EXTENDED REPORT

Acute serum amyloid A is an endogenous TLR2 ligand that mediates inflammatory and angiogenic mechanisms

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ABSTRACT

Introduction Acute-phase serum amyloid A (A-SAA) has cytokine-like properties and is expressed at sites of inflammation. We examined whether A-SAA-induced pro-inflammatory mechanisms are mediated through Toll-like receptor 2 (TLR2) in rheumatoid arthritis (RA).

Methods The effect of A-SAA on human embryonic kidney (HEK), TLR2 or TLR4 cells was quantified by nuclear factor (NF)-κB luciferase reporter assays. A-SAA-induced RASFC and dHMVEC function were performed in the presence of a specific neutralising anti-TLR2 mAb (OPN301) (1 μg/mL) and matched IgG isotype control Ab (1 μg/mL). Cell surface expression of intracellular adhesion molecule (ICAM)-1, chemokine expression, cell migration, invasion and angiogenesis were assessed by flow cytometry, ELISA, Matrigel invasion chambers and tube formation assays. MyD88 expression was assessed by real-time PCR and western blot.

Results A-SAA induced TLR2 activation through induction of NF-κB ($p<0.05$), but failed to induce NF-κB in HEK-TLR4 cells, confirming specificity for TLR2. A-SAA-induced proliferation, invasion and migration were significantly inhibited in the presence of anti-TLR2 (all $p<0.05$), with no significant effect observed for tumour necrosis factor-α-induced events. Additionally, A-SAA-induced ICAM-1, interleukin-8, monocyte chemoattractant protein-1, RANTES and GRO-α expression were significantly reduced in the presence of anti-TLR2 (all $p<0.05$), as was A-SAA induced angiogenesis ($p<0.05$). Finally, A-SAA induced MyD88 signalling in RASFC and dHMVEC ($p<0.05$).

Conclusions A-SAA is an endogenous ligand for TLR2, inducing pro-inflammatory effects in RA. Blocking the A-SAA/TLR2 interaction may be a potential therapeutic intervention in RA.

INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disease characterised by synovial proliferation, neovascularisation and leucocyte extravasation.[1 2] Activation of the synovium transforms the lining layer into a hyperplastic tumour-like 'pannus' that is capable of destroying adjacent articular cartilage and bone.[3–5] Acute-phase serum amyloid A (A-SAA) is an acute-phase protein that belongs to a closely related group of 12–14 kDa apoproteins.[6 7] During the inflammatory response, A-SAA is dramatically elevated in serum where it displaces ApoA-1 and saturates high-density lipoprotein (HDL),[8] resulting in high levels of free circulating A-SAA.[9] Unlike other acute-phase proteins that are synthesised primarily in the liver as part of the systemic acute-phase response, A-SAA expression is markedly increased at local sites of tissue inflammation.[10] Such extra-hepatic A-SAA expression has been demonstrated in brain amyloid plaques of patients with Alzheimer's disease and in atherosclerotic lesions.[11 12] A-SAA is also the precursor of AA, an insoluble degradation product, deposited in a number of major organs in the progressive fatal disease secondary amyloidosis.[13]

We and others have previously reported significantly increased expression of A-SAA and its receptors in RA synovial tissue and cells compared with OA and healthy controls.[14–17] Furthermore, A-SAA is spontaneously released from RA synovial tissue explants, with A-SAA levels at site of inflammation significantly higher than systemic circulation,[14] and A-SAA induces chemokines, adhesion molecules, angiogenesis, cytoskeletal rearrangement and matrix metalloproteinases (MMPs) in RA synovial cells in vitro.[17–22] Furthermore, A-SAA has a high affinity for the extracellular matrix components and promotes RASFC fibroblasts invasion through a β1-integrin and RhoGTPase-dependent mechanism.[22 23] A-SAA has two well-characterised receptors, lipoxin A_4 receptor/*N*-formyl peptide receptor-like-1 (FPRL-1) and Scavenger receptor class B member 1 (SR-B1) (formerly CD36 and LIMPII analogous-1 (CLA-1)) both highly expressed in RA ST compared with OA or healthy control.[16 19 24] Through FPRL-1 and SR-B1, A-SAA activates many pro-inflammatory processes, including cell migration/invasion, chemokine induction and MMP production.[16 25–28] Blockade of FRPL-1 and SR-B1 in an ex vivo RA synovial explant culture model inhibits spontaneous chemokine secretion, suggesting endogenous A-SAA is active.[14]

Recent evidence has implicated Toll-like receptor 2 (TLR2) as an alternative receptor for A-SAA,[29] TLR2 is expressed in the perivascular regions of the joint,[30] at the sites of attachment and invasion into cartilage and bone, and on synovial macrophages.[31] Increased expression of TLR2 has been demonstrated in collagen-induced arthritis.[32] Additionally, overexpression of dominant negative forms of TLR2/4 adapter molecules MyD88 and Mal/TIRAP or anti-TLR2 blockade inhibits spontaneous release of pro-inflammatory cytokines from RA synovial cells and explants.[33 34]

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In this study, we examine whether TLR2 mediates A-SAA-induced pro-inflammatory mechanisms in RA. The rapid induction of A-SAA during inflammation, its localised expression at inflammatory sites and its ability to induce many pro-inflammatory processes through TLR2 suggest that A-SAA is directly involved in the pathogenesis of inflammatory joint disease.

