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DISSERTATION

The influence of serum amyloid A on pro-inflammatory signaling in monocytes/macrophages cell lines

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Contents

Abbreviations

°C	degree centigrade
µg	microgram
µl	microliter
µmol	micromole
ACS	acute coronary syndromes
apoA	apolipoprotein A
apoE	apolipoprotein E
APP	acute-phase proteins
A-SAA	acute-phase serum amyloid A
BLT-1	block lipid transport-1
CCL	chemokine (C-C motif) ligand
CCR	C-C chemokine receptor
CKD	chronic kidney disease
CRP	c-reactive protein
C-SAA	constitutive serum amyloid A
CSF	colony stimulating factor
CT	cardiotropin
CVD	cardiovascular disease
CXCL	chemokine (C-X-C motif) ligand
DHE	dihydroethidium
DMEM	Dulbeccos´s modified eagle medium
DNA	deoxyribonucleic acid
ECM	extracellular matrix
ESRD	end stage renal disease
FBS	fetal bovine serum

FPR	formyl peptide receptor
Gp130	glycoprotein 130
HBSS	Hank's salt solution
HDL	high density lipoprotein
HKSA	heat killed staphylococcus aureus
ICUs	intensive care units
IFN	interferon
IL	interleukin
IL-6R	interleukin -6 membrane-bound receptor
L	litre
LDL	low density lipoprotein
LPS	lipopolysaccharide
MCP	monocyte chemoattractant protein
Min	minute
ml	milliliter
mmol	millimole
MMP	matrix metalloprotein
MODS	multiple organ dysfunction syndrome
NO	nitric oxide
NOS	nitric oxide synthases
OxPAPC	oxidation of 1-palmitoyl-2-arachidonoyl-snglycero-3-phosphorylcholine
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMA	phorbol 12-myristate 13-acetate
RAGE	receptor for advanced glycation end products
rhSAA	recombinant human Apo-SAA

ROI	reactive oxygen intermediates
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute
SAA	serum amyloid A
SD	standard deviations
Sgp 130	soluble form of gp130
sIL-6R	interleukin-6 soluble bound receptor
SMCs	smooth muscle cells
SR-BI	scavenger receptor class B type I
Th	T-helper cell
TLR	toll-like receptor
TNF	tumor necrosis factor
VEGF	vascular endothelial growth factor
VSMCs	vascular smooth muscle cells
WRW4	tryptophan-arginine-tryptophan-tryptophan-tryptophan-tryptophan-NH_2

Figures and tables

Zusammenfassung

Kardiovaskuläre Erkrankungen, wie beispielsweise die Atherosklerose, sind ein großes klinisches Problem und resultieren in einer hohen Morbiditäts- und Mortalitätsrate. Die Blutvergiftung (Sepsis), die eine komplexe systemische Entzündungsreaktion darstellt, ist ein Grund für die hohe Mortalitätsrate von Patienten auf Intensivstationen weltweit. Epidemiologische Studien zeigen eine Verbindung zwischen einem hohen Plasmaspiegel an Serum Amyloid A (SAA) und dem Auftreten einer Atherosklerose bzw. auch Sepsis. SAA ist ein Akutphaseprotein, dass hauptsächlich bei Verletzungen und Infektionen in Hepatozyten produziert wird. SAA stellt somit einen Entzündungsmarker dar. Die Rolle von SAA in der Entzündungsreaktion kardiovaskulärer Erkrankungen ist bislang nur unzureichend erklärt. Es ist bekannt, das Monozyten und Makrophagen eine zentrale Rolle in der Abwehrreaktion des Körpers einnehmen. Sie sind zelluläre Komponenten des sogenannten angeborenen Immunsystems. Aktivierte Makrophagen können proinflammatorische Mediatoren, wie u.a. SAA, Stickstoffmonoxid (NO), "monocyte chemoattractant protein-1" (MCP-1) und Interleukin 6 freisetzen.

Das Ziel der Arbeit war, die Rolle von SAA auf die Produktion von reaktiven Sauerstoffradikalen, Nitrit und Zytokinen in Monozyten/Makrophagen-Zelllinien (THP-1, RAW264.7), sowie die zugrundeliegenden Signalwege zu untersuchen. Die vorliegende Studie zeigt, dass SAA ein potenter endogener Stimulator der Nitritproduktion in der murinen Makrophagenzelllinie RAW264.7 ist. Dieser Effekt von SAA konnte durch Antagonisten für den "toll-like" Rezeptor (TLR) 2 und 4 sowie den SR-BI Rezeptorantagonisten blockiert werden. Außerdem wird durch Stimulation der humanen Makrophagenzelllinie THP-1 mit SAA die Produktion von Zytokinen, wie z.B. MCP-1 und IL-6, induziert. Auch hier ergaben Untersuchungen mit Agonisten/Antagonisten, dass die Aktivierung von TLR2&4 in die Signalwege involviert sind.

Zusammenfassend zeigte sich in diesen *in vitro* Untersuchungen, dass sowohl für die Nitritproduktion als auch die Zytokinproduktion in Makrophagenzelllinien die Aktivierung von TLRs vorausgehen: TLR2&4 Aktivierung für die Nitritproduktion, hauptsächlich TLR4 für die Produktion von MCP-1 und TLR2&4 für die Produktion von IL-6. Weitere Untersuchungen, insbesondere *in vivo* Tierstudien sollen nun zeigen, in wieweit diese Signalwege im Organismus pathophysiologisch relevant sind.

Abstract

Cardiovascular disease (CVD), like atherosclerosis, is one of the most common medical problems nowadays. Sepsis is one essential reason for the high mortality rates in intensive care units (ICUs) worldwide. Elevated plasma levels of serum amyloid A (SAA) were observed in these diseases. In addition, there is evidence demonstrating the relationship of SAA with the occurrence of atherosclerosis and sepsis. The acute-phase protein SAA is predominantly produced by hepatocytes in response to injury and infection. It is commonly considered as a marker for inflammatory diseases. The role of SAA on inflammatory processes and CVD has not been fully elucidated yet. It is well established that monocytes/macrophages play a central role in the first line of host defense. They are key cellular components of the innate immune system. Macrophages are able to release pro-inflammatory mediators like SAA, nitric oxide (NO), monocyte chemoattractant protein-1 (MCP-1) and interleukin 6 (IL-6) upon stimulation. However, SAA's precise functional signaling pathways are not clear.

The aim of this study was to investigate the effect of SAA on production of reactive oxygen species, nitrite, or cytokines in monocytes/macrophages. The study demonstrated that SAA is a potent endogenous stimulator of nitrite production in murine macrophages; nitrite was significantly increased upon SAA stimulation in a dose- and time-dependent fashion. The nitrite activation was blocked by antagonists of the TLR2/4-receptors or SR-BI-receptors. In addition, SAA dose-dependently induced cytokine (MCP-1 and IL-6) secretion in THP-1-derived macrophage-like cells. Here, especially activation of TLR-receptors plays a major role within the signaling pathway. In summary, SAA induced nitrite production in mouse macrophages via TLR2/4-receptors and SR-BI-receptor. Moreover, SAA induced MCP-1 secretion thorough TLR4 and induced IL-6 secretion thorough both TLR2 and TLR4 in THP-1-derived macrophage-like cells. Further work is warranted to establish animal models to investigate whether SAA exerts similar signaling *in vivo*.

1 Introduction

Atherosclerosis is one of the most common diseases in patients. It is a chronic inflammatory disease and characterized by excessive accumulation of lipoproteins within the subendothelial layer of the arterial wall.[1] Atherosclerosis is correlated with ischemic events, which are the main cause of premature deaths in western countries.[2, 3] Sepsis is an acute inflammation disorder, which is the main cause of mortality in intensive care units (ICUs) world-wide.[4] Estimated mortality of sepsis is between 30% and 50%.[4] Moreover, the incidence of sepsis increases by 1.5% per year.[5] Patients with chronic kidney disease (CKD) and end stage renal disease (ESRD) have high mortality. Nearly half of mortality is attributed to cardiovascular disease.[6] Previous studies found high levels of SAA in serum and high density lipoproteins (HDL) in ESRD patients. SAA reduces HDL's anti-inflammatory activity.[2] Accumulating evidence in large population-based studies showed that SAA is associated with inflammatory diseases.[7] The concentrations of SAA *in vivo* can increase by 1000-fold during an acute inflammatory condition (e.g., sepsis).[8] Thus, SAA may play an important role in inflammatory diseases.

1.1 Serum amyloid A

SAA is part of a family of highly conserved acute-phase proteins (APP).[9] The SAA family has been divided into acute-phase isoforms (A-SAAs) and constitutive isoforms (C-SAAs).[10]

In acute inflammation, the plasma levels of SAA can increase nearly by up to 1000 fold. SAA concentrations can even exceed 1 mg/ml over a short period of several days (<4 days).[8, 11] C-SAAs have been described in two species: human and mouse.[4] A-SAAs are mainly produced in the acute-phase response. Whereas, C-SAAs are minimally produced in the acute-phase response. SAA is mainly found in HDL, SAA (constitutive- and acute-phase) has properties of an apoliprprotein.[9, 10]

1.1.1 Mechanisms of SAA synthesis and its physiological function

The liver is the major site of A-SAAs synthesis and catabolism.[10] Besides the liver, vascular smooth muscle cells (VSMCs) and endothelial cells can also produce SAA.[12]

SAA associates with HDL when it is released into blood circulation.[13] Studies showed that SAA is also found in LDL, although SAA is mainly associated with HDL particles.[11, 14] It is known that SAA-LDL correlates with the occurrence of CVD.[14] Furlaneto CJ et al` showed that SAA performs its biological activities only when it is lipid-free.[11, 15] In contrast, it was shown that SAA-enriched HDL loses its anti-inflammatory capacity.[2] It is known that A-SAAs has important immune-related activities. SAA activates extracellular matrix (ECM)-degrading enzymes and is able to act as chemoattractant of many immune cells, such as monocytes/macrophages.[16]

To date, there are no selective SAA receptors known, but SAA interacts with a number of "unspecific" receptors. These receptors include the toll-like receptor (TLR) family such as TLR2 and TLR4,[17, 18] a class B scavenger receptor (SR-BI),[19] receptor of advanced glycation end-products (RAGE)[20] and the G-protein-coupled receptor formyl peptide receptor-like 1 (FPRL1).[21]

1.1.2 Role of SAA in pathophysiology

There is increasing evidence that SAA plays an important role in CVD.[7] SAA exerts several potential pro-atherosclerotic effects: (1) SAA induces pro-inflammatory cytokines production, such as TNF-α and IL-1; (2) SAA enhances chemotaxis of neutrophils and monocytes; (3) SAA induces production of the matrix metalloproteinases, which result in atherosclerotic plaque destabilization.[10, 11, 16] Animal studies showed that SAA is present in early atherosclerotic lesions.[22] Clinical studies found the expression of SAA in human atherosclerotic plaques.[12] Endothelial cells, VSMCs, and monocytes/macrophages produce SAA.[12] These types of cells play a very important role in different stages of atherosclerosis. Local expression and synthesis of SAA may contribute to the development of atherosclerotic lesions.

SAA can replace apoA1 and becomes the major apolipoprotein in HDL in acute-phase reaction.[2, 23] Our research group showed that SAA acts as a pro-inflammatory mediator. Then, it is one of the major apolipoprotein on HDL, and reduces HDL's anti-inflammatory activities in ESRD patients.[2]

In summary, SAA plays a pivotal role in inflammatory diseases and is a biological marker of inflammatory diseases (e.g., atherosclerosis, CKD and sepsis). Immune-related

activities of SAA may possibly be involved in the activation of the monocytes/macrophages, which produce a range of cytokines and chemokines.

1.2 Macrophages

It is well known that myelomonocytic cells, such as monocytes/macrophages, play a central role in the first line of host defense. They are the pivotal element of the innate and adaptive immune systems.[24, 25] $CD34^+$ myeloid progenitor cells are the major sites of monocyte production.[26] Monocytes enter peripheral tissues under inflammatory conditions. Then, monocytes mature into macrophages. These are characterized by a low rate of protein synthesis and oxygen consumption.[24, 26] Monocytes/macrophages respond to invading microorganisms through activating both innate and adaptive immune responses, in order to clear exogenous noxious materials and reinstate homeostasis finally.[25]

1.2.1 Physiological function of macrophages

Plasticity and diversity are the two principal characteristics of macrophages.[27] Moreover, it is well known that “plasticity” of macrophages is the response to changes of microenvironment *in vivo*.[28]

Macrophages are divided into two subtypes: (1) the classically activated macrophage phenotype, called M1; (2) the alternatively activated macrophage phenotype, called M2.[25, 27] Macrophages are able to produce a large amount of inflammatory mediators in inflammation, developing pro-inflammatory and anti-inflammatory profiles.[25] The main function of M1 macrophages is to produce pro-inflammatory factors, such as IL-6, MCP-1, NO or TNF.[29, 30] In contrast, M2 macrophages produce a number of anti-inflammatory factors, e.g., vascular endothelial growth factor (VEGF) and matrix metalloprotein-9 (MMP-9). These factors have the functions of tissue repair and remodeling. [27, 30, 31] Classification of T-helper cell 1 (Th1) and Th2 also influences the activities of monocytes/macrophages.[27]

1.2.2 Role of macrophages in pathophysiology

1.2.2.1 Macrophages in atherosclerosis

Macrophages are pivotal players in atherosclerosis.[24] Animal studies have shown that the content of M1 macrophages in plaque decreases, while M2 macrophages increase in mouse models of atherosclerosis regression.[32] In line with this, it was shown that M1 macrophages are the major phenotype associated with atherosclerosis progression.[33] These results strongly support the notion that macrophages are important elements in atherosclerosis, both progression and regression. In addition, these results indicate distinct pro-inflammatory and anti-inflammatory functions of M1 and M2 in atherosclerosis.[32] Collectively, plasticity and diversity, the key features of monocytes/macrophages, are correlated with their role in diverse processes of atherosclerosis.[25]

1.2.2.2 Macrophages in sepsis

Sepsis is a syndrome of deregulated inflammation, which is the major cause of mortality in ICUs world-wide.[4] Macrophages play one important role in this disease, and especially in response to exogenous noxious materials.[34] When macrophages are exposed to invading pathogens, a large amount of pro-inflammatory factors, such as IL-6, reactive oxygen species (ROS), TNF-α, and NO are produced by activated macrophages.[35] This reaction is necessary to clear the invading pathogens, meanwhile, activating the adaptive immune system.[35]

Macrophages can be activated by high levels of SAA, and release a large amount of pro-inflammatory factors, such as MCP-1, IL-6 and NO, in order to clear endogenous or exogenous noxious materials. Therefore, they act as the first host defence line.

