BRIEF CONCLUSIVE REPORT

Effects of serum amyloid protein A on influenza A virus replication and viral interactions with neutrophils

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Abstract
Innate immunity is vital for the early control of influenza A virus (IAV) infection. Serum amyloid A (SAA1) is an acute phase reactant produced in the liver and lung that rises dramatically during IAV infection. The potential role of SAA1 in host defense against IAV is unknown. SAA1 has been reported to directly activate neutrophils and to recruit them to the lung during infectious and inflammatory processes. Neutrophils are the most abundant cell recruited to the lung in the early phase of IAV infection. There are different forms and preparations of SAA1 that have found to have different effects on phagocyte responses, through various receptors. In this paper, we test the direct effects of various preparations of serum derived or recombinant SAA on IAV and how it modulates the interactions of IAV with neutrophils. All SAA preparations bound to IAV in vitro but caused minimal hemagglutination inhibition or viral aggregation. The human serum-derived SAA1 or the complex of SAA1 with HDL did have IAV neutralizing activity in vitro, whereas the recombinant SAA1 preparations did not. We found that different SAA preparations also had markedly different effects on neutrophil functions, with E. coli-derived SAA1 triggering some responses in neutrophils on its own or in presence of IAV whereas mammalian cell-derived SAA1 did not. This discrepancy could be explained by the reported contamination of the former preparation with bacterial components. Of interest, however, serum SAA alone, serum SAA complexed with HDL, or HDL alone potentiated some neutrophil responses to IAV. Our results suggest that SAA may play some role in host response to IAV, but further work needs to be done to clarify the role of different variants of SAA alone or complexed with HDL.

KEYWORDS
HDL, influenza, serum amyloid A

1 | INTRODUCTION

The innate immune response is critical in containing influenza A virus (IAV) replication during the first few days of infection (i.e., prior to the generation of an adaptive immune response). The innate immune response to IAV is remarkably complex as recently reviewed. Our laboratory has focused principally on the role of soluble inhibitors in respiratory lining fluids and resident or recruited phagocytes. In the current study we evaluate effects of serum amyloid A (SAA1) protein on influenza replication and its interactions with phagocytes. SAA1 is an acute phase reactant and its levels increase markedly during IAV infection in humans and other animals (e.g., mice, pigs, and ferrets). It has also been recently shown to be markedly elevated in the serum of patients with COVID-19. The effects of other acute phase reactant proteins on IAV have been studied. Surfactant protein D (SP-D) strongly inhibits strains of IAV containing high mannose sugars on their hemagglutinin (HA), through lectin mediated binding to the HA sugars. Serum amyloid P (SAP) and pentraxin both inhibit IAV through a mechanism that has been termed γ-inhibition. This mechanism involves the presence of sialic acids on SAP or pentraxin, which act as decoy ligands for the viral HA. Two other innate defense proteins that inhibit IAV by this mechanism include surfactant protein...
Despite the marked elevation of SAA during influenza infection, and proposals to use it as a biomarker of severe infection, we could find no prior studies directly addressing potential anti-influenza activity. SAA has been shown to bind to hepatitis C virus and inhibit infection by this virus through blocking viral entry.\(^{12,13}\)

SAA is a highly conserved protein through evolution. Normally, the plasma level of SAA is around 1 \(\mu\)g/ml, but it can be induced up to 1000-fold during acute phase response to infections, suggesting a protective role of SAA against acute infections. SAA1 has also been demonstrated in lung lavage at concentrations up to 100 ng/ml during inflammatory conditions.\(^{34,15}\) Infection with IAV has been shown to cause marked elevation of lung SAA1 expression.\(^{15}\) SAA1 binds to HDL and is therefore considered an apolipoprotein. It is believed that SAA1 redirects cholesterol to damaged cells during acute inflammation and injury states. The common form of SAA1 is a hexamer and it binds to heparin through positively charged patches on its surface.\(^{16}\) SAA-induced amyloidosis results from massive accumulation of fragments of SAA1 in tissues. This condition occurs in chronic inflammatory states like rheumatoid arthritis and cancer.

SAA1 has been reported to have varied effects on phagocyte activation through binding to various phagocyte receptors including formyl peptide receptor 2 (FPR2), scavenger receptors, TLR2 and TLR4, and the ATP receptor P2X7. In many cases SAA1 leads to proinflammatory responses; however, anti-inflammatory effects are also reported.\(^{17}\) SAA1 has been found to act as a chemoattractant for neutrophils by binding to FPR2,\(^{18}\) and it can induce proinflammatory cytokine production by neutrophils and macrophages.\(^{14,19–21}\) SAA1 has been shown to delay neutrophil apoptosis through a mechanism involving the P2 \(\times\) 7 receptor\(^{22}\) or FPR2\(^{23}\) in different studies. The apoptosis delaying effect of SAA1 can be overridden by lipoxins, which also bind FPR2.\(^{23}\) IL-8 induction by SAA1 in monocytes was mediated by TLR2.\(^{18,24}\) Overall these results suggest that SAA1 is a pleiotropic agent mediating effects through multiple receptors. A recent paper showed that a recombinant form of Apo-SAA1 used in many studies activates TLR2 and inflammatory responses due to bacterial lipoprotein bound to the recombinant SAA1 in this preparation. In contrast, recombinant SAA1 produced in HEK cells lacked this effect.\(^{25}\) This raises the question whether some of the described proinflammatory effects of SAA are mediated by bacterial lipoproteins bound to SAA and not SAA itself. In fact, it was found that SAA3 knockout mice have a proinflammatory phenotype suggesting that SAA may be a predominantly anti-inflammatory protein.\(^{26}\) SAA3 knockout mice also had increased mortality and viral loads in response to IAV infection,\(^{26}\) suggesting either a direct or indirect role for SAA in host defense against IAV. Given the marked rise in SAA in the lung during IAV infection and the findings with SAA knockout mice we decided to test for antiviral activity of SAA.

