Original Article

The Atherogenic Effects of Serum Amyloid A are Potentially Mediated via Inflammation and Apoptosis

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Aim: The mechanism through which SAA contributes to the pathogenesis of atherosclerosis remains unclear. The objective of this study was therefore to determine whether SAA plays a role in the onset of atherosclerosis via inflammatory and apoptotic pathways.

Methods: An array of experiments, including cell cultures, MTT assays, quantitative real-time PCR and Western blotting, were carried out in order to assess the effects of human recombinant SAA on NF- κ B activation and the role of NF- κ B in the onset of SAA-induced inflammation and apoptosis.

Results: We found that SAA (80 μ g/mL) induced 3.5- to 37.8-fold increases in the expression of targets known to play important roles in the initiation and progression of atherosclerosis (i.e., ICAM-1, MCP-1, MMP-9 and TF) via NF- κ B regulation within one hour after exposure. RAW264.7 cells treated with increasing doses of SAA showed regulation of apoptotic targets and a dose-dependent reduction in cell viability, with 69% cell viability observed following exposure to 80 μ g/mL of SAA for 24 hours.

Conclusions: Our results suggest that SAA contributes to the pathogenesis of atherosclerosis via both inflammatory and apoptotic mechanisms.

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Key words: Serum amyloid A (SAA), Atherosclerosis, Inflammation, Apoptosis, NF- κ B

Introduction

Atherosclerosis is a major cause of death, especially in developed countries. Approximately 30% of all deaths worldwide, amounting up to 19 million deaths every year, are due to atherosclerosis^{1, 2}. This condition is the main cause of coronary artery disease (CAD) and was first thought to be a passive disease resulting from cholesterol accumulation in blood vessels^{2, 3}. Today, it is known that inflammation is a prominent contributor to the pathogenesis of athero-

sclerosis with respect to both the initiation and progression of the disease^{4, 5}. Intracellular adhesion molecule-1 (ICAM-1), monocyte chemoattractant protein-1 (MCP-1), matrix metalloproteinase-9 (MMP-9) and tissue factor (TF) are inflammatory factors that are known to be pro-atherosclerotic. ICAM-1 and MCP-1 facilitate the recruitment of monocytes into lesion sites, while MMP-9 and TF promote plaque destabilization and coagulation respectively⁶⁻⁹. Serum amyloid A (SAA) levels are elevated in patients with atherosclerosis, and SAA has been postulated to be a prothrombotic and proinflammatory mediator of CAD due to its ability to induce TF and TNF- α production in peripheral blood mononuclear cells (PBMCs) among CAD patients¹⁰. However, the mechanisms through which SAA contributes to the onset and progression of atherosclerosis remain unknown.

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SAA is an 11.5-kDa major acute-phase protein that is primarily synthesized and released by the liver following tissue injury. Its expression can increase by up to 1,000-fold from baseline after the onset of inflammation¹¹. In addition to the liver, SAA synthesis has been detected in various cell types found in atherosclerotic lesions, including macrophages¹¹⁻¹⁵. Macrophages play an important role in all stages of atherosclerosis, and unstable plaques, present in patients with acute coronary syndrome, are primarily composed of macrophages¹⁶.

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) is a family of nuclear transcription factors responsible for the regulation of a wide range of genes, such as those involved in inflammation and apoptosis⁴. NF- κ B is activated by various stimuli, including cytokines and tumor necrosis factor α (TNF- α), with activation being both rapid and short-lasting^{4, 17}. SAA has also been found to stimulate NF- κ B activation¹⁸. In addition, prolonged NF- κ B activation has been detected in various diseases, including atherosclerosis^{4, 19}. In fact, inflammation plays a role in all stages of atherosclerosis⁴, and various targets of NF- κ B are known to be involved in the initiation and progression of atherosclerosis²⁰. Furthermore, SAA has been shown to induce the expression of the pro-inflammatory cytokine, IL-8, as mediated via I κ B degradation and NF- κ B activation²¹.

