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Serum Amyloid A Activates the NLRP3 Inflammasome via P2X7 Receptor and a Cathepsin B-Sensitive Pathway

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Serum amyloid A (SAA) is an acute-phase protein, the serum levels of which can increase up to 1000-fold during inflammation. SAA has a pathogenic role in amyloid A-type amyloidosis, and increased serum levels of SAA correlate with the risk for cardiovascular diseases. IL-1β is a key proinflammatory cytokine, and its secretion is strictly controlled by the inflammasomes. We studied the role of SAA in the regulation of IL-1β production and activation of the inflammasome cascade in human and mouse macrophages, as well as in THP-1 cells. SAA could provide a signal for the induction of pro–IL-1β expression and for inflammasome activation, resulting in secretion of mature IL-1β. Blocking TLR2 and TLR4 attenuated SAA-induced expression of IL1B, whereas inhibition of caspase-1 and the ATP receptor P2X7 abrogated the release of mature IL-1β. NLRP3 inflammasome consists of the NLRP3 receptor and the adaptor protein apoptosis-associated speck-like protein containing CARD (a caspase-recruitment domain) (ASC). SAA-mediated IL-1β secretion was markedly reduced in ASC−/− macrophages, and silencing NLRP3 decreased IL-1β secretion, confirming NLRP3 as the SAA-responsive inflammasome. Inflammasome activation was dependent on cathepsin B activity, but it was not associated with lysosomal destabilization. SAA also induced secretion of cathepsin B and ASC. In conclusion, SAA can induce the expression of pro–IL-1β and activation of the NLRP3 inflammasome via P2X7 receptor and a cathepsin B-sensitive pathway. Thus, during systemic inflammation, SAA may promote the production of IL-1β in tissues. Furthermore, the SAA-induced secretion of active cathepsin B may lead to extracellular processing of SAA and, thus, potentially to the development of amyloid A amyloidosis. The Journal of Immunology, 2011, 186: 6119–6128.

Serum amyloid A (SAA) is a major acute-phase protein present in serum. It is mainly produced in the liver under the regulation of IL-1, IL-6, and TNF-α (1), but its expression has also been demonstrated in other cell types, including macrophages, endothelial cells, and smooth muscle cells (2). During infection or inflammation, the serum levels of SAA may increase up to 1000-fold. Prolonged high SAA levels can lead to amyloidosis, because SAA is the precursor for the amyloid A (AA) protein deposited in tissues in AA-type amyloidosis. SAA was also shown to possess proinflammatory properties [e.g., it can induce the release of cytokines from different cell types, including THP-1 monocytes (3), human neutrophils (4, 5), and mast cells (6)]. The levels of SAA, and especially those of the low-density lipoprotein–SAA complex, correlate with the risk for cardiovascular disease (7–9). However, it is not yet clear whether SAA is only a marker of the increased risk or whether it plays a direct role in the pathogenesis of atherosclerosis.

IL-1β is a key cytokine that has been implicated in the pathogenesis of several inflammatory disorders, such as gout, rheumatoid arthritis (RA), and atherosclerosis (10, 11). The production of mature IL-1β is tightly regulated. Two signals are required for the secretion of biologically active IL-1β. First, transcription of the IL-1β gene and production of cytosolic pro–IL-1β are dependent on the activation of NF-κB via, for example, TLRs. TLRs recognize pathogen-associated molecular patterns, such as LPS (12), on the cell surface or in endosomes. The second signal is needed to activate the pathway that leads to cleavage of pro–IL-1β by caspase-1 and secretion of the mature IL-1β cytokine. Caspase-1 is activated by inflammasomes, cytoplasmic multiprotein complexes capable of sensing stress and danger signals (13). The core of the inflammasome is formed by a receptor from the nucleotide-binding domain leucine-rich-repeat containing (NLR) family, the most studied member of which is NLR pyrin-domain containing (NLRP)3. Upon activation, NLRP3 (also known as NALP3, cryopyrin, or CIAS1) binds to the adaptor protein apoptosis-associated speck-like protein containing a CARD (caspase recruitment domain) (ASC), which, in turn, recruits procaspase-1 for activation. The final outcome of the NLRP3 inflammasome assembly is the cleavage of cytosolic pro–IL-1β and pro–IL-18 into the mature proinflammatory cytokines IL-1β and IL-18 by the activated caspase-1. The danger signal needed for NLRP3 activation can be provided by extracellular ATP through stimulation of P2X7 receptor (P2X7-R) (14), potassium efflux (15), and re- cruitment of pannexin-1 channel (16). Other danger signals, such as β-glucans and biglycan, have been shown to activate NLRP3