SAA1 has been demonstrated in lung lavage of patients with COPD and its levels correlate with lung neutrophil recruitment, neutrophil elastase, and IL-8 concentrations.\(^{35}\) Human phagocytes including neutrophils, monocytes, and macrophages play important roles in host defense against IAV.\(^{27–32}\) Neutrophils predominate in the early response to IAV infection. This early neutrophilic response has been shown to contribute to viral control and also to modulation of subsequent adaptive responses in several studies.\(^{32}\) In contrast, other studies have suggested that in some settings exuberant neutrophil responses could be harmful during severe IAV infection.\(^{33}\) For these reasons we also tested the effects of SAA1 on IAV and neutrophil responses to IAV. Here again we found some discrepancies between the activities of different SAA preparations. Initial studies were done with a serum SAA preparation and the \(E.\) \(coli\)-derived recombinant Apo-SAA preparation and were started before the report on bacterial lipoproteins in the latter preparation by Burgess et al.\(^{25}\) We extended the studies to include serum SAA bound to HDL and the mammalian recombinant SAA1 preparation.

## 2 MATERIALS AND METHODS

### 2.1 Innate immune protein preparations and other reagents

The various innate immune proteins used in this study are outlined in Table 1. We tested several commercially available forms of SAA1, including native human serum SAA complexed with HDL (SAAHDL; Fisher Scientific), SAA (19-94) isolated from human serum (Abcam; Cambridge, United Kingdom, Abcam.com), recombinant SAA1 derived from \(E.\) \(coli\) (Peprotech; Rocky Hill, NJ, USA), and recombinant SAA1 derived from HEK cells (Origene, Rockville, MD, USA). The \(E.\) \(coli\)-derived form is referred to as Apo-SAA1 in this paper and has been used extensively in various studies due to its reported low endotoxin level <0.1 ng/\(\mu\)g protein (<1 endotoxin units [EU/\(\mu\)g]). However, this preparation was recently shown to contain other bacterial contaminants.\(^{25}\) The HEK cell-derived preparation is referred to as SAA1 in this paper. HDL was obtained from Sigma Corp. (St. Louis, MO) and was isolated from human plasma. The description of the various proteins used in this study is provided in Table 1. We independently tested endotoxin levels using the ToxinSensor chromogenic Limulus Amoeboocyte Lysate endotoxin assay kit from GenScript (Piscataway, NJ, USA) following manufacturer’s instructions. We tested the amount of endotoxin present in samples containing the highest concentrations of SAA or HDL proteins used in our assays (i.e., 32 \(\mu\)g/ml) to be as follows: Apo-SAA1 0.48 EU, SAA1 0.42 EU, HDL 0.07 EU, and SAA1/HDL no detectable EU (average results of 3 assays). Hence all samples had <1 EU/\(\mu\)g of protein. Wortmannin and pertussis toxin (PT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). TLR2 antibodies were purchased from Invitrogen (San Diego, CA, USA).

### 2.2 Virus preparations

Philippines 82/H3N2 (Phil82) strain was kindly provided by Dr. E. Margot Anders (University of Melbourne, Melbourne, Australia) and the PR-8 strain was kindly provided by Dr. Jon Abramson (Wake Forest University, Winston-Salem, NC, USA). These strains were grown in the chorioallantoic fluid of 10-d-old chicken eggs and purified on a discontinuous sucrose gradient as previously described.\(^{34}\) The virus...
TABLE 1  Human SAA and/or HDL proteins compared in this study

| Protein (amino acids included) | Source | Sequence | Description | Designation used in this paper |
|--------------------------------|--------|----------|-------------|--------------------------------|
| Serum amyloid A (SAA1) (19-94) | Human serum (Abcam) | RSFFSFLGAEFDGARDMWRA YSDMREANYIGSDKHYFHA GNYDAAKRGPQGVVWAE AIDARENIQRFFGHGAEDS | No N-terminal signal peptide, no C-terminus | Serum SAA |
| **SAAHDL** | Human Serum (EMD Millipore) | Serum Amyloid A Protein-Rich-High-Density Lipoprotein (262 µg/ml SAA, 1.508 µg/ml HDL) | No N-terminal signal peptide, With HDL | SAAHDL |
| Apo-SAA1 (19-122) | Recombinant E. coli (Peprotech) | MRSFFSFLGAEFDGARDMW RAYSDMREANYIGSDKHYFHA RNGYDAAKRGPQGVVWAE AIDARENIQRFFGHGAEDSLA DQAANEWGRSGKDNPNNFRPA GLPEKY | No N-terminal signal peptide | ApoSAA1 |
| **SAA1 (1-122)** | Recombinant HEK cells (Origene) | MKLTLGLVCLSVLGVSRSFF SFLGEAFGDGARDMWRYASD MREANYIGSDKHYFHAHGNYD AAKRGPGGVWAEAIASDER NQRFFGHGAEDSLADQAAAN EWGRSGKDNPNNFRPAGLPKY | Full length (contains signal peptide) | SAA1 |
| **HDL** | Human serum (Sigma-Aldrich) | | | HDL |

1 Human apolipoprotein serum amyloid A-1 gene codes for a 122 amino acid nonglycosylated polypeptide including an 18 amino acid N-terminal sequence.