Apoptosis is also known to be a key event in atherosclerosis, with the observation of a correlation between the frequency of apoptosis and stage of atherosclerotic lesions²²⁻²⁵. Due to poor efferocytosis, apoptosis of vascular cells within atherosclerotic plaques can affect the stability and structure of these lesions, resulting in the formation of unstable plaques²⁶. The effects of apoptosis on plaque stability can eventually lead to plaque rupture and thrombosis¹. Moreover, SAA has been reported to cause apoptosis via caspase 3 activation in mammalian cell lines^{27, 28}.

Taking together the association between SAA and atherosclerosis as well as the contribution of NF- κ B and apoptosis to the pathogenesis of atherosclerosis, we aimed to determine whether SAA contributes to the onset of atherosclerosis via inflammatory and apoptotic pathways.

Materials and Methods

An array of experiments, including cell cultures, MTT assays, quantitative real-time PCR and Western blotting, were carried out in order to assess the effects of SAA on NF- κ B activation and the role of NF- κ B in the onset of SAA-induced apoptosis.

Cell Culture

Murine macrophages, RAW264.7 cells (ATCC), were cultured in DMEM supplemented with 10% FBS, 100 units/mL of penicillin and 100 μ g/mL of streptomycin (all obtained from Sigma Aldrich) and maintained in 5% CO₂ at 37°C.

The cells were cultured to confluence and treated with human recombinant SAA (Peprotech) of various concentrations (0-80 μ g/mL) for one, three, six or 24 hours in serum-free medium. In the NF- κ B stimulation study, the cells were treated with 80 μ g/mL of SAA, and nuclear proteins were extracted from the cells at zero, five, 15 or 30 minutes after incubation with SAA using a nuclear extract kit (Active Motif, USA) according to the manufacturer's instructions. In the NF- κ B inhibition study, the cells were pretreated with 10 μ M of Bay11-7082 (Sigma Aldrich) for one hour before being treated with SAA. In the NF- κ B inhibition and JNK involvement study, the cells were treated with 80 μ g/mL of SAA for five, 15 or 30 minutes.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) Assay

An MTT assay was used to assess the effects on cell viability. Viable cells contain an enzyme capable of cleaving yellow MTT tetrazolium rings to produce dark blue formazan, allowing for the quantification of the percentage of viable cells via a simple colorimetric assay. Briefly, 10 μ L of MTT solution (5 mg/mL; Molecular Probes) was added to each well. After four hours of incubation, the medium was removed and 100 μ L of DMSO was added to each well. The plate was shaken to ensure that all precipitate was dissolved before being read at 570 nm on a microplate reader.

Quantitative Real-Time PCR

Total RNA was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. RNA was used to synthesize first-strand cDNA in a single step using a first strand cDNA synthesis kit (Fermentas). Real-time PCR was carried out using a LightCycler 480 system (Roche) and the LightCycler 480 SYBR Green I Master kit (Roche). The primer sequences were as follows:

ICAM-1: Forward primer 5'-TGTTTCCTGCCTCTGAAGC-3', Reverse primer 5'-CTTCGTTTGTGATCCTCCG-3';

MCP-1: Forward primer 5'-CCCACTCACCTGCTGCTACT-3', Reverse primer 5'-TCTGGACCCATTCTTCTTG-3';

MMP-9: Forward primer 5'-TGTCTGGAGATTCGACTTGAAGTC-3', Reverse primer 5'-TGAGTTCCAGGGCACACCA-3';

TF: Forward primer 5'-TCAAGCACGGGAAA-GAAAAC-3', Reverse primer 5'-CTGCTTCCTGGG-CTATTTTG-3';

A20: Forward primer 5'-AAACCAATGGTGAT-GGAAACTG-3', Reverse primer 5'-AAACCAATG-GTGATGGAAACTG-3';

