0.078$ ) (Figure 8C). Note that total macrophage area was not affected by STAT1 deficiency ( $120.0 \pm 11.8 \times 10^3$  and  $111.5 \pm 21.3 \times 10^3$   $\mu\text{m}^2$  in WT and *Stat1*<sup>-/-</sup> bone marrow recipients, respectively;  $P=0.72$ ; see Discussion).

Plaque necrosis likely results from the eventual cellular necrosis of macrophages that become apoptotic but are not subsequently cleared by phagocytes.<sup>1,2</sup> Therefore, we predicted that as the lesions in the 2 groups of mice progressed, the difference in necrotic core areas would become statistically significant, whereas apoptotic macrophages per se would become less numerous and less different between the 2 groups of mice. As shown by the data in Figure 8D, the necrotic cores were larger in the 12-week-diet mice, and a statistically significant difference was present in the necrotic core area (34% decrease in the *Stat1*<sup>-/-</sup>→*Ldlr*<sup>-/-</sup> lesions;  $P=0.02$ ) but not the number of TUNEL-positive cells. In summary, STAT1 deficiency in bone marrow-derived cells in *Ldlr*<sup>-/-</sup> mice has a substantial protective effect on apoptosis in the macrophage-rich lesions of advanced plaques and on plaque necrosis.

## Discussion

Increasing evidence from a number of laboratories suggests that an ER stress-based model of macrophage apoptosis plays an important role in advanced lesional macrophage death and plaque necrosis.<sup>4-9</sup> The work reported here adds critical new components to this model by demonstrating essential roles for STAT1 and CaMKII in macrophage apoptosis in vitro and for STAT1 in advanced lesional macrophage apoptosis and plaque necrosis in vivo.