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Abbreviations used in this article: AA, amyloid A; ASC, apoptosis-associated speck-like protein containing CARD (a caspase-recruitment domain); BMM, bone marrow-derived macrophage; FPRL1, formyl-peptide receptor-like-1; huSAA, human serum amyloid A; NLR, nucleotide-binding domain leucine-rich repeat; NLRP, nucleotide-binding domain leucine-rich-repeat family pyrin-domain containing; oATP, oxidized ATP; PTX, pertussis toxin; P2X7-R, P2X7 receptor; RA, rheumatoid arthritis; rhSAA, recombinant human serum amyloid A; SAA, serum amyloid A; SFMM, serum-free macrophage medium; siRNA, small interfering RNA.

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inflammation (17, 18). Likewise, crystalline or particulate material, such as monosodium urate crystals or calcium pyrophosphate dihydrate (19), silica (20, 21), asbestos (21), amyloid fibrils (22), and cholesterol crystals (23, 24) have been shown to activate NLRP3. The inflammasome activation by crystalline structures involves lysosomal damage and the release of cathepsin B into the cytosol (20, 22, 23), with subsequent activation of NLRP3 inflammasome via an unidentified mechanism (22). The stimulation of P2X7 and endocytosis of crystalline structures can activate NLRP3 via the production of reactive oxygen species (21, 25). However, the exact mechanism of this activation pathway is still under investigation (26).

In this article, we report that SAA is the first natural proinflammatory mediator that can provide signals for the production of pro-IL-1β and for the activation of the NLRP3 inflammasome, resulting in the secretion of mature IL-1β. This SAA-induced IL-1β secretion is mediated by cathepsin B and P2X7-R and abrogated in mouse ASC-/− macrophages. We also show that SAA induces the secretion of cathepsin B and inflammasome components from human macrophages. Because processing of SAA by cathepsin B may result in production of amyloidogenic SAA fragments (27, 28), the data also provides evidence for the possible linkage between the inflammasome cascade and amyloidogenesis. Taken together, our findings suggest that SAA has a unique and important role in coordinating inflammatory reactions via several signaling pathways.

Materials and Methods

Materials

Recombinant human SAA (rhSAA) was purchased from Peprotech EC. Its amino acid sequence corresponded to the sequence of SAA 1c, except with the exception of the addition of Met at the N terminus, substitution of Asp for Asn at position 60, and substitution of His for Arg at position 71. According to the manufacturer, the endotoxin level of the product is <0.1 ng/μl. Compound KN-62 (1-[N,O-bis(5-isouquinolinesulfonyl)-N-methyl-1-tosyl]-4-phenylpyrazine), cytochalasin D, N-galactosyl phosphate K, and polyclonal Ab for human caspase-1 were from Sigma-Aldrich. Santa Cruz supplied Z-YVAD-fmk (caspase-1 inhibitor) and polyclonal Ab for human IL-1β, mAb for mouse IL-1β was from R&D Systems. Pertussis toxin (PTX), Ca-074-Me (cathepsin B inhibitor), and polyclonal Ab for caspase B were purchased from Calbiochem, and anti-ASC rabbit polyclonal Ab was purchased from Calbiochem, and anti-ASC rabbit polyclonal Ab was purchased from BioLegend. BioLegend provided the neutralizing Ab for mouse IL-1β, huSAA (10–20 μg/ml), or LPS (10 μg/ml) at 37˚C for 2 h, after which the media were replaced with PBS and transferred to medium containing the other stimulants.