Fas: Forward primer 5'-CCCATGCACAGAAG-GGAAGGAGT-3', Reverse primer 5'-TTCCATGTT-CACACGAGGCGCAG-3';

BCL2: Forward primer 5'-GGATAACGGAGGC-TGGGATGCCT-3', Reverse primer 5'-TCGACCT-CACCTGTGGCCAG-3';

GAPDH: Forward primer 5'-GACGGCCGCA-TCTTCTTGTGC-3', Reverse primer 5'-TCGGCC-TTGAAGTGTGCGCTT-3'. The assays were performed in triplicate, and GAPDH was used to normalize each target sample.

Western Blotting

Protein (40 μ g) was loaded on and separated using a 12% SDS-PAGE gel. The rabbit anti-I κ B, -PARP, -caspase 3, -caspase 8, -caspase 9, -BCL2, -A20, -JNK, -pJNK antibodies (all obtained from Cell Signaling) were used at a dilution of 1:1,000. Rabbit anti-GAPDH antibodies (Cell Signaling) were also used at a 1:1,000 dilution. The rabbit anti-p65 antibodies (Santa Cruz) were used at a 1:400 dilution, while the mouse anti-actin antibodies (Millipore) were used at a 1:3,000 dilution. The anti-rabbit secondary antibodies (Thermo Scientific) and anti-mouse secondary antibodies (Thermo Scientific) were used at a 1:3,000 dilution. The blots were developed with SuperSignal West Pico Chemiluminescent Substrate (Pierce, USA) and exposed using a Clear Blue X-ray film (Pierce). The bands were quantified using densitometry (GeneTool, SynGene, Cambridge, UK) and normalized against GAPDH/Beta actin, as shown in the **Supplemental Fig. 1**.

Statistical Methods

The mean $\pm$ standard deviation (SD) of triplicate ($n=3$) experiments was calculated. The statistical significance of differences between the means of the test and control groups was analyzed using Student's *t*-test, with a *p* value of <0.05 being considered to be statistically significant.

Results

SAA Stimulates NF- κ B Activation in RAW264.7 Cells

RAW264.7 cells were exposed to 80 μ g/mL of SAA for various time periods before performing West-

Fig. 1. SAA activates NF- κ B.

A) Exposure to SAA induces the nuclear accumulation of p65. RAW264.7 cells were treated with 80 μ g/mL of SAA. The nuclear proteins were extracted from the cells at zero, five, 15 and 30 minutes after incubation with SAA using a nuclear extract kit (Active Motif, USA) according to the manufacturer's instructions. The protein samples were immunoblotted with anti-p65 and anti-actin antibodies. The blots are representative of three individual experiments. B) SAA activates NF- κ B via I κ B degradation, which is inhibited by Bay117082. RAW264.7 cells were pretreated with or without 10 μ M of Bay11-7082 for one hour before being incubated with 80 μ g/mL of SAA for the indicated time periods. The protein samples were immunoblotted with anti-I κ B and anti-GAPDH antibodies. The blots are representative of three individual experiments.

ern blotting. Following SAA exposure, the nuclear accumulation of p65 was observed, indicating NF- κ B activation (**Fig. 1A**). This was further confirmed by the degradation of I κ B in RAW264.7 following SAA treatment (**Fig. 1B**). The pretreatment of RAW264.7 cells with Bay11-7082 for one hour before exposure to SAA prevented I κ B degradation, demonstrating the inhibition of NF- κ B by Bay11-7082 (**Fig. 1B**).

SAA Stimulates ICAM-1, MCP-1, MMP-9 and TF Expression in RAW264.7 Cells

In order to determine the effects of SAA on inflammatory targets known to play important roles in atherosclerosis (ICAM-1, MCP-1, MMP-9 and TF), RAW264.7 cells were stimulated with various concentrations of SAA for different time periods. Quantitative real-time PCR was then carried out to determine the gene expression levels of ICAM-1, MCP-1, MMP-9 and TF. SAA was found to significantly induce the expression of ICAM-1, MCP-1, MMP-9 and TF (**Fig. 2A-2D**). The ICAM-1 level increased by up to 4.0- and 4.7-fold within one hour after treat-

Fig. 2. SAA induces the (A) ICAM-1, (B) MCP-1, (C) MMP-9 and (D) TF gene expression in RAW264.7 cells.