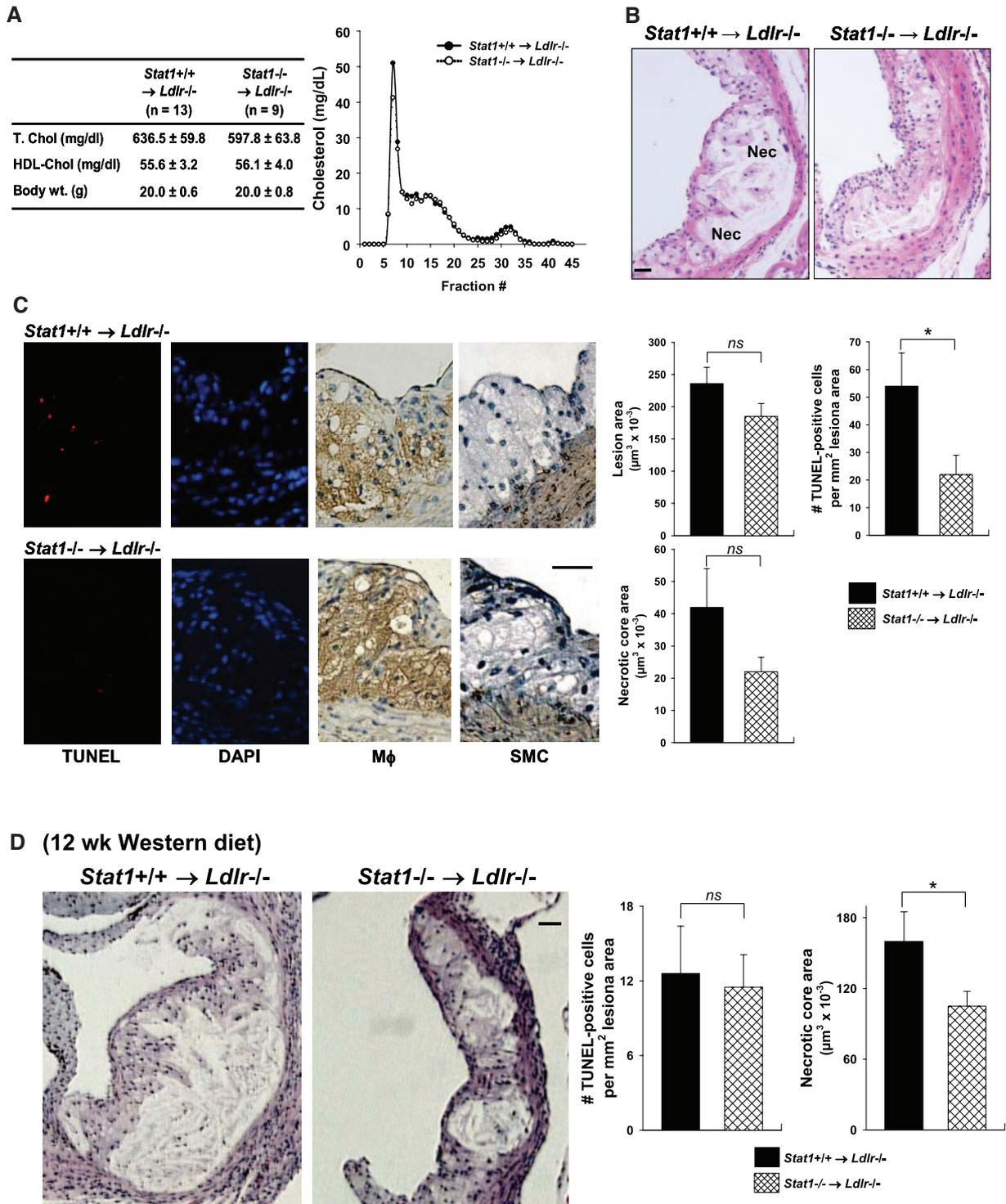


**Figure 7.** Ser-P-STAT1 is present in advanced human coronary atheromata but not in diffuse intimal thickening. The sections were stained with Movat pentachrome, anti-Ser-P-STAT1, anti-CD68, and nonimmune immunoglobulin (IgG) as indicated. **A**, Diffuse intimal thickening. The CD68 and Ser-P-STAT1 images on the right are higher magnifications of the area indicated by the box in the low-magnification Ser-P-STAT1 image. As shown in the higher-magnification images, only a few CD68-positive macrophages are present directly under the endothelium (arrowheads). Ser-P-STAT1 was not detected. Arrow indicates internal elastic lamina (IEL). **B**, Pathological intimal thickening. Ser-P-STAT1 staining coincides with CD68-positive macrophages. **C**, Fibroatheroma. Ser-P-STAT1 staining coincides with CD68-positive macrophages. Some of the Ser-P-STAT1 staining is in macrophages surrounding a necrotic area (asterisk). The lower middle and right images are higher magnifications of the area indicated by the box in the low-magnification CD68 image. The lower middle image shows the result of double immunostaining with anti-Ser-P-STAT1 (dark punctate structures) and anti-CD68 (red), demonstrating Ser-P-STAT1 in the nuclei of macrophages. The lower right image shows the result of double immunostaining with TUNEL (dark, punctate structures) and anti-CD68 (red), demonstrating apoptotic macrophages. Nuclei of nonapoptotic cells are stained green. Note that exact alignment of the nuclei is not possible because the sections are from separate tissue slices.

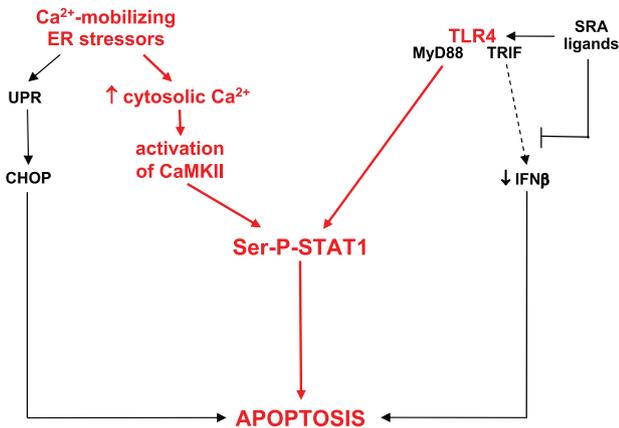
Further studies are required to define at a precise molecular level how the proapoptotic components elucidated in this study fit into the overall scheme of the multihit model of macrophage apoptosis. Our working hypothesis is depicted in Figure 9. We suggest that ER stress triggers 2 key proapoptotic processes: UPR/CHOP and another pathway in which ER stress-induced cytosolic calcium activates CaMKII, which in turn leads to serine phosphorylation of proapoptotic

STAT1. Activation of the TLR4-MyD88 pathway by SRA ligands, which is critical for apoptosis,<sup>15</sup> also contributes to STAT1 serine phosphorylation. SRA ligands additionally promote apoptosis through SRA-dependent suppression of prosurvival IFN $\beta$ .<sup>14,15</sup>

This scheme raises a number of critical issues that require further investigation. Among these is whether STAT1 serine phosphorylation per se is required for apoptosis, which is consistent



**Figure 8.** STAT1 plays a role in advanced lesional macrophage apoptosis and plaque necrosis in female *Stat1*<sup>-/-</sup>→*Ldlr*<sup>-/-</sup> mice. **A**, The table shows plasma cholesterol and body weight of *Ldlr*<sup>-/-</sup> mice transplanted with *Stat1*<sup>+/+</sup> or *Stat1*<sup>-/-</sup> bone marrow and then fed a Western-type diet for 10 weeks starting 6 weeks after transplantation. The graph shows pooled plasma samples from 3 *Stat1*<sup>+/+</sup> and 3 *Stat1*<sup>-/-</sup> recipient mice that were fractionated by fast protein liquid gel-filtration chromatography and then assayed for cholesterol. None of the differences in cholesterol, lipoproteins, or body weight were statistically significant. T. Chol indicates total cholesterol; HDL, high-density lipoprotein. **B**, Hematoxylin and eosin staining of proximal aortas from *Ldlr*<sup>-/-</sup> mice fed a Western diet for 10 weeks that were transplanted with bone marrow from *Stat1*<sup>+/+</sup> and *Stat1*<sup>-/-</sup> mice. Total lesion area was 493.7±40.5 and 380.6±24.4 μm<sup>2</sup> in *Stat1*<sup>+/+</sup>→*Ldlr*<sup>-/-</sup> and *Stat1*<sup>-/-</sup>→*Ldlr*<sup>-/-</sup> mice, respectively. Bar=20 μm. **C**, TUNEL (red), DAPI (blue), macrophage (brown), and SMC (brown) staining of lesions similar to those in **B**. Bar=20 μm. The graph shows quantification of lesion area, TUNEL-positive cells, and necrotic area (Nec) in the lesions of *Stat1*<sup>+/+</sup>→*Ldlr*<sup>-/-</sup> and *Stat1*<sup>-/-</sup>→*Ldlr*<sup>-/-</sup> mice. \**P*=0.034 by Student *t* test. **D**, Hematoxylin and eosin staining of proximal aortas from *Ldlr*<sup>-/-</sup> mice fed a Western diet for 12 weeks that were transplanted with bone marrow from *Stat1*<sup>+/+</sup> and *Stat1*<sup>-/-</sup> mice (n=18 for both groups of mice). Bar=20 μm. The graph shows quantification of TUNEL-positive cells and necrotic area in the lesions of *Stat1*<sup>+/+</sup>→*Ldlr*<sup>-/-</sup> and *Stat1*<sup>-/-</sup>→*Ldlr*<sup>-/-</sup> mice. *P*=0.02 by Student *t* test.



**Figure 9.** Integration of calcium, CaMKII, and STAT1 into the multihit pathway of macrophage apoptosis. According to this working hypothesis, ER stress-induced increase in cytosolic calcium triggers 2 proapoptotic hits: UPR/CHOP and a pathway involving CaMKII and Ser-P-STAT1. TLR4 activation also contributes to STAT1 serine phosphorylation. Ser-P-STAT1 is depicted as a separate pathway from CHOP because studies with *Chop*<sup>-/-</sup> and *Stat1*<sup>-/-</sup> macrophages showed that CHOP is neither upstream nor downstream of Ser-P-STAT1 (data not shown). See Discussion for details and for a description of the areas of uncertainty in this model.

with our data and with previous work showing a proapoptotic role of Ser-P-STAT1 in apoptosis in other systems.<sup>33</sup> However, definitive proof requires comparing SRA/ER stress-induced apoptosis in macrophages containing S727- with Y701-mutated STAT1.<sup>22,33</sup> Until then, we cannot definitively rule out the possibility that apoptosis requires Y701 phosphorylation and that Tyr-P-STAT1 in our SRA-ER stress model is below the limit of immunoblot detection. In pilot studies, we found that apoptosis induced by thapsigargin and fucoidan was markedly suppressed in peritoneal macrophages from S727A-STAT1 knock-in mice, but results with FC-induced apoptosis were difficult to interpret because of an as-yet-undefined compensatory pathway (J.M.T., W.L., T. Decker, PhD, and I.T., unpublished data, 2007). Assuming that Ser-P-STAT1 is a key apoptosis mediator in this pathway, the next goal is to elucidate whether and how serine-only phosphorylated STAT1 affects gene transcription in a manner that promotes apoptosis.<sup>33-35</sup> A related issue is the precise mechanism by which STAT1 is serine phosphorylated and how this process is linked to both CaMKII and TLR4 (Figure 9). One obvious possibility is that CaMKII directly phosphorylates STAT1, as has been described previously in other models.<sup>28</sup> If this is the case in our model, a link to TLR4 could occur through IP3 receptor-mediated elevation of cytosolic calcium,<sup>26</sup> leading to a further increase in CaMKII activation. Alternatively, TLR4 signaling may stimulate STAT1 serine phosphorylation more directly, as has been reported in other models using the TLR4 ligand lipopolysaccharide.<sup>29,30</sup>

