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Serum Amyloid A Induces Monocyte Tissue Factor


C-reactive protein (CRP) and serum amyloid A (SAA) increase in the blood of patients with inflammatory conditions and CRP-induced monocyte tissue factor (TF) may contribute to inflammation-associated thrombosis. This study demonstrates that SAA is a potent and rapid inducer of human monocyte TF. SAA induced TF mRNA in PBMC within 30 min and optimal procoagulant activity within 4 h, whereas CRP (25 μg/ml)-induced activity was minimal at this time. Unlike CRP, SAA did not synergize with LPS. Procoagulant activity was inhibited by anti-TF and was dependent on factors VII and X, and TF Ag levels were elevated on CD14+ monocytes. Responses were optimal with lymphocytes, although these were not obligatory. Inhibitor studies indicate activation of NF-κB through the ERK1/2 and p38 MAPK pathways; the cyclo-oxygenase pathway was not involved. SAA-induced TF was partially inhibited by high-density lipoprotein, but not by low-density lipoprotein or by apolipoprotein A-I. SAA is a ligand for the receptor for advanced glycation end products (RAGE), and TF generation was suppressed by high-density lipoprotein, but not by low-density lipoprotein or by apolipoprotein A-I; CRP, C-reactive protein; DSS, disuccinimidyl suberate; HDL, high-density lipoprotein; LDL, low-density lipoprotein; LAL, Limulus ameobocyte lysate; MCF, mean channel fluorescence; PCA, procoagulant activity; RA, rheumatoid arthritis; RAGE, receptor for advanced glycation end products; SI, stimulation index; sRAGE, soluble RAGE; TF, tissue factor.

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Abbreviations used in this paper: SAA, serum amyloid A; ApoA-I, apolipoprotein A-I; CRP, C-reactive protein; DSS, disuccinimidyl suberate; HDL, high-density lipoprotein; HMGBl, high mobility group box-1 protein; HSAB, N-hydroxy succinimido-4-azidobenzoate; LAL, Limulus ameobocyte lysate; LDL, low-density lipoprotein; MCF, mean channel fluorescence; PCA, procoagulant activity; RA, rheumatoid arthritis; RAGE, receptor for advanced glycation end products; SI, stimulation index; sRAGE, soluble RAGE; TF, tissue factor.

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was many orders of magnitude greater than the CRP-induced response. SAA may also modulate platelet adhesion at sites of vascular injury. It can reduce thrombin-activated platelet aggregation (23), but supports platelet adhesion to fibronectin, thus suggesting a regulatory role in thrombosis and hemostasis (24). Together with its effects on platelets, we propose that SAA plays a key role in thrombus formation and fibrin deposition in inflammatory lesions.

Materials and Methods

Reagents

Recombinant human apolipoprotein SAA and ApoA-I (both from PeproTech) were tested. Both were expressed in Escherichia coli, and following folding and purification by ion exchange chromatography, the lyophilized proteins or aliquots were stored at −80°C until used for cell stimulation. The majority of the rSAA was in the monomeric or dimeric forms (determined by Western blotting and silver staining; see Fig. 6D). Recombinant high mobility group box-1 protein (HMGB1) expressed in E. coli, LPS (E. coli 055:B5), or CRP purified from human plasma (all from Sigma-Aldrich) were also used as stimulants for culture in 96-well flat-bottom plates or in Nunc minisorb tubes (Nunc). RPMI 1640 and HBSS were from Invitrogen Life Technologies; medium prepared with pyrogen-free distilled water (Travenol Labs) was filtered through Zetapore membranes (0.2 μm; AMO-MUNO) into heated glass (3 h at 250°C). All reagents and medium were tested for endotoxin levels using the Limulus amebocyte lysate (LAL) buffer and endotoxin standards from Associates of Cape Cod, visualized with Spectrozyme LAL (American Diagnostica). Reagents were not used if they contained endotoxin levels of >5 pg/ml; SAA contained 2.53 pg of endotoxin in 250 ng.

For measurement of TF activity, factors VII, X, Xa chromogenic substrate, a neutralizing mAb to human TF, and FITC-conjugated mouse anti-human TF mAb were all from American Diagnostica. Mouse IgG anti-human TF mAb was from Serotec. FITC-labeled anti-human CD14 IgG was from BD Pharmingen. SB202190, PD98059, and BAY-11-70821 were obtained from Calbiochem.

HDL and low-density lipoprotein (LDL) were prepared from normal human plasma, as previously described, using stringent endotoxin minimized procedures (25, 26).

Soluble RAGE42–59 (sRAGE) and the reverse peptide were synthesized by manual solid-phase synthesis using 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate-mediated couplings with Boc in situ neutralization chemistry (27) and purified by preparative reverse-phase HPLC using a C4 column. Fractions containing the pure proteins were rerun on analytical HPLC, and masses were confirmed by electrospray ionization mass spectrometry (21, 765.5 ± 1.0; calculated: 717.72).

Cross-linking studies

sRAGE (final concentration 100 μM) was added to SAA (10 μM) or HMGB1 (2 μM) in 20 mM HEPES, 150 mM NaCl, and 1 mM CaCl2, and incubated for 30 min at room temperature. The heterobivalent reagent N-hydroxysuccinimidy 4-azidobenzoate (HSAB; Pierce) or the amine-reactive disuccinimidyl carbonate (DSS; Pierce) was added from fresh stocks prepared in dimethyl formamide, to final concentrations of 0.5 mM. Mixtures were incubated in a dark room temperature for 30 min in the dark, after which samples with DSS were quenched by addition of SDS-PAGE sample buffer. Completion of cross-linking in samples with HSAB was achieved by irradiation with a handheld UV lamp (6W-314-nm tube; Vildber Lourberg, Sweden) with or without 4% TCA quenching. Samples were resolved on 4-15% gradient polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and incubated in blocking buffer with 10% nonfat dry milk, 0.1% Tween 20, and 10 mM Tris-HCl, pH 7.4. Membranes were incubated with 1:1000 dilution of anti-RAGE serum (Chemicon International) at room temperature for 1 h, followed by three washes with blocking buffer. Membranes were then incubated with 1:5000 dilution of goat anti-rabbit IgG (Pierce) horseradish peroxidase-conjugated antibody. After washing three times with blocking buffer, membranes were incubated with 1:10 000 dilution of ECL western blotting reagent (Amersham Pharmacia Biotech) for 1 h, washed three times with blocking buffer, and exposed to film. Films were scanned and bands were quantitated using the ImageQuant program (Molecular Dynamics, Sunnyvale, CA). Cell-associated radioactivity was measured using a Packard Top Count liquid scintillation spectrometer (Packard Instruments, Downers Grove, IL).

Mononuclear cell culture

Peripheral citrated blood from normal healthy donors, or when large numbers of cells were required, donors with hemochromatosis not receiving medication were used to separate PBMC by density-gradient centrifugation, as described (28). Duplicate samples of PBMC (5 × 106) in a total volume of 0.2 ml of serum-free RPMI 1640 with SAA, LPS, CRP, or other stimulants in 96-well plates were incubated at 37°C in 5% CO2 in air for the times given. To test whether TF induction by SAA was lymphocyte dependent, mononuclear cells were allowed to adhere to serum-coated wells for 2 h, nonadherent cells were removed, and adherent cells were washed three times with warm HBSS. For blocking experiments, PBMC were preincubated with goat anti-receptor for advanced glycation end products (RAGE) IgG (Chemicon International) or control IgG (Sigma-Aldrich) for 1 h, or with ApoA-I (1 μg/ml) for 1 h, and then SAA (10 or 100 ng/ml) was added, cells were cultured for 2 h, and procoagulant activity (PCA) was measured.

Measurement of PCA and verification of TF activity

At the end of culture, plates were centrifuged at 1400 rpm for 10 min, supernatants were discarded, and cells were resuspended in 0.2 ml of RPMI 1640 and subjected to two cycles of freeze (−70°C)-thawing (37°C). After the final thaw, PCA of PBMC (100 μl) was measured using a one-stage plasma procoagulability test with a chromogenic coagurometer (Diagnostica Stago), as described (19). PCA was calculated from a standard curve using dilutions of rabbit brain extract (Sigma-Aldrich) as standard; results are expressed as mU TF/106 cells or as stimulation index (SI) (TF-SI: mU TF of stimulated PBMC divided by mU TF of unstimulated PBMC).

Chromogenic assays were performed to confirm the dependence of PCA on factors VII and X. Triplicate samples of PBMC stimulated with SAA (100 ng/ml) or LPS (100 ng/ml) for 2 h were washed with 0.2 ml of phenol red-free HBSS, and supernatants were discarded. Factor VII (2 ng/well), factor X (1 μg/well), and factor Xa fluorogenic substrate (1.6 mM) were added, and TF activity was quantitated at 37°C by measuring fluorescence at λex = 360/40 nm and λem = 460/40 nm using a CytoFluor multwell plate reader system (PerSeptive Biosystems). Dependence on factors VII and X was confirmed in assays performed in the presence of factor VII alone and factor X alone. Activity is given as the rate in absolute fluorescence units per minute. Blocking studies using a neutralizing mAb to TF were also performed to confirm induction of TF. Anti-TF IgG (2 μg/ml) or isotype-matched mouse IgG was added to cells that had been stimulated for 2 h with SAA (100 ng/ml) or LPS (100 ng/ml), and then cells were incubated on ice for 1 h and washed, and supernatants were discarded.

Chromogenic assays were performed, as described (19).

Flow cytometry

PBMCs (2 × 106) were incubated in RPMI 1640, or RPMI 1640 with SAA (100 ng/ml) for 2 h in minisorb tubes, and then tubes were gently vortexed and centrifuged for 5 min at 1300 rpm, and supernatants were discarded. Heated (56°C, 30 min) human serum (5%) in Ca2+/Mg2+-free HBSS was incubated with the cells for 20 min at 4°C to block nonspecific binding, and cells were washed with cold HBSS (Ca2+/Mg2+-free) and resuspended in FITC-labeled anti-human CD14, FITC-conjugated mouse IgG, or FITC-conjugated anti-TF mAb (all 10 μg/ml). After 40 min on ice, cells were washed twice with Ca2+/Mg2+-free HBSS and resuspended in 0.3 ml of 0.5% formaldehyde/1% sucrose in Ca2+/Mg2+-free HBSS. Flow cytometry was performed using a FACScan (BD Biosciences); CD14-positive monocytes were used to set the monocyte gate for analysis. Mean channel fluorescence (MCF) relative to that of control IgG was used to established fold increases in TF on SAA-stimulated or unstimulated monocytes.

RT-PCR and real-time RT-PCR

After stimulation for the times indicated, total RNA was extracted using the single-step guanidinium thiocyanate-phenol-chloroform method (29). To detect expression of TF and MCP-1 mRNA, RT-PCR was performed using primer pairs for TF (forward, 5′-GGT ACC TCA CGG AGA AGA TT-3′; reverse, 5′-CCG AGG TTT GTC TCC AGG TA-3′); MCP-1 (forward, 5′-CCC CAC TCA CTT GCT ATT AT-3′; reverse, 5′-TGG AAT CCT GAA CCC ACT TC-3′); and GAPDH (forward, 5′-ACC ACA GTG CAT GCC ATC AC-3′; reverse, 5′-TCC ACC ACC CTG TTG CTG TA-3′) mRNA. PCR products were subjected to agarose gel electrophoresis, and bands were visualized under UV.

To quantitated TF mRNA production, forward (5′-CCT GCT CGG CTT CTT-3′) and reverse TF primers (5′-CAG TCA CCT GCT ATT G-3′) were used to yield a 78-bp PCR product that was detected by a 6-FAM-labeled probe (5′-GGC GCC CCC TTC AGG CAC T-3′) using the real-time ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Levels of GAPDH mRNA were used as internal positive control, using GAPDH primers and VIC-labeled probe (Applied Biosystems). TF mRNA was calculated by comparison of its threshold cycle and that of GAPDH and values expressed as relative levels.

Rheumatoid arthritis (RA) patients

PBMC from 12 patients with RA by American College of Rheumatology criteria (30) (average age 60 ± 5 years; 6 male, 6 female) were used to compare TF induction by SAA with PBMC from 11 normal donors (average age 52 ± 5 years; 6 male, 5 female). Eleven of the RA patients were receiving low dose (<10 mg/day) prednisone, and 11 were being treated.
with between 1 and 2 disease-modifying antirheumatic drugs, most commonly methotrexate (9 of 12). Serum CRP levels of 4 RA patients were >4 mg/L; the remainder were all 2 mg/L or less. Of the normals, one donor had a CRP level of 4 mg/L, and the remainder had levels <2 mg/L.

Statistical analysis

Data are expressed as the mean ± SEM and were analyzed using paired t test for paired comparisons or unpaired t test for nonpaired comparisons; significance was set at p < 0.05.

Results

SAA induces PCA with properties of TF

SAA increased basal PCA over the range of 10–500 ng/ml, in a dose-dependent manner. Cells harvested after 2-h culture had high activity, although less than after 5-h culture (Fig. 1A). When responses to SAA were compared with the same doses of LPS (Fig. 1B), SAA rapidly induced PCA, with increases above basal levels evident within 1 h for SAA. Maximal activity induced by SAA was evident after 4 h and was maintained over the next 6 h, and then gradually declined; at 16 h, PCA was 30% of maximum, but still ~10-fold greater than basal levels (data not shown). PCA induced by 100 ng/ml LPS was evident 2 h after stimulation and followed a time course similar to SAA (Fig. 1B). Compared with SAA, higher amounts of CRP were needed to directly induce TF on PBMC; we used a predetermined amount of 25 μg/ml (19) in this study. In marked contrast to SAA, the low levels of PCA induced by CRP were apparent only after 4- to 6-h culture (Fig. 1B) and continued to increase over 10 h and then declined over the next 6 h (data not shown).

To confirm induction of the TF gene by SAA, mRNA from PBMC was detected by RT-PCR and further quantitated by real-time RT-PCR. Fig. 2A shows minimal expression of the gene in unstimulated cells, whereas significant induction by SAA or LPS was seen at 2 h. A 2.6-fold increase in mRNA levels was evident after 30-min incubation with SAA, whereas the effect of LPS was not apparent at this time point. TF mRNA levels increased ~5-fold after 1-h stimulation with LPS, whereas SAA-induced levels increased ~57-fold after 1 h and increased to ~380-fold after 2 h (Fig. 2B). When the same cells were tested for PCA, activity induced by SAA was evident at 1 h, whereas negligible activity was detected in response to LPS at this time (Fig. 2C). After 2-h incubation, TF mRNA and procoagulant levels induced by SAA and LPS were both markedly elevated (Fig. 2, B and C).

To confirm that TF mRNA induced by SAA was translated, TF Ag levels were assessed by flow cytometry. Fig. 2D shows that unstimulated normal blood monocytes cultured for 2 h expressed negligible TF Ag (MCF 1.5 compared with control IgG (MCF = 1)), and MCF of monocytes stimulated with SAA increased to 2.84. Next, SAA-induced PCA was tested for dependence on factors VII and X. Fig. 2E shows that the factor Xa generated by monocytes stimulated with SAA or LPS was largely dependent on both factors. In the absence of factor VII, the SAA-induced activity was significantly reduced by 86%; no activity was observed in the absence of factor X. Furthermore, treatment of SAA- or LPS-stimulated monocytes with anti-TF significantly reduced factor Xa generation by 90% (data not shown).

Endotoxin contamination does not contribute to TF induction by SAA

All reagents used in cell culture experiments were tested for endotoxin levels using the LAL assay and were not used if they contained endotoxin levels of >5 pg/ml in culture medium. SAA contained 2.53 pg of endotoxin/250 ng of protein, or ~1 pg/100 ng/ml SAA. To test whether this trace amount of endotoxin contributed to SAA induction of monocyte TF, first, responses to increasing doses of LPS were tested. Fig. 3A shows that LPS at 50 pg/ml or less did not induce significant TF activity, and 100 pg/ml only induced a 2-fold increase above basal levels, suggesting that TF induction by SAA was not due to endotoxin contamination. To address this further, activities induced by SAA and LPS were compared following boiling for 15, 30, and 45 min. PCA induced by SAA was significantly reduced by 49% even after 15 min, and only 14% residual activity was present after boiling for 45 min. In marked contrast, LPS retained its activity when treated for 15 and 30 min, and after 45 min this was reduced by only 13% (Fig. 3B). To rule out the possibility that boiling the SAA preparation eliminated LPS by precipitating it, PBMC from another cohort of normal donors was stimulated with a combined preparation of SAA and LPS both boiled for 45 min. This yielded similar TF induction as boiled LPS in the absence of SAA (Fig. 3C), excluding the possibility that contaminating LPS can precipitate with SAA, rendering SAA unable to induce TF. Taken together, these results confirm that endotoxin contamination did not play a measurable contribution to SAA induction of TF.

SAA does not synergize with CRP or LPS

One of the features of TF induction by CRP is its ability to act in synergy with LPS (19). Induction of TF by suboptimal levels of SAA was tested in the presence of increasing concentrations of LPS or CRP. CRP (0.5–10 μg/ml) did not alter the SAA-induced response at any dose tested (5.8 ± 0.9 TF-SI with 25 ng/ml SAA, and 5.9 ± 1.3 TF-SI with SAA + 10 μg/ml CRP). Concentrations of LPS of up to 50 pg/ml did not induce TF after 2-h incubation, and these and higher doses (to 10 ng/ml) had no effect on SAA-induced activity (10.5 ± 3.0 TF-SI with 50 ng/ml SAA, and 9.7 ± 3.8 TF-SI with SAA + 50 pg/ml LPS).