RAW264.7 cells were treated with 40 or 80 $\mu\text{g/mL}$ of SAA for one, three, six or 24 hours. Total RNA was extracted, and quantitative real-time PCR was carried out in order to determine the mRNA expression levels of each gene. GAPDH was used as an endogenous control to normalize each target sample. The results of three separate experiments are expressed relative to that of the control group and presented as the mean $\pm$ sd. **denotes a significant difference with a p -value of <0.05 as compared to the control.

ment with 40 and 80 $\mu\text{g/mL}$ of SAA, respectively (**Fig. 2A**). MCP-1 was induced by up to 5.5- and 26.2-fold, while MMP-9 increased by 28.7- and 53.5-fold after exposure to 40 and 80 $\mu\text{g/mL}$ of SAA respectively, for six hours (**Fig. 2B, 2C**). The TF level was also increased by 13.8- and 37.7-fold within one hour following exposure to 40 and 80 $\mu\text{g/mL}$ of SAA, respectively (**Fig. 2D**).

The Effects of SAA on ICAM-1, MCP-1, MMP-9 and TF are Regulated via NF- κ B

As ICAM-1, MCP-1, MMP-9 and TF are known to be downstream targets of NF- κ B, we next sought to determine whether the regulation of these four targets by SAA is mediated via NF- κ B using Bay11-7082, an irreversible inhibitor of NF- κ B. Cells pretreated with Bay11-7082 were subsequently treated with various concentrations of SAA for different time periods, after which the gene expression levels of the four targets were quantified. Changes were observed

in the levels of ICAM-1, MCP-1, MMP-9 and TF, with and without NF- κ B suppression (**Fig. 3A-3D**). The 4.0- and 4.7-fold increases in ICAM-1 at one hour after treatment with 40 and 80 $\mu\text{g/mL}$ of SAA, respectively, were reduced to 1.2- and 1.3-fold in the absence of NF- κ B (**Fig. 3A**). The induction of MCP-1 following treatment with 40 and 80 $\mu\text{g/mL}$ of SAA for six hours was significantly reduced to 1.3- and 1.7-fold respectively, while that of MMP-9 was reduced to 3.2- and 8.1-fold, respectively (**Fig. 3B, 3C**). In the absence of NF- κ B, the increase in TF level was similarly reduced to 1.7- and 2.5-fold following treatment with 40 and 80 $\mu\text{g/mL}$ of SAA respectively (**Fig. 3D**). The reductions in the MCP-1, ICAM-1, MMP-9 and TF expression levels observed in the absence of NF- κ B demonstrate the importance of NF- κ B activity in the induction of all four targets.

SAA Induces Apoptosis in RAW264.7 Cells

Apoptosis plays an important role in atheroscle-

Fig. 3. SAA induces (A) ICAM1, (B) MCP1, (C) MMP-9 and (D) TF gene expression in RAW264.7 cells via NF- κ B regulation.

RAW264.7 cells were pretreated with or without 10 μ M of Bay11-7082 for one hour before being treated with 40 or 80 μ g/mL SAA for one, three or six hours. Total RNA was extracted, and quantitative real-time PCR was carried out in order to determine the mRNA expression levels of each gene. GAPDH was used as an endogenous control to normalize each target sample. The results of three separate experiments are expressed relative to that of the control group and presented as the mean $\pm$ sd. **denotes a significant difference with a p -value of <0.05 as compared to the control.

rotic progression via its effects on plaque stability. In order to determine whether SAA contributes to the pathogenesis of atherosclerosis via apoptosis, the effects of SAA on cell viability were first assessed using an MTT assay. SAA reduced the level of cell viability in a dose-dependent manner, with significant reductions to 79.6% and 69.6% after treatment with 40 and 80 μ g/mL of SAA respectively, for 24 hours (Fig. 4A). As milliQ water (mq) was used as the diluent of SAA, cells were also treated with milliQ water only as a control. The treatment of cells with milliQ water did not result in any significant changes in RAW264.7 viability (Fig. 4A).