The impetus for this study was to explore pathways that may be involved in promoting macrophage apoptosis in atherosclerosis. The ultimate significance of lesional macrophage apoptosis likely depends on lesion stage.<sup>1,2</sup> In early lesions, rapid and efficient phagocytic clearance of apoptotic

macrophages appears to limit lesion cellularity and progression. Of interest, STAT1 may have a separate role in these early lesions that is independent of macrophage death because STAT1 deficiency in *ApoE*<sup>-/-</sup> mice blocks foam cell formation and early lesion development.<sup>36</sup> In advanced lesions, however, evidence exists that clearance of apoptotic cells is defective, leading to postapoptotic macrophage necrosis, inflammation, and eventually overall plaque necrosis.<sup>1,2</sup> In this context, the multihit model of macrophage apoptosis is likely most relevant to advanced lesions. For example, immunoblots have shown that CHOP is expressed only in advanced lesions<sup>7</sup> and that manipulation of ER stress in vivo is positively associated with advanced lesional necrosis, not negatively associated with early lesion progression.<sup>5,37</sup> In the case of STAT1 deficiency, a clear trend toward decreased plaque necrosis was present. However, the maximum effect on plaque necrosis may lag behind that of macrophage apoptosis because plaque necrosis likely results from the progressive coalescence of apoptotic macrophages after they become secondarily necrotic.<sup>1,2</sup> Another prediction from this idea and from the fact that the anti-macrophage antibody used in our study recognizes pre-necrotic apoptotic macrophages is that total macrophage area should be similar in *Stat1*<sup>+/+</sup>→*Ldlr*<sup>-/-</sup> and *Stat1*<sup>-/-</sup>→*Ldlr*<sup>-/-</sup> lesions, exactly as we observed experimentally. More fundamentally, we clearly did not observe an increase in lesion area in the *Stat1*<sup>-/-</sup> group, which is what is found when early lesional macrophage apoptosis is blocked.<sup>38</sup> In terms of other studies linking STAT1 to advanced plaque progression, in vivo data suggest that interleukin-10, which suppresses STAT1 activity,<sup>39</sup> may protect advanced atheromata from macrophage apoptosis and plaque necrosis.<sup>40,41</sup> Moreover, Koga et al<sup>25</sup> reported that blocking the function of the STAT1 activator IFN $\gamma$  stabilized advanced plaques in *ApoE*<sup>-/-</sup> mice. Thus, pending further in vivo studies, local inhibition of STAT1 activity may represent a potentially promising therapeutic strategy to prevent the progression of relatively benign lesions to those with increased macrophage apoptosis and plaque necrosis.

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## Disclosures

None.

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### CLINICAL PERSPECTIVE

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 luminal 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 is necrotic cores, which likely promote plaque breakdown and acute thrombosis. Necrotic cores are "graveyards of dead macrophages," a prominent cell type in atherosclerosis. This study 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 2 molecules, calcium/calmodulin-dependent protein kinase II and signal transducer and activator of transcription-1 (STAT-1). Both mouse and human advanced atheromata have activated STAT-1. Most important, 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, so the calcium/calmodulin-dependent protein kinase II–STAT1 pathway represents only 1 piece of the puzzle. Second, the mouse is a poor model of plaque disruption and acute thrombosis. Thus, additional studies are needed to explore other pathways involved in advanced lesional macrophage death, and improved mouse models are required to prove the hypothesis that macrophage death and plaque necrosis promote plaque disruption and acute thrombosis. Nonetheless, this study provides important new molecular-cellular information related to the progression of advanced atherosclerotic lesions—information that someday may be translated into therapy designed to block benign-to-vulnerable plaque transformation.