For cytokine and ATP-release assays, human and mouse macrophages were incubated overnight at 37˚C in fresh SFMM containing rhSAA (0.25–30 μg/ml), huSAA (10–20 μg/ml), or a combination of rhSAA (5 μg/ml) with PTX (500–1500 ng/ml), KN-62 (0.5–10 μM), apyrase (0.4–4 U/ml), cytochalasin D (1 μg/ml), Ca-074Me (12.5 μM), or Z-YVAD-fmk (20 μM). For oATP pretreatment, human macrophages were incubated with oATP (300–600 μM) for 2 h, after which they were washed with PBS and transferred to medium containing the other stimulants. For endotoxin control, rhSAA (5 μg/ml) or LPS (1 μg/ml) was incubated in新鲜 SFMM containing proteinase K (50 μg/ml) or polymyxin B (25 μg/ml) at 37˚C for 1 h, after which the mixtures were added to the macrophages. After the stimulations, the cell-culture media were collected, and cytokine concentrations were determined using commercial ELISA kits for human IL-1β and TNF-α (R&D Systems) or for mouse IL-1β (BioLegend). To study ATP release from human macrophages, the cell-culture media collected from stimulated macrophages were subjected to luminescence-based assay for ATP (CellTiter-Glo Luminescent Cell Viability Assay; Promega), as described (32). Intracellular ATP was measured as a control, following the instructions provided by the manufacturer. To study cytokotixicity and induced cell death, lactate dehydrogenase activity and the amount of nucleosomes were measured from supernatants and cell lysates, respectively, using commercial assays (Roche), as instructed by the manufacturer.

For Western blotting of cell-culture media, human or mouse macrophages were incubated overnight at 37˚C in fresh RPMI 1640 medium supplemented with 10 μM HEPEs, 1% t-glutamine, and 1% penicillin-streptomycin. For Western blotting of cell-culture media, human or mouse macrophages were incubated overnight at 37˚C in fresh RPMI 1640 medium supplemented with 10 μM HEPEs, 1% t-glutamine, and 1% penicillin-streptomycin and containing rhSAA (3–10 μg/ml). After stimulation, the cell-culture media were collected and concentrated by filter centrifugation (Millipore) and cut-off ≤10,000 nominal M. The concentrated medium samples were then purified, further concentrated using a commercial precipitation kit (2D Clean-up Kit; GE Healthcare), and subjected to 15% SDS-PAGE, followed by immunoblotting with Abs for caspase-1, cathepsin B, and ASC (dilution 1:500).

For analysis of mRNA expression levels, macrophages were incubated with rhSAA (3–30 μg/ml), LPS (1 μg/ml), huSAA (10 μg/ml), Bay-10782 (20 μM), TRLR2 Ab (10 μg/ml), TRLR4 Ab (20 μg/ml), or CD36 Ab FA6-152 (10 μg/ml), or combinations thereof, for 6 h at 37˚C. TRLR2, TRLR4, and CD36 Abs were added 1 h prior to the addition of stimulants.

Small interfering RNA experiments

PMA-differentiated THP-1 macrophages were transfected with 100 nM nontargeting control small interfering RNA (siRNA) (AllStars Negative Control siRNA; Qiagen) or with 50 nM two nLNP3 siRNAs (CIA1S,6 and CIA1S,9; Qiagen), combined, with the HiPerFect Transfection Reagent (Qiagen) as instructed by the manufacturer. PMA was present in the cell-culture medium throughout the transfection (22 h). The medium was subsequently replaced, the cells were stimulated with SAA (3 μg/ml) for 6 h, the cell-culture medium was collected for IL-1β and TNF-α ELISA analysis, and the cells were harvested for total RNA purification and quantitative RTPCR (NLRP3), as described below.

Staining of caspase B on THP-1 macrophages

For caspase B staining, differentiated THP-1 macrophages were incubated in the presence or absence of SAA (3 μg/ml) for 6 h. Thereafter, the cells were stained with the fluorescent caspase B substrate z-Arg-Arg-cresyl violet with a commercial CV-Cathepsin B Detection Kit (BIOKOL), following the instructions from the manufacturer. Hoechst stain was used to
label the cell nuclei, and acridine orange was used to identify lysosomes. Cells were visualized with a Nikon Eclipse E600 microscope.

Quantitative RT-PCR analysis

Transfected and stimulated THP-1 macrophages or stimulated human macrophages were harvested, and their total cellular RNA was purified using RNeasy columns (Qiagen). The RNA was converted to cDNA using Moloney murine leukemia virus reverse transcriptase and random hexamers (Promega). For quantitative RT-PCR, the cDNA was amplified in duplicates using TaqMan Universal PCR Master Mix (Applied Biosystems) with gene-specific primers and fluorogenic TaqMan probes on an ABI PRISM 7500 sequence detector system (Applied Biosystems) with the following program: a 10-min preincubation at 95°C and 40 cycles of 15 s at 95°C and 1 min at 60°C. The data were normalized relative to the ribosomal protein 18S as an endogenous control. PCR assays were conducted with predeveloped TaqMan assay primers and probes (NLRP1: Hs00248187_m1, NLRP2: Hs00215284_m1, NLRP3: Hs00366461_m1, Hs00918082_m1; Applied Biosystems) or were self-developed with the following oligonucleotide sequences (shown in 5’–3’ orientation): IL-1β sense primer (forward): 5’-TTACAGTGCAATGAGGATGAC-3’, anti-sense primer (reverse): 5’-GTTTGCTACAACATGGGCTACAG-3’, and TNF-α probe FAM-CCCAGCGATCATCTTCTGGA-BHQ1; NALP3 (forward): 5’-GGAGAGACCTTTATGAGAAAGCAA-3’, NLRP3 (reverse): 5’-GCTGTCTTCCTGCCATACACA-3’, and NLRP3 probe FAM-AACAGATGAAGTGCTCCTTCCAGGACC-BHQ1.

Statistical analysis

Calculations were performed using GraphPad InStat3 for Macintosh software. Overall significance level between the stimulated and control groups or between stimulated and inhibited groups was analyzed using one-way ANOVA, with the Dunnett multiple comparisons when appropriate. Statistical significance was set at p < 0.05. The data are shown as mean ± SEM.

Results

SAA induces expression and secretion of TNF-α and IL-1β in human macrophages

rhSAA induced a strong expression of IL1B and TNFA in human macrophages (Fig. 1A). It also induced dose-dependent secretion of these proteins (Fig. 1B), suggesting that SAA alone is able to

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**FIGURE 1.** SAA induces the transcription and secretion of IL-1β from human macrophages. 

A. Human macrophages were incubated in the presence of rhSAA (30 µg/ml) for the indicated times. After the incubations, the cells were harvested and analyzed for IL1B and TNFA mRNA levels by quantitative real-time RT-PCR. Data are expressed as fold-change compared to control (relative fold increase); mean values shown are from two independent experiments. 

B. Human macrophages were incubated with rhSAA (0–30 µg/ml) for 18 h. After the incubation, the media were analyzed for TNF-α and IL-1β concentrations by ELISA. 

C. Human macrophages isolated from three buffy coat donors were incubated with rhSAA (3 µg/ml) for 18 h. After incubation, the cell-culture media were pooled, concentrated, and analyzed for secreted IL-1β by Western blotting. 

D. Human macrophages were incubated with huSAA (0–20 µg/ml) for 18 h. After the incubation, the cell-culture media were analyzed for IL-1β concentrations by ELISA. huSAA (10 µg/ml) induced IL-1β concentrations varying between 21 and 203 pg/ml, whereas huSAA (20 µg/ml) induced IL-1β concentrations between 64 and 353 pg/ml. Data are expressed as relative fold increase compared to nonstimulated cells (control); the means shown are from three independent experiments. *p < 0.05, **p < 0.01.
provide the signal for the synthesis of pro–IL-1β and the activation of the inflammasome, with ensuing caspase-1 activation, proteolytic cleavage of pro–IL-1β, and secretion of IL-1β. The appearance of mature IL-1β in the cell-culture medium was verified by immunoblotting. A clear band corresponding to mature IL-1β could be observed in rhSAA-stimulated cells (Fig. 1C). In addition to rhSAA, plasma-derived huSAA (29) induced the secretion of IL-1β (Fig. 1D), implying that the observed effects are not inherent to properties of the recombinant protein.