Quantitative real-time PCR of apoptotic targets was carried out in order to assess whether the reduction in cell viability following SAA treatment was due to apoptosis. The transcript level of Fas, a death receptor for the extrinsic pathway, was found to be upregulated, ranging from 2.9- to 13.2-fold (Fig. 4B), while

Bcl-2, an anti-apoptotic protein found on mitochondria, was downregulated following SAA treatment (Fig. 4C). Western blotting of apoptotic proteins and targets was then performed in order to further confirm the apoptotic effects of SAA. Such effects were evident via the activation of executioner caspase 3 and degradation of PARP, as evidenced by the downregulation of uncleaved caspase 3 and uncleaved PARP in association with an increasing exposure time to SAA (Fig. 4D and 4E, respectively). However, despite the activation of caspase 3, initiator caspase 8 and caspase 9 remained uncleaved (Fig. 4F). These results indicate that SAA has a negative effect on cell viability via apoptosis, although its initiation is apparently independent of the actions of caspases 8 and 9 (Supplemental Fig. 2).

NF- κ B Plays a Role in the SAA-Induced Apoptosis of RAW264.7 Cells

Given the role of NF- κ B in apoptosis, we next

Fig. 4. SAA induces apoptosis in RAW264.7 cells.

A) SAA reduces cell viability in a dose-dependent manner. RAW264.7 cells were treated with SAA at various concentrations (5–80 µg/mL) for 24 hours. MTT solution was added, and the percentage of viable cells was quantitated at 570 nm. SAA B) induces the Fas and C) reduces the BCL2 gene expression in RAW264.7 cells. RAW264.7 cells were treated with 40 or 80 µg/mL of SAA for one, three, six or 24 hours. Total RNA was extracted, and quantitative real-time PCR was carried out in order to determine the mRNA expression levels of each gene. GAPDH was used as an endogenous control to normalize each target sample. The results of three separate experiments are expressed relative to that of the control group and presented as the mean $\pm$ sd. SAA induces D) caspase 3 activation and E) PARP cleavage. No changes were detected in the protein levels of F) caspase 8 or caspase 9. RAW264.7 cells were incubated with 80 µg/mL of SAA for the indicated time periods. The protein samples were immunoblotted with anti-caspase 3, anti-PARP, anti-caspase 8, anti-caspase 9 and anti-GAPDH antibodies. The blots are representative of three individual experiments. **denotes a significant difference with a p -value of < 0.05 as compared to the control.

examined the effects of NF- κ B suppression on SAA-induced apoptosis. As described above, RAW264.7 cells were pretreated with Bay11-7082 prior to SAA exposure. The reduction in uncleaved caspase 3 and PARP was abolished with NF- κ B suppression. Similarly, no reduction in the uncleaved caspase 8 and 9 levels was observed following SAA treatment with inhibition of NF- κ B (**Fig. 5**). These findings demonstrate the important role of NF- κ B in SAA-induced cell death (**Supplemental Fig. 3**).