1.3 Cytokines

Cytokines are small and secreted proteins 8-14 kDa in weight. They play an important role in the regulation of homeostatic and pathological processes *in vivo*. In addition, chemokines play a key role in migration control of monocytes/macrophages and leukocytes in response to innate and adaptive immune reaction.[36, 37]

Chemokines are grouped into four subgroups, the CC, CXC, C and CX3C families according to their genetic organization and the position at the N-terminus.[37] It is well accepted that chemokines have distinct inflammatory and homeostatic profiles based on their mode of action.[38] Until now, five members of MCPs were identified within CC chemokine family, which comprises MCP-1, MCP-2, MCP-3, MCP-4 and MCP-5.[39] Their related peptides have also been found, CCL2, CCL7, CCL8, CCL12 and CCL13.[40, 41]

1.3.1 Definition and occurrence of MCP-1

The MCP-1 is a member of the CC chemokine family and one of the best characterized chemokines to date. MCP-1 attracts circulatory monocytes into inflammatory tissues and controls migration of monocytes.[41] The human MCP-1 was first found in tumor cell lines.[42] MCP-1 can be produced by various cell types, such as monocytes/macrophages, endothelial, smooth muscle cells (SMCs), etc.[2, 43, 44] Nevertheless, the major sites of MCP-1 production are monocytes/macrophages.[43]

1.3.2 Mechanisms of MCP-1 production

A previous study found that in atherosclerotic lesions, macrophages can produce a large amount of MCP-1.[39] MCP-1 is mainly produced by tissues during inflammatory situations. *In vitro*, a variety of cell types can produce MCP-1. Usually, pro-inflammatory mediators like IL-1, TNF-α, or endotoxins stimulate the cells.[39]

In vivo, the most important function of MCP-1 is to attract monocytes and dendritic cells, but not neutrophils to inflammatory sites.[45] Similarly, *in vitro*, the key activity of MCP-1 is necessary to attract monocytes into chemotaxis chambers.[43] Animal studies have indicated that MCP-1 plays a pivotal role in controlling monocytes infiltrate into tissues under inflammatory condition.[40] In line with this, animal studies of MCP-1-deficient mice models have found that there is less lesion formation and macrophages recruitment in MCP-1 gene knockout mice compared to control.[46] Additionally, overexpression of MCP-1 in ApoE-deficient mice led to increased lesion formations characterized by enhanced deposition of macrophages and oxidized lipid.[47] These studies strongly indicate that MCP-1 is a pivotal player of atherosclerosis.

1.3.3 Physiological function of MCP-1

The main functional receptor of MCP-1 is C-C chemokine receptor type 2 (CCR2).[48]
Two isoforms occur within CCR2, of which are CCR2A and CCR2B, they are generated by the same gene via splicing.[49] In human monocytes, the main isoform of CCR2 is CCR2B, gene expression of CCR2 (A and B) is decreased after the maturation of monocytes into macrophages upon stimulation.[50] CCR2 has also been identified on VSMCs, so that MCP-1 may possibly interact with these cells. Indeed, MCP-1 can activate nuclear factor-κB to increase production of IL-6, integrins and tissue factors in SMCs.[51, 52] A previous animal study showed that deletion of CCR2 gene can significantly relieve atherosclerotic lesions.[53] These results suggested that MCP-1 and its receptor (CCR2) play an important role in atherosclerosis.

1.3.4 Role of MCP-1 in pathophysiology

In 2009, a large population-based study showed that elevated plasma MCP-1 is significantly positively correlated with atherosclerosis.[54] Additionally, clinical studies also indicated that high levels of plasma MCP-1 are associated with complications of atherosclerosis, e.g., increased mortality, myocardial infarction, ischemic stroke, and so on.[54] Furthermore, other studies showed elevated plasma MCP-1 to be related with increased mortality after acute coronary syndromes (ACS).[55] A clinical study with focus on bone marrow transplantation also showed that MCP-1 overexpression in macrophages resulted in increased risk of atherosclerosis formation.[47]
Infiltration of macrophages into arterial subendothelium is an important event in atherosclerosis. It is well established that atherosclerosis is a chronic inflammatory disease, and monocytes/macrophages are key players.[24] MCP-1 controls migration of monocytes, therefore, MCP-1 is a pivotal player of early atherosclerosis.[56] The feature of early atherosclerotic lesions is fatty streaks, which are made by lipid-laden macrophages. Which are called foam cells.[57, 58] Previous studies found that these foam cells are derived from circulating monocytes.[57] Furthermore, animal studies of atherosclerosis models showed that MCP-1 or its receptor CCR2 gene-knockout in mice resulted in a significant attenuation of lesion formations.[46]
MCP-1 and its receptor (CCR2) are key modulators in inflammatory diseases, such as atherosclerosis.

1.3.5 Definition and occurrence of IL-6

IL-6 is a further important element in inflammatory diseases.[59] IL-6 was identified in 1980 as a small secreted glycoprotein.[60] It is a member of the IL-6 family. Within this family, there are several cytokines, of which are cardiotropin-1 (CT-1), IL-6, IL-27, or IL-11.[61]

1.3.6 Physiological function of IL-6

In general, there are two different signaling pathways of IL-6: (1) classic-signaling, IL-6 activates cells via binding its membrane-bound receptor (IL-6R); (2) trans-signaling, IL-6 activates cells via binding its soluble IL-6R (sIL-6R).[62, 63] IL-6 exerts biological activities only in combination with IL-6R or sIL-6R, afterwards activating the glycoprotein 130 (gp130), its signal-transducing receptor.[62] Classic-signaling of IL-6 is correlated with anti-inflammatory functions.[63] In contrast, trans-signaling is responsible for the pro-inflammatory activities of IL-6.[60]

An animal study of a murine sepsis model showed that blockade of sIL-6R significantly decreased mortality.[64] In addition, another study showed that IL-6 induces MCP-1 secretion in endothelial cells through trans-signaling.[65] This strongly indicated that trans-signaling accounts for the pro-inflammatory functions of IL-6.

1.3.7 Role of IL-6 in pathophysiology

A number of cell types are able to synthesize and release IL-6, including macrophages, endothelial cells, etc.[66] Normally, the plasma levels of IL-6 are around 1-5 pg/ml in healthy individuals.[60] However, IL-6 acts as an inflammatory factor, then high levels can be found in most human diseases. The concentration of IL-6 *in vivo* can increase by thousand-fold during the acute inflammation as found in sepsis and trauma.[67]

IL-6 is an important element in infectious diseases and autoimmunity.[68] Under inflammatory conditions, although a variety of pro-inflammatory factors are able to induce hepatocytes to produce other APPs, such as C-reactive protein (CRP) and SAA, but IL-6 is a central one.[69, 70] Moreover, IL-6 participates in modulating migration, proliferation, and differentiation of targeted cells under inflammatory conditions.[68, 71]

Clinical studies of sepsis patients in ICU have shown that high plasma levels of IL-6 were positively correlated with mortality.[72] Other studies in CKD patients demonstrated an

increased mortality with high levels of IL-6 in kidneys and urine.[73] In the context of atherosclerosis, elevated plasma IL-6 is an independent risk of mortality.[74] Furthermore, animal studies have shown that IL-6 gene knockout animals results in attenuated tissues lesions in response to acute-phase inflammation.[75, 76]

Taking these results together, IL-6 plays an important role in inflammatory diseases.

1.4 Nitric oxide

1.4.1 Definition and occurrence of nitric oxide synthase

NO was identified in the 1980s.[77] *In vivo*, it is a pivotal signaling molecule which participates in various biological functions, including regulation of tissue oxygenation and blood flow.[77, 78] Abnormal production and transport of NO in blood vascular systems can result in cardiovascular diseases, e.g., atherosclerosis or hypertension.[79] NO is generated by various cell types, such as monocytes/macrophages, endothelial cells, neuronal cells, etc.[18, 80]

1.4.2 Mechanisms of nitric oxide synthesis

NO is generated by the following three different NO synthase (NOS) isoforms: (1) endothelial NOS (eNOS), also named NOS3, expressed in the endothelium; (2) neuronal NOS (nNOS), also named NOS1, expressed in the neural tissue; (3) inducible NOS (iNOS), also named NOS2, that is expressed by activated macrophages and injured tissues.[78, 81]

NOS1 and NOS3 are constitutive enzymes. Their up-regulation is dependent on intracellular Ca^{2+}/-calmodulin.[81] In contrast, NOS2 is an inducible enzyme expressed by the immune system. Production of NOS2 is correlated with gene transcription in reaction to inflammatory factors.[77]

1.4.3 Physiological function of nitric oxide synthase

Due to the feature that NO non-specifically impacts on various targets, normal cells and tissues could be damaged when increased NO concentration is expressed *in vivo*.[82]

Generally, NO generated by NOS1 is correlated with signaling of neuronal cells. Additionally, NOS1 expression can be induced by inflammatory stimuli.[83] NOS3 is a

constitutive enzyme. A range of stimuli can regulate NOS3 production.[84] Studies showed that human macrophages also express NOS3 under stimulation.[85] An *in vitro* study of murine macrophages showed that NO generated by NOS3 plays an important role in the incapacitation of macrophage activation.[86]

It is well known that NOS2 is involved in immune response. Studies have shown that NOS2 participated in clearing pathogens together with reactive oxygen intermediates (ROI).[87] NOS2 is expressed in macrophages at a low concentration. Expression of NOS2 in macrophages increased after stimulation.[78] Furthermore, human macrophages can produce NOS2, and NOS2 expression is correlated with many human diseases, such as atherosclerosis.[88]

It is well established that NO from NOS1 acts as a neurotransmitter.[77] The major sites of NO production from NOS1 or NOS3 are non-immunological cells, such as endothelium, muscle, and neurons.[89] In contrast, NO in the immune system is produced by, including macrophages. Therefore, the quantity of NO derived from NOS2 is higher than that generated by NOS1 and NOS2 NO. In addition, it was considered that both NOS1 and NOS3 have less immunological functions compared with NOS2. [84]

1.4.4 Role of nitric oxide synthase in pathophysiology

The incidence and mortality of sepsis is rising. Various therapies have been developed in recent decades.[5] Based on previous animal and clinical studies, it became increasingly clear that sepsis is a complicated syndrome, neither a single inflammatory factor nor a special signaling pathway accounts for its pathophysiology.[5] Animal studies found that mortality decreased in sepsis and hypovolaemic shock animal models through vagus nerve mediate cholinergic pathway to regulate production of cytokines and NO.[90] In line with this, a further animal study showed that inhibition of NO generation can prevent, even revert the septic shock induced by lipopolysaccharide (LPS) or TNF.[91]

High levels of cardiac output and persistent irreversible hypotension characterize septic shock. In 1986, NO was identified as one vasodilating factor accounting for vasodilatation in septic shock.[92] Furthermore, many studies proved that NOS2 is the main isoform of increased NO production in septic shock patients, and that NOS2 is generated from activated macrophages.[77, 93]

NO from NOS2 is an important element in the immune system, which is involved in clearing exogenous noxious materials.[93] Under inflammatory conditions, a variety of cytokines are correlated with the regulation of NOS2 synthesis, including IL-6, IL-1, and TNF-α.[93, 94]

NOS2 is also found in human atherosclerosis plaque.[95] In addition, animal study of ApoE-KO mice showed that NOS2 gene-deficient mice models have a significant reduction of atherosclerosis formation compared with control cohorts.[96] Elevated levels of NOS2 in the vascular system are associated with an increased production of peroxynitrite.[97] Peroxynitrite is involved in progression of atherosclerosis.[78] These results indicate that NOS2 plays a pivotal role in the pathogenesis of atherosclerosis. Furthermore, it is well known that NOS2 is an important pathogenic mechanism in inflammatory diseases.[79]

NO plays a central role in inflammatory diseases, such as atherosclerosis and septic shock. Therefore, the generation of NO must be tightly regulated and controlled.

A summary of possible pathogenesis of SAA in inflammatory diseases is illustrated in a schematic diagram (Figure 1).

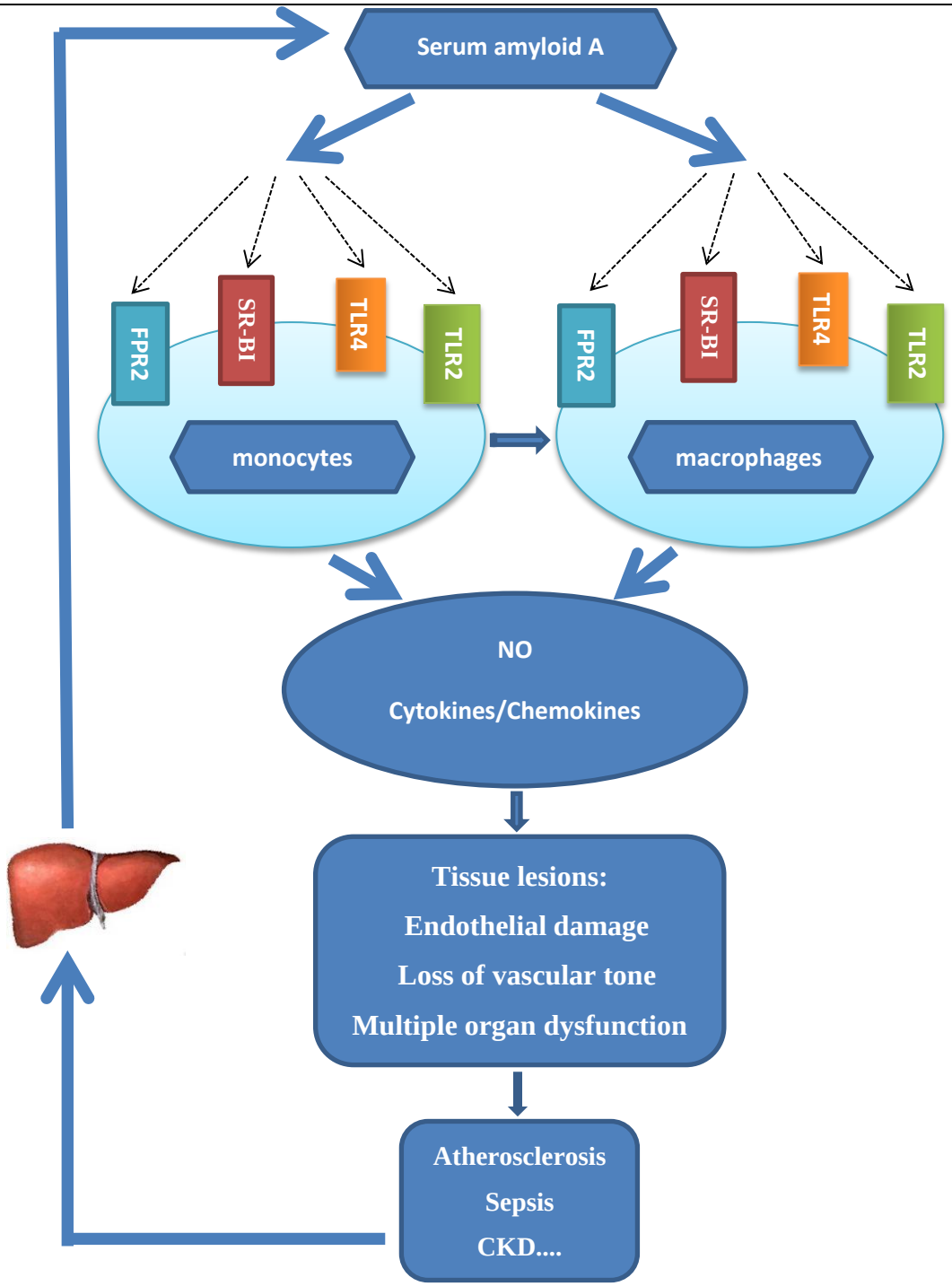


Figure 1. Possible pathogenesis of inflammatory diseases mediated by SAA
Inflammatory diseases, such as atherosclerosis, CKD, and sepsis characterized by high levels of plasma SAA. SAA possibly contribute to the progression of these diseases via stimulation of monocytes/macrophages to produce a large amount of pro-inflammatory factors, including NO, MCP-1 and IL-6. The potential signaling pathways involved in SAA-induced production of pro-inflammatory factors in monocytes/macrophages possibly via cell surface receptors TLR2/4, FPR2, SR-BI and RAGE. Overexpression of these pro-inflammatory factors leads to tissue lesions, such as loss of vascular tone, endothelial damage, and MODS. A particularly vicious circle is thus formed.

1.5 Objective of the study

The purpose of the current study was to assess the impact of SAA on signaling pathways in murine and human monocytes/macrophages. Previous research has shown that SAA is an acute phase protein known to accumulate during acute as well as chronic inflammatory disease, e.g., in septic patients, CKD patients or patients with diabetes mellitus. The impact of SAA on vascular disease and its signaling pathways in these patient cohorts is less well understood. High SAA plasma level correlates with less anti-inflammatory potential of endogenous protective components. It is hypothesized that SAA has direct pro-inflammatory potential by receptor activation and down-stream inflammatory reaction.

Therefore, this study aims to investigate down-stream pro-inflammatory mediators like NO, MCP-1 and IL-6 upon SAA stimulation of monocytes/macrophages. In addition, the cell surface receptors known to be activated by SAA were investigated using receptor agonist and antagonist.

The findings provide evidence that NO production in murine macrophages is mainly dependent on TLR2 and TLR4 and SRBI. MCP-1 is induced by TLR4 activation in THP-1-derived macrophage-like cells, whereas both TLR2 and TLR4 are responsive to IL-6 secretion of THP-1-derived macrophage-like cells.

2 Methodology

2.1 Reagents and materials

Table 1. Reagents and materials

Agarose	Life Technologies, Oregon, USA
Dihydroethidium (DHE)	Life Technologies, Oregon, USA
Dimethylsulfoxide	Sigma Aldrich, MO, USA
Juglone	Sigma Aldrich, MO, USA
Roti® Load DNA Stain	Carl Roth, Karlsruhe, Germany
TAE buffer	Sigma Aldrich, MO, USA

2.1.1 Cell culture materials

All cell culture materials were used in sterile condition. The media and buffers are listed in Table 2. All cell culture flasks and plates were Nuclon®-cell-culture-treated (Thermo Scientific, MA, USA). All other disposables were obtained from Nunc™, TPP and Falcon (Thermo Scientific, MA, USA).

Table 2. Materials for cell culture

Dulbeccos´s modified eagle medium (DMEM)	Biochrom AG, Berlin, Germany
Fetal bovine serum (FBS)	Biochrom AG, Berlin, Germany
Hank's salt solution (HBSS)	Sigma Aldrich, MO, USA
L-glutamine	Biochrom AG, Berlin, Germany
Penicillin/Streptomycin	Biochrom AG, Berlin, Germany
Phosphate buffered saline (PBS)	Biochrom AG, Berlin, Germany
Roswell Park Memorial Institute (RPMI) 1640	Biochrom AG, Berlin, Germany

2.1.2 Agonists, antagonists

Human recombinant SAA (rhSAA) was obtained from Peprotech (London, UK). rhSAA is an apolipoprotein molecule with a consensus sequence of human SAA1 including an N-terminal amino acid substitution as present in human SAA2. According to the manufacturer's instruction, the level of endotoxin within rhSAA is below 0.1 ng per mg protein. For the characterization of the receptors involved in the signaling pathway, the receptor agonists and antagonists are listed in Table 3.

Table 3. Receptor agonists and antagonists

Receptors	Agonists		Antagonists	
formyl peptide receptor like-1 (FPR 2)	MMK-1	Tocris , MO, USA	WRW4	Tocris, MO, USA
A class B scavenger receptor (SR-BI)			BLT-1	Merck Millipore, Darmstadt, Germany
Toll-like receptor 2 (TLR 2)	HKSA	InvivoGen, San Diego, USA	CLi-095, OxPAPC	InvivoGen, San Diego, USA
Toll-like receptor 2/4 (TLR 2/4)	LPS	InvivoGen, San Diego, USA	OxPAPC	InvivoGen, San Diego, USA

2.1.3 Software

Table 4. Software information

Ascent, version 2.6
Bioplex, version 4.1.1
ChemiCapt 3000, version 2.4
Endnote X7
GraphPad prism, version 5.0
Microsoft Office 2013
MikroWin, version 4.41
Skanlt, version 2.4.4

2.2 Cell culture, differentiation and stimulation

All cell culture work was done in sterile conditions with sterile media, buffer and cell culture disposables.
The Neubauer Hemocytometer was used to determine the cell number for experimental settings.

2.2.1 Human THP-1 cells

The human monocytic cell line THP-1 (ATCC 202-TIB) was obtained from the American Type Culture Collection (Manassas, VA, USA). The cells, were grown in RPMI-1640 medium supplemented with 10% FBS, 1% penicillin-streptomycin and L-glutamine (2 mmol/L) at 37 °C in a humidified atmosphere containing 5% CO_2 . The culture medium was changed twice a week with a cell density set to 3-6*10^5 cells/ml. Figure 2A shows a representative picture of cultured THP-1 monocytes.
To induce mature macrophages, the cells were treated with 100 ng/ml PMA (Sigma, MO, USA) for 24 h. Accordingly, the THP-1 adhere to the culture flask (Figure 2B). 24 h after cell activation, adherent cells were washed twice with medium and incubated with fresh medium lacking PMA. THP-1-derived macrophage-like cells (1*10^6 cells/ml) were seeded in 96-well plates in serum-free condition, and were exposed to varying concentrations of rhSAA (0.5, 1, 5, and 10 µg/ml) for various periods of time (24 h and 48 h). Subsequently, the supernatants were collected for cytokine measurements. In the signaling pathway studies, THP-1-derived macrophage-like cells were incubated with and without agonist or antagonist for investigated receptor activation in co-stimulation with rhSAA (10 µg/ml) for 24 h, respectively. Afterwards, the supernatants were collected for cytokine measurements, too.

2.2.2 Murine RAW264.7 cells.

The RAW264.7, murine monocytes/macrophages cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in DMEM (4.5 g/L glucose) supplemented with 10% FBS, 1% penicillin-streptomycin and L-glutamine (2 mmol/L) in a humidified atmosphere at 37 °C with 5% CO_2. The cells

were sub-cultured every 2-3 days. Prior to treatment, the cells were grown for 24 h. In Figure 2C, a representative picture of cultured RAW264.7 cells is shown.

For nitrite measurements, RAW264.7 cells were seeded in a cell density of $1*10^6$ cells/ml in a 96-well plate and treated with varying concentrations of rhSAA (0.5, 1, 5, and 10 µg/ml) and LPS (0.1, 1, and 5 µg/ml). After various periods of stimulation time (24 h and 48 h), the supernatants were collected for nitrite measurements. In the signaling pathway studies, RAW264.7 cells were incubated with and without agonist or antagonist for receptor activation in co-stimulation with rhSAA (1 µg/ml) for 24 h and 48 h.

A B C

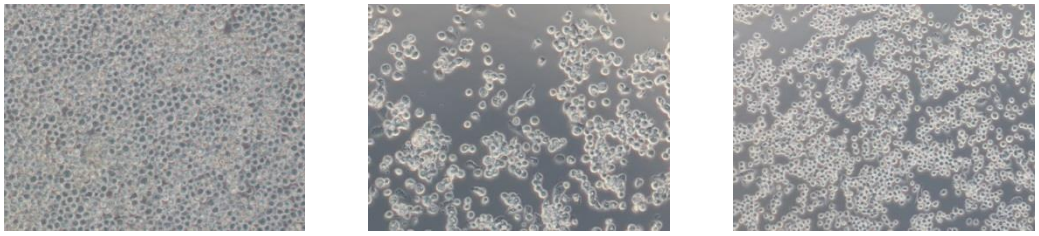


Figure 2. Cultured cells
Representative microscopic images (magnification 200X) of cultured (A) THP-1 monocytes, (B) THP-1 cell after PMA (100 ng/ml) treatment for 24 h and (C), RAW264.7 cells are shown.

2.2.3 Freezing and unfreezing of cells

For long-term storage, the cells were frozen in a freezing medium containing 90% FCS and 10% dimethylsulfoxide. The cell pellet was resuspended within the freezing medium, transferred to a cryo tube and subsequently cooled down with a frost container filled up with isopropanol. After at least 24 h at -80°C, the cryo tube with cells was transferred to a cell container with liquid nitrogen, where the cells were stored in the gas phase.

2.3 Mycoplasma test

Mycoplasma contamination is one of the most common problems of cultured cells. About 87% of cell lines are contaminated with mycoplasma.[98] In addition, continuous sub-culturing of cells increases mycoplasma contamination to around 15-35%.[99] The functions and activities of mycoplasma containing cells are influenced and could result in

unreliable experimental results.[98] Thus, RAW264.7 and THP-1 cells were routinely tested for mycoplasma contamination.

To test for mycoplasma contamination, the commercially available LookOut™ Mycoplasma PCR Detection Kit (Sigma Aldrich, MO, USA) was used according to the manufacturer's instructions.

The PCR products were separated via agarose gel (1%) electrophoresis. Roti® GelStain (Carl Roth, Germany) was used to visualize bands of the PCR products. The Quick-Load® 100 bp DNA ladder (New England Biolabs, USA) was used as molecular weight marker. Each sample, including the positive and negative control, contains an internal control resulting in a PCR product of 481 bp (Figure 3). All samples positive for mycoplasma contamination give an additional band of 259 bp, as shown for the positive control (Figure 3, lane 2). Always, the cells were free of mycoplasma contamination (Figure 3, representative image).

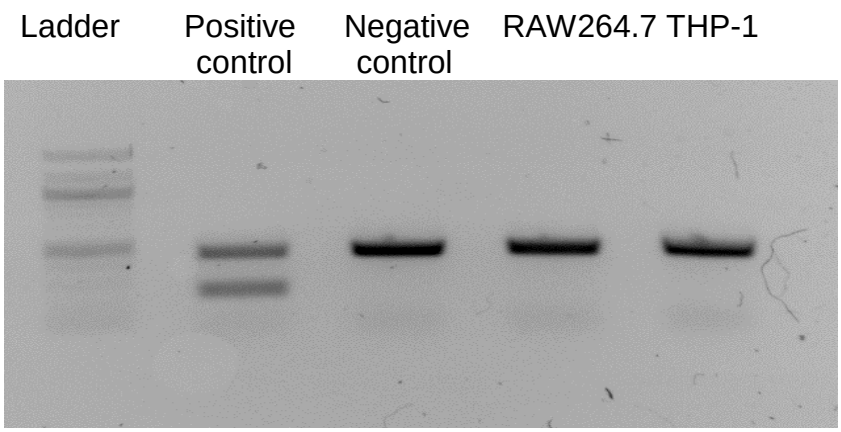


Figure 3. Mycoplasma test for THP-1 and RAW264.7 cells
Cell culture supernatants from RAW264.7 and THP-1 cell lines were collected and mycoplasma PCR were performed. The PCR products were separated via a 1% agarose gel stained with a fluorescence dye. A 100 bp ladder serves as molecular weight standard. Samples positive for mycoplasma result in a PCR band of 259 bp. All samples contain an internal standard resulting in a PCR product of 481 bp. The image shows a representative result of routinely performed experiments (n=3).

2.4 Determination of protein concentration

The commercial Pierce™ BCA Protein Assay Kit assay kits (Thermo Scientific, USA) were used to determinate the protein concentration of cells. The cells were lysed in NP40 Cell Lysis buffer (Life Technologies, Oregon, USA) supplemented with a protease

inhibitor mix (Roche Diagnostics Corporation, Indianapolis, USA). Afterwards, the lysed cells were centrifuged at 4°C, 1200 x g for 10 min for clarification of debris. Afterwards, 10 µl samples were mixed with 200 µl working solution and incubated for 30 min at room temperature. The absorbance was measured at 495 nm wavelength using a 96-well plate reader (Labsystems iEMS Reader MF, Thermo Scientific, USA). According to the manufacturer's instructions, the concentration of protein was calculated using an albumin standard curve.

2.5 Detection of MCP-1 and IL-6 secretion in THP-1-derived macrophage-like cells

For the detection of cytokine secretion, the Luminex™ technology in a Bio-Plex 100 device (Bio-Rad, Muenchen, Germany) was used. For data acquisition and analysis, the Bio-Plex (version 4.1.1) software was used. The levels of cytokine secretion in the cell supernatant were determined with commercially available assay kits (Millipore, Schwalbach, Germany) for MCP-1 and IL-6 detection. The measurement was done according to the manufacturer's instructions. Each assay included a standard curve. The measurements were run as multiplex assay for MCP-1 and IL-6. The results were normalized to the cell protein content. For determination of the protein content, the cells were washed twice with ice-cold PBS and then lysed in NP40 Cell Lysis buffer (Life Technologies, Oregon, USA) on ice for 5 min. After centrifugation at 2000 x g for 10 min, the protein content of the cells were determined using the Pierce™ BCA detection kit (see also 2.4).

THP-1-derived macrophage-like cells were seeded in a cell density of $1*10^6$ cells/ml in 96-well plates in serum-free condition for 24 h. Cells were stimulated with different concentrations of rhSAA (0.5, 1, 5, and 10 µg/ml) or agonists for 24 h or 48 h. If antagonists were used, the cells were co-stimulated with rhSAA (10 µg/ml) and antagonists in indicated concentrations. Afterwards, supernatants were collected and centrifuged at 1200 x g for 10 min for clarification of debris. The levels of cytokine secretion were measured by the Luminex^{TM} technique (Millipore, Schwalbach, Germany) according to the manufacturer’s directions. The results were normalized by cell protein content of each well.

2.6 Measurement of NO production in RAW264.7 cells

NO levels generated from monocytes/macrophages were determined by measuring nitrite, a stable degradation product of NO. Using the Griess system (NO Assay kit, Promega, USA), nitrate is reduced to nitrite by nitrate reductase. Therefore, NO levels can be represented by measured nitrite levels.[100]

RAW264.7 cells were seeded at a density of $1*10^6$ cells/ml in 96-well plates. The cells were serum-starved for 24 h before stimulation. Afterwards, the cells were stimulated with rhSAA (0.5, 1, 5, and 10 µg/ml) and LPS (0.1, 1, and 5 µg/ml) in serum-free media for 24 h and 48 h.

The supernatants were collected and centrifuged at 1200 x g for 10 min for clarification of debris. 50 µl aliquots of supernatant was mixed with 100 µl of the Griess reagent. After an incubation period for 5-10 min at room temperature, the absorbance was measured at 540 nm using a 96-well plate reader (Multiskan Spectrum, Thermo Electron Corporation, USA). Each assay contains a nitrite standard curve for calculation of the nitrite amount.

2.7 Cell cytotoxicity by MTT assay

The viability of THP-1-derived macrophage-like cells and RAW264.7 after varying stimuli was determined using CellTiter 96® AQueous One Solution Cell Proliferation Assay kit (Promega, USA).

The cells were seeded (THP-1-derived macrophage-like cells: $1*10^6$ cells/ml, RAW264.7: $1*10^6$ cells/ml) in 96-well plates (100 µl per well) and serum-starved for 24 h before stimulation. The cells were stimulated in serum-free media with rhSAA (10 µg/ml) in co-stimulation with antagonists for 24 h and 48 h. The viability assay was performed according to the manufacturer´s directions by adding 20 µl of the CellTiter 96® AQueous One Solution Reagent. The plate was incubated for 1 hour at 37°C in a humidified atmosphere containing 5% CO_2. Afterwards, the absorbance was measured at 490 nm using a 96-well plate reader (MultiSkan Spectrum, Thermo Electron Corporation, USA).

2.8 Detection of intracellular ROS

Dihydroethidium (DHE, Ex/Em = 518 nm/605 nm), is a fluorescent probe which specifically reacts with ROS.[101] THP-1 monocytes, THP-1-derived macrophage-like cells and RAW264.7 cells were seeded in 96-well plates (3.5×10^4 cells/well in 200 µl) containing serum-free medium incubated with rhSAA and LPS for 24 h. After incubation, cells were washed twice with PBS followed by cell resuspension in HBSS. DHE (final concentration of 20 µmol/L) was added to each well and incubated for 30 min at 37°C in the dark. Fluorescence intensity expressed the levels of ROS and was measured using a Mithras plate reader (Ex/Em=530 nm/620 nm) (Berthold Technologies, Bad Wildbad, Germany).

2.9 Statistical analyses

The experiments were conducted in at least three independent experiments to confirm the reproducibility of the results. The individual number of experiments is given in the figure legend. Data are presented as means with standard deviations (SD), unless otherwise indicated. The statistical significance was determined using the GraphPad Prism software 5.0 (GraphPad Software Inc, La Jolla, CA, USA). A P value<0.05 was considered as statistically significant.

3 Results

In the course of this project, the impact of SAA on the pro-inflammatory reactions of monocytes/macrophages as well as the receptors involved were investigated *in vitro*. Therefore, the production of NO or ROS and the cytokine secretion of MCP-1 or IL-6 were studied. To determine the receptor activation in macrophages upon rhSAA stimulation, agonists and antagonists for various receptors activated by SAA were used. For this purpose, the murine monocyte/macrophage cell line RAW264.7 and the human monocytic cell line THP-1 were investigated in the following study. Beside THP-1 monocytes, the macrophage-like state of this cell line was investigated upon PMA treatment for 24 h.

3.1 rhSAA and LPS did not induce superoxide generation in monocytes/macrophages

As already known in literature, inflammatory cells like monocytes/macrophages produce ROS as a defense mechanism.[102] Therefore, the impact of ROS production should be investigated upon rhSAA treatment of RAW264.7 and THP-1 cells.

The production of superoxide, one of the ROS molecules, could be measured via DHE labeling of the cells. Juglone, a generator of ROS,[103] was used as positive control. The impact of rhSAA (0.01, 0.1, and 1 µg/ml) and LPS (0.1, 1, and 5 µg/ml) was tested within this assay for various periods of time. However, neither rhSAA nor LPS induced superoxide anion production in RAW264.7, THP-1 monocytes and THP-1-derived macrophage-like cells (data not shown). Each experiment was run with juglone as positive control. Juglone induced superoxide production in a dose- and time-dependent manner (Figures 4 and Figure 5) in all investigated cells.

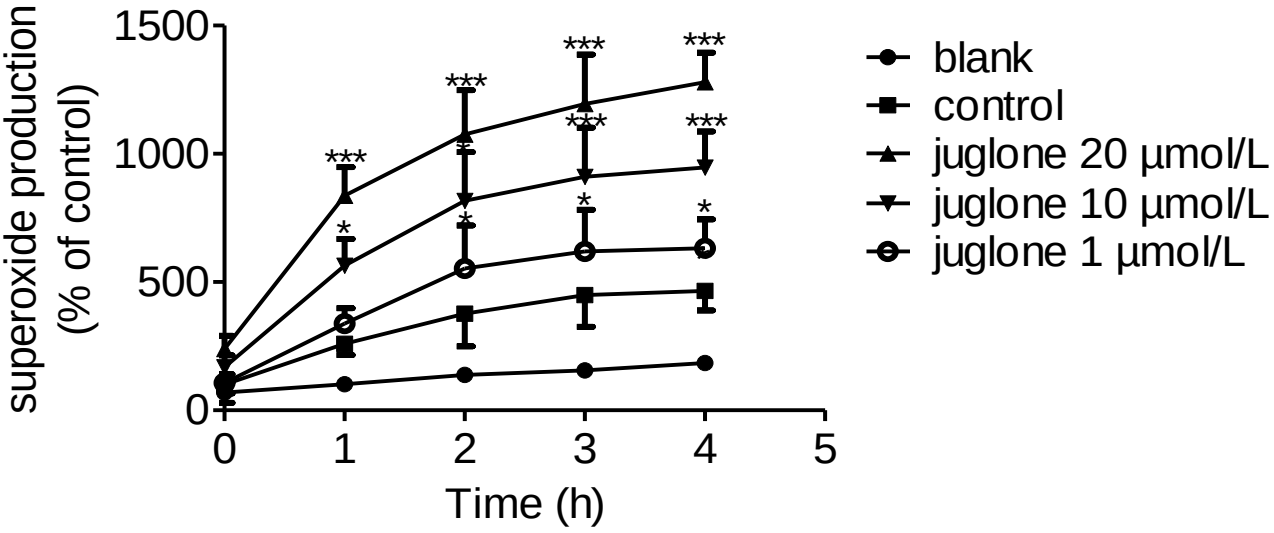
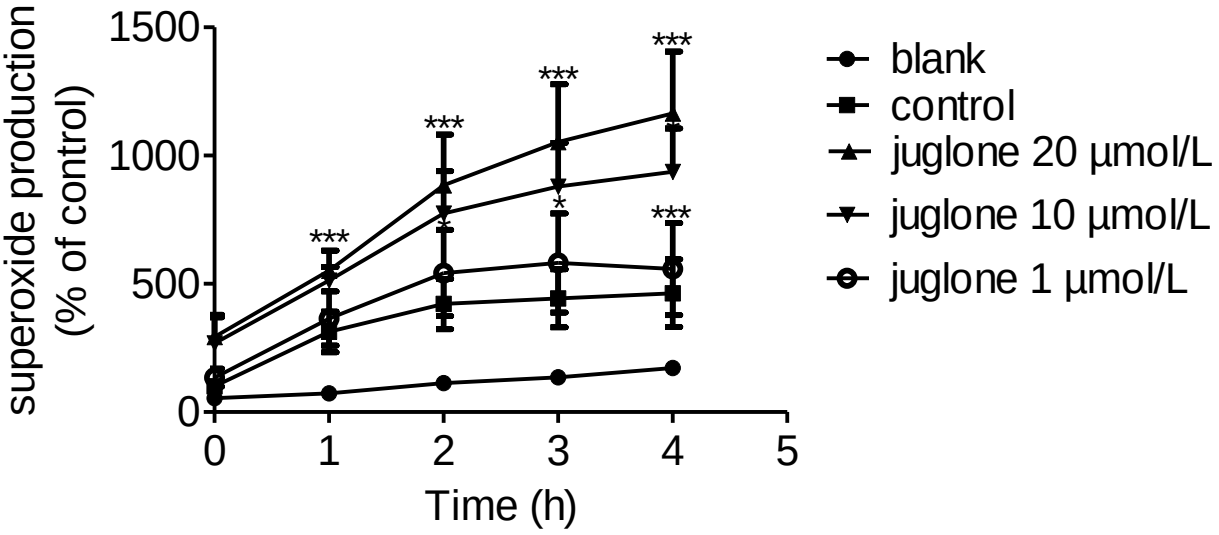


Figure 4. Juglone induced superoxide generation in RAW264.7 cells
RAW264.7 cells were treated with indicated concentration of juglone. Superoxide production is expressed as percentage to control. Untreated cells (control) were set to 100%. PBS was used as blank. Values represented as mean ± SD of three independent experiments. * $p<0.05$; *** $p<0.001$ significant change vs` control. Mann–Whitney U test.

A



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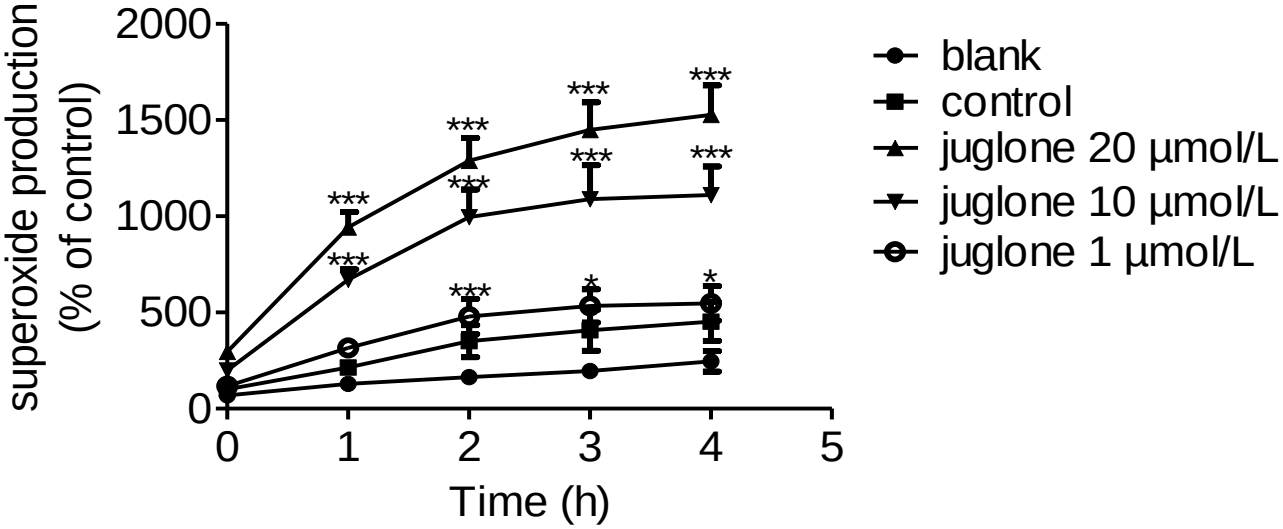


Figure 5. Juglone induced superoxide generation in THP-1 monocytes and THP-1-derived macrophage-like cells

(A) THP-1 monocytes and (B) THP-1-derived macrophage-like cells were treated with indicated concentration of juglone. Superoxide production is expressed as percentage to control. Untreated cells (control) were set to 100%. PBS was use as blank. Values represented as mean ± SD of three independent experiments. * $p<0.05$; *** $p<0.001$ significant change vs` control. Mann–Whitney U test.

3.2 LPS and rhSAA induced nitric oxide production in RAW264.7 cells

A previous study has shown that LPS induces NO production in human monocytes/macrophages.[104] Consistent with the previous report, the present results show that LPS stimulated nitrite production in RAW264.7 cells in a dose- and time-dependent manner (Figure 6). Therefore, LPS (1 µg/ml) was used as positive control in this study. To investigate the potential of rhSAA inducing nitrite production, RAW264.7 cells were exposed to different concentrations of rhSAA (0.1, 0.5, 1, and 10 µg/ml). With 24 h and 48 h incubation period, rhSAA dose-dependently and significantly induced nitrite production. Moreover, the induction of nitrite production by rhSAA was time-dependent. Compared to the 24 h incubation period, nitrite production increased around 70-150% in various rhSAA concentrations at 48 h incubation period (Figure 7).

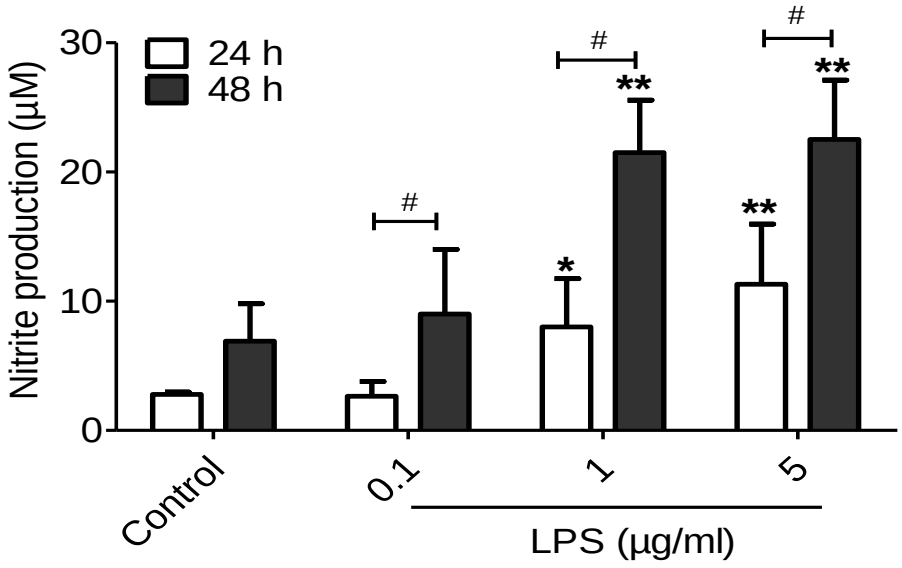


Figure 6. LPS induced nitrite production in RAW264.7 cells
Cells were stimulated at indicated concentrations of LPS for 24 h and 48 h. Nitrite production was measured using Griess reagent. Untreated cells were used as controls. Values represented as mean ± SD (n(24 h)=5,n (48 h)=4). * $p<0.05$; ** $p<0.01$ significant change vs control. In all panels, there is a significant difference between 24 h and 48 h incubation time (#, $p<0.05$). Mann–Whitney U test.

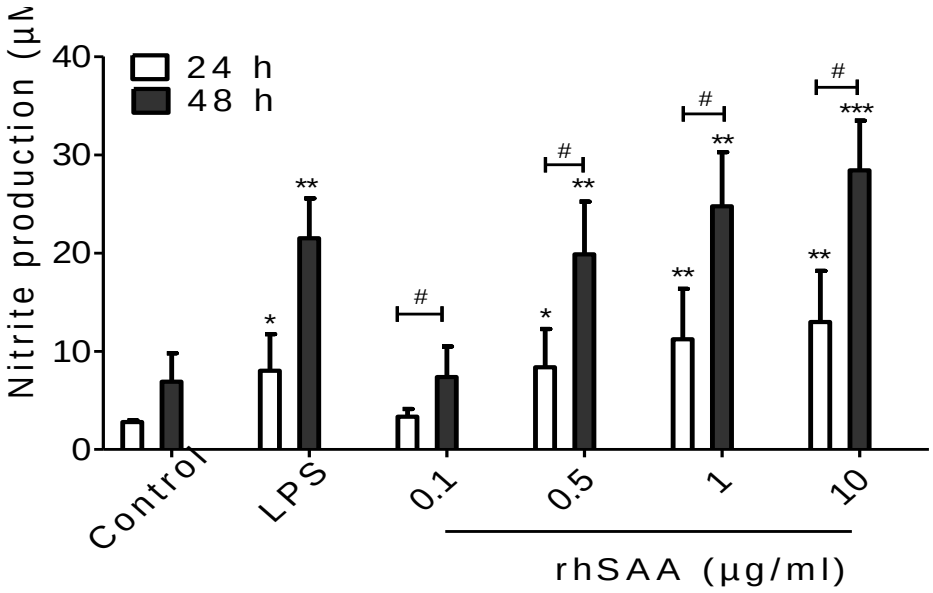


Figure 7. rhSAA and LPS induced nitrite production in RAW264.7 cells
Cells were stimulated at indicated concentrations of rhSAA for 24 h and 48 h. Nitrite production was measured using Griess reagent. Untreated cells were used as controls. LPS (1 µg/ml) was used as the positive control in nitrite production induced by rhSAA. Values represented as mean ± SD (n (24h)=5, n (48h)=4). * $p<0.05$; ** $p<0.01$, *** $p<0.001$ significant change vs control. In all panels, there is a significant difference between 24 h and 48 h incubation time (#, $p<0.05$). Mann–Whitney U test.

In order to determine whether rhSAA and LPS have same effects on THP-1 monocytes and macrophages, cells were treated with varying concentrations of rhSAA (0.5, 1, 5, and 10 µg/ml) and LPS (0.1, 1, and 5 µg/ml) for various periods of time (24 h or 48 h). There was no detectable nitrite production, neither in THP-1 monocytes nor in THP-1-derived macrophage-like cells (data not shown). The results imply that rhSAA and LPS did not induce nitrite production in THP-1 monocytes/macrophages.

3.3 rhSAA induced the secretion of MCP-1 and IL-6 secretion in THP-1-derived macrophage-like cells

To examine whether rhSAA have direct effects on the formation of pro-inflammatory cytokines like MCP-1 and IL-6, the THP-1-derived macrophage-like cells were exposed to different concentrations of rhSAA (0.5, 1, 5, and 10 µg/ml) for 24 h and 48 h. Protein concentrations of MCP-1 and IL-6 in the culture supernatants were measured by Luminex^{TM} technique. After 24 h incubation time, rhSAA significantly induced MCP-1

secretion in THP-1-derived macrophage-like cells in a dose-dependent manner. MCP-1 production further increases to around 20% by prolonging the incubation time to 48 h; however, the data did not reach statistical significance (Figure 8). As shown in Figure 9, rhSAA also induced IL-6 secretion in THP-1-derived macrophage-like cells in a dose-dependent manner. The highest concentration of rhSAA (10 µg/ml) induced IL-6 secretion in a time-dependent manner. In comparison with MCP-1 secretion, basal secretion of IL-6 is lower, close to the detection limit of the assay (3.2 pg/ml). The results suggested that rhSAA was a strong inductor of pro-inflammatory cytokines in THP-1-derived macrophage-like cells.

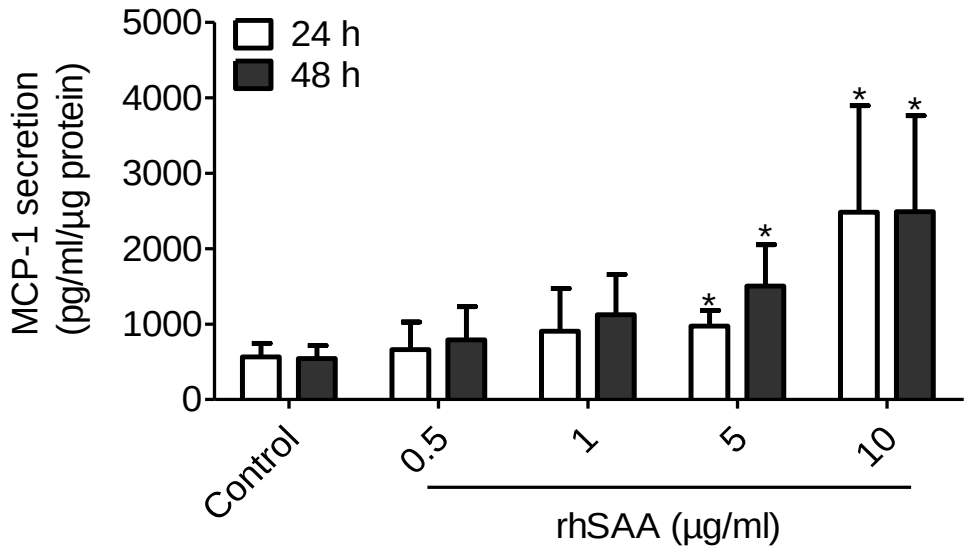


Figure 8. rhSAA induced the secretion of MCP-1 in THP-1-derived macrophage-like cells

Cells were stimulated at indicated concentrations of rhSAA for 24 h and 48 h. MCP-1 secretion was measured by Luminex^{TM} technique. Untreated cells were used as control. Values represented as mean ± SD (n (24h)=5, n (48h)=4). * $p<0.05$ significant change vs control. Mann–Whitney U test.

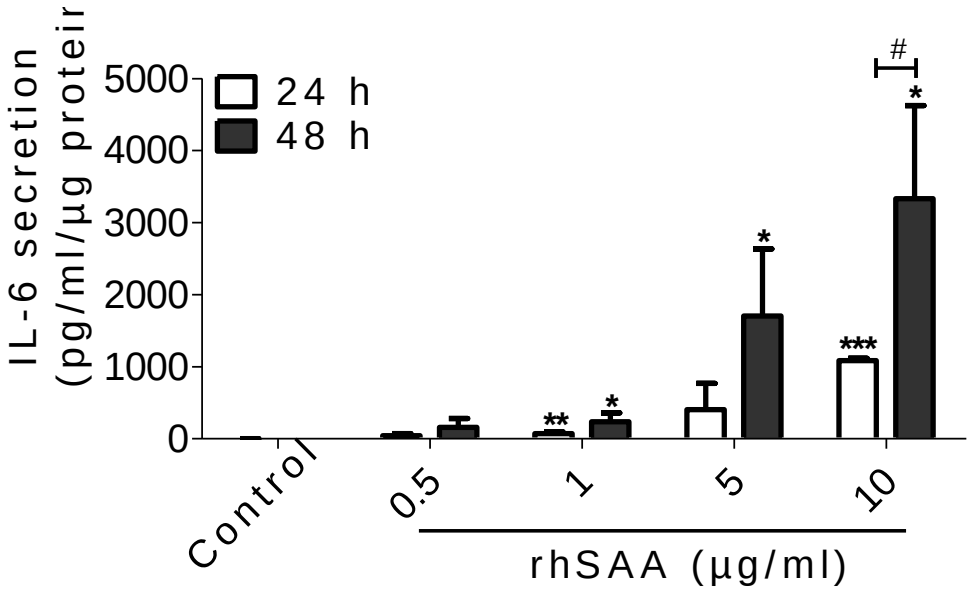


Figure 9. rhSAA induced the secretion of IL-6 in THP-1-derived macrophage-like cells
Cells were stimulated at indicated concentrations of rhSAA for 24 h and 48 h.IL-6 secretion was measured by Luminex™ technique. Untreated cells were used as control. Values represented as mean ± SD (n=3). * p<0.05; ** p<0.01; *** p<0.001 significant change vs control. IL-6 secretion induced by rhSAA (10 µg/ml), there is a significant difference between 24 h and 48 h incubation time (#, p<0.05). Mann–Whitney U test.

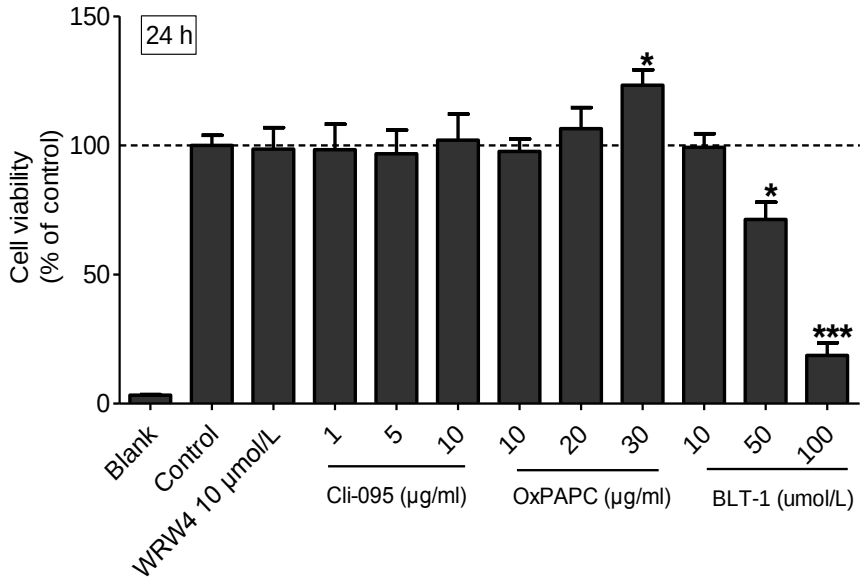
3.4 Mechanism of rhSAA-induced production of nitrite and cytokines in macrophages

SAA has been shown to interact with several cell-surface receptors, including TLR2, TLR4,[17, 18] SR-BI,[19] and FPR2.[21] Moreover, these receptors are expressed in human and murine macrophages.[17, 18, 105, 106] In the present study, OxPAPC, Cli-095, WRW4 and BLT-1 were used as selective antagonists for TLR2&4, TLR4, FPR2 and SR-BI, respectively. HKSA, LPS and MMK-1 were used as selective agonists for TLR2, TLR4 and FPR2, respectively. These ligands were used in the following signaling experiments.

3.4.1 Cell viability

To test the potential cytotoxicity of the above antagonists to the cells, CellTiter 96® AQueous One Solution Cell Proliferation Assay kit (Promega, USA) was used. The RAW 264.7 cells were treated with rhSAA (1 µg/ml) in co-stimulation with these antagonists: WRW4 (10 µmol/L), OxPAPC (10, 20, and 30 µg/ml), Cli-095 (1, 5, and 10 µg/ml), or BLT-1 (10, 50, and 100 µmol/L) for 24 h (A) and 48 h (B), respectively.

A



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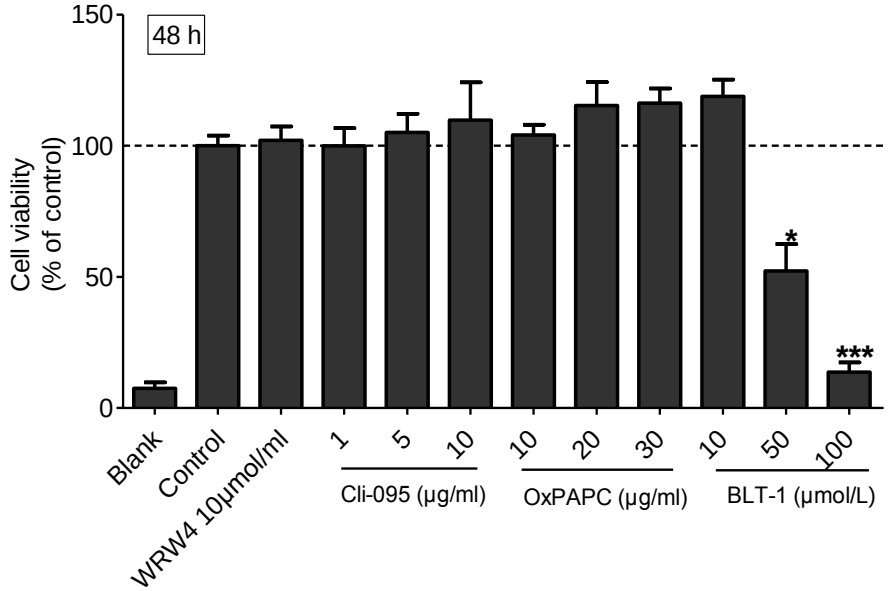


Figure 10. Effect of the antagonists of SAA receptors on cell viability-RAW264.7 cells

The RAW264.7 cells were stimulated with rhSAA (1 µg/ml) in co-stimulation with the antagonists of SAA receptors (concentration as indicated) for (A) 24 h and (B) 48 h. Cell viability is expressed as percentage to control. Untreated cells (control) were set to 100%. PBS was use as blank. Values represented as mean ± SD (n=4). * $p<0.05$; *** $p<0.001$ significant change vs control. Mann–Whitney U test.

MTT assay revealed that the cell viability was similar to that of the control cells, under the treatment with WRW4, OxPAPC (10 µg/ml), Cli-095 and the lowest concentration of BLT-1 (10 µmol/L). However, higher concentrations of BLT-1 (50 and 100 µmol/L) resulted in a decrease in viability by nearly 20% and 75%, respectively at 24 h, and even more at 48 h. In addition, high concentrations of OxPAPC (30 µg/ml) had a proliferating effect on RAW264.7 cells (Figure 10A and 10B).

Thus, 10 µmol/L of BLT-1, and 10 µg/ml of OxPAPC, 1 µg/ml Cli-095 and 10 µmol/L WRW4 were selected for the further evaluation of their inhibitory effects on the signaling pathway in RAW264.7 cells.

THP-1-derived macrophage-like cells were exposed to rhSAA (10 µg/ml) with the antagonists, WRW4 (10 µmol/L), OxPAPC (30 µg/ml), Cli-095 (10 µg/ml) and BLT-1 (20, 50, and 100 µmol/L) for 24 h. No obvious effects was observed after treatment with these antagonists except that high concentrations of BLT-1 (50 and 100 µmol/L), where a significant reduction in the cell viability, by nearly 21% and 70%, respectively was observed (Figure 11).

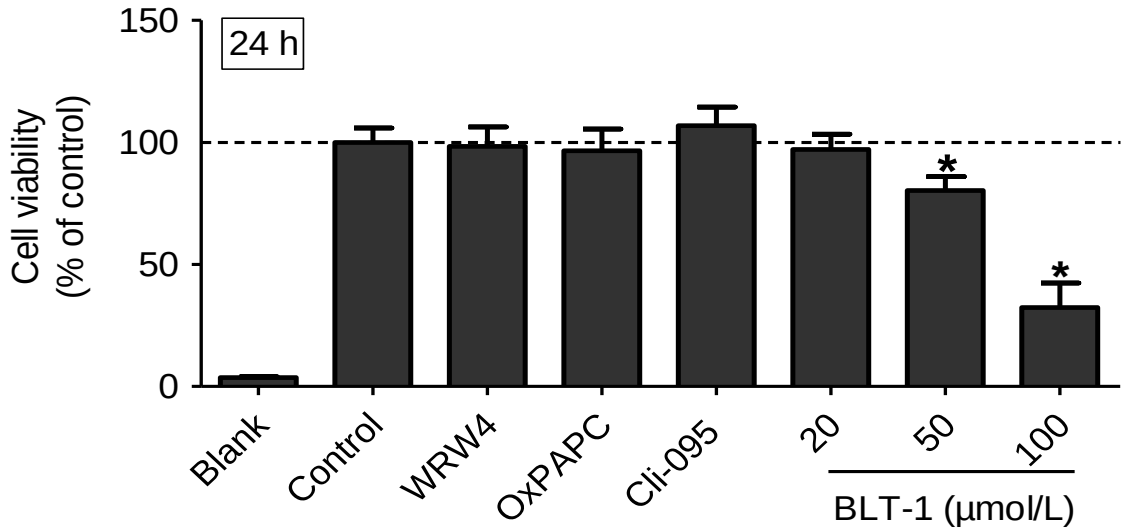


Figure 11. Effect of the antagonists of SAA receptors on cell viability-THP-1-derived macrophage-like cells

The THP-1-derived macrophage-like cells were stimulated with rhSAA (10 µg/ml) in co-stimulation with the antagonists of SAA receptors (OxPAPC (30 µg/ml), Cli-095 (10 µg/ml), WRW4 (10 µmol/L) and different concentrations of BLT-1 for 24 h. Cell viability is expressed as percentage to control. Untreated cells (control) were set to 100%. PBS was used as blank. Values represented as mean ± SD (n=12). * $p<0.05$ significant change vs control. Mann–Whitney U test.

Ultimately, the optimal concentrations for following analyses of these antagonists in THP-1-derived macrophage-like cells are as follows: WRW4 (10 µmol/L), OxPAPC (30 µg/ml), Cli-095 (10 µg/ml) and BLT-1 (20 µmol/L).

3.4.2 rhSAA induced nitrite production in RAW264.7 via TLR2/4 and SR-BI

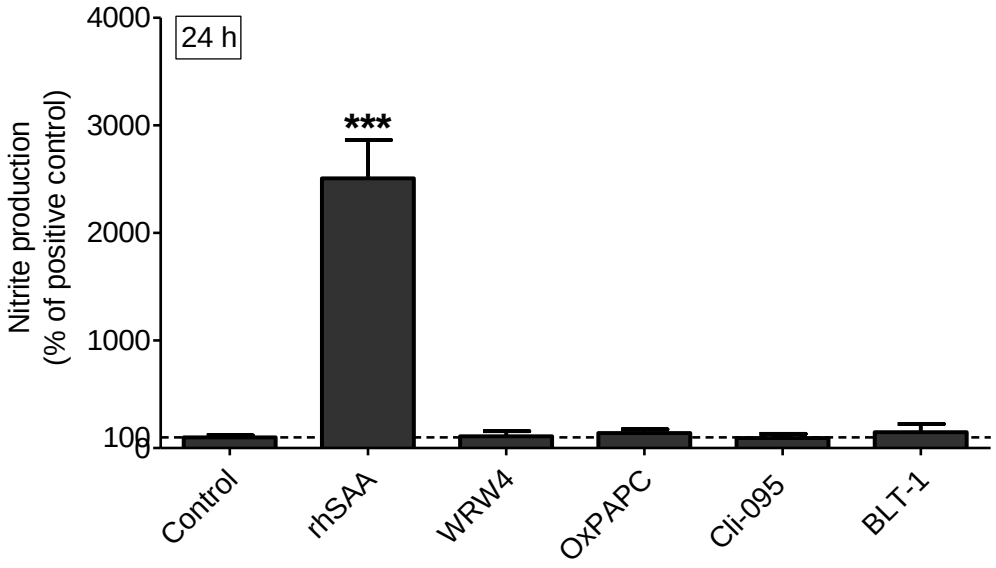
To identify the functional receptors which mediate the rhSAA-induced nitrite production in RAW264.7 cells, the antagonists WRW4, OxPAPC, Cli-095 and BLT-1 were tested in this study. Firstly, the antagonists themselves had no impact on the nitrite production in RAW264.7 cells (Figure 12) over 24 h and 48 h of incubation time.

Next, RAW264.7 cells were treated with rhSAA (1 µg/ml) in co-stimulation with antagonists for 24 h and 48 h. After 24 h, OxPAPC, the inhibitor of both TLR2 and TLR4, produced a significant inhibition (about 40%) of rhSAA-stimulated nitrite production. The inhibitory potential of Cli-095, the antagonist of TLR4, is comparable inhibitory potential as OxPAPC. These results suggest that at least TLR4 is required for nitrite production in RAW264.7 cells in response to rhSAA (Figure 13). To further support this point, the agonists of TLR2 and TLR4, HKSA and LPS were used in the present study, respectively. HKSA (10^8 Z/ml) and LPS (1 µg/ml), induced nitrite production (Figure 14). These results strongly indicated that TLR2 and TLR4 signaling is involved in rhSAA-stimulated nitrite production in RAW264.7 cells.

BLT-1, the antagonist of SR-BI, also plays a significant role in the rhSAA-induced nitrite production (Figure 13). BLT-1 inhibited nitrite production by nearly 40% in RAW264.7 cells too.

In contrast, WRW4, the antagonist of FPR2, could not inhibit the rhSAA-stimulated nitrite production in RAW264.7 cells (Figure 13). In line with this, MMK-1, the agonist of FPR2, failed to induce nitrite production (Figure 14). These results suggest that FPR2 does not play a significant role in rhSAA-stimulated nitrite production in RAW264.7 cells. The results from 48 h incubation showed comparable results to those from 24 h incubation time (Figure 14 B).

A



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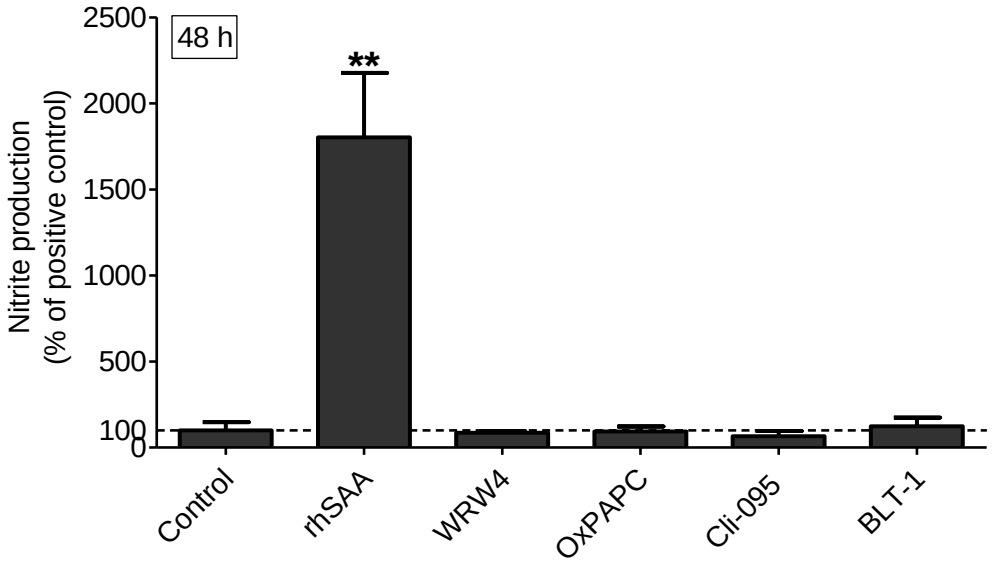
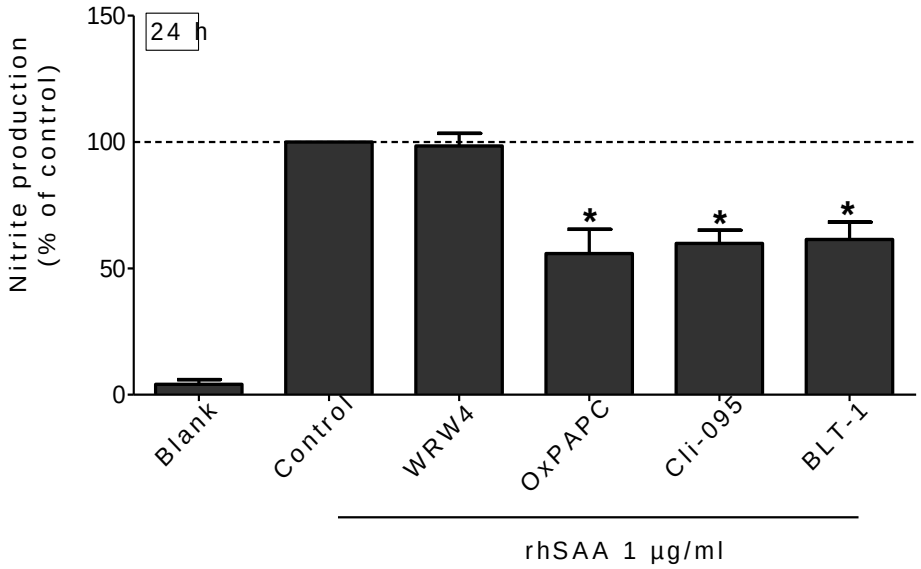


Figure 12. SAA antagonists induced nitrite oxide production in RAW264.7 cells
The cells were treated with rhSAA (10 µg/ml), and SAA antagonists including WRW4 (10 µmol/L), OxPAPC (30 µg/ml), Cli-095 (10 µg/ml) and BLT-1 (10 µmol/L) for (A) 24 h and (B) 48 h. Nitrite production is expressed as percentage to control. Untreated cells (control) were set to 100%. rhSAA (1 µg/ml) treatment group was used as positive control. Values represented as mean ± SD of three independent experiments. ** $p<0.01$, *** $p<0.001$ significant change vs control. Mann–Whitney U test.

A



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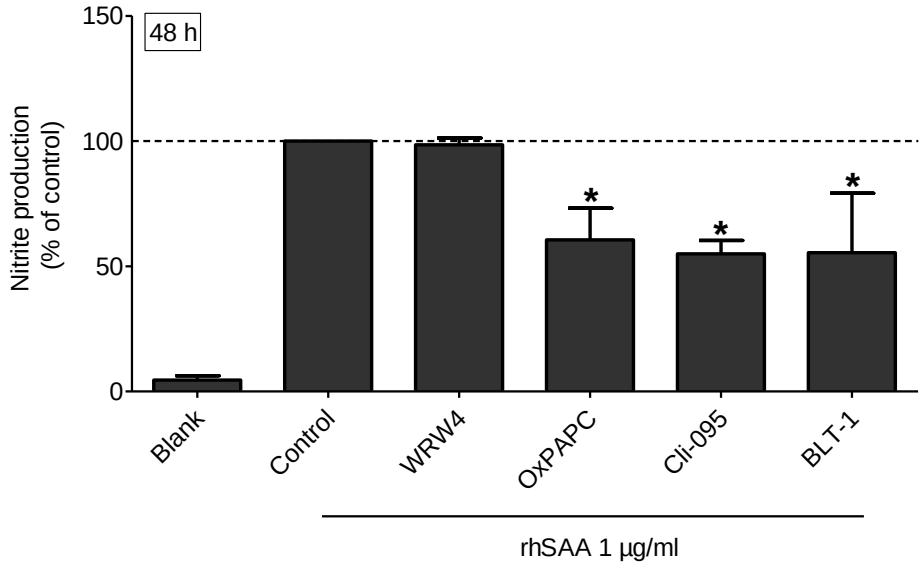
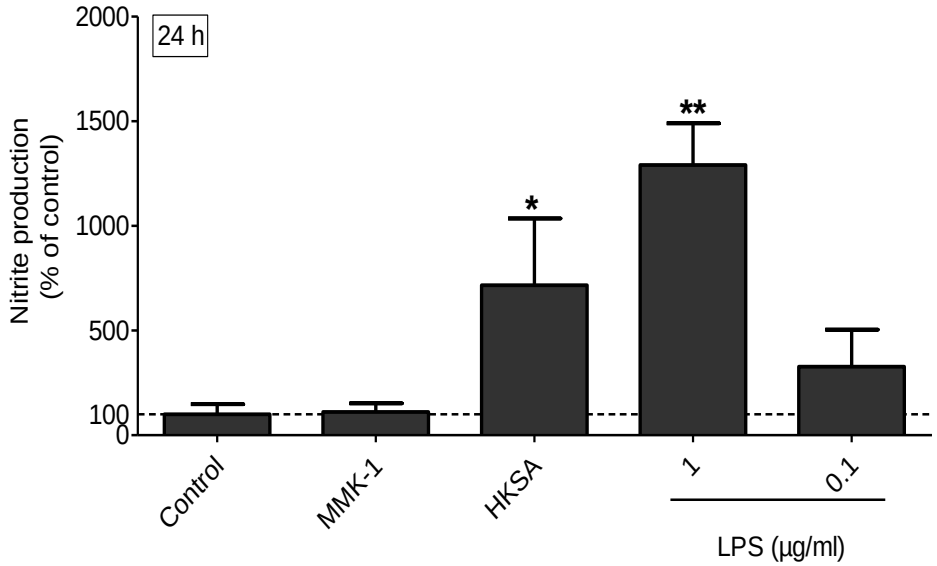


Figure 13. SAA antagonists inhibits rhSAA-induced nitrite production in RAW264.7 cells

The cells were treated with rhSAA (1 µg/ml) alone or in co-stimulation with the antagonists (WRW4 (10 µmol/L), OxPAPC (10 µg/ml), Cli-095 (1 µg/ml) and BLT-1 (10 µmol/L)) for (A) 24 h and (B) 48 h. Nitrite production is expressed as percentage to control. Cells treated with rhSAA (control) were set to 100%. Untreated group was used as blank. Values represent mean ± SD (n=6). * $p<0.05$ significant change vs control. Mann–Whitney U test.

A



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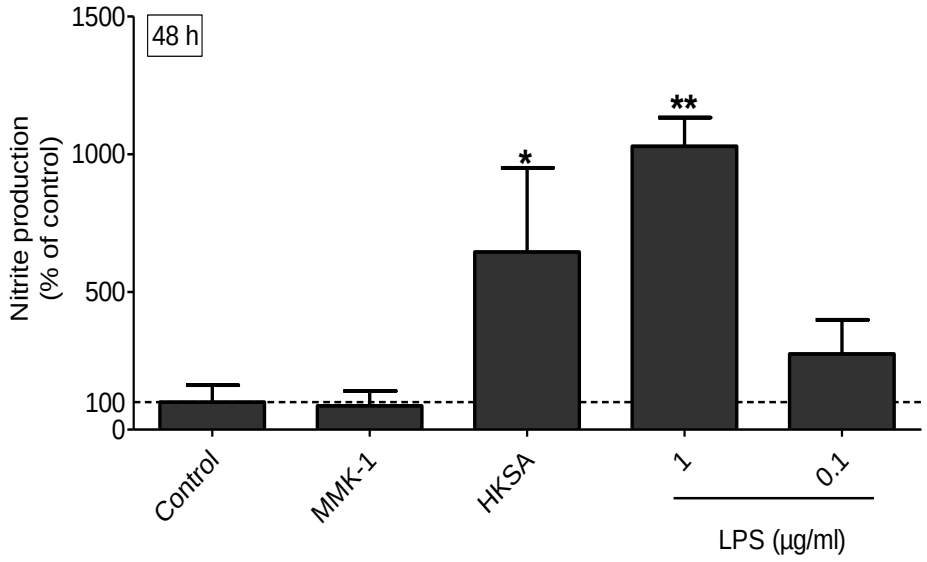
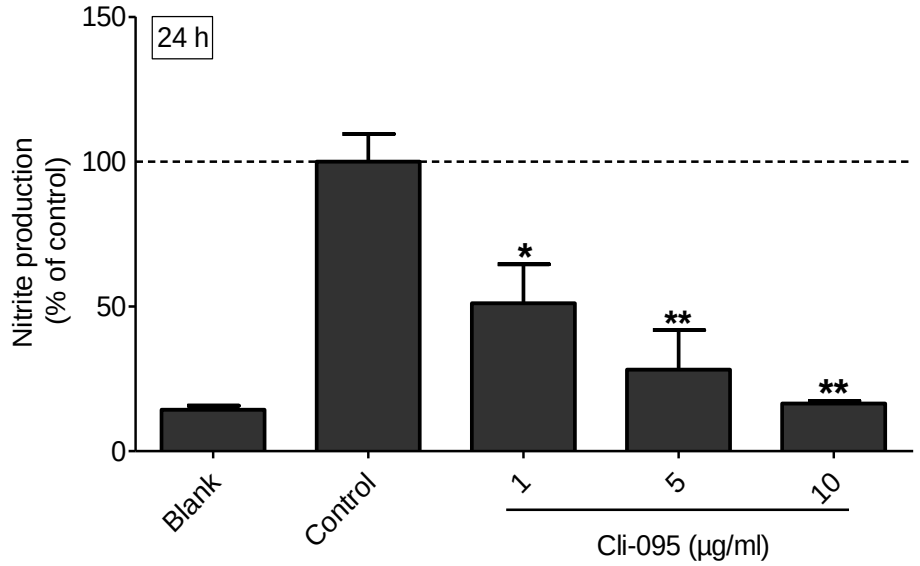


Figure 14. Production of nitrite in RAW264.7 induced by agonists
The cells were stimulated with MMK-1 (10 µg/ml), HKSA (10^8 Z/ml), and LPS (concentration as indicated) for (A) 24 h and (B) 48 h. Nitrite production is expressed as percentage to control. Untreated cells (control) were set to 100%. Values represented as mean ± SD (n (24 h)=5, n (48 h)=4). * $p<0.05$; ** $p<0.01$ significant change vs control. Mann–Whitney U test.

A



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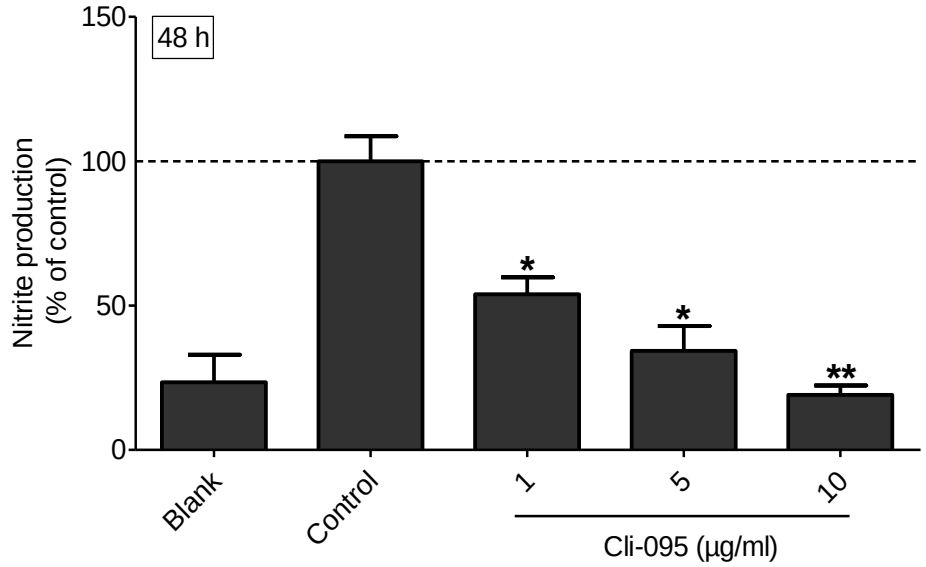
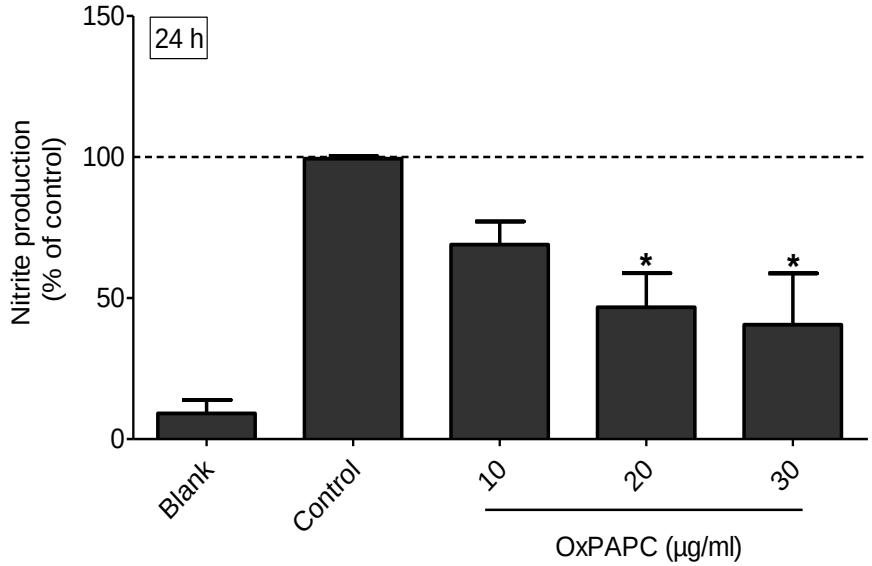


Figure 15. Cli-095 inhibit rhSAA-induced nitrite production in RAW264.7 cells
The cells were treated with rhSAA and increasing concentrations of Cli-095 for (A) 24 h and (B) 48 h. Values represent mean ± SD (n=3). * $p<0.05$; ** $p<0.01$ significant vs control. Mann–Whitney U test.

A



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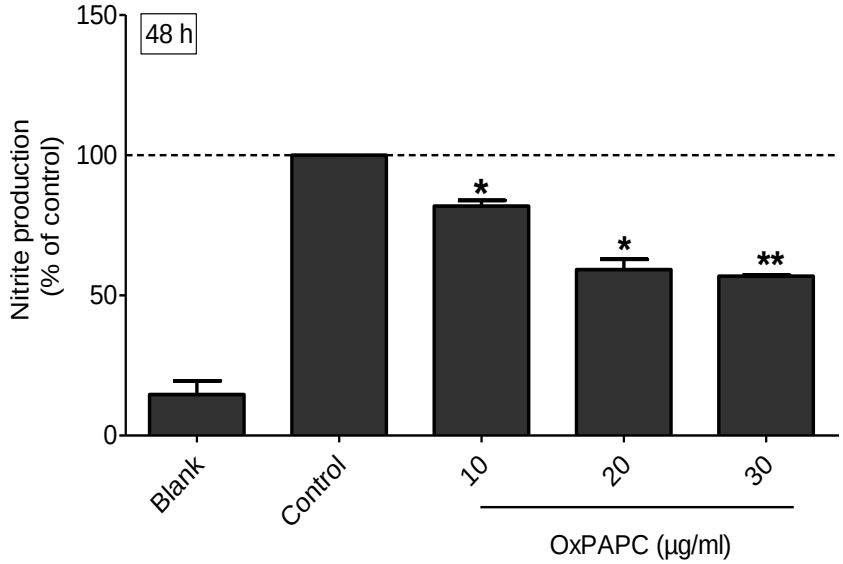


Figure 16. OxPAPC inhibit rhSAA-induced nitrite production in RAW264.7 cells
The cells were treated with rhSAA and increasing concentrations of OxPAPC for (A) 24 h and (B) 48 h. Values represent mean ± SD (n=3). * $p<0.05$; ** $p<0.01$ significant vs control. Mann–Whitney U test.

The inhibitory effects of increasing concentrations of OxPAPC (10, 20, and 30 µg/ml) and increasing concentration of Cli-095 (1, 5, and 10 µg/ml) were examined. Both SAA antagonists reduced rhSAA-stimulated nitrite production in a dose-dependent manner. The highest concentration of Cli-095 (10 µg/ml) almost completely abolished nitrite production (Figure 15). In addition, OxPAPC (30 µg/ml) reduced nitrite production by nearly 70% (Figure 16).

Taken together, these results suggest that several signaling cascades are simultaneously involved in the rhSAA-stimulated nitrite production in RAW264.7 cells. Activation of TLR2/4 and SR-BI is the most prominent signaling pathway. Results from the current study did not show any significant role of FPR2 signaling in the rhSAA-stimulated nitrite production in RAW264.7 cells.

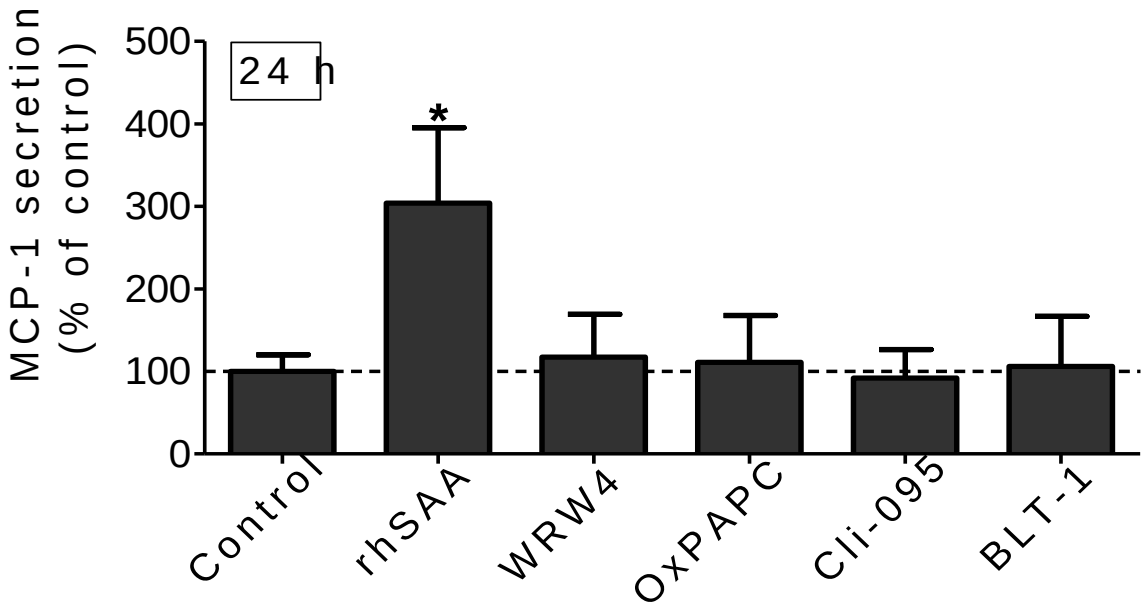
3.4.3 rhSAA induces MCP-1 secretion via TLR4, and induces IL-6 secretion via both TLR2&4 in THP-1-derived macrophage-like cells

In further experiments, it was elucidated by which receptor the cytokine (MCP-1 and IL-6) secretion in THP-1-derived macrophage-like cells is mediated in response to rhSAA. Firstly, the antagonists themselves were used as stimulus to check whether they themselves have effects on induction of MCP-1 and IL-6 secretion in macrophages. THP-1-derived macrophage-like cells were exposed to antagonists for 24 h and MCP-1 and IL-6 secretion were measured (Figure 17).

Next, THP-1-derived macrophage-like cells were treated with rhSAA (10 µg/ml) and its antagonists for 24 h. As shown in Figure 18, OxPAPC (TLR2&TLR4 antagonist) and Cli-095 (TLR4 antagonist) significantly blocked rhSAA-induced MCP-1 secretion, by 40% and 50%, respectively. OxPAPC and Cli-095 inhibited rhSAA-induced IL-6 secretion by 70% and 65%, respectively. Thus, TLR4 activation seemed to play a major role in rhSAA-induced MCP-1 and IL-6 secretion in THP-1-derived macrophage-like cells.

To verify this observation, the role of the agonists of TLR2 (HKSA) and TLR4 (LPS) on cytokine secretion in macrophages was tested. Both agonists had effects on cytokine secretion in THP-1-derived macrophage-like cells by induction of MCP-1 and IL-6 secretion, respectively (Figure 19). IL-6 secretion increased significantly after treatment with LPS and HKSA. MCP-1 secretion increased significantly after treatment of LPS. Although MCP-1 increased after HKSA treatment, there was no significant difference compared to control. Prolonging incubation time to 48 h had no further effects and the results were comparable to those of 24 h incubation period (Figure 20).

A



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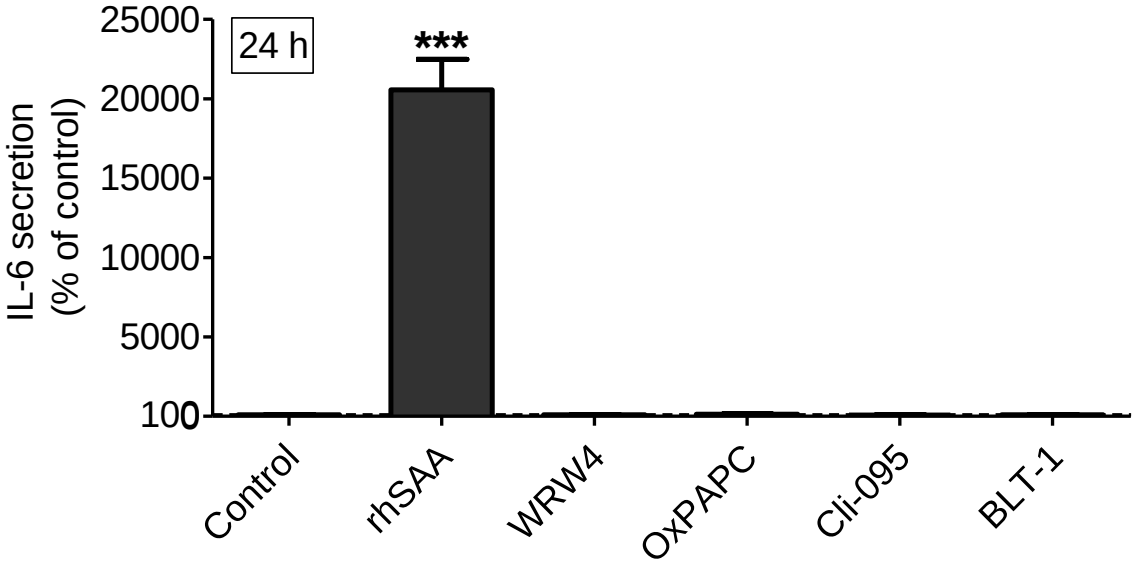
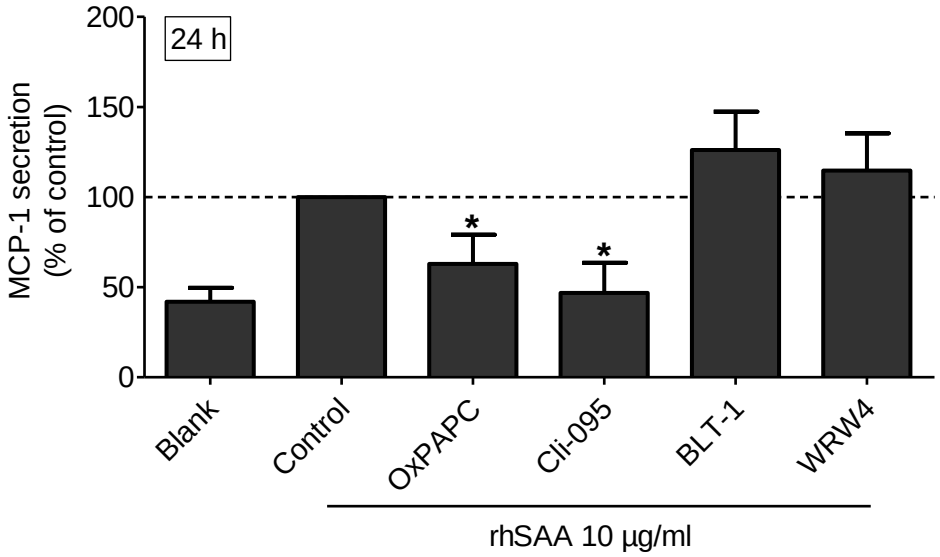


Figure 17. Antagonists induced cytokines secretion in THP-1-derived macrophage-like cells

The cells were treated with rhSAA (10 µg/ml), WRW4 (10 µmol/L), OxPAPC (30 µg/ml), Cli-095 (10 µg/ml) and BLT-1 (20 µmol/L). (A) MCP-1 and (B) IL-6 secretion was measured and expressed as percentage to control. Untreated cells (control) were set to 100%. Cells treated with rhSAA were used as positive control. Values represented as mean ± SD of three independent experiments. * $p<0.05$; *** $p<0.001$ significant change vs control. Mann–Whitney U test.

A



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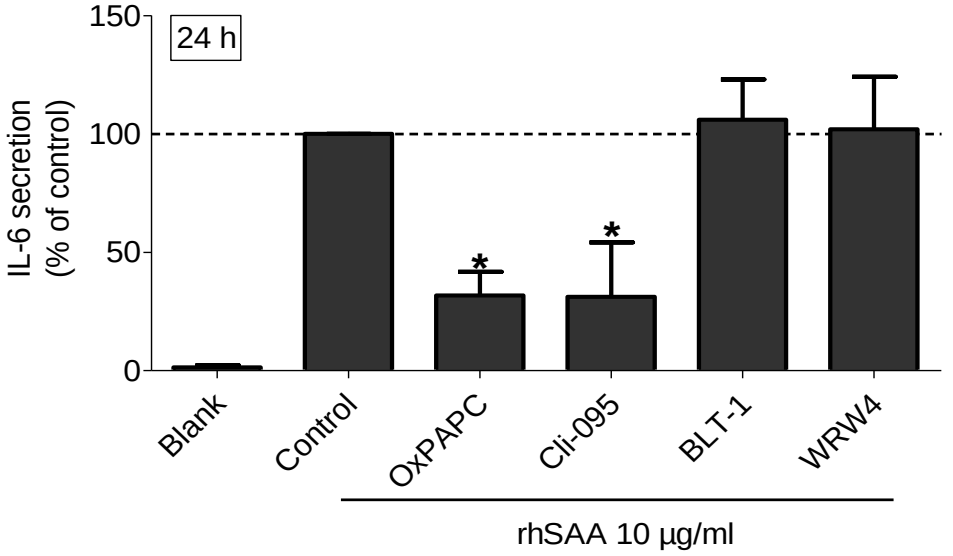
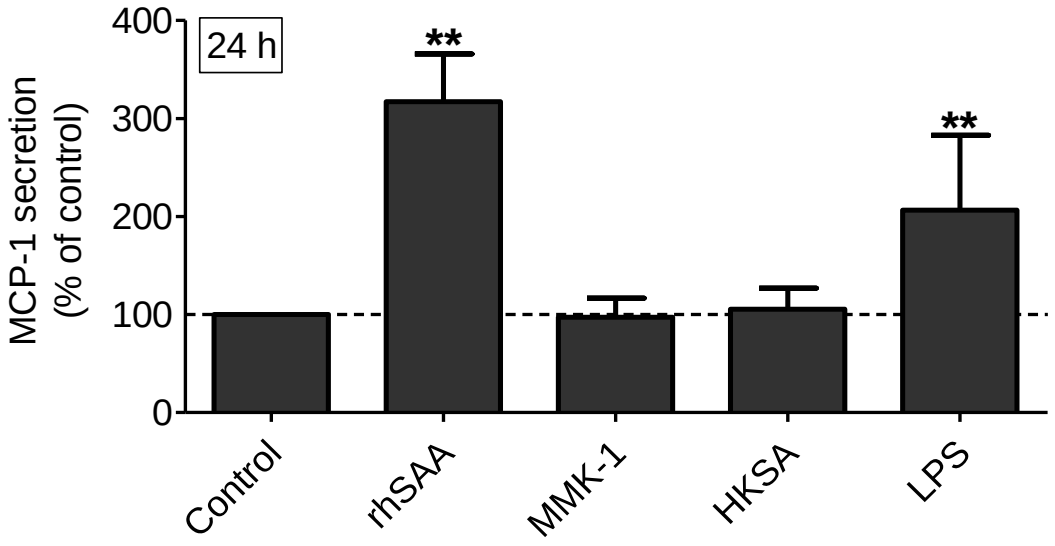


Figure 18. Inhibition of rhSAA induced cytokines secretion in THP-1-derived macrophage-like cells by antagonists

The cells were treated with rhSAA (10 µg/ml) in co-stimulation with antagonists. OxPAPC (30 µg/ml), Cli-095 (10 µg/ml), BLT-1 (10 µmol/L) and WRW4 (10 µmol/L) for 24 h. (A) MCP-1 and (B) IL-6 are expressed as percentage to control. Cells treated with rhSAA (control) were set to 100%. Untreated cells were used as blank. Values represented as mean ± SD (n (A)=10, n (B)=5). * $p<0.05$ significant change vs control. Mann–Whitney U test.

A



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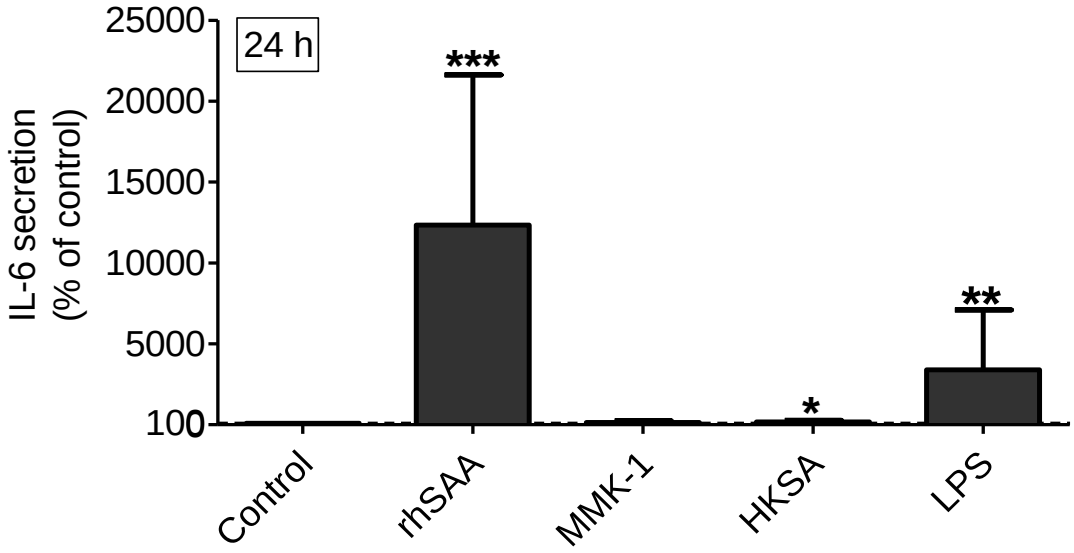
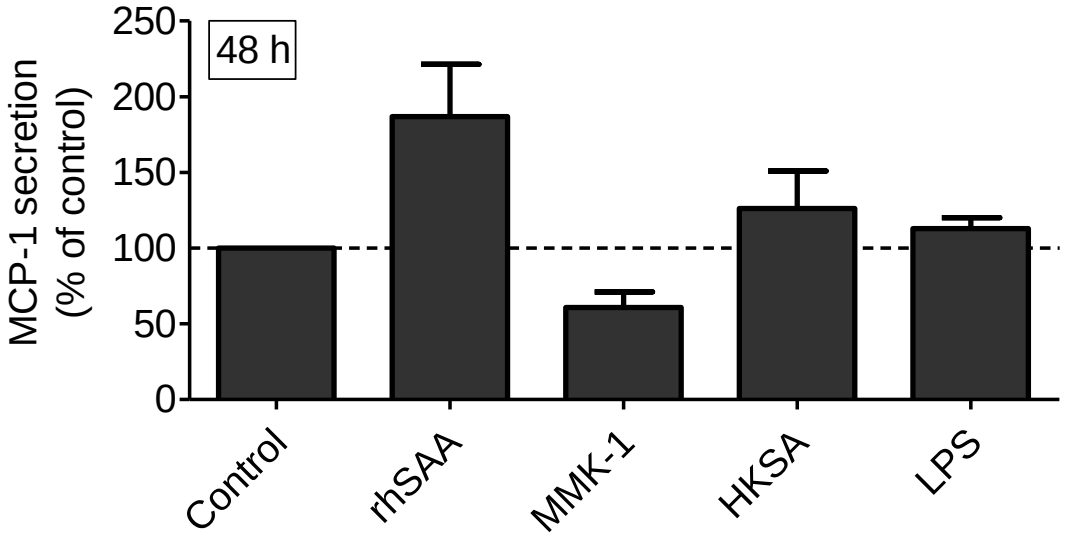


Figure 19. Secretion of cytokines induced by agonists in THP-1-derived macrophage-like cells (24 h)

The cells were stimulated with rhSAA (10 µg/ml), MMK-1 (10 µg/ml), HKSA (10^8 Z/ml) and LPS (10 µg/ml) for 24 h. (A) MCP-1 and (B) IL-6 are expressed as percentage to control. Cells untreated (control) were set to 100%. Cells treated with rhSAA were used as positive control. Values represented as mean ± SD (n (A)=10, n (B)=4). * $p<0.05$; ** $p<0.01$; ***$p<0.001$ significant change vs control. Mann–Whitney U test.

A



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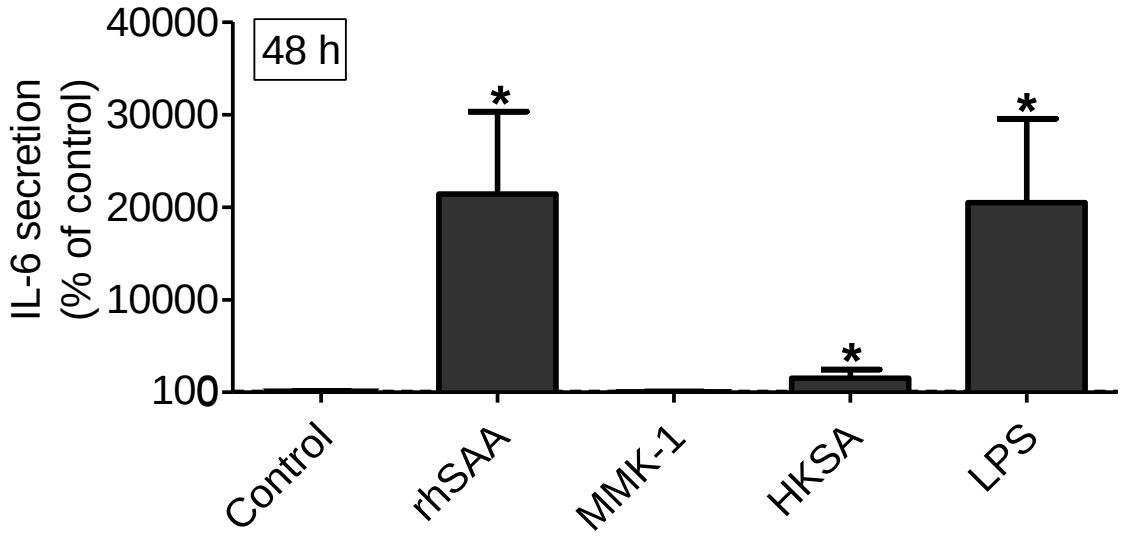

Figure 20. Secretion of cytokines induced by agonists in THP-1-derived macrophage-like cells (48 h)

The cells were stimulated with rhSAA (10 µg/ml), MMK-1 (10 µg/ml), HKSA (10^8 Z/ml) and LPS (10 µg/ml) for 48 h. (A) MCP-1 and (B) IL-6 are expressed as percentage to control. Untreated cells (control) were set to 100%. Cells treated with rhSAA were used as positive control. Values represented as mean ± SD (n=4). * $p<0.05$ significant change vs control. Mann–Whitney U test.

These results confirmed that TLR4 signaling is essential for rhSAA-induced MCP-1 secretion in THP-1-derived macrophage-like cells. The results also indicated that rhSAA-induced IL-6 secretion in THP-1-derived macrophage-like cells is mediated via both TLR2 and TLR4 signaling pathways.

In contrast, WRW4 failed to inhibit the rhSAA-induced MCP-1 and IL-6 secretion in THP-1-derived macrophage-like cells (Figure 18). Moreover, MMK-1, the agonist of FPR2, was unable to induce cytokines secretion (Figure 19). Similarly, BLT-1, antagonist of SR-BI, had no effect either (Figure 18). These results suggest that FPR2 and SR-BI are not required for cytokines secretion (MCP-1, IL-6) in THP-1-derived macrophage-like cells in response to rhSAA.

The results imply that TLR4 is the most prominent signaling pathway in rhSAA-induced MCP-1 secretion, and both TLR2 and TLR4 are essential for rhSAA-induced IL-6 secretion in THP-1-derived macrophage-like cells. However, under these experimental conditions, FPR2 and SR-BI did not seem to be involved in rhSAA-induced MCP-1 and IL-6 secretion.

4 Discussion

A number of studies suggested that the acute-phase protein SAA plays a pivotal role in inflammatory diseases, including atherosclerosis, CKD and sepsis.[7, 8] SAA acts as cytokine and induces the secretion of pro-inflammatory mediators, including NO, MCP-1 and IL-6, in various cell types.[12] Indeed, clinical and animal studies have shown that local production of SAA in atherosclerotic sites is associated with the progression of atherosclerosis.[12, 22]

Migration of macrophages to the site of inflammation is a key event of the innate immune system. This is an essential response to invading microorganisms.[35] But also in sterile inflammatory diseases, such as atherosclerosis and CKD, there is a migration of macrophages.[29]

The current work investigated the effects of rhSAA on the regulation of pro-inflammatory mediators from macrophages as well as the corresponding functional receptors mediating rhSAA's pro-inflammatory response. The experiments were performed using murine macrophages (RAW264.7 cells) and a human monocyte cell line (THP-1 cells). Both cell lines are repetitively used in other studies to investigate inflammatory responses.[107, 108] rhSAA induced dose- and time-dependent production of nitrite in RAW264.7. Moreover, rhSAA induced dose-dependent secretion of MCP-1 and IL-6 in THP-1 cells. These findings are comparable with previous studies,[18, 107] which also found that rhSAA induced nitrite and cytokine production in macrophages. These results indicate that SAA secreted during the inflammatory response can activate innate immune reaction to pathogens in part through activation of macrophages. Activation and accumulation of macrophages in tissues leads to tissue damage.

The results from this study show a potential mechanism underlying the role of elevated plasma SAA and inflammatory diseases, such as atherosclerosis,[7] sepsis[109] and CKD.[2] SAA is possibly involved in the progression of these diseases through induce ROS, nitrite and cytokines (such as MCP-1 and IL-6).

4.1 rhSAA could not induce superoxide generation in monocyte/macrophage cell lines

Many studies have shown that overproduction of ROS by macrophages led to abnormal inflammatory responses.[110] Some studies showed superoxide generation in

THP-1-derived macrophage-like cells or murine macrophages after stimulation. In the current study it was shown that rhSAA induces superoxide. However, in the present study, it was not possible to generate superoxide upon stimulation with rhSAA and LPS. Here, both human THP-1 monocytes and THP-1-derived macrophage-like cells (after PMA stimulation) were tested. Whereas superoxide can be induced by juglone. In addition, the murine monocyte/macrophage cell line RAW264.7 was also investigated. However, these results were not in line with previous studies in literature. Investigations on the effects of different stimuli on superoxide generation may be required in different cell types. This gives the impression that rhSAA is not able to induce superoxide in monocytes/macrophages. In literature, there is no hint that SAA should produce superoxide.

4.2 rhSAA induced nitrite production in RAW264.7 cells in a dose- and time-dependent manner

Abnormal production and transport of NO is related with CVDs, such as atherosclerosis, and it is also a key element of vasodilatation in septic shock.[77, 78] NO can be produced by macrophages[18] after induction of NOS2.[80]

A previous study demonstrated that LPS induces nitrite in THP-1-derived macrophage-like cells.[111] Thus, the effect of rhSAA and LPS on nitrite production in THP-1 monocytes/macrophages was investigated. However, neither rhSAA nor LPS induced nitrite production in human monocytes and macrophages in the present study. To exclude a possible problem of the cell line during culturing within laboratory, a different THP-1 cell line was ordered from ATCC. However, here again, no increases in nitrite after treating THP-1-derived macrophage-like cells with rhSAA and LPS were observed. Currently, it is not clear why these results are contrary to literature.

To resolve this issue, the effects of rhSAA and LPS on nitrite production were tested in a different macrophage cell line. RAW264.7 was used in previous studies as well for nitrite production.[18, 107] In line with literature, the experiments resulted in a dose- and time-dependent induction of nitrite production upon rhSAA and LPS treatment. In comparison with LPS, rhSAA induced a more robust production of nitrite. This finding is in agreement with a previous study by Silvana Sandri, which reported that rhSAA

induced nitrite in a dose-dependent manner in resident peritoneal macrophages from mice.[18]

4.3 The potential effects of contaminating LPS in the rhSAA are not responsive to the activities of rhSAA

It is known that bacterial contamination of rhSAA can make data interpretation complicated when investigating ligands of endogenous TLRs.[17] rhSAA with a low level of endotoxin contamination was used in the study. Taken precautions of whether the contaminating materials like LPS are involved in the action attributed to rhSAA. As previously shown, heat shock of the rhSAA protein abolished the rhSAA-induced effect, whereas a heat shock of LPS did not influence its stimulatory potential.[2]

4.4 Mechanism of rhSAA-induced nitrite production in macrophages

4.4.1 Potential receptors of SAA

Up to now, no specialized receptors for SAA have been identified. SAA can interact with several types of receptors: These are the formyl peptide receptor-like-1 (FPRL1/FPR2),[21] the receptor for advanced glycosylation end products (RAGE),[20] SR-BI[19] and TLR2/4.[17, 18] These receptors are expressed on various cell types, such as dendritic cells, macrophages, and epithelial cells. Through these potential receptors, SAA can participate in the innate or adaptive immune responses with respective effects.

4.4.2 rhSAA-induced nitrite production in RAW264.7 cells via SR-BI

In the current study, the results suggested that rhSAA induced nitrite production in murine macrophages via SR-BI.

Usually, scavenger receptors are expressed on the cell surface. Depending on the structure of scavenger receptors, they are divided into several “class”, labeled by capital letters, e.g., class A, class B. Within a class, a capital roman numeral was used to distinguish individual receptor types.[112] SR-BI was isolated from a Chinese hamster ovary, and its proteins share 75-80% homology between human, murine, and rat.[112] SR-BI is an HDL receptor, a member of the CD36 superfamily, and also expressed on macrophages.[106]

Previous studies showed that SR-BI regulates the HDL-dependent activation of NO synthase in endothelial cells.[113] It seemed that SR-BI participates in the regulation of pro-inflammatory mediator production.[113] In the present study, it has been shown that rhSAA induces nitrite production in murine macrophages via SR-BI. It was shown for the first time that rhSAA induced nitrite signaling mechanisms of SR-BI. