Journal Articles

2015

Serum Amyloid A Stimulates PKR Expression and HMGB1 Release Possibly through TLR4/RAGE Receptors

W. Li
Northwell Health

S. Zhu
Northwell Health

J. Li
Northwell Health

J. D'Amore
Northwell Health

J. D'Angelo
Hofstra Northwell School of Medicine

See next page for additional authors

Authors

W. Li, S. Zhu, J. Li, J. D'Amore, J. D'Angelo, H. Yang, P. Wang, K. J. Tracey, and H. Wang

Molecular Medicine

Serum Amyloid A Stimulates PKR Expression and HMGB1 Release Possibly through TLR4/RAGE Receptors

Wei Li,[1,2*] Shu Zhu,[1,2*] Jianhua Li,[2] Jason D'Amore,[1] John D'Angelo,[1] Huan Yang,[2] Ping Wang,[2] Kevin J Tracey,[2] and Haichao Wang[1,2]

[1]Department of Emergency Medicine, North Shore University Hospital, Manhasset, New York, United States of America; and [2]The Feinstein Institute for Medical Research, Manhasset, New York, United States of America

Serum amyloid A (SAA) proteins are known to be surrogate markers of sepsis, but their pathogenic roles remain poorly elucidated. Here we provide evidence to support a possible role of SAA as a pathogenic mediator of lethal sepsis. In a subset of septic patients for which serum high mobility group box 1 (HMGB1) levels paralleled the clinical scores, some anti-HMGB1 antibodies detected a 12-kDa protein belonging to the SAA family. In contrast to the most abundant SAA1, human SAA induced double-stranded RNA-activated protein kinase R (PKR) expression and HMGB1 release in the wild-type, but not toll-like receptor 4/receptor for advanced glycation end products (TLR4/RAGE)-deficient, macrophages. Pharmacological inhibition of PKR phosphorylation blocked SAA-induced HMGB1 release, suggesting an important role of PKR in SAA-induced HMGB1 release. In animal models of lethal endotoxemia and sepsis, recombinant SAA exacerbated endotoxemic lethality, whereas SAA-neutralizing immunoglobulins G (IgGs) significantly improved animal survival. Collectively, these findings have suggested SAA as an important mediator of inflammatory diseases. Highlights of this study include: human SAA is possibly only expressed in a subset of septic patients; SAA induces HMGB1 release via TLR4 and RAGE receptors; SAA supplementation worsens the outcome of lethal endotoxemia; whereas SAA-neutralizing antibodies confer protection against lethal endotoxemia and sepsis.
Online address: http://www.molmed.org
doi: 10.2119/molmed.2015.00109

INTRODUCTION

Despite recent advances in antibiotic therapy and intensive care, sepsis remains a significant problem in critically ill patients with >225,000 victims in the U.S. alone. The pathogenesis of sepsis remains poorly understood, but is attributable to dysregulated immune responses orchestrated by innate immune cells including macrophages/monocytes (1). Macrophages/monocytes are equipped with various pattern recognition receptors (PRRs) (such as the toll-like receptors [TLRs] TLR2, TLR4 and TLR9), which can recognize various pathogen-associated molecular patterns (PAMPs) (such as bacterial lipoproteins, endotoxins and CpG-DNA) (2). Upon PRR–PAMP engagement, innate immune cells sequentially release early (for example, tumor necrosis factor [TNF], interleukin [IL]-1, interferon [IFN]-γ and cold-inducible RNA-binding protein [CIRP]) (3,4) and late (for example, nitric oxide [NO] or high mobility group box 1 [HMGB1]) proinflammatory mediators (5,6). If dysregulated, the excessive release of these late mediators adversely contributes to the pathogenesis of lethal sepsis (4,7–9).

In addition to stimulating macrophages/monocytes to release late proinflammatory mediators, early cytokines also alter the expression of liver-derived acute-phase proteins that similarly participate in the regulation of inflammatory responses. For instance, TNF, IL-1β and interferon (IFN)-γ induce the expression of serum amyloid A (SAA) in hepatocytes (10) and macrophages/monocytes (11), resulting in subsequent SAA secretion upon cleaving off the signal sequence. The human SAA family is comprised of multiple members, including the most abundant SAA1, and other isoforms such as SAA, SAA2α, SAA2β and SAA3. Members of the SAA family share >95–98% identity within species, with >75% sequence homology between human and rodents. During endotoxemia, circulating SAA levels are significantly elevated (up to 1,000-fold) within 16–24 h as a result of *de novo* expression of early cytokine inducers and subse-

quent synthesis and secretion of SAAs (12,13). Clinically, SAAs have been implicated as biomarkers in cardiovascular disorders (14), ulcerative colitis (15) and sepsis (16). Extracellular SAA signals via a family of receptors including the receptor for advanced glycation end products (RAGE) (17), TLR2 (18,19) and TLR4 (20) to activate NLRP3 inflammasome (21) and to induce various cytokines and chemokines (22–25).

Previously, we demonstrated that a ubiquitous nuclear protein, HMGB1, is released from macrophages/monocytes in response to exogenous PAMPs (for example, lipopolysaccharide [LPS] and CpG-DNA) (6,26) or endogenous cytokines (for example, IFN-γ or CIRP) (4,27). The nucleus-to-cytoplasm translocation of HMGB1 is mediated by the STAT1-mediated acetylation of the HMGB1 nuclear-localization sequences (28). The extracellular HMGB1 release is regulated by caspase 1- and the double-stranded RNA-activated protein kinase R (PKR)-dependent inflammasome activation (29,30), pyroptosis (31) or necroptosis (32). For instance, pharmacological inhibition of PKR interaction with pyroptosome components (for example, apoptosis-associated speck protein [ASC]) by the 7-desacetoxy-6,7-dehydrogedunin (7DG) (31) results in the interruption of pyroptosis. Similarly, the suppression of PKR-mediated phosphorylation of necrosome components (for example, the death domain receptor–interacting protein 1 kinase [RIP1] and RIP3) by kinase inhibitors (for example, C16) (32) leads to the impairment of necroptosis. It was previously unknown, however, whether SAA can induce PKR expression to stimulate HMGB1 release.

In this study, we report a possibility that SAA was expressed only in a subset of septic patients and stimulated the expression of PKR and caused HMGB1 release in wild-type, but not in TLR4/RAGE-deficient, macrophages. Pharmacological inhibition of PKR phosphorylation inhibited SAA-induced HMGB1 release, and administration of

SAA-neutralizing immunoglobulins G (IgGs) significantly improved animal survival in sepsis. Collectively, these findings have suggested a possible role of SAA as an important mediator for lethal inflammatory diseases.

MATERIALS AND METHODS

Materials

Crude bacterial endotoxin (LPS, *E. coli 0111:B4*; catalog no. L4130), mouse anti-β-actin antibodies (catalog no. A1978) were Sigma-Aldrich products. Calcein-AM (catalog no. C3099), CM-DiI (catalog no. C7001), Dulbecco modified Eagle medium (DMEM) (catalog no. 11995-065), penicillin/streptomycin (catalog no. 15140-122), fetal bovine serum (FBS) (catalog no. 26140079) and Trypan blue (catalog no. 15250-061) were Invitrogen products. Anti-PKR antibody (catalog no. sc-6282) and horseradish peroxidase (HRP) conjugated goat anti-mouse IgG (catalog no. sc-2060) were Santa Cruz Biotechnology products. Anti-phosphorylated PKR antibody (T451; catalog no. 07-886) was a Millipore product. Rabbit anti-p-STAT1 (Y701; catalog no. 7649S) was a Cell Signaling product. Rabbit anti-TLR9 (catalog no. NBP1-76680) was a Novus Biologicals product. Recombinant human SAA (catalog no. 300-13) and SAA1 (catalog no. 300-53) were PeproTech products. HRP-conjugated donkey anti-rabbit IgG was a GE Healthcare product (catalog no. NA934). HMGB1-specific polyclonal antibodies were generated in rabbits as previously described (6). TLR2, TLR4 and RAGE KO mice and TLR2/RAGE and TLR4/RAGE-double KO mice on a C57BL/6 genetic background were maintained at The Feinstein Institute for Medical Research as previously described (33). SAA1/2 knockout mice were generated by targeted deletion of the exon 2 of both *Saa1* and *Saa2* genes as previously described (34), and the knockout mice were backcrossed into a C57BL/6 genetic background. Because the KO mice were derived from C57BL/6 mice, small colonies of wild-type C57BL/6 (The Jackson Laboratory) were maintained under the same conditions.

Preparation of Recombinant HMGB1

The cDNA encoding for rat HMGB1 was cloned onto a pCAL-n vector, and the recombinant calmodulin-binding protein (CBP)–tagged HMGB1 (rHMGB1) was expressed in *E. coli* BL21 (DE3) pLysS cells as previously described (6). The rHMGB1 containing an ~3-kDa CBP tag (CBP-HMGB1 fusion protein, 33 kDa) was expressed in *E. coli* and purified to remove contaminating endotoxin by Triton X-114 extraction, as previously described (35).

Cell Culture

Murine macrophage-like RAW 264.7 and human monocytic U937 cells were obtained from the American Type Culture Collection (ATCC). Primary peritoneal macrophages were isolated from Balb/C mice (Taconic; male, 7–8 wks, 20–25 g) at 2–3 d after intraperitoneal injection of 2 mL thioglycollate broth (4%), as previously described (36,37). RAW 264.7 macrophages, U937 monocytes and primary macrophages were cultured in DMEM supplemented with 1% penicillin/streptomycin and 10% FBS. Adherent macrophages or monocytes were gently washed with, and cultured in, DMEM before stimulating with LPS (0.5 μg/mL), HMGB1 (0.5 μg/mL) or human SAA, in the absence or presence of PKR inhibitors or SAA-neutralizing IgGs for 16 h. Subsequently, the cell-conditioned culture media were analyzed respectively for levels of HMGB1, nitric oxide and other cytokines by Western blotting analysis, the Griess reaction and cytokine antibodies arrays as previously described (38,39).

Western Blotting

The levels of HMGB1 in the culture medium were determined by Western blotting analysis as previously described (6,27). The levels of total PKR, phosphorylated PKR (P-PKR, T451) or phosphorylated STAT1 (P-STAT1, Y701) in primary macrophage lysates were determined by Western blotting analysis with reference to β-actin. Briefly, equal amounts of cellular proteins were resolved on sodium dodecyl sulfate (SDS)-

polyacrylamide gel electrophoresis
(PAGE) gels and transferred to
polyvinylidene difluoride membranes.
After blocking with 5% nonfat milk, the
membrane was incubated with respec-
tive antibodies overnight [anti-PKR,
1:250; anti-phospho-PKR (T451), 1:1,000;
anti-P-STAT1 (Y701), 1:500; anti-β-actin,
1:5,000]. Subsequently, the membrane
was incubated with the appropriate sec-
ondary antibody, and the immunoreac-
tive bands were visualized by chemilu-
minescence technique.

Nitric Oxide Assay

The levels of NO in the culture me-
dium were determined indirectly by
measuring the NO^{2-} production with a
colorimetric assay on the basis of the
Griess reaction (36,40). NO^{2-} concentra-
tions were determined with reference to
a standard curve generated with sodium
nitrite at various dilutions.

Cytokine Antibody Array

Human Cytokine Antibody Array C3
(catalog no. AAH-CYT-3-4) and Murine
Cytokine Antibody Arrays (catalog no.
M0308003, RayBiotech Inc., which re-
spectively detect 42 and 62 cytokines on
one membrane, were used to determine
cytokine levels in human serum or cell
culture medium as previously described
(36,40). Briefly, the membranes were se-
quentially incubated with equal volumes
of human serum (10 µL, mixed with
90 µL buffer) or cell culture medium
(200 µL), primary biotin-conjugated
antibodies and horseradish peroxidase–
conjugated streptavidin. After exposing
to X-ray film, the relative signal intensity
was determined by using the Scion
Image software.

Clinical Characterization of Septic Patients

As per the approval by the Feinstein
Institute for Medical Research institu-
tional review board ethics committee,
blood samples (10 mL) were collected at
various time points (0, 12, 24, 48 and
72 h) after the diagnosis of patients with
septic shock, severe sepsis or sepsis, in

the Department of Emergency Medicine,
North Shore University Hospital. The
American College of Chest Physicians/
Society of Critical Care Medicine Con-
sensus Conference definitions of sepsis
and septic shock were used for the diag-
nosis of these patients (41). As controls,
blood samples (10 mL) were also col-
lected from eight healthy individuals.
Serum samples were analyzed for levels
of HMGB1 and 42 other cytokines by
Western blotting and cytokine antibody
arrays, respectively.

MALDI-TOF Mass Spectrometry

To identify the 12-kDa band that cross-
reacted with anti-HMGB1 antibodies,
serum samples were resolved by SDS-
PAGE, and the corresponding 12-kDa
band was subjected to MALDI-TOF mass
spectrometry analysis. Briefly, the 12-kDa
band was excised from the SDS-PAGE gel
and subjected to in-gel trypsin digestion.
The mass of the tryptic peptides was
measured by matrix-assisted laser des-
orption/ionization–time of flight mass
spectrometer (MALDI-TOF-MS) and then
subjected to peptide mass fingerprinting
database analysis to identify the 12-kDa
protein ("P12") in septic patients.

Analysis of Contaminating Bacterial Products

The recombinant human SAA and
SAA1 were analyzed for contaminating
bacterial products (such as lipoproteins,
CpG-DNAs) by SDS-PAGE, followed by
Coomassie blue or ethidium bromide
staining. Recombinant HMGB1 protein
was used as a control for comparison.
Recombinant SAAs were tested for LPS
content by the chromogenic *Limulus*
amebocyte lysate assay (Endochrome;
Charles River), and the endotoxin con-
tent was expressed as nanograms endo-
toxin per microgram of SAAs.

Animal Models of Endotoxemia and Polymicrobial Sepsis

This study was approved and per-
formed in accordance with the guidelines
for the care and use of laboratory ani-
mals at the Feinstein Institute for Med-

ical Research, Manhasset, New York. To
evaluate the role of SAA in lethal sepsis,
Balb/C mice (male, 7–8 wks, 20–25 g)
were subjected to lethal endotoxemia or
sepsis induced by cecal ligation and
puncture (CLP) as previously described
(38,39,42). Briefly, the cecum of Balb/C
mice was ligated at 5.0 mm from the
cecal tip and then punctured once with a
22-gauge needle. Recombinant SAA or
SAA-neutralizing antibodies were in-
traperitoneally administered into endo-
toxemic or septic mice at indicated doses
and time points, and animal survival
rates were monitored for up to 2 wks.

Generation of Anti-SAA Polyclonal Antibodies

Female New Zealand white rabbits
were repetitively immunized with re-
combinant human SAA in combination
with the Freund's complete adjuvant,
and blood was collected on 3-wk cycles
of immunization and bleed. The anti-
body titers were determined by direct
SAA ELISA, and total IgGs were purified
from the serum using a Protein A affinity
column as previously described (6).
Briefly, rabbit serum was prebuffered
with PBS and slowly loaded to a protein
A column to allow sufficient binding of
IgGs. After washing with 1× PBS to re-
move nonbound serum components,
the IgGs were eluted with acidic buffer
(0.1 mol/L glycine-HCl, pH 2.8) and then
immediately dialyzed into 1× PBS buffer
at 4°C overnight.

Peptide Dot Blotting

A library of 19 overlapping peptides
(18-mer, offsite by 6) corresponding to the
human SAA sequence were synthesized
and spotted (1.0 µg in 2.5 µL) onto nitro-
cellulose membrane (Thermo Scientific,
catalog no. 88013). Subsequently, the
membrane was probed with IgGs from
different rabbits following a standard
protocol.

Statistical Analysis

Data are expressed as mean ± SEM of
two independent experiments in tripli-
cates (n = 2). One-way analyses of vari-

A Western blots (anti-HMGB1)
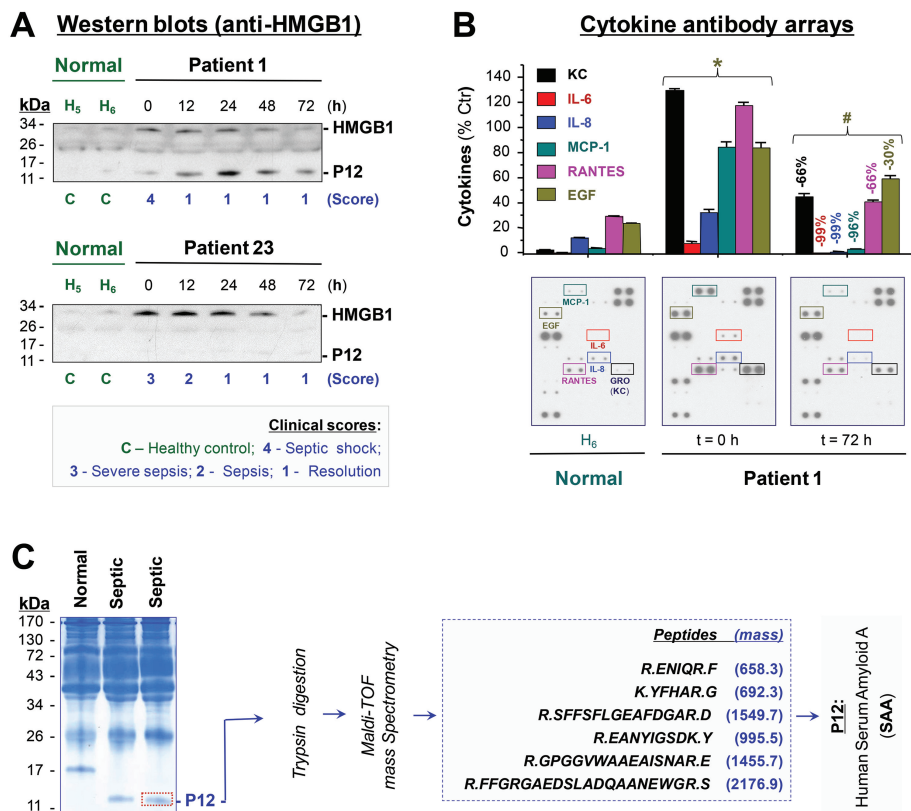

B Cytokine antibody arrays

C

Figure 1. Identification of SAA as an anti-HMGB1 IgG cross-reacting protein in a subset of septic patients. (A) Western blots of serum proteins. Note that certain polyclonal anti-HMGB1 IgGs recognized both the 30-kDa HMGB1 and a 12-kDa protein (termed "P12") in a subset of septic patients. (B) Immunoassay of multiple cytokines and chemokines by cytokine antibody arrays. Note that the serum levels of several sepsis surrogate markers (IL-6, MCP-1 and RANTES) paralleled with the clinical scores. *$P < 0.05$ versus normal healthy controls; #$P < 0.05$ versus $t = 0$ h (diagnosis of septic shock). (C) Mass spectrometry analysis of P12. The identity of the 12-kDa protein (P12) in septic serum was determined by mass spectrometry after gel excision and in-gel trypsin digestion.

ance (ANOVAs) followed by the Tukey's test for multiple comparisons were used to compare between different groups. The Kaplan-Meier method was used to compare the differences in mortality rates between groups. A P value <0.05 was considered statistically significant.

RESULTS

Identification of Human SAA, but not SAA1, as an HMGB1 Inducer

To understand the role of cytokines in sepsis, we characterized the kinetics of circulating HMGB1 and other cytokines

in a group of septic patients admitted to the Emergency Medicine Department at North Shore University Hospital. In 8 of 23 septic patients, serum HMGB1 levels paralleled with the clinical scores; circulating HMGB1 levels returned toward baseline when these patients recovered from septic shock or severe sepsis (Figure 1A, data not shown). In a subset of septic patients, the anti-HMGB1 IgGs cross-reacted with a 12-kDa protein (denoted as "P12") (Figure 1A), which paralleled with the serum levels of HMGB1 (Figure 1A) and several sepsis biomarkers (for example, IL-6, growth regulated

oncogene-alpha/keratinocyte chemoattractant (GRO/KC), monocyte chemoattractant protein-1 (MCP-1) and regulated upon activation, normal T-cell expressed and secreted (RANTES); Figure 1B). To uncover the possible role of P12 in HMGB1 release, septic serum proteins were resolved by SDS-PAGE, and the corresponding 12-kDa band was subjected to in-gel trypsin digestion and mass spectrometry analysis. Peptide mass fingerprinting analysis identified this protein as a member(s) of the human SAA family (Figure 1C).

In agreement with the high homology between human and murine SAAs (Figure 2A), polyclonal antibodies generated against human SAA cross-reacted with both human SAA and SAA1 on Western blots (Figure 2B, *bottom panel*). Remarkably, the anti-HMGB1 IgGs that detected the P12 in a subset of septic patients, also specifically recognized human SAA, but not SAA1 (Figure 2B), suggesting an antigenic distinction between human SAA and SAA1 despite their high sequence homology (Figure 2A). Moreover, there is a dramatic difference in their biological activities. At physiologically relevant concentrations (0.1–10 μg/mL), human SAA effectively stimulated murine macrophages and human monocytes to release HMGB1 (Figure 2C) in a dose- and time-dependent fashion (Figure 2D). In contrast, human SAA1 completely failed to induce HMGB1 release even when given at concentrations up to 10 μg/mL (Figure 2C). Recombinant SAA and SAA1 were not contaminated with bacterial lipoproteins or CpG-DNA (Figure 2E) and occasionally contained low levels of endotoxins (Figure 2F). This trivial endotoxin contamination was not likely the underlying cause for SAA-mediated HMGB1 release, because endotoxin-neutralizing agents (for example, polymyxin B) failed to impair SAA-induced HMGB1 release (data not shown). Furthermore, many SAA preparations that were totally free from endotoxin contamination could still effectively induce HMGB1 release.

A

Amino acid sequence of human and murine SAAs

B

C

Western blot
(Anti-HMGB1, PAb)

D

Western blot
(MAb)

E

Lipoprotein
(Coomassie blue)

CpG-DNA
(Ethidium bromide)

F

Endotoxin
(LAL assay)

(Mean ± S.E.M. of
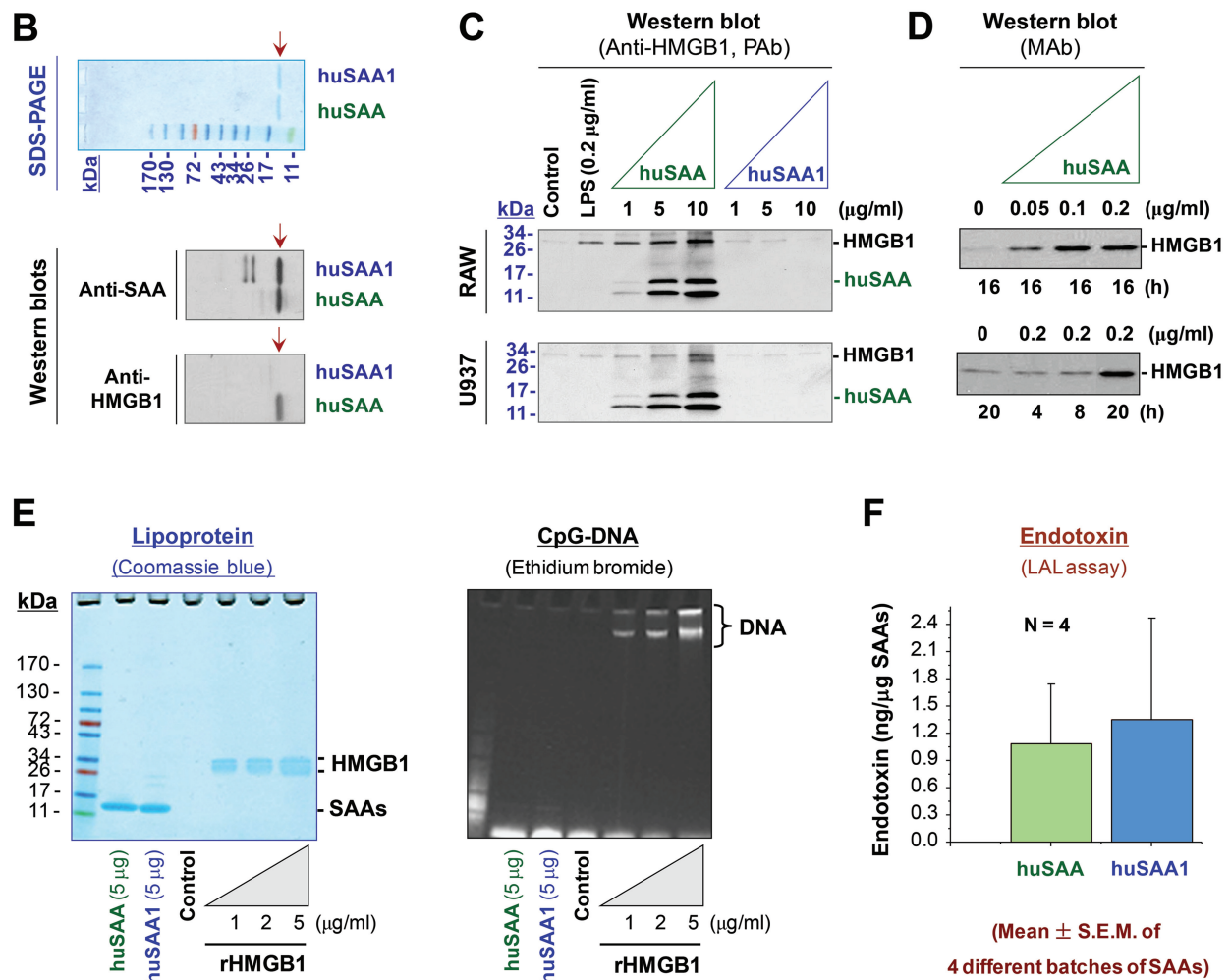**4 different batches of SAAs)**

Figure 2. Human SAA, but not SAA1, induced HMGB1 release. (A) Amino acid sequence of human and murine SAAs. Bar (|), identical residues; colon (:), homologous residues. The bold letters denote the two residues that distinguish between human SAA and SAA1. (B) Western blots of human SAA and SAA1. Note that the anti-HMGB1 IgGs that recognized the P12 in septic patients also specifically cross-reacted with huSAA, but not huSAA1. (C) HuSAA, but not huSAA1, induced HMGB1 release. Murine RAW 264.7 macrophages and human U937 monocytes were stimulated with huSAA or huSAA1 for 16 h, and extracellular HMGB1 levels were determined by Western blots. (D) Dose-response and time course of SAA-induced HMGB1 release. RAW 264.7 macrophages were stimulated with huSAA at indicated doses and for indicated time periods, and extracellular HMGB1 levels were determined by Western blotting analysis by using monoclonal anti-HMGB1 antibody. (E) Detection of bacterial lipoproteins and DNAs by gel electrophoresis. Large amounts of SAAs and rHMGB1 were resolved on SDS-PAGE and stained with Coomassie blue or ethidium bromide to detect contaminating bacterial lipoprotein or DNA. (F) LAL endotoxin assay. Four separate batches of recombinant huSAA and huSAA1 were assayed for endotoxin content.
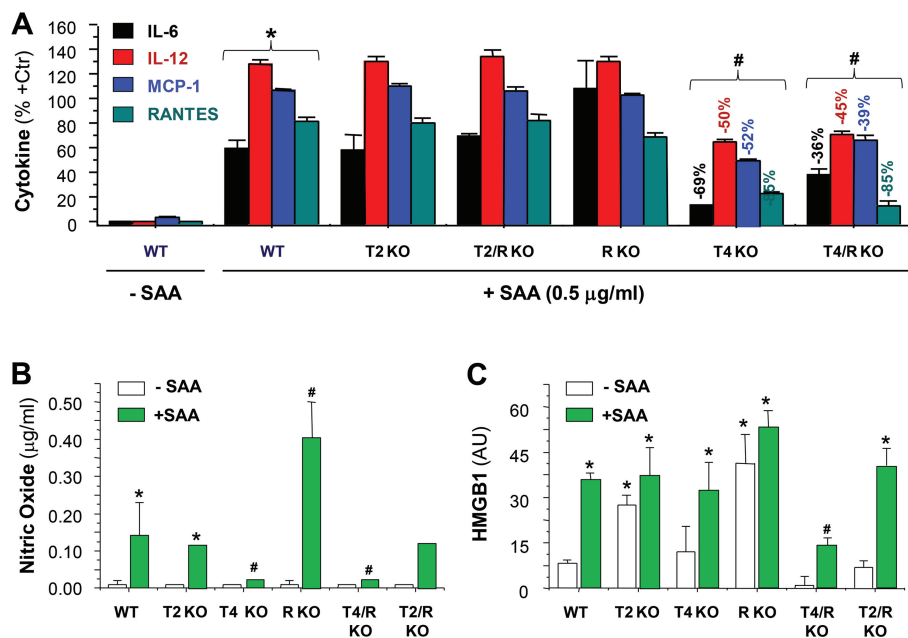
Figure 3. Distinct roles of TLR4 and RAGE receptors in SAA-induced cytokines/chemokines, NO and HMGB1. Thioglycollate-elicited primary macrophages were isolated from wild-type or mutant mice respectively deficient in TLR2, TLR4, RAGE, TLR2/RAGE or TLR4/RAGE. After stimulation with huSAA (0.5 μg/mL) for 16 h, the extracellular levels of cytokines, chemokines, NO and HMGB1 were determined by cytokine antibody arrays (A), Griess reaction (B) and Western blotting (C), respectively. The disruption of TLR4 completely abolished SAA-induced NO production, and partly impaired several cytokines/chemokines, but did not affect SAA-induced HMGB1 release. The concurrent knockout of both TLR4 and RAGE completely abrogated SAA-induced HMGB1 release (C). *$P < 0.05$ versus –SAA controls; #$P < 0.05$ versus WT + SAA.

Requirement of TLR4/RAGE Receptors in SAA-Induced HMGB1 Release

SAAs can signal via multiple receptors including the TLR2, TLR4 (18–20,43) and RAGE (17,25) to activate macrophages and monocytes. To identify the receptor(s) responsible for SAA-mediated HMGB1 release, we compared the levels of SAA-induced HMGB1 release between primary peritoneal macrophages from wild-type and mutant mice, respectively, deficient in TLR2, TLR4, RAGE, TLR2/RAGE or TLR4/RAGE. We found that human SAA effectively induced the release of cytokines (for example, IL-6 and IL-12), chemokines (for example, MCP-1 and RANTES) and NO (Figures 3A, B). Surprisingly, the disruption of TLR2, RAGE or TLR2/RAGE did not obviously impair SAA-induced cytokines/chemokines (Figure 3A), NO (Figure 3B) and HMGB1 re-

lease (Figure 3C). In contrast, the knock-out of TLR4 partially impaired SAA-induced cytokines/chemokines (for example, IL-6, IL-12, MCP-1 and RANTES) (Figure 3A) and completely abolished SAA-induced NO production (Figure 3B). However, the disruption of TLR4 alone did not impair SAA-induced HMGB1 release, indicating the involvement of distinct signaling pathways in NO production and HMGB1 release. Deletion of both TLR4 and RAGE resulted in an almost complete impairment of HMGB1 release (Figure 3C), indicating a critical requirement for both TLR4 and RAGE in SAA-induced HMGB1 release.

Requirement of TLR4/RAGE Receptors for SAA-Induced PKR Upregulation

Emerging evidence has revealed an important role for PKR (30) and STAT1 (28)

in LPS-induced HMGB1 release. To evaluate this mechanism of SAA-mediated HMGB1 release, we examined the effects of TLR4/RAGE deficiency on SAA-induced activation of these signaling molecules. Consistent with the capacity of SAA in stimulating NLRP3 inflammasome activation (21,44), we observed that SAA significantly upregulated PKR expression (Figures 4A, B) and induced the phosphorylation of both PKR (Figure 4A) and STAT1 (Figure 4C). However, the disruption of TLR4 led to impairment of SAA-induced phosphorylation of PKR (Figure 4B) and STAT1 (Figure 4C). Because it only partly reduced SAA-induced PKR upregulation (Figure 4B), it is therefore plausible that another receptor(s) participates in SAA-induced PKR upregulation. Double deletion of both TLR4 and RAGE significantly impaired SAA-induced PKR upregulation (Figure 4B), suggesting similarly important roles of these receptors in SAA-induced PKR expression and HMGB1 release.

To verify the role of PKR phosphorylation in SAA-induced HMGB1 release, we examined the effects of two classes of PKR kinase inhibitors on PKR phosphorylation and HMGB1 release. At the concentrations that almost completely abolished SAA-induced PKR phosphorylation, 2-AP and C16 also effectively attenuated SAA-induced STAT1 phosphorylation (Figure 5A). However, at the concentrations that dramatically suppressed PKR and STAT1 phosphorylation, these PKR kinase inhibitors also inhibited SAA-induced HMGB1 release (Figure 5B), supporting an important role for PKR phosphorylation in SAA-induced HMGB1 release.

Characterizing SAA-Neutralizing Polyclonal Antibodies

SAA contains several distinct functional domains respectively responsible for: (a) binding to HDL (D1, residue 1–28) (45); (b) inhibiting immune cell adhesion (D2, residue 29–42); (c) as-yet-undefined function (D3, residue 43–76); (d) a protease cleavage site between residues 76 and 77 (46); and (e) activating
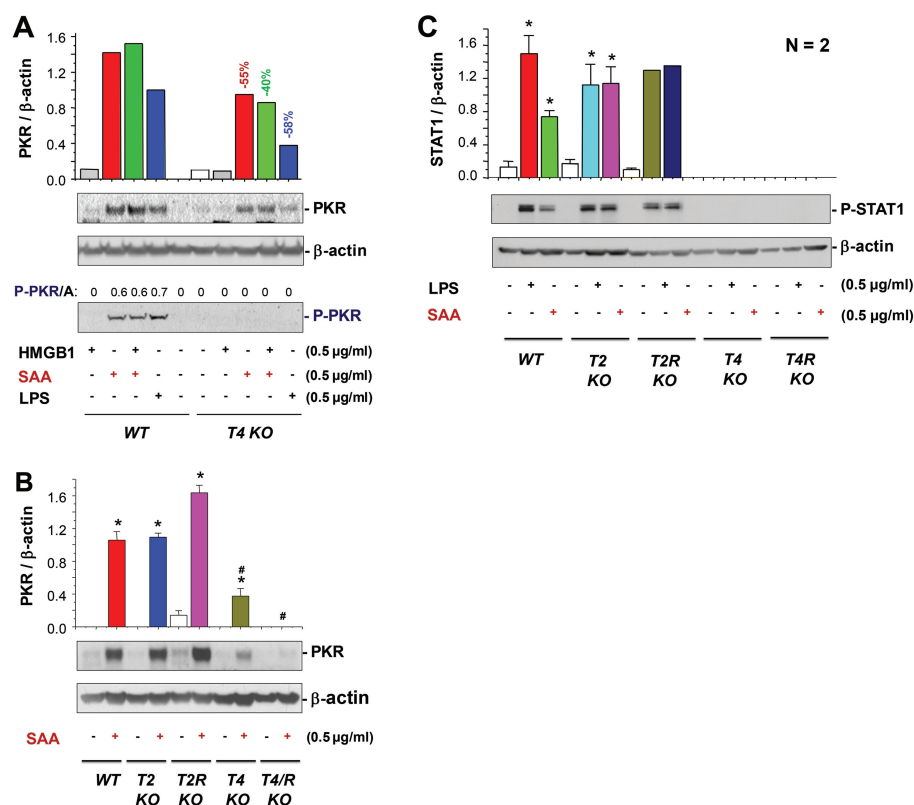
Figure 4. Divergent roles of TLR4 and RAGE receptors in SAA-induced expression and phosphorylation of PKR and STAT1. Thioglycollate-elicited primary macrophages were isolated from wild-type or mutant mice deficient in TLR2, TLR4, TLR2/RAGE or TLR4/RAGE. After stimulation with LPS, HMGB1 or huSAA for 16 h, the intracellular levels of total PKR (A, B), phosphorylated PKR (A) or STAT1 (C) were determined by Western blotting analysis. The disruption of TLR4 abolished SAA-induced PKR and STAT1 phosphorylation, but only partly impaired SAA-induced PKR upregulation. The concurrent knockout of both TLR4 and RAGE completely abrogated SAA-induced PKR upregulation. *P < 0.05 versus –SAA or –LPS controls; #P < 0.05 versus WT + SAA.

Figure 5. Inhibition of PKR phosphorylation partly impaired SAA-induced HMGB1 release. Thioglycollate-elicited peritoneal macrophages were stimulated with crude LPS or SAA for 16 h in the absence or presence of PKR kinase inhibitors (2-AP and C16), and the cellular levels of phosphorylated PKR and STAT1 (A) and HMGB1 (B) were determined by Western blotting analysis. *P < 0.05 versus –SAA or –LPS controls; #P < 0.05 versus +SAA or +LPS.

T cells (D4, residue 77–104; Figure 6A). Specifically, the D2 domain contains the YIGSD laminin-related and RGN fibronectin-related motifs that may be responsible for inhibiting T cell and platelet adhesion to extracellular matrixes (47,48). The cleavage between 76-77 residues leads to the liberation of: 1) the 8.5-kDa amyloid A (AA) that integrate into pathogenic amyloid aggregates; and 2) the 3.5-kDa fragment (D4) that may activate CD4 T cells to produce proinflammatory cytokines (for example, IFN-γ) (49). To understand the roles of SAA in lethal sepsis, we generated human SAA-specific polyclonal antibodies in rabbits, and characterized their SAA-reacting and -neutralizing

properties. Consistent with recent findings that SAA autoantibodies were detected in both healthy individuals and patients with various autoimmune diseases (50,51), we found that before immunization, all rabbits produced small amounts of anti-SAA autoantibodies, which were immunoreactive to human, and fortunately not to murine, SAAs (data not shown). After repetitive immunizations, the titers of anti-SAA IgGs were dramatically elevated in most rabbits (for example, R1 and R3), but remained extremely low in others (for example, R2; Figure 6B). Dot blotting analysis revealed that these SAA-reacting IgGs recognized several overlapping peptides (for example, P7, P8
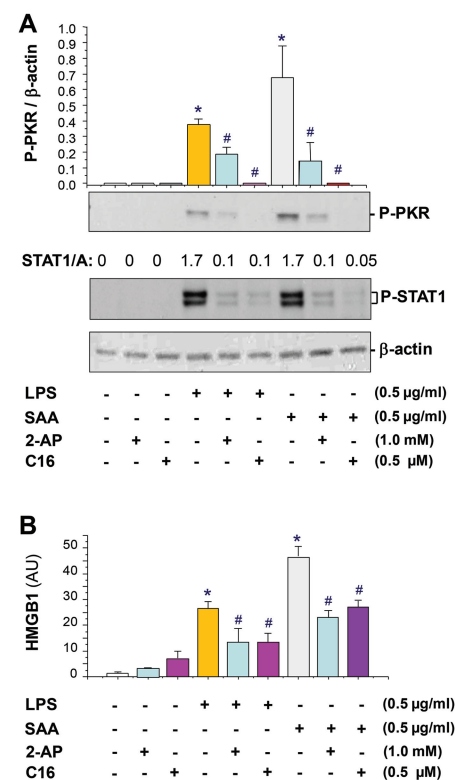
and P18), that corresponded to the SAA D1/2 and D4 domains (Figure 6C). Notably, most SAA-reacting IgGs (R1 and R3) efficiently impaired SAA-induced HMGB1 release (Figure 6D), whereas these neutralizing activities were impaired by a preincubation with excessive amounts of cross-reacting peptide (for example, P8, Figure 6D), suggesting that the neutralization depended on specific antigen interactions.

Roles of SAA in Animal Models of Lethal Endotoxemia and Sepsis

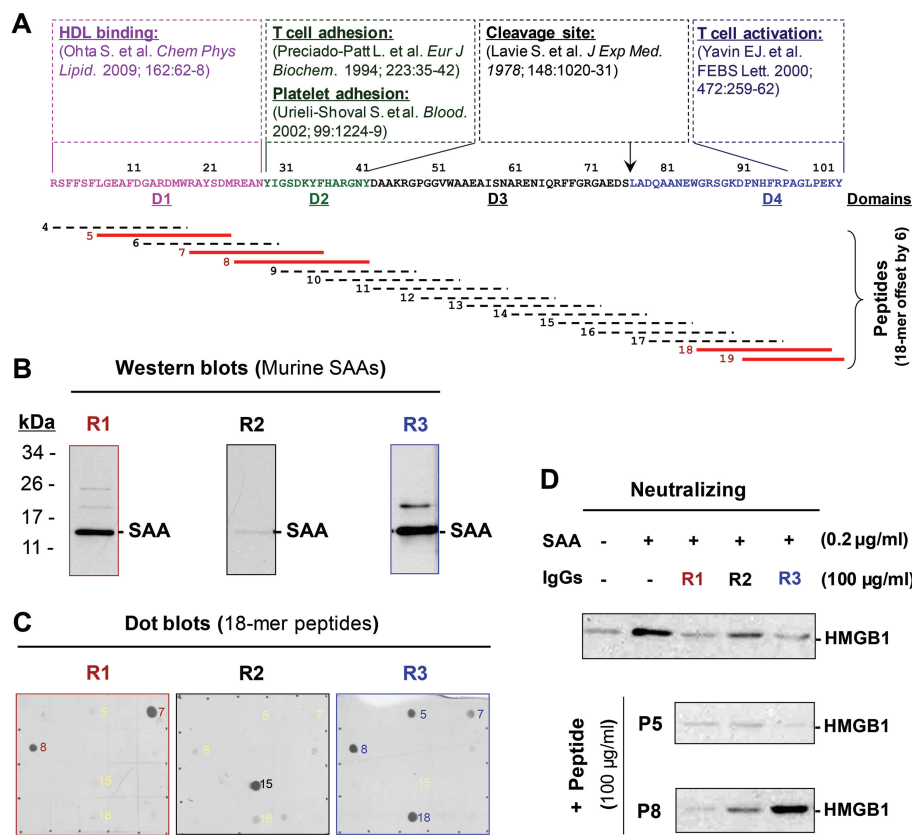Given the capacity of SAA in activating PKR and inducing NO and HMGB1

Figure 6. Characterization of SAA-specific polyclonal antibodies. (A) Sequence of synthetic SAA peptides. The reported functional domains were marked. (B) Western blotting assay of SAA immunoreactivities. Blood was collected from septic mice at 24 h after CLP, and serum proteins (1.0 µL/lane) were resolved by SDS-PAGE and immunoblotted with total IgGs isolated from serum of SAA-immunized rabbits (R1, R2, R3). (C) Dot blot epitope mapping. Synthetic peptides (P4–P19) were sequentially dotted on nitrocellulose membranes, and each membrane was respectively immunoblotted with anti-SAA IgGs from each rabbit. (D) Effect of anti-SAA IgGs on SAA-induced HMGB1 release. Macrophages were stimulated with SAA in the absence or presence of different (R1, R2, R3) IgGs, and HMGB1 release was assayed by Western blotting. In parallel experiments, anti-SAA IgGs were premixed with excessive amounts of different peptides, and the SAA-neutralizing activities were assessed. References cited in (A): Ohta S, *et al.* (45); Lavie G, *et al.* (46); Preciado-Patt L, *et al.* (47); Urieli-Shoval S, *et al.* (48); Yavin EJ, *et al.* (49).

Figure 7. Divergent effects of SAA and SAA-neutralizing IgGs on lethal endotoxemia and sepsis. (A) SAA exacerbated LPS-induced animal lethality. Balb/C mice were given LPS (5 mg/kg, i.p.) either alone or in combination with SAA (0.9 or 2.8 mg/kg) to determine their effects on animal survival rates. *P* < 0.05 versus saline (LPS alone) control. (B) SAA-specific IgGs protected mice against lethal endotoxemia. Balb/C mice were given LPS (15 mg/kg) in combination with either control IgGs (50 mg/kg, R2) or SAA-neutralizing IgGs (R1 and R3, 50 mg/kg) at +0.5 and +24 h after LPS, and animal survival rates were monitored for <2 wks. *P* < 0.05 versus control IgGs. (C) SAA-neutralizing IgGs rescued mice from lethal sepsis. Balb/C mice were subjected to sepsis by CLP and irrelevant ("control," 50 mg/kg) or SAA peptide–specific IgGs (50 mg/kg, R1 or R3) were given at +6, +24 and +48 h after CLP surgery. Shown in the figure was a summary of two independent experiments with similar results. *P* < 0.05 versus control IgGs.

release, we sought to further understand the role of SAA in lethal systemic inflammation by using recombinant SAA protein or SAA-neutralizing IgGs. In animal models of lethal endotoxemia, concurrent administration with SAA dose dependently exacerbated LPS-induced animal lethality, significantly decreasing animal survival rates from 90% to 60% (Figure 7A). In contrast, repetitive administration of SAA-neutralizing IgGs (at +0.5 and +24 h after LPS) conferred a significant protection against lethal endotoxemia, increasing animal survival from 0% to 50–75% (Figure 7B). In a clinically relevant animal model of CLP-induced sepsis, repetitive administration of SAA-neutralizing IgGs (at +6, 24 and 48 h after CLP) significantly improved animal survival rates (Figure 7C), even when the first dose was given in a delayed fashion. Collectively, these findings suggested a pathogenic role for SAA in animal models of lethal endotoxemia and sepsis.
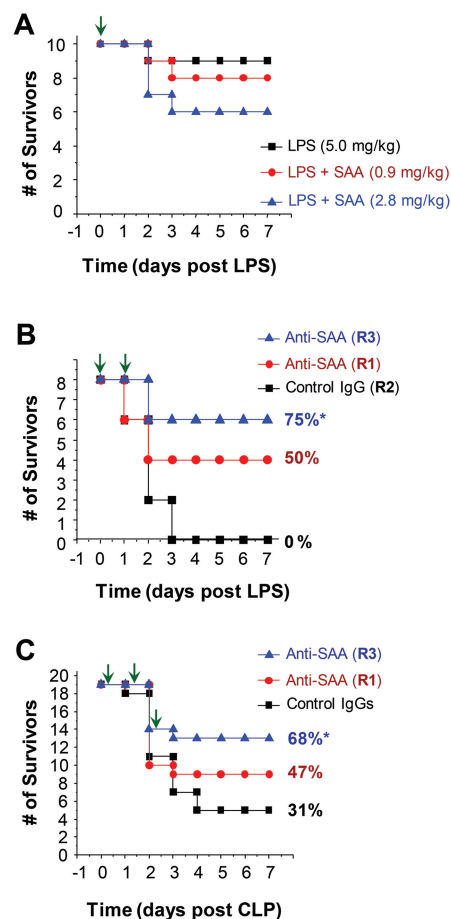
DISCUSSION

Despite high homology between human SAA and SAA1, their capacities in inducing HMGB1 release are dramatically different. The infrequently expressed SAA, unlike the most abundant SAA1, significantly induced HMGB1 release in TLR4/RAGE- and PKR-dependent mechanisms. These novel findings were consistent with several studies that echoed their dramatic differences in stimulating other cytokines/chemokines (22–25). Although SAA contained minute amount of endotoxins, this trivial contamination was not likely the underlying cause for SAA-mediated HMGB1 release, because (a) Ultrapure LPS (free from bacterial proteins and nucleic acids; catalog # tlrl-pelps, InvivoGen) fails to trigger HMGB1 release even when given up to 10 μg/mL (29,30); (b) the similarly prepared SAA1 that contained comparable amounts of endotoxin still failed to induce HMGB1 release; (c) endotoxin-neutralizing agent (for example, polymyxin B) effectively abrogated LPS-induced (0.5 μg/mL), but not SAA-induced (0.5 μg/mL), HMGB1 release; and (d) the disruption of TLR4 receptor impaired crude LPS-, but not SAA-induced, HMGB1 release. Finally, consistent with the capacity of HDL in capturing SAA (52) and blocking its chemokine activities (53), we found that HDL (catalog no. L8039; Sigma-Aldrich) effectively attenuated SAA-induced HMGB1 release (data not shown), further supporting SAA as an inducer of HMGB1 release.

SAA signals via a family of receptors (for example, TLR2, TLR4 and/or RAGE) to activate NLRP3 inflammasome (21,44) and to induce various cytokines/chemokines and NO (22–25). However, the signaling pathways leading to different proinflammatory mediators appear to be distinguishable. For instance, TLR4 is both necessary and sufficient for SAA-induced PKR and STAT1 phosphorylation and consequent NO production, reinforcing the critical role for PKR (54) and STAT1 (55) phosphorylation in iNOS upregulation (Figure 8). Notably, these PKR/STAT1-activating properties are shared by other key HMGB1 stimuli (for

example, IFN-γ and crude LPS) (27,30,32,54,56–58). Although LPS may not directly activate STAT1, it can trigger indirect STAT1 activation through intermediate production of IFNs (55) that are capable of inducing HMGB1 release (27,28,59). It is presently not yet known whether SAA activates PKR/STAT1 to induce HMGB1 release directly by itself or indirectly through inducing IFNs or other intermediates. In contrast, both TLR4 and RAGE are critically needed for SAA-mediated PKR upregulation and HMGB1 release, because double deletion of TLR4 and RAGE resulted in a complete impairment of both processes, reinforcing a critical role for PKR in the regulation of HMGB1 release (Figure 8).

Both genetic and pharmacological approaches are routinely used to assess the divergent roles of inflammatory mediators in sepsis. However, caution should be exercised when using gene knockout approaches to evaluate the pathogenic roles of any particular mediators that are still critically needed for maintaining beneficial physiological functions. For instance, despite the well-established pathogenic role of HMGB1 in infection- and injury-elicited inflammatory diseases (60,61), the disruption of HMGB1 expression adversely renders animals more susceptible to infectious (62) or injurious insults (63,64), reinforcing the dramatically distinct roles of HMGB1 in health and disease (65). Similarly, in an animal model of dextran sodium sulfate (DSS)-induced colitis, SAA1/2 knockout mice appeared to be more susceptible to colitis, possibly because the intestinal epithelia-derived SAAs are still critically needed for bactericidal activities (34). Thus, we used pharmacological approaches to evaluate the role of SAA in animal models of lethal systemic inflammation. Consistent with the notion that early PAMP-elicited early inflammatory responses might still be needed for the innate immunity against infection (66), it was previously shown that prophylactic treatment with two anti-SAA IgGs (mc29 and mc4; 1:1, beginning at 12 h before CLP) reduced animal survival rates from 60% to 20% (67). In the
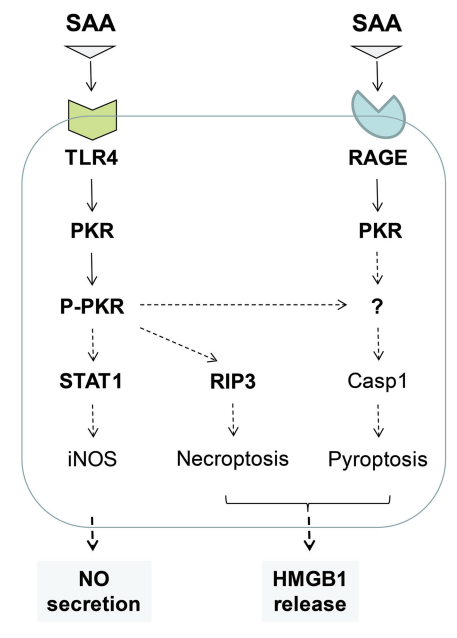

Figure 8. Proposed model for a pathogenic role of SAA in lethal sepsis. In a subset of septic patients, SAA is excessively accumulated and activates both TLR4 and RAGE signaling pathways that contribute to PKR upregulation and phosphorylation. PKR phosphorylation does not just enable STAT1 activation and the resultant iNOS expression and NO production. It is not yet known if PKR regulates HMGB1 release possibly through facilitating RIP3-dependent necroptosis or caspase 1–dependent pyroptosis. The SAA-induced excessive release of NO and HMGB1 may contribute to the pathogenesis of lethal sepsis. Future studies are needed to evaluate the influence of SAA-neutralizing antibodies on systemic accumulation of NO or HMGB1 in animal models of sepsis.

present study, we found that delayed and repetitive administration of SAA-neutralizing polyclonal antibodies conferred significant protection against lethal sepsis, suggesting a pathogenic role of SAA during a late stage of sepsis. Consistently, after lethal endotoxemia (10 mg/kg), SAA1/SAA2 knockout mice exhibited a higher lethality (ratio of animal death: KO/WT = 6/4) within 24 h, followed by a lower mortality (KO/WT = 2/5) between 24 and 48 h, supporting a pathogenic role of SAAs during a late stage of sepsis.

In septic patients (68), multiple SAA isoforms have been found including the most abundant SAA1, as well as several scarce variants such as SAA, SAA2α and SAA2β. The SAA2s differ from each other only at position 71 (H versus R), but differ from SAA1 at seven to eight other positions, indicating a possible polymorphism (69). Indeed, the frequencies of the α and β alleles at the SAA2 locus varied among different populations (for example, the Turkish versus the Azerbaijan and Kazakh) (70). At present, it is not yet known why human SAA was only detected in a subset of septic patients. Although human SAA and SAA1 differ from each other only at position 60 (D versus N) and 71 (H versus R), it will be important to investigate whether a possible polymorphism is associated with SAA and SAA1. This is important because genetic polymorphisms of proinflammatory cytokines (for example, TNF) can influence their circulating levels and consequently influence the outcomes of sepsis (71).

CONCLUSION

Here we provided some evidence to support the possibility that SAA might be expressed only in a subset of septic patients. Unlike the most abundant SAA1, SAA significantly upregulated PKR and stimulated HMGB1 release in a TLR4/RAGE-dependent fashion. Pharmacological inhibition of PKR phosphorylation inhibited SAA-induced HMGB1 release, supporting an important role of PKR in SAA-induced HMGB1 release and NO production. In animal models of lethal endotoxemia and experimental sepsis, recombinant SAA exacerbated LPS-induced animal lethality, whereas SAA-neutralizing IgGs significantly improved animal survival. Collectively, these findings have suggested a possible role of SAA as an important mediator and potential therapeutic target for lethal inflammatory diseases.

ACKNOWLEDGMENTS

DISCLOSURE

REFERENCES

1. Vincent JL, Opal SM, Marshall JC, Tracey KJ. (2013) Sepsis definitions: time for change. *Lancet.* 381:774–5.
2. Akira S, Takeda K. (2004) Toll-like receptor signalling. *Nat. Rev. Immunol.* 4:499–511.
3. Tracey KJ, *et al.* (1987) Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteraemia. *Nature.* 330:662–4.
4. Qiang X, *et al.* (2013) Cold-inducible RNA-binding protein (CIRP) triggers inflammatory responses in hemorrhagic shock and sepsis. *Nat. Med.* 19:1489–95.
5. MacMicking JD, *et al.* (1995) Altered responses to bacterial infection and endotoxic shock in mice lacking inducible nitric oxide synthase. *Cell.* 81:641–50.
6. Wang H, *et al.* (1999) HMG-1 as a late mediator of endotoxin lethality in mice. *Science.* 285:248–51.
7. Vincent JL, Zhang H, Szabo C, Preiser JC. (2000) Effects of nitric oxide in septic shock. *Am. J. Respir. Crit. Care Med.* 161:1781–5.
8. Yang H, *et al.* (2004) Reversing established sepsis with antagonists of endogenous high-mobility group box 1. *Proc. Natl. Acad. Sci. U. S. A.* 101:296–301.
9. Qin S, *et al.* (2006) Role of HMGB1 in apoptosis-mediated sepsis lethality. *J Exp. Med.* 203:1637–42.
10. Ramadori G, Sipe JD, Dinarello CA, Mizel SB, Colten HR. (1985) Pretranslational modulation of acute phase hepatic protein synthesis by murine recombinant interleukin 1 (IL-1) and purified human IL-1. *J. Exp. Med.* 162:930–42.
11. Urieli-Shoval S, Meek RL, Hanson RH, Eriksen N, Benditt EP. (1994) Human serum amyloid A genes are expressed in monocyte/macrophage cell lines. *Am. J. Pathol.* 145:650–60.
12. Hudgins LC, *et al.* (2003) A single intravenous dose of endotoxin rapidly alters serum lipoproteins and lipid transfer proteins in normal volunteers. *J. Lipid Res.* 44:1489–98.
13. McAdam KP, Sipe JD. (1976) Murine model for human secondary amyloidosis: genetic variability of the acute-phase serum protein SAA response to endotoxins and casein. *J. Exp. Med.* 144:1121–7.
14. Liuzzo G, *et al.* (1994) The prognostic value of C-reactive protein and serum amyloid a protein in severe unstable angina. *N. Engl. J. Med.* 331:417–24.
15. Niederau C, Backmerhoff F, Schumacher B, Niederau C. (1997) Inflammatory mediators and acute phase proteins in patients with Crohn's disease and ulcerative colitis. *Hepatogastroenterology.* 44:90–107.
16. Ng PC, *et al.* (2010) Host-response biomarkers for diagnosis of late-onset septicemia and necrotizing enterocolitis in preterm infants. *J. Clin. Invest.* 120:2989–3000.
17. Yan SD, *et al.* (2000) Receptor-dependent cell stress and amyloid accumulation in systemic amyloidosis. *Nat. Med.* 6:643–51.
18. Cheng N, He R, Tian J, Ye PP, Ye RD. (2008) Cutting edge: TLR2 is a functional receptor for acute-phase serum amyloid A. *J. Immunol.* 181:22–6.
19. He RL, *et al.* (2009) Serum amyloid A induces G-CSF expression and neutrophilia via Toll-like receptor 2. *Blood.* 113:429–37.
20. Sandri S, *et al.* (2008) Is serum amyloid A an endogenous TLR4 agonist? *J. Leukoc. Biol.* 83:1174–80.
21. Niemi K, *et al.* (2011) Serum amyloid A activates the NLRP3 inflammasome via P2X7 receptor and a cathepsin B-sensitive pathway. *J. Immunol.* 186:6119–28.
22. Patel H, Fellowes R, Coade S, Woo P. (1998) Human serum amyloid A has cytokine-like properties. *Scand. J. Immunol.* 48:410–8.
23. Song C, *et al.* (2009) Serum amyloid A induction of cytokines in monocytes/macrophages and lymphocytes. *Atherosclerosis.* 207:374–83.
24. Sandri S, *et al.* (2008) Serum amyloid A induces CCL20 secretion in mononuclear cells through MAPK (p38 and ERK1/2) signaling pathways. *Immunol. Lett.* 121:22–6.
25. Cai H, *et al.* (2007) Serum amyloid A induces monocyte tissue factor. *J. Immunol.* 178:1852–60.
26. Ivanov S, *et al.* (2007) A novel role for HMGB1 in TLR9-mediated inflammatory responses to CpG-DNA. *Blood.* 110:1970–81.
27. Rendon-Mitchell B, *et al.* (2003) IFN-gamma induces high mobility group box 1 protein release partly through a TNF-dependent mechanism. *J. Immunol.* 170:3890–7.
28. Lu B, *et al.* (2014) JAK/STAT1 signaling promotes HMGB1 hyperacetylation and nuclear translocation. *Proc. Natl. Acad. Sci. U.S.A.* 111:3068–73.
29. Lamkanfi M, *et al.* (2010) Inflammasome-dependent release of the alarmin HMGB1 in endotoxemia. *J. Immunol.* 185:4385–92.
30. Lu B, *et al.* (2012) Novel role of PKR in inflammasome activation and HMGB1 release. *Nature.* 488:670–4.
31. Hett EC, *et al.* (2013) Chemical genetics reveals a kinase-independent role for protein kinase R in pyroptosis. *Nat. Chem. Biol.* 9:398–405.
32. Thapa RJ, *et al.* (2013) Interferon-induced RIP1/RIP3-mediated necrosis requires PKR and is licensed by FADD and caspases. *Proc. Natl. Acad. Sci. U.S.A.* 110:E3109–18.
33. Yang H, *et al.* (2010) A critical cysteine is required for HMGB1 binding to toll-like receptor 4 and ac-

tivation of macrophage cytokine release. *Proc. Natl. Acad. Sci. U.S.A.* 107:11942–7.

34. Eckhardt ER, *et al.* (2010) Intestinal epithelial serum amyloid A modulates bacterial growth in vitro and pro-inflammatory responses in mouse experimental colitis. *BMC Gastroenterol.* 10:133. doi: 10.1186/1471–230X-10–133.

35. Zhu S, *et al.* (2009) Spermine protects mice against lethal sepsis partly by attenuating surrogate inflammatory markers. *Mol. Med* 15:275–82.

36. Li W, *et al.* (2007) A major ingredient of green tea rescues mice from lethal sepsis partly by inhibiting HMGB1. *PLoS One.* 2:e1153.

37. Zhang Y, *et al.* (2012) Tanshinone IIA sodium sulfonate facilitates endocytic HMGB1 uptake. *Biochem. Pharmacol.* 84:1492–500.

38. Li W, *et al.* (2011) A hepatic protein, fetuin-A, occupies a protective role in lethal systemic inflammation. *PLoS One.* 6:e16945.

39. Li W, *et al.* (2011) EGCG stimulates autophagy and reduces cytoplasmic HMGB1 levels in endotoxin-stimulated macrophages. *Biochem. Pharmacol.* 81:1152–63.

40. Li W, *et al.* (2007) A cardiovascular drug rescues mice from lethal sepsis by selectively attenuating a late-acting proinflammatory mediator, high mobility group box 1. *J. Immunol.* 178:3856–64.

41. Levy MM, *et al.* (2003) 2001 SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions Conference. *Crit. Care Med.* 31:1250–6.

42. Li W, *et al.* (2012) Use of animal model of sepsis to evaluate novel herbal therapies. *J. Vis. Exp.* (62):e3926.

43. Chen ES, *et al.* (2010) Serum amyloid A regulates granulomatous inflammation in sarcoidosis through toll-like receptor-2. *Am. J. Respir. Crit. Care Med.* 181:360–73.

44. Ather JL, *et al.* (2011) Serum amyloid A activates the NLRP3 inflammasome and promotes Th17 allergic asthma in mice. *J. Immunol.* 187:64–73.

45. Ohta S, *et al.* (2009) Defining lipid-binding regions of human serum amyloid A using its fragment peptides. *Chem. Phys. Lipids.* 162:62–8.

46. Lavie G, Zucker-Franklin D, Franklin EC. (1978) Degradation of serum amyloid A protein by surface-associated enzymes of human blood monocytes. *J. Exp. Med.* 148:1020–31.

47. Preciado-Patt L, *et al.* (1994) Inhibition of cell adhesion to glycoproteins of the extracellular matrix by peptides corresponding to serum amyloid A: toward understanding the physiological role of an enigmatic protein. *Eur. J. Biochem.* 223:35–42.

48. Urieli-Shoval S, *et al.* (2002) Adhesion of human platelets to serum amyloid A. *Blood.* 99:1224–9.

49. Yavin EJ, *et al.* (2000) Serum amyloid A-derived peptides, present in human rheumatic synovial fluids, induce the secretion of interferon-gamma by human CD(4)(+) T-lymphocytes. *FEBS Lett.* 472:259–62.

50. Lakota K, *et al.* (2011) Could antibodies against serum amyloid A function as physiological regulators in humans? *Autoimmunity.* 44:149–58.

51. Lakota K, *et al.* (2011) Antibodies against acute phase proteins and their functions in the pathogenesis of disease: a collective profile of 25 different antibodies. *Autoimmun. Rev.* 10:779–89.

52. Hoffman JS, Benditt EP. (1982) Secretion of serum amyloid protein and assembly of serum amyloid protein-rich high density lipoprotein in primary mouse hepatocyte culture. *J. Biol. Chem.* 257:10518–22.

53. Badolato R, *et al.* (1994) Serum amyloid A is a chemoattractant: induction of migration, adhesion, and tissue infiltration of monocytes and polymorphonuclear leukocytes. *J. Exp. Med.* 180:203–9.

54. Hsu LC, *et al.* (2004) The protein kinase PKR is required for macrophage apoptosis after activation of toll-like receptor 4. *Nature.* 428:341–5.

55. Ohmori Y, Hamilton TA. (2001) Requirement for STAT1 in LPS-induced gene expression in macrophages. *J. Leukoc. Biol.* 69:598–604.

56. Decker T, Kovarik P. (2000) Serine phosphorylation of STATs. *Oncogene.* 19:2628–37.

57. He S, Liang Y, Shao F, Wang X. (2011) Toll-like receptors activate programmed necrosis in macrophages through a receptor-interacting kinase-3-mediated pathway. *Proc. Natl. Acad. Sci. U. S. A.* 108:20054–9.

58. Nystrom S, *et al.* (2013) TLR activation regulates damage-associated molecular pattern isoforms released during pyroptosis. *EMBO J.* 32:86–99.

59. Kim JH, *et al.* (2009) Bacterial endotoxin induces the release of high mobility group box 1 via the IFN-beta signaling pathway. *J. Immunol.* 182:2458–66.

60. Lu B, *et al.* (2014) Molecular mechanism and therapeutic modulation of high mobility group box 1 release and action: an updated review. *Expert. Rev. Clin. Immunol.* 10:713–27.

61. Wang H, Ward MF, Sama AE. (2014) Targeting HMGB1 in the treatment of sepsis. *Expert Opin. Ther. Targets.* 18:257–68.

62. Yanai H, *et al.* (2013) Conditional ablation of HMGB1 in mice reveals its protective function against endotoxemia and bacterial infection. *Proc. Natl. Acad. Sci. U. S. A.* 110:20699–704.

63. Huang H, *et al.* (2014) Hepatocyte-specific high-mobility group box 1 deletion worsens the injury in liver ischemia/reperfusion: a role for intracellular high-mobility group box 1 in cellular protection. *Hepatology.* 59:1984–97.

64. Kang R, *et al.* (2014) Intracellular Hmgb1 inhibits inflammatory nucleosome release and limits acute pancreatitis in mice. *Gastroenterology.* 146:1097–107.

65. Kang R, *et al.* (2014) HMGB1 in health and disease. *Mol. Aspects Med.* 40:1–116.

66. Yang H, *et al.* (2015) MD-2 is required for disulfide HMGB1-dependent TLR4 signaling. *J. Exp. Med.* 212:5–14.

67. Sander LE, *et al.* (2010) Hepatic acute-phase proteins control innate immune responses during infection by promoting myeloid-derived suppressor cell function. *J. Exp. Med.* 207:1453–64.

68. Steinkasserer A, Weiss EH, Schwaeble W, Linke RP. (1990) Heterogeneity of human serum amyloid A protein: five different variants from one individual demonstrated by cDNA sequence analysis. *Biochem. J.* 268:187–93.

69. Kluve-Beckerman B, Dwulet FE, Benson MD. (1988) Human serum amyloid A: three hepatic mRNAs and the corresponding proteins in one person. *J. Clin. Invest.* 82:1670–5.

70. Tastan H, Osmanagaoglu O, Tuzun A. (2005) The frequencies of the serum amyloid A2 alleles in healthy (Turkish, Azerbaijan and Kazakh) populations. *Genetika.* 41:986–9.

71. Stuber F, Petersen M, Bokelmann F, Schade U. (1996) A genomic polymorphism within the tumor necrosis factor locus influences plasma tumor necrosis factor-alpha concentrations and outcome of patients with severe sepsis. *Crit. Care Med.* 24:381–4.