The Extrinsic Cell Death Pathway is Unlikely to Play a Role in the SAA-Induced Apoptosis of RAW264.7 Cells

A20 is an anti-apoptotic protein that has been found to inhibit caspase 8 activation and shown to be expressed in response to stimuli activating NF- κ B. Therefore, since SAA stimulated the NF- κ B expression, as shown in **Fig. 1**, we next determined the effects of SAA on the A20 expression. The A20 expression following SAA treatment with or without NF- κ B inhibition was investigated using quantitative real-time PCR and Western blotting. As shown in **Fig. 6**, both the transcript and protein expression levels of A20 were elevated following SAA treatment. In addition, the A20 gene expression showed a sharp increase of 46.7-fold within the first hour of exposure to 80 μ g/mL SAA (**Fig. 6A**). The inhibition of NF- κ B activation, on the other hand, suppressed the induction of A20 at both the transcript and protein levels (**Fig. 6B** and **6C** respectively).

JNK has been shown to play a critical role in extrinsic apoptotic pathways by upregulating the levels of pro-apoptotic genes. In addition, the expression of A20 has been found to inhibit TNF-induced JNK activation. In order to determine whether JNK is involved in SAA-induced apoptosis, a Western blot analysis of SAA-treated RAW264.7 cells was carried out, with or without Bay11-7082 pretreatment. Without NF- κ B inhibition, JNK activation was markedly suppressed (**Fig. 7**). JNK showed transient activation at 5 and 15 minutes, then returned to baseline at 30 minutes following SAA treatment under NF- κ B inhibition (**Fig. 7**). The absence of prolonged JNK activation in both cases further suggests the unlikelihood of the extrinsic apoptotic pathway being involved in SAA-induced apoptosis.

The Intrinsic Cell Death Pathway is Likely to Play a Role in the SAA-Induced Apoptosis of RAW264.7 Cells

As one of the mitochondrial proteins involved in apoptosis, the expression of Bcl-2 following SAA treat-

Fig. 5. The apoptotic effects of SAA on RAW264.7 cells are mediated via NF- κ B.

RAW264.7 cells were incubated with 10 μ M of Bay11-7082 for one hour before being exposed to 80 μ g/mL of SAA for one, three or six hours. The protein samples were immunoblotted with anti-PARP, -caspase 3, -caspase 8, -caspase 9, -GAPDH antibodies. The blots are representative of three individual experiments.

ment in the absence or presence of Bay11-7082 was investigated. As mentioned above, following exposure of RAW264.7 cells to SAA, the Bcl-2 gene expression was downregulated (**Fig. 4C**). The protein level of Bcl-2 was also downregulated following SAA exposure under normal conditions (**Fig. 8B**). However, with the inhibition of NF- κ B, the downregulation of Bcl-2 following SAA exposure was eliminated at both the transcript and protein levels (**Fig. 8A** and **8B**, respectively). The lack of Bcl-2 downregulation when NF- κ B is inhibited is consistent with the abolition of uncleaved caspase 3 degradation, as shown in **Fig. 5**. Bcl-2 is thus likely to play a role in the SAA-induced apoptosis of RAW264.7 cells.

Discussion

SAA has been shown to be associated with inflammation and is regarded as a possible marker of cardiovascular events²⁹. In this study, we demonstrated that SAA stimulates NF- κ B activation (**Fig. 1**) and induces the expression of pro-atherosclerotic factors, such as ICAM-1, MCP-1, MMP-9 and TF (**Fig. 2**), in a mouse macrophage cell line, RAW264.7. Moreover, we showed that the SAA-induced expression of ICAM-1, MCP-1, MMP-9 and TF is regulated via NF- κ B activation (**Fig. 3**).

ICAM-1 and MCP-1 have been reported to be involved in the recruitment of monocytes into atherosclerotic lesions^{6, 7}. The induction of ICAM-1 and MCP-1 in RAW264.7 cells by SAA provides an indi-

Fig. 6. SAA induces the A20 expression via NF- κ B.