SAA-induced monocyte TF: partial dependence on lymphocytes

To test whether lymphocytes were required for TF up-regulation by SAA, monocytes in PBMC were enriched by adherence and
stimulated for 2 h with SAA or LPS. SAA-induced TF activity was reduced by 48% following lymphocyte depletion (Fig. 4). As expected, the LPS-induced PCA response was also reduced to 65.6% of that of total PBMC; lymphocytes alone had no PCA before or after SAA stimulation (Fig. 4). In addition, THP-1 monocytic cells responded directly to SAA (data not shown), confirming that lymphocytes are not essential for TF induction.

Can HDL alter TF induction by SAA?

Because SAA circulates in association with HDL, its effects on SAA induction of monocyte TF were tested (Fig. 5). HDL did not

stimulated for 2 h with SAA or LPS. SAA-induced TF activity was reduced by 48% following lymphocyte depletion (Fig. 4). As expected, the LPS-induced PCA response was also reduced to 65.6% of that of total PBMC; lymphocytes alone had no PCA before or after SAA stimulation (Fig. 4). In addition, THP-1 monocytic cells responded directly to SAA (data not shown), confirming that lymphocytes are not essential for TF induction.

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Because SAA circulates in association with HDL, its effects on SAA induction of monocyte TF were tested (Fig. 5). HDL did not

representative of three separate experiments. D, TF Ag on monocytes stimulated with SAA, PBMC incubated for 2 h with medium (broken line) or SAA (100 ng/ml, solid black line) were stained with anti-TF mAb or with control IgG (solid thin line) and analyzed by flow cytometry. The monocyte gate was set using FITC anti-CD14-labeled cells. Monocytes stimulated with 100 pg/ml LPS were TF negative (data not shown). A representative experiment of three separate analyses is shown. The average increase in MCF for TF (n = 3) was 2.8-fold above that of unstimulated monocytes. E, PBMC were incubated with medium (□), SAA (100 ng/ml; ■), or LPS (100 ng/ml; □) for 2 h, and factor Xa generation was measured in the presence/absence of factors VII and X. Results represent the mean rates of factor Xa generation ± SEM of cells from four different donors tested in triplicate.

AFU, Absolute fluorescence units.
induce TF when tested at concentrations up to 50 μg/ml. HDL (0.2 μg/ml) cultured with SAA reduced the SAA-induced response by 22%, and suppression reached 48% with 5 μg/ml HDL. Fig. 5A shows a dose-dependent suppression of SAA induction of TF by HDL with PBMC from seven donors. Inhibition with 0.05–50 μg/ml was statistically significant (all p < 0.008) and optimal with 5 μg/ml. Interestingly, HDL weakly enhanced LPS-induced activity by almost 2-fold when used at amounts of 5 μg/ml or greater, but increases failed to reach statistical significance (data not shown). LDL had no effect on TF induction in the presence or absence of SAA (data not shown). To determine the effect of autologous serum on SAA-induced TF, PMBC were stimulated in 1–10% serum. Although responses between individuals were vari-
tologous serum on SAA-induced TF, PMBC were stimulated in
but increases failed to reach statistical significance (data not

Like SAA, ApoA-I is another major apolipoprotein of HDL. In
contrast to SAA, ApoA-I (100 ng/ml to 1.5 μg/ml) did not induce TF activity over a time course of 16 h (data not shown). To test whether ApoA-I might alter SAA induction of TF, PBMC were preincubated with ApoA-I and then stimulated with SAA. Fig. 5B shows that ApoA-I pretreatment had no effect on SAA induction of PCA.

Mechanisms of TF induction by SAA

RAGE is a receptor for the amyloidogenic form of SAA (31), and RAGE-AA interactions on a monocyte cell line up-regulate NF-κB target genes (31). The selective NF-κB pathway inhibitor BAY-11-70821 inhibited TF induction by SAA by 77.9 ± 1.5 and 94.5 ± 1.3% when used at 1 or 10 μM, respectively (Fig. 6A). The MAPK pathway can also mediate TF induction (32) and is triggered by SAA (11); therefore, the inhibitors of ERK-1/ERK-2 (PD98059) and p38/SAPK-2 (SB202190) phosphorylation were tested. PD98059 partially inhibited the SAA-induced response (54 ± 4.8% at 10 mM), whereas SB202190 reduced activity by 75 ± 0.7% (Fig. 6A). These results indicate converging pathways involving NF-κB and MAPK in TF induction by SAA. Because most of the RA patients were on corticosteroid treatment, dexamethasone was used to determine whether steroids had any effect on SAA induction of TF. Although dexamethasone can enhance LPS induction of TF (33), it had no significant effect on SAA-induced TF in concentrations from 10⁻⁷ to 10⁻⁵ M. The cyclo-
oxigenase inhibitor indomethacin also failed to affect TF induction significantly (Fig. 6A).