MATERIALS AND METHODS

Arthroscopy, isolation of RA synovial fibroblasts and reagents

See online supplementary file 1.

Luciferase reporter gene assay

HEK293-TLR2 and HEK293-TLR4 cells were grown in Dulbecco's modified Eagle medium with GlutaMAX (Gibco-BRL) supplemented with 10% fetal calf serum (FCS), penicillin-streptomycin, noromycin and 200 µg/mL of G418 and maintained at 37°C in a humidified atmosphere of 5% CO_2.[36] HEK293-TLR2 and HEK293-TLR4 cells (2×10^4 cells/well; 96-well plate) were transfected with 80 ng/well luciferase reporter gene plasmid for nuclear factor (NF)-κB,[37] using Lipofectamine 2000 as described by the manufacturer (Invitrogen). In all cases, 40 ng/well of phRL-TK reporter gene was co-transfected to normalise data for transfection efficiency. After 24 h, cells were stimulated with A-SAA (0.01–10 µg/mL), Pam_2CSK_4 (1 µg/mL), Pam_3CSK_4 (1 µg/mL) or lipopolysaccharide (LPS) (0.01–10 µg/mL) for 24 h. Cell lysates were prepared and reporter gene activity was measured using the Dual Luciferase Assay system (Promega) as previously described.[38] Data are expressed as the mean fold induction±SEM relative to control levels from a minimum of three separate experiments.

Human microvascular endothelial cell culture and cell growth assays

See online supplementary file 1.

dHMVEC tubule formation in vitro

Matrigel (50 µL) (Becton Dickinson, Mountain View, California, USA) was plated in 48-well culture plates after thawing on ice and allowed to polymerise for 1 h at 37°C in humidified air with 5% CO_2. dHMVEC were removed from culture, trypsinised and resuspended at a concentration of 4×10^4 cells/mL in EGM. In total, 500 µL of cell suspension was added to each chamber in the presence of A-SAA (10 µg/mL), tumour necrosis factor (TNF)-α (10 ng/mL)±OPN301 (1 µg/mL), or IgG-matched control (1 µg/mL). The chambers were then incubated for 24 h at 37°C in humidified air with 5% CO_2. Endothelial cell tubule formation was assessed using phase-contrast microscopy and quantified as previously described.[18]

Analysis of cell surface ICAM-1 expression

RASFC or dHMVEC were plated to a density of 5×10^4 in 12-well plates (Falcon, Franklin Lakes, New Jersey, USA) for 48 h in medium plus supplements. Cells were stimulated with A-SAA (10 µg/mL)±OPN301 (1 µg/mL) for 6 h, then incubated with 0.3 µg/mL of phycoerythrin-conjugated mouse monoclonal anti-intracellular adhesion molecule (ICAM)-1 or isotype-matched IgG control (Becton Dickinson) for 30 min at 4°C. Cells were then washed twice and fixed in 1% paraformaldehyde, and analysed by flow cytometry (FACS) using a FACScan flow cytometer and Lysis II software (both from Becton Dickinson).

Cytokine measurement in RASFC and dHMVEC

To assess the effect of TLR2 blockade on chemokine expression, RASFC and dHMVEC were serum starved for 24 h, followed by stimulation with A-SAA (10 µg/mL) in the presence or absence of OPN301 (1 µg/mL) for a further 24 h. Supernatants were harvested and protein levels of interleukin (IL)-8, monocyte chemoattractant protein (MCP)-1, IL-6, RANTES and GRO-α were measured by ELISA (R&D Systems) according to the manufacturer's protocol.

Wound repair assay

RASFC and dHMVEC were plated in 48-well plates, grown to confluency, growth arrested for 24 h in serum-free Roswell Park Memorial Institute medium or 1% EGM and scraped with a sterile pipette tip to create a linear wound across the well. Cells were subsequently stimulated for 24 h with A-SAA (10 µg/mL) and TNF-α (10 ng/mL) in the presence or absence of OPN301 (1 µg/mL) or isotype-matched IgG (1 µg/mL) control antibody. Migration was determined by visual assessment of cells that had repopulated wound margins using phase-contrast microscopy.

Transwell invasion assay

Biocoat Matrigel Invasion Chambers (Becton Dickinson, UK) were used to assess cell invasion in response to A-SAA ±OPN301. dHMVEC were seeded at a density of 2.5×10^4 per well on 8 µm membranes pre-coated with Matrigel. EGM containing A-SAA (10 µg/mL) or TNF-α (10 ng/mL) in the presence or absence of OPN301 (1 µg/mL) or IgG-matched control antibody was added to the lower wells. Cells were allowed to migrate for 24 h in EGM media containing 1% FCS. Migrating cells attached to the lower membrane were fixed with 1% glutaraldehyde and stained with 0.1% crystal violet. To assess the average number of migrating cells, cells were counted in five random high-power fields.