A) SAA induces the A20 gene expression. RAW264.7 cells were treated with 40 or 80 μ g/mL of SAA for one, three, six or 24 hours. B) The regulation of the A20 gene expression is inhibited in the presence of Bay11-7082. RAW264.7 cells were pretreated with or without 10 μ M of Bay11-7082 for one hour before being treated with 40 or 80 μ g/mL of SAA for one, three or six hours. Total RNA was extracted, and quantitative real-time PCR was carried out in order to determine the mRNA expression levels of each gene. GAPDH was used as an endogenous control to normalize each target sample. The results of three separate experiments are expressed relative to that of the control group and presented as the mean $\pm$ sd. C) SAA induces the A20 protein expression via NF- κ B. RAW264.7 cells were pretreated with or without 10 μ M of Bay11-7082 for one hour before being incubated with 80 μ g/mL of SAA for the indicated time periods. The protein samples were immunoblotted with anti-A20 and anti-GAPDH antibodies. The blots are representative of three individual experiments. **denotes a significant difference with a p -value of < 0.05 as compared to the control.

cation that SAA facilitates the accumulation of macrophages at these sites. MMPs breakdown the extracellular matrix, promoting plaque destabilization and eventually leading to plaque rupture. MMP-9, in particular, is considered a marker of unstable plaque⁸⁾. TF, on the other hand, is known for its role in the activation of the coagulation cascade⁹⁾. Therefore, the ability of SAA to induce MMP-9 and TF to significantly higher levels also supports the role of SAA in contributing to the development of atherosclerosis. As apoptosis is a key event in plaque progression, we also investigated the ability of SAA to induce atherosclerosis via apoptosis. SAA was shown to reduce cell viability and cause apoptosis via the actions of executioner caspase 3 (Fig. 4). The activation of caspase 3 was further confirmed by the degradation of PARP, a known downstream target of caspase 3 with a DNA repair function³⁰⁾. The overexpression of SAA has previously been reported to cause apoptosis as well as an increase in

the caspase 3 activity in HC11 mammary gland epithelial cells²⁷⁾. Despite activation of the downstream executioner caspase 3, no activation was detected for the upstream initiator caspases 8 and 9. Although uncommon, initiator caspase-independent apoptosis has been reported³¹⁻³³⁾.

While generally known for its role in promoting cell survival^{34, 35)}, NF- κ B has also been implicated to be involved in apoptosis³⁶⁾. The pro- or anti-apoptotic effects exerted by NF- κ B have been found to be mediated via different apoptotic stimuli³⁷⁾. In the present study, we showed that NF- κ B plays a role in SAA-induced apoptosis, given that apoptosis was inhibited when the cells were treated with the inhibitor Bay11-7082, as evidenced by the lack of caspase 3 activation and PARP degradation (Fig. 5).

A20 is a downstream target of NF- κ B and has been reported to inhibit apoptosis by inhibiting caspase 8 activation³⁸⁾. The elevation in A20 after SAA

Fig. 7. SAA inhibits JNK activation.

RAW264.7 cells were pretreated with or without 10 μ M of Bay11-7082 for one hour before being incubated with 80 μ g/mL of SAA for the indicated time periods. The protein samples were immunoblotted with anti-phospho JNK and -JNK antibodies. The blots are representative of three individual experiments.

treatment in the presence of NF- κ B observed in this study may explain the lack of caspase 8 cleavage following SAA treatment (**Fig. 6** and **Supplemental Fig. 4**). Prolonged JNK activation, which has been linked to extrinsic cell death³⁹, was also found to be inhibited by SAA (**Fig. 7** and **Supplemental Fig. 5**), further suggesting the lack of caspase 8 participation in SAA-induced apoptosis.

Bcl-2 inhibits apoptosis by blocking mitochondrial pores and preventing the release of apoptotic proteins into the cytosol⁴⁰. Following exposure to SAA, Bcl-2 was downregulated (**Fig. 8** and **Supplemental Fig. 6**), disrupting the pro- and anti-apoptotic molecule balance, thus favoring cells undergoing apoptosis. The downregulation of Bcl-2 has been shown to enhance apoptosis induced by mitochondrial dysfunction⁴¹ and the release of pro-apoptotic proteins, such as Smac/DIABLO, from mitochondria has been reported to alleviate the caspase 3 inhibition caused by inhibitor-of-apoptosis protein (XIAP)⁴².