**SAA induces synthesis of pro–IL-1β through TLR2 and TLR4**

First, we elucidated the signal-transduction pathways by which SAA induces the synthesis of pro–IL-1β. SAA has been shown to interact with several cell-surface receptors, including formylpeptide receptor-like 1 (FPRL1) (5, 33, 34), TLR4 (35), TLR2 (36–38), and the scavenger receptor CD36 (39). The ability of SAA and TLR4 agonist LPS to induce IL1B expression was studied first. As demonstrated in Fig. 2A, rhSAA was a more potent inducer of IL1B mRNA than was LPS. The combination of rhSAA and LPS did not increase the expression of IL1B more than either substance alone, suggesting that SAA and LPS may act as TLR4 agonists in human macrophages. This was further supported by the finding that the addition of TLR4 Ab to the cell cultures significantly attenuated the rhSAA-induced IL1B mRNA response (Fig. 2B). Blocking TLR2 signaling by a TLR2 Ab produced a similar inhibition of IL1B expression as did the blocking of TLR4. Adding TLR2 and TLR4 Abs together inhibited IL1B expression by nearly 90% (Fig. 2B), implicating TLR2 and TLR4 in SAA-mediated IL1B induction. This was further verified by Bay11-7082, an NF-κB inhibitor (40). Activation of NF-κB represents the final step in TLR signaling (41–43) and, indeed, Bay11-7082 decreased the effects of LPS and rhSAA. In contrast, CD36-neutralizing Ab (FA6-152) could not inhibit the rhSAA-mediated IL1B induction. Also, the addition of TLR2, TLR4,
and CD36 Abs together resulted in only a slightly more effective inhibition of IL-1B expression than did the combination of TLR2 and TLR4 Abs, suggesting a minor role for CD36 signaling in SAA-mediated IL-1B induction (Fig. 2B). Similar results were obtained using huSAA, with the exception that it also seemed to stimulate IL-1B expression through the CD36 scavenger receptor (Fig. 2C). However, the significance of this finding is not clear, because the presence of plasma remnants in the huSAA preparation cannot be excluded. Finally, the effect of PTX, which has been shown to block the G protein-coupled receptors (44), was tested. However, as shown in Fig. 2D, PTX did not decrease the release of IL-1β. This suggests that FPRL1 does not possess a significant role in SAA-mediated pro–IL-1β production and maturation.

To exclude possible confounding effects of contaminating endotoxins in the rhSAA preparation, proteinase K and polymyxin B preincubations were performed before cell stimulations. As demonstrated in Fig. 2D, proteinase K almost completely abrogated IL-1β release by rhSAA, whereas polymyxin B, inactivator of endotoxins, had no effect. In contrast, treatment of LPS in a similar manner produced the opposite effects (data not shown).

Taken together, the results suggest that several signaling cascades are simultaneously involved in the SAA-mediated induction of pro–IL-1β production. Activation of TLR2/TLR4 signaling is most likely the most prominent pathway, although CD36 may be involved, but to a clearly lesser extent. Under our experimental conditions, FPRL1 signaling did not seem to play a significant role in the SAA-induced IL-1β processing.

SAA-induced secretion of IL-1β is caspase-1 dependent, and activated caspase-1 is secreted from SAA-stimulated macrophages

Inflammasome activation results in the recruitment and activation of caspase-1, the key enzyme in the processing of pro–IL-1β into the mature IL-1β. Also, other caspases have been implicated in pro–IL-1β processing (45). To confirm the role of caspase-1 and the inflammasome pathway in SAA-induced IL-1β secretion, caspase-1 was inhibited by Z-YVAD-fmk. As shown in Fig. 3A, a dramatic decrease in IL-1β secretion was observed in the presence of Z-YVAD-fmk, confirming that maturation of IL-1β induced by rhSAA was mediated through caspase-1.

Caspase-1 activation involves autoproteolytic cleavage of the protein. Because caspase-1, together with other inflammasome components, is secreted after inflammasome activation in many cases, we analyzed the cell-culture media from rhSAA-stimulated macrophages for the presence of caspase-1 by immunoblotting. As shown in Fig. 3B, no secretion of caspase-1 was detected in control cells. However, the secretion and proteolytic processing of caspase-1 were evident in the supernatants of rhSAA-stimulated macrophages, indicating that SAA induces the proteolytic processing of caspase-1.

SAA-induced inflammasome activation is mediated through P2X7R

We next elucidated the mechanisms by which SAA activates caspase-1 and the inflammasome. ATP released from damaged cells has been shown to activate the NLRP3 inflammasome through the ATP receptor P2X7 (14, 46). To study whether the effect of SAA is mediated through P2X7R, we inhibited P2X7 signaling by compound KN-62 (47) and oATP. As demonstrated in Fig. 3A, oATP and KN-62 significantly reduced the IL-1β release induced by rhSAA, suggesting that P2X7R signaling is involved in SAA-induced inflammasome activation. However, because LPS has been shown to induce the release of ATP from human macrophages (48), it is theoretically possible that SAA does not bind directly to P2X7R but instead induces the release of ATP through TLR4. Therefore, we also studied the possible involvement of ATP release in SAA-induced inflammasome activation.
inflammasome activation by using apyrase, an ATP/ADP-hydrolyzing enzyme, as well as by measuring the ATP concentration in the cell-culture media of rhSAA-stimulated macrophages. As depicted in Fig. 3A, apyrase did not diminish rhSAA-mediated IL-1β release, even at relatively high concentrations. In accordance with this, no increase in ATP release in response to rhSAA was observed. Untreated and rhSAA-stimulated cells released ~4 pmol ATP/10^6 cells during the experiments, whereas the intracellular ATP content, measured from lysed macrophages and used as a positive control, was ~1.3 nmol ATP/10^6 cells (data not shown). Thus, the results suggest that SAA-mediated inflammasome activation is mediated by a direct interaction of SAA with P2X7R and that it is not associated with the release of ATP or ADP.

**SAA-induced inflammasome activation is dependent on cathepsin B**

Phagocytosis of fibrillar material has been shown to induce lysosomal destabilization and leakage of cathepsin B into the cytoplasm, which results in the activation of the NLRP3-inflammasome by an unidentified mechanism (22). As shown in Fig. 3A, inhibition of cathepsin B activity with Ca-074Me almost completely abrogated the rhSAA-induced release of IL-1β (Fig. 3A), implying that the effect of SAA is dependent on cathepsin B. However, treatment of macrophages with cytochalasin D had no effect on the IL-1β release by SAA (Fig. 3A), which strongly argues against extracellular SAA fibril formation and phagocytosis-induced inflammasome activation. However, it has been reported that cytochalasin D-independent uptake of SAA by macrophages and subsequent formation of intracellular amyloid fibrils can take place when high SAA concentrations (>100 μg/ml) are used (49). Therefore, we studied whether SAA could induce lysosomal destabilization and leakage of cathepsin B into cytoplasm. As demonstrated in Fig. 4A, lysosomes retained their acidity and morphology in SAA-treated cells. Furthermore, staining of macrophages with a fluorescent cathepsin B substrate did not suggest leakage of cathepsin B into the cytoplasm after stimulation with SAA (Fig. 4A). These findings strongly suggest that intracellular fibril formation is not involved nor required for SAA-mediated inflammasome activation. Finally, because inflammasome activation has been associated with the release of cathepsin B from the cell (50), we examined the cell-culture media for the presence of cathepsin B. As shown in Fig. 4B, significant secretion of cathepsin B was observed in SAA-stimulated and control macrophages (Fig. 4B). Importantly, only the SAA-stimulated macrophages secreted the active form of the enzyme.

In summary, the results suggest that the observed SAA-induced inflammasome activation depends on active cathepsin B but is not mediated through fibril formation or lysosomal destabilization. Rather, the results agree well with previous reports that show that cathepsin B is associated with maturation of IL-1β in the endolysosomal compartment (51) and that stimulation of P2X7R induces secretion of cathepsin B (50, 52).

**SAA activates the NLRP3 inflammasome**

NLRP3 inflammasome consists of the NLRP3 receptor and an adaptor protein ASC. To assess whether SAA mediates its effect through NLRP3 inflammasome, we first studied the role of ASC by examining peritoneal and BMMs from ASC-deficient (31) and wild-type mice. As shown in Fig. 5, significant release of IL-1β was observed in wild-type macrophages in response to SAA. In contrast, SAA induced only minor IL-1β secretion in ASC−/− macrophages. Furthermore, mature IL-1β was observed in the culture medium of wild-type macrophages but not in the ASC−/− macrophages (Fig. 5B). Secretion of IL-1β, together with the inflammasome component ASC, in response to activation of P2X7R has been described (50). As demonstrated in Fig. 5C, SAA markedly increased the release of the ASC isoforms into cell-culture media.

Because ASC is also involved in the function of several other inflammasomes, we verified the role of the NLRP3 inflammasome by silencing the gene encoding NLRP3 receptor in THP-1 macrophages. As assessed by fluorescence-labeled control siRNA, the transfection efficiency was estimated to be 69% (data not shown). The siRNA protocol used in this study has been used earlier in studying cholesterol crystals (24), an established NLRP3 activator (23, 24). Treatment of cells with NLRP3-targeted siRNA diminished NLRP3 mRNA levels by 68% (Fig. 6A). Importantly, as depicted in Fig. 6B, knocking down NLRP3 expression led to a significant decrease in rhSAA-induced IL-1β secretion. This reduction was specific for IL-1β, because secretion of TNF-α remained unaffected, implicating NLRP3 as the SAA-responsive inflammasome.

The induction of NLRP3 expression via activation of NF-κB is a critical checkpoint for NLRP3 inflammasome activation (53). As shown in Fig. 6C, a very rapid increase in NLRP3 mRNA was observed after stimulation of human macrophages with rhSAA. The expression of NLRP3 was transient; within 3 h, the mRNA expression had returned to baseline level. This effect was specific for NLRP3 because NLRP1 or NLRP2 did not respond to SAA. This further supports a key role for SAA in controlling inflammasome activation.
Discussion

SAA is an acute-phase protein with pleiotropic cytokine-like properties. In this study, we found that SAA is also a potent activator of the NLRP3 inflammasome. In fact, to our knowledge, SAA is the first physiological proinflammatory mediator that can provide signals needed for expression of pro–IL-1β (Fig. 1) and activation of the inflammasome cascade, resulting in activation of caspase-1 and secretion of mature IL-1β (Fig. 3). Induction of pro–IL-1β expression by SAA was mediated through TLR2 and TLR4 (Fig. 2). These findings are consistent with previous reports, which used TLR2- and TLR4-deficient mice to show that SAA can activate TLR2 and TLR4 receptors (35–38). The scavenger receptor CD36 has been recently shown to function as a receptor for SAA and to mediate the expression of IL-8 in response to SAA (39). In our case, blockage of the CD36 receptor did not decrease rhSAA-induced IL1B expression (Fig. 2A). Furthermore, because the expression level of CD36 can vary markedly between individuals, and CD36 signaling may be overtaken by other pathways (39, 54), the role of CD36 in terms of SAA response, is likely to be less significant than that of TLR2 or TLR4. Indeed, blocking the CD36 receptor, in addition to TLR2 and TLR4-deficient mice, did not further decrease IL1B expression, suggesting a minor role for CD36 in SAA-induced pro–IL-1β production.

The release of IL-1β by SAA was dependent on caspase-1 activity, suggesting the activation of the inflammasome cascade. This was verified by showing that rhSAA did not induce secretion of IL-1β in ASC−/− macrophages (Fig. 5). Silencing of NLRP3 resulted in a clearly decreased IL-1β release (Fig. 6), further confirming the inflammasome-mediated mechanism and identifying NLRP3 as the SAA-responsive inflammasome. Induction of NLRP3 expression has been suggested to be a prerequisite and a checkpoint for NLRP3 inflammasome activation (53). The fact that rhSAA induced a very rapid expression of NLRP3 mRNA (Fig. 6C) further supports the role of SAA as an important activator of NLRP3 inflammasome. This induction was specific for NLRP3, because no induction of NLRP1 or NLRP2 was observed.

Inhibition of P2X7R by KN-62 or oATP significantly reduced the secretion of IL-1β (Fig. 3), suggesting that the effect of SAA is mediated, at least in part, through P2X7R. This is also supported by previous reports showing that SAA can elicit signaling through P2X7R (55). rhSAA did not induce the release of ATP from macrophages, and the ADP/ATP-hydrolyzing enzyme apyrase did not diminish the effect of rhSAA. These observations suggest that the interaction between SAA and P2X7R is direct rather than mediated through the release of ATP, as has been described for LPS (48).

Activation of inflammasome by SAA was dependent on cathepsin B activity, as inhibition of cathepsin B activity strongly reduced the secretion of IL-1β (Fig. 3), suggesting that the effect of SAA is mediated, at least in part, through P2X7R. This is also supported by previous reports showing that SAA can elicit signaling through P2X7R. Endocytosed substances, such as silica (20), amyloid-β structures (22), and cholesterol crystals (23, 24), have been shown to induce lysosomal destabilization, followed by the release of cathepsin B into the cytoplasm. SAA at high concentrations (>100 μg/ml) has been shown to form intracellular amyloid (49, 56); theoretically,
these intracellular amyloid structures could cause lysosomal destabilization. However, as demonstrated in Figs. 3A and 4A, we did not find any evidence of SAA uptake or changes in lysosome morphology at low SAA concentrations (3 μg/ml). Instead, SAA induced secretion of caspase-1, cathepsin B, and ASC (Figs. 3B, 4B, 5C), events that have been shown to occur after stimulation of P2X7-R (50, 52). Thus, these results suggest that inflammasome activation by SAA is not mediated through phagocytosis and the subsequent amyloid formation and lysosomal destabilization but that it instead proceeds via activation of P2X7, resulting in cathepsin B-dependent activation of the NLRP3 inflammasome.

Cathepsin B has been implicated in the pathogenesis of inflammatory diseases and in many aspects of atherosclerosis (57, 58). Furthermore, there is evidence for the involvement of cathepsin B in AA-amyloidosis (28, 59). Amyloidosis is a severe disease that may develop in association with chronic inflammatory diseases, such as RA and chronic infection, which are associated with constantly high levels of SAA. Proteolysis of SAA into amyloidogenic fragments by cathepsin B has been demonstrated in vitro (27, 28). Intracellular processing of SAA in macrophages/monocytes has been suggested to be a key event in the formation of amyloid fibrils and in the development of AA amyloidosis (56, 60). However, the present results show that SAA also induces the secretion of active cathepsin B from human macrophages. Thus, a potential novel pathway for amyloidogenesis can be envisioned: increased levels of SAA during inflammation induce the secretion of cathepsin B from macrophages, resulting in the processing of SAA into amyloidogenic fragments and formation of pathological extracellular amyloid fibrils. Somewhat similar extracellular proteolytic processing of SAA into amyloidogenic fragments has been described for mast cells and mast cell tryptase (6).

During inflammation, serum SAA levels may reach concentrations up to 1000 μg/ml (i.e., considerably higher levels than used in the current study). Considering the above-described powerful and robust proinflammatory potential of SAA, this would lead to extensive immune activation if there were no additional regulatory mechanisms. One such mechanism is likely to be the interactions between SAA and lipoproteins. In serum, SAA is associated with lipoproteins, high-density lipoprotein in particular, and the level of free SAA is typically very low (61). The fact that the proinflammatory properties of SAA, such as the ability to induce cytokine production, are reduced when SAA is bound to high-density lipoprotein (4, 33, 62) also suggests the importance of lipoproteins in the regulation of the activity of SAA. Locally, lipoprotein-free SAA is secreted by inflammatory cells, such as macrophages; under these conditions, SAA is likely to exert a strong local immune activation.

Patients with inflammatory diseases, RA in particular, have a greatly increased risk for cardiovascular diseases (63, 64). The mechanisms whereby the presence of systemic inflammation translates into increased risk for atherosclerosis are largely unknown. Inflammation in the arterial wall is recognized as a major driving force in atherogenesis, and several findings suggest that IL-1β is one of the key players in the pathogenesis of atherosclerosis (65). In this article, we showed that SAA alone is capable

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**FIGURE 6.** SAA activates IL-1β secretion through NLRP3 inflammasome. A, PMA-differentiated THP-1 monocytes were transfected with nontargeting control siRNA (NT-i) or NLRP3 siRNAs (NLRP3-i) for 22 h and subsequently stimulated with rhSAA (3 μg/ml) for 6 h. The cells were analyzed for NLRP3 mRNA levels by quantitative real-time RT-PCR. Transfection efficiency was 69%, as measured by fluorescent-labeled NT-i (data not shown). For data expression, rhSAA-induced NLRP3 mRNA levels without silencing were set to 100%. The data are means from three independent experiments. B, The transfected and stimulated cells described in A were analyzed for IL-1β and TNF-α secretion by ELISA. For data expression, IL-1β/TNF-α secretion without silencing was set to 100%. The data are means from three independent experiments. C, Human macrophages were incubated in the presence of rhSAA (30 μg/ml) for the indicated times. After the incubations, the cells were harvested and analyzed for NLRP1, NLRP2, and NLRP3 mRNA levels by quantitative real-time RT-PCR. The mRNA levels are shown as relative fold difference compared with time 0 h (control). Values are means from two (NLRP1, NLRP2) or three (NLRP3) individual experiments. **p < 0.01.
of activating the inflammasome and inducing the secretion of mature IL-1β. Patients with RA have constantly elevated levels of SAA. Thus, it is feasible that increased SAA levels in RA promote IL-1β expression and inflammation in the arterial wall and, thus, could explain, at least in part, the increased risk for cardiovascular diseases in RA. The important role of SAA in atherosclerosis is further supported by the observation that serum low-density lipoprotein–SAA level is an independent risk factor for cardiovascular diseases (9).

To conclude, SAA induced the expression of pro–IL-1β and activated the NLRP3 inflammasome via a cathepsin B- and P2X7-dependent manner. SAA also induced the secretion of active cathepsin B from human macrophages, a pathway that could potentially lead to extracellular proteolytic processing of SAA and formation of amyloidogenic fragments. This could represent a novel pathway for AA amyloidosis. As an acute-phase protein with pleiotropic proinflammatory properties, SAA may represent an important link between systemic inflammation and the local inflammatory response in tissues, such as the rheumatic joint and the atherosclerotic arterial wall.

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Disclosures

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References