RNA extraction from RASFC and dHMVEC

RASFC and dHMVEC were stimulated with A-SAA (10 µg/mL) or TNF-α (10 ng/mL) in serum-free medium for 3–24 h prior to RNA extraction using an RNeasy Mini Kit according to the manufacturer's protocol (Qiagen, Crawley, UK). The integrity of RNA samples was assessed using a bioanalyzer (Agilent Technologies, Cork, Ireland). Samples with a 260:280 nm ratio of ≥1.8 and an RNA Integrity Number between 7 and 10 were used in subsequent experiments. Isolated RNA was stored at −80°C until further use.

RT-PCR

Total RNA (1 µg) was added to a 25 µL reaction volume containing 200 U Superscript II in reverse transcriptase buffer 100 mM dithiothreitol (supplied with RT-enzyme), 40 units RNasin Ribonuclease Inhibitor (Promega, Dublin, Ireland), 1.25 mM each of dATP, dCTP, dGTP and dTTP (Promega), RNase-free water and 500 ng of oligo dTs (Promega). Reverse transcription was performed at 42°C for 50 min. The reaction was terminated by incubation at 95°C for 15 min. PCR primers and probes for MyD88, GAPDH and β-actin (endogenous controls) were designed by Applied Biosystems (Assays-on-Demand, Applied Biosystems). Amplification reactions contained 1 µL cDNA, 12.5 µL of Universal Taqman 2X PCR Mastermix (Applied Biosystems, Cheshire, UK), 1.25 µL of primer and probe mix and were brought to a total volume of 25 µL by the addition of RNase-free water. All reactions/negative controls were performed in triplicate using 96-well plates on the Roche

LC480 (Roche Diagnostics, West Sussex, UK). Relative changes in gene expression were determined using the C_t method.

Western blot for MyD88

RASFC and dHMVEC were incubated with A-SAA (10 μg/mL) or TNF-α (10 ng/mL) for 15 min prior to lysis. Cell lysates were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Western blotting was performed using an anti-MyD88 rabbit polyclonal AbMyD88 (Abcam, Cambridge, UK) according to the manufacturer's protocol. Blots were developed using ECL (Pierce, Rockford, Illinois, USA) for detection of horseradish peroxidase (HRP).

Statistical analysis

Statistical analysis was performed using SPSS 11 for Windows (SPSS, Chicago, Illinois, USA). For comparisons between treated and untreated RASFC, non-parametric Wilcoxon signed-rank test for paired samples was performed. Parametric Student's t tests were used for analysis of dHMVEC and HEK293 cell data. A p value of <0.05 was considered significant.

RESULTS

TLR2 is a functional receptor for A-SAA

The ability of A-SAA to drive TLR2-mediated NF-κB induction was investigated using NF-κB luciferase reporter gene assays. A-SAA induced TLR2-mediated NF-κB luciferase reporter gene activity in a dose-dependent manner with maximum activity observed at 10 and 50 μg/mL (see online supplementary figure S1A). A-SAA induced a 15.7-fold increase (10 μg/mL) or 14.6-fold increase (50 μg/mL) in TLR2-mediated NF-κB luciferase reporter gene activity compared with control (p<0.05) (figure 1A). Additionally, TLR2-mediated NF-κB luciferase reporter gene activity was significantly enhanced following stimulation with the TLR2 agonists, Pam_2CSK_4 and Pam_3CSK_4 (p<0.05). LPS-induced TLR4 mediated NF-κB reporter gene activity in a dose-dependent manner (see online supplementary figure S1B), with no effect observed for A-SAA (figure 1B).

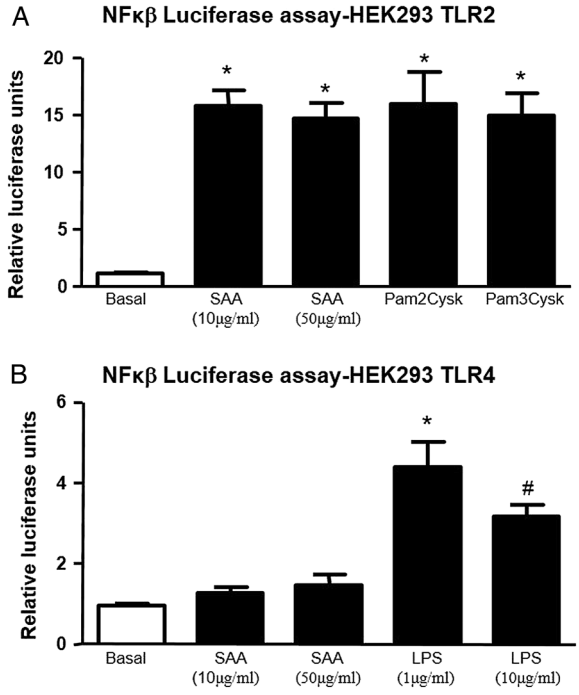

Figure 1 Acute-phase serum amyloid A (A-SAA) is a functional ligand for Toll-like receptor 2 (TLR2). The effect of A-SAA on nuclear factor (NF)-κb luciferase reporter activity in (A) HEK293-TLR2 cells and (B) HEK293-TLR4 cells. Results are shown as the mean±SEM (n=3). *p<0.01, #p<0.05 compared with basal control. LPS, lipopolysaccharide.

SAA-induced cell growth and angiogenesis is mediated via TLR2

RASFC proliferation significantly increased by 314% following stimulation with A-SAA (10 μg/mL) (p=0.02) and by 399% in response to TNF-α stimulation (p=0.005) (see online supplementary figure S2A). A-SAA-induced cell growth was inhibited by 37.4% in the presence of OPN301, with no significant effect observed on TNF-α-induced cell growth (see online supplementary figure S2A). Similar effects were observed in dHMVEC where anti-TLR2 significantly reduced A-SAA cell growth (p=0.002) (figure 2B), but had no effect on TNF-α (see online supplementary figure S2A).

A significant increase in the formation of tube-like structures was demonstrated in the presence of A-SAA alone (p<0.05) (figure 2C, D), an effect that was inhibited in the presence of OPN301 (p<0.05). Figure 2C shows representative images of increased tube formation following A-SAA stimulation, as demonstrated by an increase in the number of connecting branches between two endothelial cells. This effect was inhibited to near basal in the presence of anti-TLR2.

TLR2 mediates A-SAA-induced chemokine expression in RASFC and dHMVEC

A-SAA significantly increased IL-6, IL-8, MCP-1, GRO-α and RANTES expression in both RASFC (figure 3A) and dHMVEC (figure 3B) (p<0.05). Co-incubation of cells with SAA and OPN301 resulted in significant reductions in SAA-induced cytokine activity (p<0.05). In contrast, OPN301 had no effect on TNF-α-induced cytokine production in either RASFC or dHMVEC (data not shown).

TLR2 mediates A-SAA-induced adhesion, invasion and migration

Figure 4A shows representative histograms demonstrating increased RASFC surface expression of ICAM-1 in response to A-SAA compared with basal control, an effect that was inhibited by OPN301 (figure 4A, i). A-SAA significantly increased RASFC ICAM-1 expression from a mean fluorescent intensity (MFI) of 24.71±5.44 to 88.27±18.68 (p<0.05), which was significantly reduced to 36.34±10.96 in the presence of OPN301 (figure 4A, ii) (p<0.05). Similarly, A-SAA significantly increased cell surface ICAM-1 expression on dHMVEC, from an MFI of 209.83±109.34 to 724.58 ±74.5 (p<0.05), an effect that was significantly inhibited by OPN301 to 255.49 (p<0.01) (data not shown).

dHMVEC invasion was significantly induced by A-SAA compared with control (figure 4B), an effect similar to stimulation with TNF-α. Blockade of TLR2 significantly decreased A-SAA-induced invasion (p<0.05) (figure 4B, C), but had no effect on TNF-α-induced invasion (see online supplementary figure S2B). Figure 4D shows representative images of wound repair where repopulation of wound margins was observed in response to A-SAA, an effect that was inhibited in the presence of OPN301.

A-SAA induces MyD88 expression

As all known effector functions of TLR2 signalling depend on the MyD88 adaptor protein, we next examined whether MyD88 could be induced by A-SAA. As shown in figure 5, A-SAA

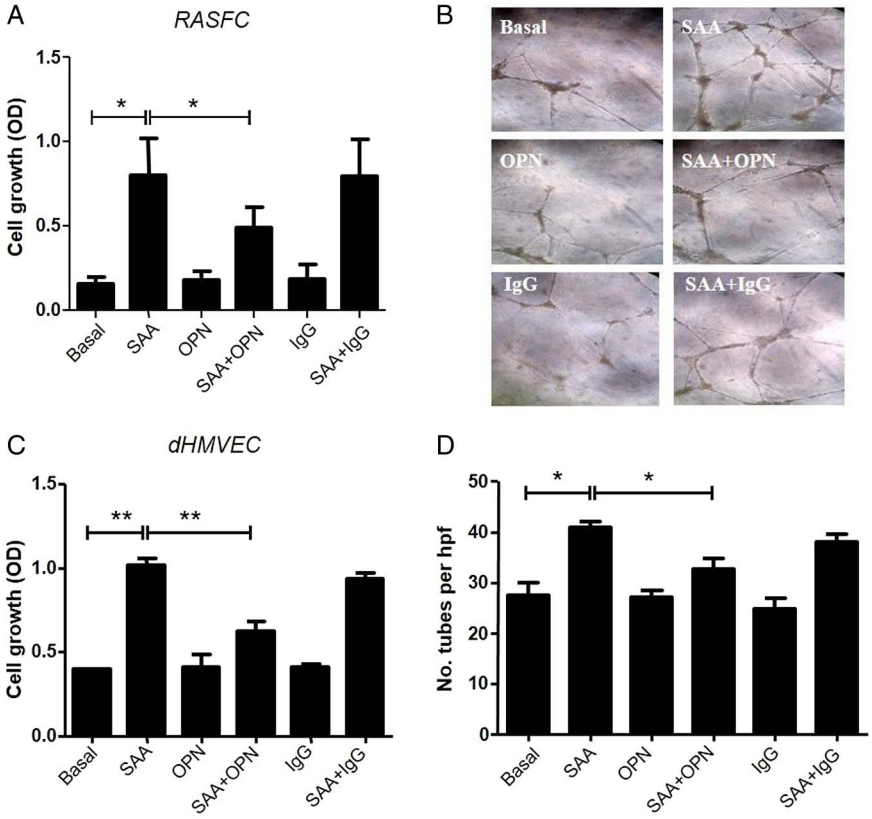

Figure 2 Acute-phase serum amyloid A (A-SAA)-induced cell growth and angiogenesis is Toll-like receptor 2 (TLR2) dependent. (A) Rheumatoid arthritis (RA) synovial fibroblasts (RASFC) and (B) microvascular endothelial cells (dHMVEC) were stimulated with SAA (10 μg/mL) in the presence or absence of OPN301 (1 μg/mL) or IgG control (1 μg/mL) for 72 h and cell growth was assessed by crystal violet assay. (C) Representative photomicrographs of dHMVEC tubule formation on Matrigel matrix following 24 h of incubation with SAA (10 μg/mL)±OPN301 (1 μg/mL). Quantitative analysis of the number of connecting branches at baseline and in response to A-SAA±OPN301 (1 μg/mL) or IgG (1 μg/mL) control shown in (D). The tube analysis was determined from five sequential fields (magnification ×40) focusing on the surface of the Matrigel. Results are shown as the mean±SEM (n=5–7). *p<0.05 significantly different.

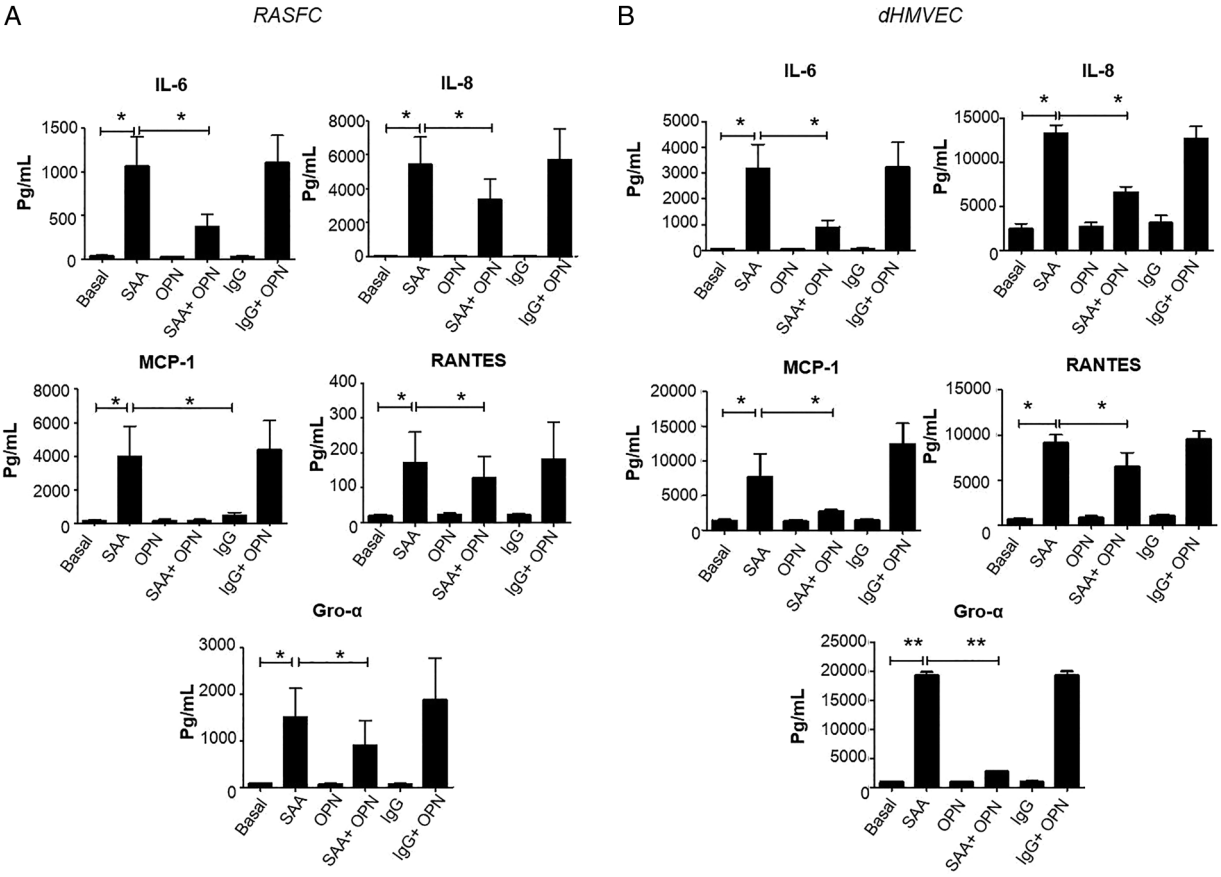

Figure 3 Toll-like receptor 2 (TLR2) mediates acute-phase serum amyloid A (A-SAA)-induced chemokine expression. (A) Rheumatoid arthritis synovial fibroblasts (RASFC) or (B) microvascular endothelial cells (dHMVEC) were stimulated with SAA (10 μg/mL) in the presence or absence of OPN301 (1 μg/mL) or IgG control (1 μg/mL) for 24 h. Supernatants were harvested and quantified for interleukin (IL)-6, IL-8, monocyte chemoattractant protein (MCP)-1, RANTES and GRO-α by ELISA. Results are shown as the mean±SEM (n=4) *p<0.05, **p<0.01 significantly different.

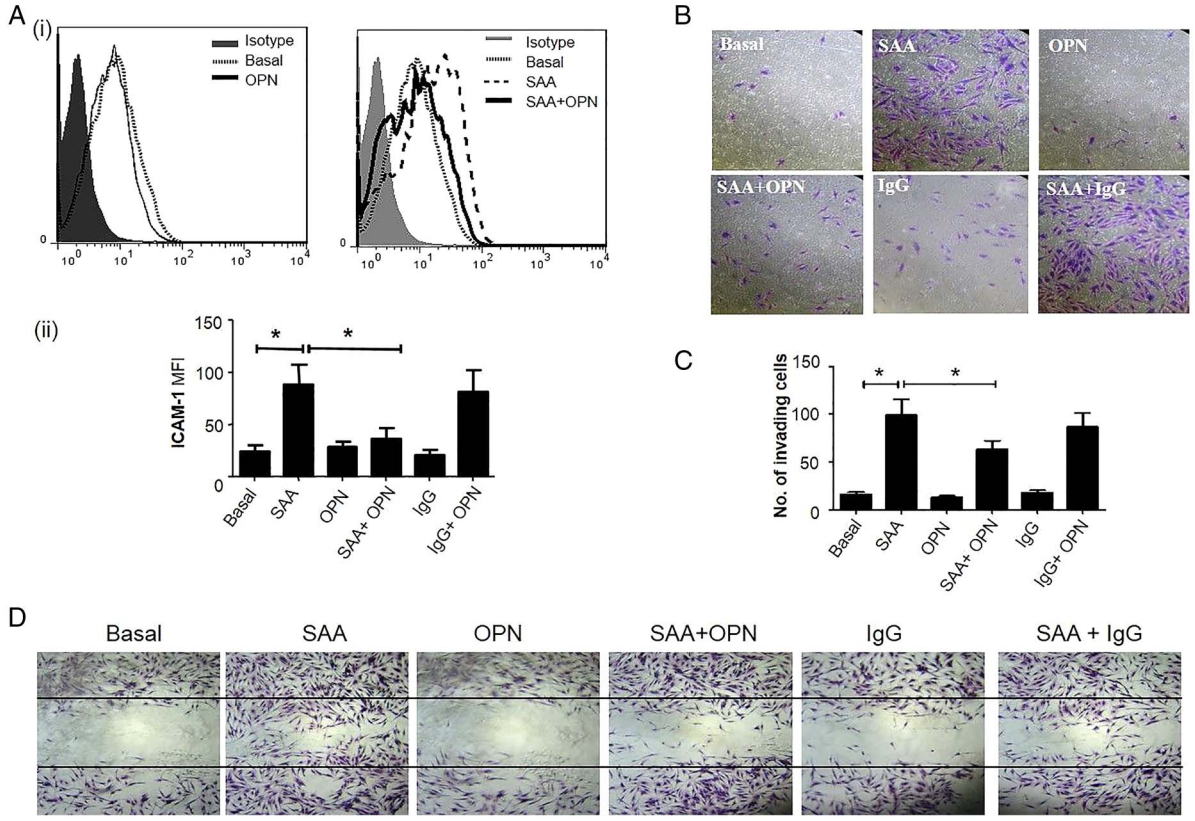

Figure 4 Toll-like receptor 2 (TLR2) mediates acute-phase serum amyloid A (A-SAA)-induced adhesion, invasion and migration. (A) RASFC were stimulated with A-SAA (10 μg/mL)±OPN301 (1 μg/mL) or IgG control (1 μg/mL) and expression of ICAM-1 was detected by flow cytometric analysis. (A)(i) Representative histogram showing intercellular adhesion molecule 1 (ICAM-1) on RA synovial fibroblasts (RASFC) following stimulation with SAA (10 μg/mL)±OPN301 (1 μg/mL) for 24 h. (A)(ii) Quantification of ICAM-1 expression in RASFC, data represented as mean fluorescent intensity (n=5). (B) Representative photomicrograph showing microvascular endothelial cell (dHMVEC) invasion. Cells were stimulated with SAA (10 μg/mL) in the presence or absence of OPN301 (1 μg/mL) or IgG control (1 μg/mL) for 24 h. Invading cells attached to the lower membrane were fixed (1% glutaraldehyde) and stained (1% crystal violet) (original magnification ×40). (C) Bar graph quantifying dHMVEC invasion (n=4). (D) Representative images showing RASFC repopulating the wound in response to A-SAA (10 μg/mL), an effect that was blocked in the presence of OPN301 (1 μg/mL), with no effect observed for IgG control (1 μg/mL) (n=3). Results are shown as the mean±SEM. *p<0.05 significantly different.

increased transcript levels of MyD88 in both RASFC (figure 5A) and dHMVEC (figure 5B), reaching maximal levels at 24 and 6 h, respectively. Additionally, A-SAA increased expression of MyD88 protein in both cell types, as demonstrated by western blot (figure 5A, B, ii). In contrast, TNF-α had no effect on MyD88 expression (see online supplementary figure S3).

DISCUSSION

In this study, we demonstrate that A-SAA is capable of driving NF-κB through TLR2, but not TLR4. At a functional level, A-SAA-induced proliferation, invasion and migration were significantly inhibited in the presence of OPN301 (anti-TLR2) with no effect on TNF-α-induced mechanisms. Additionally,

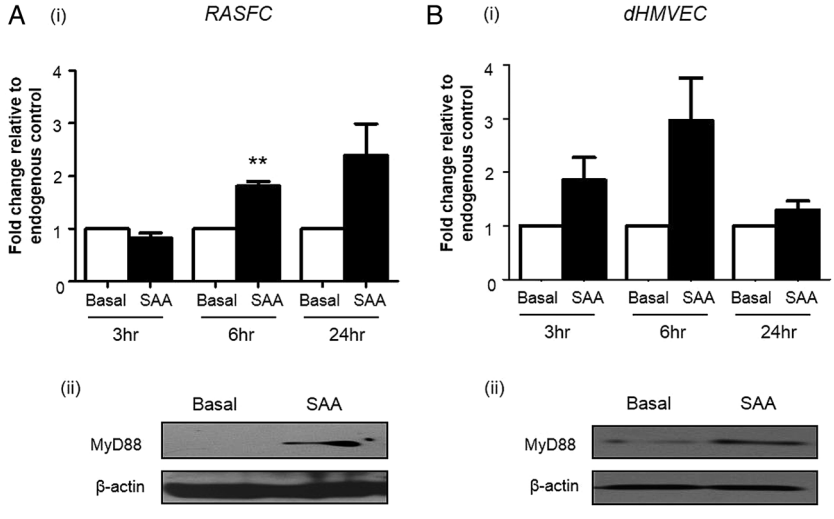

Figure 5 Acute-phase serum amyloid A (A-SAA) induces MyD88. MyD88 mRNA expression was quantified in RASFC and HMVEC following stimulation with A-SAA (10 μg/mL) by real-time PCR. Results are shown as the mean±SEM from n=4 (HMVEC) or n=5 (RASFC) experiments (A, B) (i) Western blot analysis for MyD88 in RASFC A (ii) and dHMVEC B (ii) following A-SAA (10 μg/mL) stimulation for 24 h (n=3). *p<0.05, **p≤0.01 significantly different.

A-SAA-induced angiogenesis, ICAM-1, IL-6, IL-8, MCP-1, RANTES and GRO-α expression in RASFC and dHMVEC were significantly reduced in the presence of OPN301. Finally, we show that A-SAA induces MyD88 signalling in RASFC and dHMVEC. Thus, A-SAA-induced pro-inflammatory mechanisms in RA are mediated, in-part, via the TLR2 receptor.

A-SAA, a major acute-phase protein, is highly elevated both in circulation and locally in tissues during various pathological conditions.[12 39 40] In addition, elevated A-SAA levels correlate with an increased risk of adverse cardiovascular endpoints including myocardial infarction[41] and is strongly expressed in unstable atherosclerotic plaques.[12] Additionally, A-SAA plays a central role in lipid metabolism and in acute inflammation, associates with HDL, displacing Apo-AI, resulting in increased HDL saturates, where it renders HDL pro-atherogenic. Furthermore, serum A-SAA levels correlate closely with RA disease activity and can differentiate RA from other arthropathies in a cohort of patients with early arthritis.[42] Together, this suggests that A-SAA is actively involved in innate immunity and inflammation and suggests a common role for A-SAA as an inflammatory mediator, and an important protein in lipid metabolism and transport.

In this study, we demonstrate that A-SAA-induced pro-inflammatory effects are in part mediated through TLR2 activation with no effect observed for TLR4. Furthermore, we demonstrate in RASFC that A-SAA-induced proliferation, adhesion and migration is mediated by TLR2. Previous in vitro studies have demonstrated the cytokine-like properties of A-SAA, which induces the release of several cytokines in RASFC, dHMVEC, human monocytes and neutrophils.[14 43 44] Furthermore, A-SAA promotes leucocyte recruitment and matrix degradation, key processes in the pathogenesis of RA.[18 19 45] Additional receptors for A-SAA, FPRL-1 and SR-B1 have been demonstrated in the RA joint and mediate A-SAA-induced pro-inflammatory mechanisms.[15 16]

TLRs have been implicated in the pathogenesis of RA with studies demonstrating increased expression of TLR2 in the RA joint, and TLR2 activation in RA synovial explants, RASFC and macrophages. Functional significance of TLR2 has been demonstrated in collagen-induced arthritis models and in TLR2-deficient mice. Several potential TLR2 ligands have been implicated, including heat shock protein/GP96, fibronectin fragments, hyalauronidase oligosaccharides and HMBG-1, all of which are highly expressed in RA synovial fluid.[14 46–48] The existence of a ligand is further supported by studies showing that conditioned media from RA synovial explants can activate macrophages in a MyD88 and Mal-dependent manner.[30] Given that TLR2 is a major pattern recognition receptor, that SAA is spontaneously released from RA tissue and that its functional effects are in part mediated by TLR2, this suggests that SAA may act as an endogenous ligand for TLR2 in the RA joint and contributes to inflammation through persistent activation of this receptor.

We and others have demonstrated A-SAA can directly induce angiogenic processes, adhesion molecules and chemokines expression in vitro and in vivo,[14] effects that are mediated through FRPL-1 and SR-B1.[16 17 24] However, this is the first study to show a role for TLR2 in mediating A-SAA-induced angiogenic mechanisms. Specifically, we showed that OPN301 significantly inhibited A-SAA-induced tube formation, invasion and chemokine expression. This is supported by studies showing TLR2 activation promotes angiogenesis, cell adhesion and invasion in RA, effects that are in part mediated through the key angiogenic signalling pathway Ang2/Tie2.[49] In addition, TLR2 is expressed in SM perivascular regions,[50] and in vitro TLR2 activation induces VEGF/IL-8 expression in synovial fibroblasts and chondrocytes,[51 52] and MMP-9 in corneal epithelial cells and THP-1 macrophages.[53] Furthermore, we have demonstrated using whole-tissue RA synovial explant cultures that OPN301 significantly inhibits spontaneous release of pro-inflammatory cytokines IL-1β, TNF-α, IFN-γ and IL-8, [34] and MMP-3, MMP-2 and MMP-9.[54] In addition, culture of RASFC with conditioned media from OPN301-treated RA explants inhibited RASFC migration and invasion compared with IgG control.[54] Therefore, the ability of TLR2 to mediate A-SAA-induced changes in vascularity, chemokine expression and adhesion further support the hypothesis that A-SAA engages TLR2 to act locally in the RA joint.

Finally, we demonstrated in RASFC and dHMVEC that SAA significantly induced MyD88, an adaptor protein for TLR2. Signalling from MyD88 to the activation NF-κB and activator protein-1 regulates inflammatory responses and a number of studies have demonstrated induced MyD88/MAL pathway in RA synovial fibroblasts, macrophages and tissue.[30–34 50] Previous reports have shown that mice deficient for the MyD88 adaptor molecule for IL-1R/TLR signalling failed to develop joint inflammation after induction of streptococcal cell wall arthritis, confirming an essential role for MyD88 in arthritis.[53] Furthermore, RA synovial explant conditioned media can activate macrophages in a MyD88-dependent manner.[30] As Myd88-mediated signalling pathways mainly stimulate the activation of NF-κB, our results suggest that MyD88 and NF-κB mediate the pro-inflammatory and angiogenic functions of A-SAA in RA.

A-SAA has traditionally been viewed as an acute-phase reactant and potential biomarker in inflammation, similar to erythrocyte sedimentation rate or C reactive protein. In this study, we have described critical pro-inflammatory functions in human cells to suggest that A-SAA is a functionally relevant pro-inflammatory molecule that exerts its effects via TLR2 signalling. The responses to human endothelium and RA cells provide a strong, possibly critical link, between A-SAA and the pathogenesis of vascular and joint inflammation. In addition, these data further support a role for A-SAA in other diseases where high A-SAA expression at site of inflammation has been demonstrated including atherosclerosis, Alzheimer's disease, type II diabetes and metabolic syndrome.[8–13 55 56] A better understanding of A-SAA/TLR2-mediated inflammatory pathways may lead to novel treatment strategies for inflammatory diseases.

Contributors All authors made substantial contributions to the conception or design of the work, or the acquisition, analysis or interpretation of data. All authors were involved in drafting manuscript and approved final version submitted.

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Competing interests None declared.

Ethics approval St Vincent's University Hospital Ethics Committee.

Provenance and peer review Not commissioned; externally peer reviewed.

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