In this study, human recombinant SAA (Pepro-Tech) was used at a concentration of up to 80 μ g/mL, which is higher than the average physiological concentration of 20 μ g/mL observed in normal healthy controls⁴³. However, SAA has also been found to be significantly correlated with hs-CRP and elevated by 1,000-fold under conditions of acute inflammation^{44, 45}. Furthermore, the SAA levels in transplant patients with CAD have been reported to be 203 ± 181 μ g/mL, which is 5-fold higher than those observed in patients without CAD (36 ± 16 μ g/mL)⁴⁶. Therefore, the concentration of up to 80 μ g/mL used in our study simulates a pathological state of inflammation in which the level of SAA is elevated. Despite the use of human recombinant SAA (PeproTech) in several inflammatory studies^{21, 47-49}, some groups have shown that, unlike purified endogenous SAA, human recombinant SAA induces neutrophil activation and inflam-

Fig. 8. SAA reduces the BCL2 expression via NF- κ B.

A) The regulation of the BCL2 gene expression is inhibited in the presence of Bay117082. RAW264.7 cells were pretreated with or without 10 μ M of Bay11-7082 for one hour before being treated with 40 or 80 μ g/mL of SAA for one, three or six hours. Total RNA was extracted, and quantitative real-time PCR was carried out in order to determine the mRNA expression levels of each gene. GAPDH was used as an endogenous control to normalize each target sample. The results of three separate experiments are expressed relative to that of the control group and presented as the mean $\pm$ sd. B) SAA reduces the BCL2 protein expression via NF- κ B. RAW264.7 cells were pretreated with or without 10 μ M of Bay11-7082 for one hour before being incubated with 80 μ g/mL of SAA for the indicated time periods. The protein samples were immunoblotted with anti-BCL2 and anti-GAPDH antibodies. The blots are representative of three individual experiments.

matory cytokine secretion^{50, 51}. Therefore, one limitation of this study is the use of human recombinant SAA (PeproTech) in examining the effects of SAA on inflammation and apoptosis.

Franco *et al.* found that SAA induces the production of TNF- α in both serum-free medium and lipoprotein-deficient serum medium, while no significant induction of TNF- α by SAA is observed in whole serum medium. The SAA-induced TNF- α production in both serum-free and lipoprotein-deficient medium is inhibited upon the addition of high-density lipoprotein (HDL)⁵². Although the interaction between HDL and SAA may affect the SAA-induced cytokine production, our aim was to determine the effects of SAA in its free form on inflammation; thus, SAA in

serum-free medium was used.

In conclusion, this study demonstrated the possible role of SAA in the pathogenesis of atherosclerosis due to its ability to initiate apoptosis and inflammation via NF- κ B activation. However, the detailed mechanisms by which apoptosis occurs remain to be elucidated.

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Conflicts of Interest

None.

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Supplemental Fig. 1.**1A: p65 normalized against beta-actin** **1B: IκB (with Bay11-7082) normalized against GAPDH**

The data are expressed as the mean $\pm$ SD ($n=3$). The statistical analysis was performed using Student's *t*-test, and * denotes a significant difference with a *p*-value of <0.05 as compared to the control.

Supplemental Fig. 2.**2A: Caspase 3 normalized against GAPDH** **2B: PARP normalized against GAPDH** **2C: Caspase 8 and caspase 9 normalized against GAPDH**

Supplemental Fig. 3.

PARP, caspase 3, caspase 8 and caspase 9 (with Bay11-7082) normalized against GAPDH

Supplemental Fig. 4.

A20 (with Bay11-7082) normalized against GAPDH

Supplemental Fig. 5.

pJNK (with Bay11-7082) normalized against JNK

Supplemental Fig. 6.

Bcl2 (with Bay11-7082) normalized against GAPDH