was dialyzed against PBS to remove sucrose, aliquoted and stored at −80°C until needed. Post thawing the viral stocks contained ~5 × 10^8 infectious focus forming units/ml. The California 2009 H1N1 pandemic strain (Cal09) and the New York 2001 H1N1 (NY01) seasonal strain were prepared by reverse genetics and propagated in Madin Darby Canine Kidney (MDCK) cells as described.35

2.3 Binding of SAA to IAV

Binding of SAA preparations to IAV was tested by ELISA. ELISA plates were coated with 2 µg/ml IAV or 0.05% gelatin as background blocker in sodium bicarbonate buffer (15 mM Na2CO3, 35 mM NaHCO3, pH 9.6). Standard curves of SAA were also set up for each form of SAA studied by coating plates with SAA. Plates were incubated overnight at 4°C. The next morning, plates were washed three times with PBS and blocked with 1% gelatin for 3 h at 37°C. Plates were again washed three times with PBS and doses of SAA in protein buffer (PBS + 0.04% gelatin) were added to IAV or gelatin coated rows. Just protein buffer was added to the standard curves. Plates were then incubated for 30 min at 37°C. Plates were then washed with wash buffer (PBS + 0.02% Tween 20) three times. Primary detection antibodies were then added. Anti-SAA antibody (Abcam; used 1 µg/ml) was added and incubated for 30 min at 37°C. Plates were washed and secondary peroxidase conjugated donkey anti-mouse antibody was added and incubated for 30 min at 37°C. Absorbance readings were taken at 450 nm on a POLARstar optima ELISA plate reader after adding TMB is tetramethylbenzidine peroxidase and 1N H2SO4. For detection of SAA binding using the anti-Aspartic Acid, Aspartic Acid, Lysine 50 antibody. Aspartic Acid, Lysine 50 µ/well 0.1 µg/ml anti-DDK mouse monoclonal Ab, clone OT14C5 (Origene) was added in PBS + 0.04% gelatin and 0.02% Tween 20 instead of the anti-SAA antibody. For HDL binding mouse anti-HDL Ab 0.005 µg/ml (My Biosource, San Diego, California) was used.

2.4 Hemagglutination assays

Hemagglutination inhibition was measured serially diluting SAA1 in round-bottom 96-well plates (Serocluster U-Vinyl plates; Costar, Cambridge, MA, USA) using PBS as a diluent and then adding IAV to each well. After this human type O red cells were added. Hemagglutination titers were assessed by counting the number of well in which red cell pellets did not form as described.36 Hemagglutination titers were also assessed on samples taken from the viral aggregation assays. In this case aliquots of the aggregation assay samples were serially diluted followed by addition of red cells.

2.5 Fluorescent focus assay of IAV infectivity (neutralization assay)

MDCK cell monolayers were prepared in 96-well plates and grown until confluent. These layers were then infected with diluted IAV preparations for 45 min at 37°C in PBS. MDCK cells were tested for presence of IAV infected cells after 18 h of virus addition using a monoclonal antibody directed against the IAV nucleoprotein (EMD Millipore, Burlington, MA) as previously described.36 IAV was preincubated for 30 min. at 37°C with various concentrations of SAA1 or control buffer, followed by addition of these viral samples to the MDCK cells. These cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and propagated in the undifferentiated state in standard tissue culture flasks.

2.6 Measurement of viral aggregation by SAA1

Viral aggregation caused by SAA1 was measured by assessing light absorbance at 350 nM by suspensions of IAV. This was done using a Perkin Elmer Lambda 35 UV/Vis spectrophotometer as described.37
2.7 | Neutrophil preparation

Neutrophils from healthy volunteers were isolated to >95% purity by using dextran precipitation, followed by Ficoll-Paque gradient separation for the separation of mononuclear cells (layering above the Ficoll-Paque) and neutrophils (below the Ficoll-Paque). The neutrophils were purified further by hypotonic lysis to eliminate any contaminating erythrocytes, as previously described.34 Cell viability was determined to be >98% by trypan blue staining. The isolated neutrophils were resuspended at the appropriate concentrations in PBS and used within 2 h. Neutrophil collection was done with informed consent as approved by the Institutional Review Board of Boston University School of Medicine. For some studies, neutrophils were pretreated with or without PT (1 μg/ml) for 2 h, or wortmannin (1 μM) for 10 min, or TLR2 antibody (5 μg/ml) for 10 min.

2.8 | Measurement of neutrophil H₂O₂ production

H₂O₂ production was measured by assessing reduction in scopoletin fluorescence as previously described.38 In brief, neutrophils were added to a mixture of scopoletin, sodium azide, cytochalasin B, and horseradish peroxidase, which were previously shown to maximize detection of IAV induced H₂O₂. As previously reported IAV induced respiratory burst occurs at an intracellular location and superoxide anion is not detected,39 although oxygen consumption and nitroblue tetrazolium assays occur in parallel with H₂O₂ production.38 Measurements were made using a POLARstar OPTIMA fluorescent plate reader (BMG Labtech, Durham, NC, USA).

2.9 | Measurement of IAV uptake by neutrophils

FITC-labeled IAV (Phil82 strain) was prepared and uptake of virus by neutrophils was measured by flow cytometry as described.40 In brief, IAV was treated with various doses of SAA1 for 30 min at 37°C. Then it was incubated with cells for 45 min at 37°C in presence of control buffer. Trypan blue (0.2 mg/ml) was added to these samples to quench extracellular fluorescence. Following washing, the neutrophils were fixed with 1% paraformaldehyde and neutrophil associated fluorescence was measured using flow cytometry. The mean cell fluorescence (2000 cells counted per sample) was measured.

2.10 | Assessment of neutrophil IL-8 (CXCL8) production

Neutrophils were incubated with either Phil82 virus, SAA1, both IAV and HSAA1, or control buffer for 45 min in a CO₂ incubator in PBS buffer. No serum was present during the infection with virus. After this the cells were pelleted and then resuspended and cultured in RPMI 1640 with 10% heat-inactivated autologous serum for 4 h after which the supernatant was collected and assayed for IL-8 using a commercially available ELISA kit (BD Biosciences, San Diego, CA, USA) according to the manufacturer’s instructions. Cells without any stimulus were used as negative controls in the experiment.

2.11 | Measurement of intracellular calcium responses of neutrophils

Intracellular calcium responses of neutrophils was measured using the Fura-2 AM (Thermofisher Scientific). Briefly 1 × 10⁷ cells/ml are loaded with Fura-2 AM for 30 min at 37°C then washed with PBS to remove unincorporated Fura-2 AM. After this stimuli (fMLP, SAA1 alone, or IAV with or without SAA1) are added and the fluorescence signal is obtained by taking the ratio of the excitation at 340 nm and 380 nm with emissions at ~510 nm using a POLARStar OPTIMA fluorescent plate reader (BMG Labtech). fMLP was used as a control and the final concentration used was 10⁻⁷ M.

2.12 | Neutrophil caspase 3 assay

Human neutrophils were treated with indicated proteins for 5 h. For experiments including two peptides, the peptides were pre-incubated together for 30 min at 37°C prior to the experiments. Samples were then centrifuged for 5 min at 400 xg, and pellets were collected. Collected cells were then washed with PBS and lysed with lysis buffer. The lysis buffer contained Triton X 100 at 2% of assay medium. Caspase 3 activity of each sample was measured with EnzChek Caspase-3 Assay Kit (Invitrogen Molecular Probes). Ac-aspartyl-glutamylvalyl-aspartine-CHO inhibitor was provided in the kit to ensure the observed signal is due to the activity of caspase-3-like proteases.

2.13 | Statistics

Statistical comparisons were made using Student’s paired, 2-tailed t-test or ANOVA with post hoc test (Tukey’s). ANOVA was used for multiple comparisons to a single control.

3 | RESULTS

3.1 | Direct interactions of SAA with IAV

We tested binding of the SAA preparations to IAV by ELISA. We included two recombinant SAAs called Apo-SAA1 and SAA1, which were produced, respectively, in E.coli and HEK cells. As shown in Figure 1A, the two recombinant SAA1 preparations and serum SAA-HDL bound to the Phil 82 strain of IAV. To exclude carbohydrate dependence of SAA binding to IAV we tested the effect of maltose on binding using SAA1 and found no effect (Fig. 1B). Note that SAA1 (HEK-derived recombinant preparation) showed somewhat less binding than the other two preparations. Because SAA1 has a DDK tag we also tested binding using and anti-DDK antibody and this confirmed binding of SAA1 to IAV (Fig. 1C). In fact, using this method binding of SAA1 to IAV was comparable to binding of the other preparations. In contrast, HDL alone did not bind to IAV (Fig. 1D).

We next tested various preparations of SAA for viral neutralizing activity. For these studies we included a human serum-derived purified SAA preparation containing amino acids 1–76 of the mature peptide and without the C-terminal tail. Human serum SAA caused dose-related inhibition of infectivity of the Phil82 and PR-8 strains of IAV in
FIGURE 1  Binding of SAA preparations to influenza A virus (IAV)—Binding of SAAs to the Phil82 strain of IAV was tested by ELISA as described in Section 2 (“Materials And Methods”) using an anti-serum amyloid A (anti-SAA1) antibody (panel A). Binding of SAA1 was also tested in presence of maltose (panel B) and using an anti-DDK antibody for detection (SAA1 has a DDK tail) (panel C). HDL binding to IAV was tested as well (panel D). 

MDCK cells (Fig. 2A). Unfortunately we were not able to do additional experiments with this preparation because it was no longer produced by Abcam after we completed these assays. The serum SAAHDL preparation also caused neutralization of several strains of IAV (Fig. 2B), whereas HDL alone did not (Fig. 2C). For these assays we included two additional viral strains, including two grown in MDCK cells rather than eggs (Cal09 and NY01) to be sure that the observed inhibition of infectivity could be seen in various viral strains including those prepared under different condition. The Cal09 H1N1 strain is of particular interest because it represents the 2009 pandemic H1N1 and is not inhibited by other innate inhibitors like SP-D and LL-37. {41,42} In fact HDL alone cause significant increases in viral infectivity in these assays for two of the strains. We next tested activity of the recombinant SAA1 preparations Apo-SAA1 and SAA1 (Fig. 2D) with the Phil82 strain of IAV, which was strongly inhibited by the serum SAA preparations. In contrast to the serum preparations, the recombinant preparations had no viral neutralizing activity for IAV (Fig. 2D).

We next tested if SAA preparations were able to induce viral aggregation. Using a light transmission assay we found a slight amount of viral aggregation induced by SAA1 but not by the Apo-SAA1 or SAAHDL (or HDL alone) (Fig. 3A). For comparison we included SP-D, which is strong aggregator of IAV. We measured HA titers on the virus samples treated with SAAs used in the aggregation assays. SAA1 caused some slight aggregation and reduction of HA titer on the treated samples but Apo-SAA1, SAAHDL and HDL did not (Fig. 3B).

3.2  Effects of SAA on neutrophil responses either directly of when combined with IAV

IAV alone caused H₂O₂ generation by neutrophils (Fig. 4) as previously reported. Apo-SAA1 caused dose-related increases in these responses (Fig. 4A). SAA1 did not cause any similar increase (Fig. 4B). SAAHDL caused a slight increase in the IAV-induced H₂O₂ response at the highest concentration tested (10 µg/ml) (Fig. 4C). Unexpectedly, HDL alone caused a significant increase in the H₂O₂ response to IAV. The SAA and/or HDL preparations (apart from the Abcam serum SAA1, which was not available to test) did not induce neutrophil H₂O₂ generation on their own (i.e., in absence of IAV; data not shown; n = 3). We also tested if pre-incubation of IAV with SAAs increased the ability of neutrophils to take up the virus (Fig. 5). The only SAA preparation to
Neutrophil intracellular calcium release is a precursor to neutrophil activation for many membrane-acting stimuli. As shown in Figure 6 (with comparison to the classic neutrophil stimulus, fMLP), Apo-SAA1, SAAHDL, and HDL alone caused rises in neutrophil intracellular calcium as measured by Fura-2 fluorescence. In contrast, no calcium response was elicited by SAA1. IAV alone triggered intracellular calcium release as previously reported. The peak IAV induced response was increased by pre-incubating the virus with Apo-SAA1, SAAHDL, or HDL but not by SAA1 (Fig. 7). The Apo-SAA1 also stimulated neutrophil IL-8 (CXCL8) production in the absence of IAV (Fig. 8A), whereas SAAHDL did not and SAA1 caused a minimal response only at the highest concentration tested. For these experiments an OD of 1 was equal to 800 pg/ml and an OD of 0.65 was equal to 400 pg/ml of IL-8. We also tested the effects of lower concentrations of Apo-SAA1 alone or in combination with IAV (Fig. 8B). As previously reported IAV alone caused robust production of IL-8. Apo-SAA1 had additive effect with IAV at the highest concentration of Apo-SAA1 tested in this assay. We assessed the role of TLR2 receptors or other signaling mechanisms in the neutrophil responses triggered by Apo-SAA1 as shown in Table 2. Apo-SAA1 again appeared to increase neutrophil H2O2 responses to IAV and neutrophil uptake of IAV and these effects were partially or fully blocked by anti-TLR2 antibodies (significant for uptake assay). PT and wortmannin inhibited direct calcium responses of neutrophils to Apo-SAA1.

To test the effects of SAA on neutrophil apoptosis we measured neutrophil caspase 3 activity. IAV alone caused acceleration of neutrophil apoptosis as previously described (Fig. 8C). Apo-SAA and SAAHDL did not alter caspase 3 activation caused by IAV. However, when IAV was pre-incubated with SAA1 or HDL the virus no longer caused a significant increase in caspase 3 activation as compared to control media.

4 | DISCUSSION

We show for the first time that various preparations of SAA bind to IAV. Binding was consistently found for the three SAA preparations tested and not inhibited by maltose. Viral binding by the SAAHDL preparation...
could not be accounted for by the HDL component because HDL alone did not bind. We also demonstrated viral neutralization for the serum SAA preparations (alone or combined with HDL), although not for the two recombinant SAAs. Most of the experiments in this study involved the seasonal Phil82 H3N2 virus, which we have characterized extensively in terms of its glycans structure\textsuperscript{44} and interactions with neutrophils.\textsuperscript{45} However, we confirmed neutralizing activity found with serum SAA and SAAHDL against several other strains as well (including a pandemic strain), because this was a novel finding. Note that again the HDL component in SAAHDL could not account for antiviral activity because HDL alone actually increased viral infectivity in this assay. The mechanism through which HDL increased viral infectivity is not clear at this point and requires further study, but note that it also increased neutrophil $H_2O_2$ and calcium responses to the virus. HDL has been reported to reduce some proinflammatory effects of SAA (e.g., activation of inflammasomes)\textsuperscript{46} so it is of interest that it did not block SAAs viral neutralizing activity in this study.

We cannot at present explain the differences in direct antiviral activity of the serum and recombinant preparations, although prior studies of proinflammatory activities have found some differences between serum-derived SAA1 and recombinant SAA1,\textsuperscript{17,47} The various SAA preparations differ in length (i.e., the serum SAA preparation obtained from Abcam is 76 amino acids and lacks the C-terminus) and the two recombinant preparations are of different lengths (103 for Apo-SAA1 and 122 for SAA1). The HEK cell-derived SAA1 contains the signal peptide, which would be cleaved in vivo. These features could possibly have affected observed antiviral activity. In any case the observed antiviral activity of the serum preparations suggests a possible role of SAA in limiting viral replication, which may in part account for the finding of more severe IAV infection in SAA3 knockout mice.\textsuperscript{26}

As noted in the introduction, there have been divergent findings regarding the ability of SAA to activate phagocytes.\textsuperscript{17} A striking example of this is a recent report by Zhou et al. in which a fragment of SAA composed of amino acids 11–58 had anti-inflammatory activity, including suppression of neutrophil recruitment and proinflammatory cytokine responses to LPS.\textsuperscript{48} SAA11-58 also did not induce chemotaxis or calcium flux through FPR2. Christenson et al. recently found that endogenous serum SAA did not have direct activity to activate neutrophils based on use of SAA-rich serum from arthritis patients.\textsuperscript{47} Many recent studies have used the Apo-SAA1 recombinant preparation because it has minimal endotoxin contamination. However, the recent study by Burgess et al. showed that this preparation is contaminated with other bacterial products.\textsuperscript{25} We began our studies with Apo-SAA1 prior to that report and basically confirmed and extended on their findings.

We found that the Apo-SAA1 had numerous neutrophil activating effects either alone or in combination with IAV. On its own it induced neutrophil intracellular calcium release and IL-8 production (the latter was also reported by Burgess et al.).\textsuperscript{25} Apo-SAA1 also increased neutrophil respiratory burst and calcium responses to IAV and increased neutrophil uptake of the virus. In contrast, none of these effects were seen with HEK cell-derived SAA1. We think the most likely reason for these differences is the presence of bacterial lipoprotein products in Apo-SAA1, likely due to preparation in E. coli. Experiments with antibodies to TLR2 and metabolic inhibitors were consistent with this interpretation. Note that the HEK cell-derived preparation contains the signal peptide of the protein, which could have impacted on results and future studies using mammalian recombinant or serum-derived SAA1 without the signal protein will be important to fully understand its biologic activities. In addition, we did note variation in neutrophil responses among our assays and also variation in neutrophil stimulating effects of different virus stocks used. These variations are commonly observed even with highly consistent methodology but should be taken into account when comparing results of neutrophil respiratory burst assays especially. Nonetheless, Apo-SAA1 differed from the other preparations in a range of neutrophil assays and the results remain consistent with presence of bacterial products in this preparation. Because SAA1 is known to bind bacterial products, rigorous studies of its effects with and without such products will be of interest (e.g.,
the effects of SAA1 combined to bacteria or viruses may also be relevant in vivo).

The situation becomes more complicated when we evaluate SAAHDL, which did cause increases in neutrophil intracellular calcium response alone or with IAV and also slight increases in respiratory burst response to IAV. However, in this case these responses were also found with HDL alone. In fact, the responses were more obvious with HDL alone. It may be, therefore, that the neutrophil effects of SAAHDL could be accounted for by the HDL component. The responses to HDL were unexpected and further research will need to be done to explain them. HDL binds to scavenger receptors, which could mediate responses of phagocytes.

SAA has been reported to inhibit apoptosis of various cells including neutrophils and dendritic cells. We have previously reported that IAV accelerates apoptosis of neutrophils and that this effect is linked to respiratory burst activation. We here show that SAA1 and HDL were able to blunt the caspase 3 activating effect of IAV, whereas Apo-SAA1 and SAAHDL were not. In the case of Apo-SAA1 as compared to SAA1 (HEK cell derived), this might reflect increased activation of the cells by the former preparation. Further research on effects

**FIGURE 4** Stimulation of neutrophil hydrogen peroxide production by SAA and or HDL—Neutrophil $\text{H}_2\text{O}_2$ production was measured through decline in scopoletin fluorescence as described in Section 2 (“Materials And Methods”). Influenza A virus (IAV; Phil82 strain) was pre-incubated with the indicated concentrations of SAA and/or HDL preparations and added to suspensions of neutrophils at time 0. Results of unstimulated cells (PBS) are included for comparison. Instances where serum amyloid A (SAA1) proteins increased IAV-induced $\text{H}_2\text{O}_2$ production are noted by * or ** in legend, where * = $P < 0.05$ and ** = $P < 0.01$ compared to control. Results represent mean ± SEM for 3 to 5 experiments

**FIGURE 5** Effects of SAA and or HDL on neutrophil uptake of influenza A virus (IAV)—FITC-labeled IAV (Phil82 strain) was pre-incubated with the indicated concentrations of SAA preparations and then added to neutrophils and viral uptake was measured by flow cytometry as described, using trypan blue to quench extracellular fluorescence. Significant increases in viral uptake are indicated by * = $P < 0.05$, ** = $P < 0.01$ and *** = $P < 0.001$. Results represent mean ± SEM for 3 to 5 experiments.
**Calcium Flux without Virus**

**FIGURE 6** Effects of SAA and or HDL on neutrophil calcium metabolism—Calcium flux caused by SAA and or HDL preparations were assessed by Fura-2 fluorescence. Responses to FMLP were tested in parallel as a positive control. Increases in calcium responses compared to PBS control alone are indicated by * symbols in the legend, where * $= P < 0.05$ and ** $= P < 0.01$. Results represent mean ± SEM for 3 to 5 experiments.

**Calcium Flux**

**FIGURE 7** Effects of SAA and or HDL on neutrophil calcium response to influenza A virus (IAV)—Intracellular calcium flux was measured with Fura-2 fluorescence as in Figure 6. In these experiences, IAV alone or IAV pre-incubated with the indicated concentrations of SAA and or HDL preparations were added to neutrophils. Instances where the IAV induced calcium response was significantly further increased by SAA and or HDL are indicated by * symbols in the legend. * $= P < 0.05$ and ** $= P < 0.01$ compared to virus alone. Results represent mean ± SEM for 3 to 5 experiments.
of SAA1 (not associated with bacterial products) on neutrophil apoptosis will be of interest.

As noted in the introduction, serum SAA rises markedly during IAV infection and expression in the lung is also increased in this context. Despite this, to date the physiologic role of SAA during IAV infection has been unclear. We provide some evidence that serum forms of SAA have some antiviral activity and can modulate neutrophil responses to the virus. Clearly considerable uncertainty remains regarding whether SAA truly has proinflammatory activity, but when we exclude results obtained with the Apo-SAA1 preparation we were not able to show modulating effects of SAA alone on neutrophils. Prior studies of the proinflammatory effects of SAA should be reevaluated in view of the recent finding by Burgess et al. of bacterial contamination in the commonly used Apo-SAA1 preparation.25 Recent findings with SAA3 knockout mice suggest that at least that form of SAA has predominantly anti-inflammatory effects.26 It is tempting to speculate that the marked rise in SAA during IAV or SAR-CoV 2 infection may contribute to dampening excessive inflammation, but further in vivo studies would be needed to test this hypothesis.

FIGURE 8 Effect of SAA and or HDL on neutrophil IL-8 production or caspase activity—In panel A, IL-8 production by neutrophils was measured by ELISA as described in Section 2 (“Materials And Methods”). ** = P < 0.01 compared to control. Results represent mean ± SEM for 3 to 5 experiments. Panel B shows IL-8 production in response to influenza A virus (IAV) alone or IAV combined with Apo-serum amyloid A (Apo-SAA1). Panel C shows caspase 3 activation by IAV alone or IAV combined with Apo-SAA1, SAAHDL (SAA complexed with HDL), SAA1, or HDL. * = P < 0.05 compared to results with neutrophils in media alone. Results are mean ± SEM of 4 experiments.

TABLE 2 Effects of TLR2 blocking antibodies, pertussis toxin (PT), and wortmannin of neutrophil responses to Apo-serum amyloid A (Apo-SAA1)

| Assay                  | Inhibitor | IAV | Apo-SAA1 | Apo-SAA1 + IAV |
|------------------------|-----------|-----|----------|---------------|
| H2O2                   | Control   | 79 ± 2.4 | 83 ± 1.7 | 64.5 ± 5      |
| Anti-TLR2              | Control   | 79 ± 1.8 | 81.4 ± 1.4 | 69 ± 1.4     |
| Virus uptake           | Control   | 125 ± 3.6 |           |               |
| Anti-TLR2              |           | 124 ± 8  |           |               |
| Peak calcium response  | Control   | 90 ± 5.2 |           |               |
| PT                     |           | 101 ± 3.6 |           |               |
| Wortmannin             |           | 98 ± 2 |           |               |

Assays were performed as described in Section 2 (“Materials And Methods”). * = P < 0.05 and ** = P < 0.01 compared to control and results represent mean ± SEM for 4 to 5 experiments with separate neutrophil donors. For H2O2 assay the mean percentage fluorescence at 1600 s after addition of stimulus. Note that a lower number at this time point indicates increased production of H2O2. The peak calcium response indicates fluorescence value obtained at 15 s after addition of stimulus. IAV: influenza A virus.

AUTHORSHIP
M.R.W. conceived of and performed the majority of experiments, analyzed the data, prepared figures, and wrote much of the article; I-N.H. and X.D. performed some experiments and participated in interpreting data and in the article review; and K.L.H. oversaw the project, helped in its conception and design of experiments, and writing and editing the article.

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DISCLOSURES
The authors declare no conflicts of interest.

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REFERENCES
1. Tripathi S, White MR, Hartshorn KL. The amazing innate immune response to influenza A virus infection. Innate Immun. 2015;21:73-98.
2. Vollmer AH, Gebre MS, Barnard DL. Serum amyloid A (SAA) is an early biomarker of influenza virus disease in BALB/c, C57BL/2, Swiss-Webster, and DBA.2 mice. Antiviral Res. 2016;133:196-207.
16. Sun L, Ye RD. Serum amyloid A: structure, function and gene polymorphism. Gene. 2016;583:48-57.

17. Ye RD, Sun L. Emerging functions of serum amyloid A in inflammation. J Leukoc Biol. 2015;98:923-929.

18. De Buck M, Berghmans N, Portner N, et al. Serum amyloid A1alpha induces paracrine IL-8/CXCCL8 via TLR2 and directly synergizes with this chemokine via CXCR2 and formyl peptide receptor 2 to recruit neutrophils. J Leukoc Biol. 2015;98:1049-1060.

19. Migita K, Izumi Y, Jiuchi Y, et al. Serum amyloid A induces NLRP3-mediated IL-1beta secretion in neutrophils. PLoS One. 2014;9:e96703.

20. Sun L, Zhu Z, Cheng N, Yan Q, Ye RD. Serum amyloid A induces interleukin-33 expression through an IRF7-dependent pathway. Eur J Immunol. 2014;44:2153-2164.

21. Lee HY, Kim SD, Shim JW, Lee SY, Yun J, Bae YS. LL-37 inhibits serum amyloid A-induced IL-8 production in human neutrophils. Exp Mol Med. 2009;41:325-333.

22. Christenson K, Bjorkman L, Tangemo C, Bylund J. Serum amyloid A inhibits apoptosis of human neutrophils via a P2X7-sensitive pathway independent of formyl peptide receptor-like 1. J Leukoc Biol. 2008;83:139-148.

23. Kebir E, D J, L K, et al. Aspirin-triggered lipoxins override the apoptosis-delaying action of serum amyloid A in human neutrophils: a novel mechanism for resolution of inflammation. J Immunol. 2007;179:616-622.

24. Ji YR, Kim HJ, Bae KB, Lee S, Kim MO, Ryoo ZY. Hepatic serum amyloid A1 aggravates T cell-mediated hepatitis by inducing chemokines via Toll-like receptor 2 in mice. J Biol Chem. 2015;290:12804-12811.

25. Burgess EJ, Hoyt LR, Randall MJ, et al. Bacterial lipoproteins constitute the TLR2-stimulating activity of serum amyloid A. J Immunol. 2018;201:2377-2384.

26. Ather JL, Dienz O, Boyson JE, Anathy V, Amiel E, Poynter ME. Serum amyloid A3 is required for normal lung development and survival following influenza infection. Scientific reports. 2018;8:16571.

27. Gong D, Farley K, White M, Hartshorn KL, Benarafa C, Remold-O'Donnell E. Critical role of serpinB1 in regulating inflammatory responses in pulmonary influenza infection. J Infect Dis. 2011;204:592-600.

28. Lin KL, Sweeney S, Kang BD, Ramsburg E, Gunn MD, CCR2-antagonist prophylaxis reduces pulmonary immune pathology and markedly improves survival during influenza infection. J Immunol. 2011;186:508-515.

29. Lin KL, Suzuki Y, Nakano H, Ramsburg E, Gunn MD, CCR2 monocyte-derived dendritic cells and exudate macrophages produce influenza-induced pulmonary immune pathology and mortality. J Immunol. 2008;180:2562-2572.

30. Hufford MM, Richardson G, Zhou H, et al. Influenza-infected neutrophils within the infected lungs act as antigen presenting cells for anti-viral CD8(+) T cells. PLoS One. 2012;7:e46581.

31. Tate MD, Brooks AG, Reading PC, Mintern JD. Neutrophils sustain effective CD8(+) T-cell responses in the respiratory tract following influenza infection. Immunol Cell Biol. 2012;90:197-205.

32. Tate MD, Ioannidis LJ, Croker B, Brown LE, Brooks AG, Reading PC. The role of neutrophils during mild and severe influenza virus infections of mice. PLoS One. 2011;6:e17618.

33. Ichikawa A, Kuba K, Morita M, et al. CXCL10-CXCR3 enhances the development of neutrophil-mediated fulminant lung injury of viral and nonviral origin. Am J Respir Crit Care Med. 2013;187:65-77.

34. Hartshorn KL, Collamer M, Auerbach M, Myers JB, Pavlotsky N, Tauber AI. Effects of influenza A virus on human neutrophil calcium metabolism. J Immunol. 1988;141:1295-1301.

35. Qi L, Kash JC, Dugan VG, et al. The ability of pandemic influenza virus hemagglutinins to induce lower respiratory pathology is associated with decreased surfactant protein D binding. Virology. 2011;412:426-434.

36. Hartshorn KL, Crouch EC, White MR, et al. Evidence for a protective role of pulmonary surfactant protein D (SP-D) against influenza A viruses. J Clin Invest. 1994;94:311-319.

37. Doss M, Ruchala P, Tecle T, et al. Hapivirins and diprovirins: novel theta-defensin analogs with potent activity against influenza A virus. J Immunol. 2012;188:2759-2768.

38. Hartshorn KL, Collamer M, White MR, Schwartz JH, Tauber AI. Characterization of influenza A virus activation of the human neutrophil. Blood. 1990;75:218-226.

39. Kazhdan M, White MR, Tauber AI, Hartshorn KL. Human neutrophil respiratory burst response to influenza A virus occurs at an intracellular location. J Leukoc Biol. 1994;56:59-64.

40. Hartshorn KL, White MR, Shepherd V, Reid K, Jensenius JC, Crouch EC. Mechanisms of anti-influenza activity of surfactant proteins A and D: comparison with serum collectins. Am J Physiol. 1997;273:L1156-66.

41. Tripathi S, Wang G, White M, Qi L, Taubenberger J, Hartshorn KL. Antiviral activity of the human cathelicidin, LL-37, and derived peptides on seasonal and pandemic influenza A viruses. PLoS One. 2015;10:e0124706.
42. Nikolaidis NM, White MR, Allen K, et al. Mutations flanking the carbohydrate binding site of surfactant protein D confer antiviral activity for pandemic influenza A viruses. Am J Physiol Lung Cell Mol Physiol. 2014;306:L1036-44.

43. Colamussi ML, White MR, Crouch E, Hartshorn KL. Influenza A virus accelerates neutrophil apoptosis and markedly potentiates apoptotic effects of bacteria. Blood. 1999;93:2395-2403.

44. Khatri K, Klein JA, White MR, et al. Integrated omics and computational glycomics reveal structural basis for influenza A virus glycan microheterogeneity and host interactions. Mol Cell Proteomics. 2016;15:1895-1912.

45. Tecle T, White MR, Gantz D, Crouch EC, Hartshorn KL. Human neutrophil defensins increase neutrophil uptake of influenza A virus and bacteria and modify virus-induced respiratory burst responses. J Immunol. 2007;178:8046-8052.

46. Shridas P, De Beer MC, Webb NR. High-density lipoprotein inhibits serum amyloid A-mediated reactive oxygen species generation and NLRP3 inflammasome activation. J Biol Chem. 2018;293:13257-13269.

47. Christenson K, Bjorkman L, Ahlin S, et al. Endogenous acute phase serum amyloid A lacks pro-inflammatory activity, contrasting the two recombinant variants that activate human neutrophils through different receptors. Front Immunol. 2013;4:92.

48. Zhou H, Chen M, Zhang G, Ye RD. Suppression of lipopolysaccharide-induced inflammatory response by fragments from serum amyloid A. J Immunol. 2017;199:1105-1112.

49. Ather JL, Fortner KA, Budd RC, Anathy V, Poynter ME. Serum amyloid A inhibits dendritic cell apoptosis to induce glucocorticoid resistance in CD4(+) T cells. Cell Death Dis. 2013;4:e786.

50. Engelich G, White M, Hartshorn K. Influenza A virus markedly potentiates neutrophil apoptosis induced by bacteria: role of respiratory burst. Blood. 1999;94:211a.

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