To determine whether induction may occur via RAGE, SAA or LPS was preincubated with sRAGE42–59 peptide antagonist containing the RAGE ligand-binding domain, and PBMC was stimulated 2 h later. Fig. 6B shows that 1 nM sRAGE suppressed TF induction by both concentrations of SAA (100 or 250 ng/ml) to 40–50%, and inhibition increased to 60–65% with 10 nM levels. In contrast, LPS (10, 100, or 250 ng/ml) induction of TF activity was not affected by sRAGE. Interestingly, HMGB1 (10 ng/ml–1 μg/ml), another proinflammatory RAGE ligand (34), failed to induce PCA when PBMC were stimulated for up to 24 h (Fig. 6B), and stimulation of PBMC with HMGB1 and LPS (10 pg/ml–100 ng/ml) did not show any additive effect above LPS alone (data not shown). The functional capacity of HMGB1 was confirmed by its ability to up-regulate MCP-1 mRNA; LPS induced both the TF and MCP-1 genes (Fig. 6C).

Several approaches were used to confirm the involvement of RAGE in the SAA response. Chemical cross-linking demonstrated that the sRAGE decoy peptide bound SAA, SAA was cross-linked with sRAGE, and complexes were detected by silver staining (Fig. 6D-A) and Western blotting with a specific Ab to sRAGE (Fig. 6D-B). SAA was cross-linked to sRAGE with DSS (lane 1) and HSAB (lane 2). The majority of SAA was monomeric (Mr ~13 kDa) or dimeric, indicating that it is not in the fibrillar form when used to stimulate PBMC. HMGB1 also formed sRAGE cross-links (Fig. 6E).

Neutralizing anti-RAGE IgG was used to block SAA-RAGE binding; Fig. 6F shows that anti-RAGE significantly inhibited SAA induction of TF activity by up to ~85% compared with control IgG.

Monocytes from patients with RA express more TF in response to SAA

SAA is used as a marker of disease activity in RA (5), so the responsiveness of PBMC from patients with RA (n = 12) was
centrations of inhibitors of NF-
Pathways of TF induction by SAA.
FIGURE 6. From duplicates of three experiments.
(100 or 250 ng/ml) or LPS (100 ng/ml). Results are expressed as TF-SI
sRAGE with DSS ( ), or diluent control ( ), lane 2
lane 1
lane 3

stimulation of PBMC for 2 h. Data representative of three independent
MCP-1, but not TF mRNA, whereas LPS (100 ng/ml) induced both, after
stimulation of PBMC for 2 h. Data representative of three independent
experiments. D, SAA binds sRAGE. SAA was chemically cross-linked to
sRAGE with DSS (lane 1), HSAB (lane 2), or diluent control (lane 3),
followed by detection with silver staining (gel A) and Western blotting (gel B).
E, HMGB1 binds sRAGE. HMGB1 was chemically cross-linked to
sRAGE with DSS (lane 1) or diluent control (lane 2) and bands visualized

compared with cells from normal subjects (n = 11). Fig. 7 shows that in response to 100 ng/ml SAA, the average TF activity on
PBMC from normal donors increased from 20.7 ± 3.1 to 249.4 ± 43.5 mU TF/10⁶ cells (p = 0.0003). PBMC from patients had elevated basal levels of activity with ~3-fold more activity than
normal PBMC (p = 0.006), indicating that mononuclear cells from rheumatoid patients may be preactivated in vivo. PBMC from
RA patients were similarly responsive to SAA; average PCA in-
creased from 62.4 ± 12.2 to 532.1 ± 95.7 mU TF/10⁶ cells (p = 0.0004). Importantly, the activity on monocytes induced by SAA
was significantly greater in RA patients than that induced on cells from normal subjects (p = 0.02).

Discussion
TF is a key regulator of thrombosis and hemostasis and the principal initiator of thrombin generation, which contributes signifi-
cantly to inflammatory processes (reviewed in Ref. 35). Earlier
studies implicate CRP in induction of TF expression on blood
monocytes (19, 36, 37), and because of the association of plasma
CRP levels with increased risk of cardiovascular events in patients
with unstable coronary syndromes, this suggested a prothrombotic
role in the pathogenesis of atherosclerosis. Raised plasma levels of
SAA are also associated with the pathogenesis of numerous
chronic or recurrent inflammatory diseases, including amyloidosis,
RA, and atherosclerosis (38, 39). Because the TF pathway is im-
plicated in the pathogenesis of atherothrombosis (35) and in po-
tentiation of inflammatory arthritis such as RA (40), particularly
when expressed on macrophages, we investigated whether SAA
could regulate TF induction.

In this study, we demonstrate that SAA is a potent and rapid
inducer of TF expression on normal human monocytes. Using
PBMC, SAA dose and time dependently induced TF mRNA and
PCA that was dependent on factors VII and X and blocked by
anti-TF Abs. The induction was not due to trace amounts of en-
dotoxin contaminating the recombinant reagent (~1 pg/ml in cul-
ture with 100 ng/ml SAA) because of the following: 1) LPS at
much higher concentrations (50 pg/ml) did not significantly induce
TF activity when PBMC were stimulated for 2 h; 2) heat denatur-
ation destroyed the ability of SAA to induce TF, whereas the
activity of LPS was largely retained; 3) a heat-treated mixture of
by silver staining (gel A) and Western blotting (gel B). F, Anti-RAGE
blocks SAA induction of TF. PBMC from three normal donors were pre-
treated with anti-RAGE or control IgG (both 1:50 v/v) for 1 h and stim-
ulated with SAA (100 ng/ml) for 2 h, and TF activity was measured.
SAA and LPS yielded similar PCA levels as boiled LPS in the absence of SAA; and 4) SAA induction of PCA was partially inhibited by sRAGE, whereas LPS induction was not.

A contrasting feature of TF induction by SAA compared with most other stimulants was the very rapid response. TF mRNA was evident within 30 min, whereas induction by LPS was not evident at this time, and at 2 h TF mRNA induced by SAA was higher than that induced by LPS. In contrast, CRP induction of TF mRNA was later and first evident 4 h poststimulation (36). It is noteworthy that the TF activity induced by SAA (100 ng/ml) was significantly higher than that induced by CRP (25 μg/ml) after 6-h stimulation (Fig. 1B), the time reported as optimal for TF induction by CRP (36), emphasizing the differences in the potencies and kinetics of these stimulants. Moreover, CRP synergizes with LPS to induce monocyte TF (19), whereas SAA did not synergize with either LPS or CRP.

Expression of TF by monocytes can be regulated by lymphocytes. Cell-mediated immunity via cytokines originating from lymphocytes and direct monocyte-lymphocyte interaction via CD40 ligation are known to participate in the process (18, 41). We and others demonstrated that lymphocytes are absolutely required for monocyte TF induction by CRP, and contribute, in part, to LPS induction of monocyte TF (19, 42). Depletion of lymphocytes from PBMC reduced SAA and LPS-induced PCA by ~50%, indicating that lymphocytes contribute to optimal TF induction by SAA. Our finding that SAA directly induced TF on monocytic THP1 cells confirmed the lack of an absolute requirement for lymphocytes (C. Song, Y. Shen, E. Yamen, K. Hsu, W. Yan, O. Akbar Ali, P. Witting, C. Geczy, and S. Freedman, manuscript in preparation). The role of lymphocytes is unclear; SAA could also activate lymphocytes via RAGE to generate cytokines that may potentiate TF induction, although the rapidity of the response suggests alternate mechanisms such as coactivation through cell-cell contact via CD40-CD40L interactions. In contrast, the slower kinetics of the CRP-induced response may be mediated by lymphocyte products such as IFN, which can synergize with CRP to induce TF (19). SAA, unlike CRP, can also directly activate monocyte TF expression. Thus, in inflammatory lesions, the local production of SAA by activated macrophages (43, 44) could generate a positive feedback loop even in the absence of lymphocytes.

Circulating SAA is largely present as an apolipoprotein of HDL, mainly HDL3. It can account for as much as 2.5% of total protein produced by the liver and can replace ApoA-I as the major HDL apolipoprotein during the acute-phase response (45). SAA has several proposed functions involved in cellular cholesterol transport and clearance, and the HDL scavenger receptor CLA-1, which is expressed on monocytes and macrophages, was proposed recently to be a potential SAA receptor. In this study, we show that HDL did not alter basal TF activity, but increasing amounts, to 10 μg/ml, inhibited procoagulant induction by SAA by ~50% with no further increases with higher HDL concentrations (Fig. 5). Another study showed that HDL inhibits the chemotactic activity of SAA (13), and it is possible that HDL either competes for receptor binding sites, thereby reducing SAA triggering, or the HDL-SAA complex is a less effective activator. Direct effects on the generation of factor Xa are unlikely because in pure systems, TF-factor VIIa activity is inhibited by HDL and by LDL (46), whereas LDL had no effect in our studies. Moreover, and in keeping with studies by Schlichting et al. (47), TF activity induced by LPS was not suppressed by HDL, confirming that HDL did not directly alter the generation of activated coagulation factors. The SAA response was increased in 1–5% autologous serum, but this decreased in 10% serum, suggesting that factors present in serum, or the ratio of lipid-free to HDL-bound SAA, may modulate TF induction. Basal and LPS-induced TF activities are also augmented by serum (48, 49), and other factors, such as traces of thrombin, TF pathway inhibitor levels, etc., could positively or negatively regulate net PCA. Interestingly, although both SAA and ApoA-I are mainly associated with HDL in blood and ApoA-I can be replaced by SAA as the major apolipoprotein in HDL during the acute-phase response, ApoA-I itself did not induce TF and did not block the activity of SAA, indicating a unique function for SAA.

NF-κB is a key transcription factor for expression of many inflammatory mediators, including TF in monocytes (50). SAA induction of monocyte TF was almost totally inhibited by the NF-κB pathway inhibitor, BAY-11-70821, confirming its involvement. Inhibition studies confirmed that the ERK1/2 and p38 MAPK pathways also mediate TF induction by SAA, similar to reports for LPS (51). Some stimulants such as arachidonic acid, but not others (e.g., LPS (52)), increase monocyte TF via production of cyclooxygenase metabolites (53), but indomethacin did not influence induction even though SAA increases cyclooxygenase metabolites in activated human monocytes (54). Glucocorticoids are clinically useful in treating inflammatory disorders, but, as in other systems, dexamethasone did not reduce TF levels on monocytes stimulated with SAA, and unlike its effect on LPS (33), no enhancement was observed.

RAGE, a multiligand receptor proposed to propagate inflammation, is also a receptor of SAA (31). Interaction of RAGE with its ligands activates NF-κB through the ERK1/2 and p38 MAPK pathways, resulting in induction of proinflammatory genes and TF (55, 56). We confirmed a requirement for these pathways. SAA-induced monocyte TF was suppressed by ~50% by sRAGE, a peptide competitor spanning the RAGE binding domain and which bind SAA in vitro cross-linking studies. Importantly, SAA-induced monocyte TF was markedly inhibited by anti-RAGE IgG, confirming RAGE involvement. In contrast, another proinflammatory RAGE ligand, HMGB1 (34, 57), although capable of binding sRAGE (Fig. 6E), and of inducing MCP-1 mRNA (Fig. 6C), did not induce monocyte TF mRNA or PCA and failed to enhance LPS-induced monocyte TF. HMGB1 binds receptors other than RAGE, including TLR 2 and 4, but its proinflammatory domain does not contain the RAGE-binding sequence (58), suggesting multiple functional interactions. It is possible that the outcome of RAGE signaling may be determined by a ligand binding to a particular second receptor to mediate tailored responses.

SAA is a better marker of disease activity than CRP or erythrocyte sedimentation rate in patients with recent onset arthritis, and high levels are found in the circulation of patients with RA (59). In addition, links between inflammation and atherosclerosis/thrombosis may underlie the reported increase in cardiovascular mortalities in patients with RA, to almost double that of an age-matched population without RA (60). In this study, we demonstrate that PBMC from patients with RA expressed significantly higher basal TF activity and were more responsive to SAA induction of TF than controls. Thus, SAA could contribute to a hypercoagulable state, particularly if free to HDL-bound SAA levels were elevated. Studies also show increased basal TF levels in patients with cardiovascular disease (21), and, as SAA levels are also elevated in these patients (61), we propose that SAA, as a potent and rapid inducer of TF, may be a new link between inflammation and thrombosis. Importantly, blood-borne TF, in the form of microparticles, may contribute to thrombus formation and to vessel damage (62), although the factors that induce TF in the circulation of patients with inflammatory diseases are unclear. SAA, particularly when HDL levels are reduced, may contribute to this process. Because RAGE-positive macrophages and T cells are found in RA synovial tissue
(63), and SAA is produced by macrophages and fibroblasts in inflammatory lesions. SAA-RAGE interactions may contribute to localized pathogenesis by directly contributing to fibrin deposition and thrombosis in inflammatory states.

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References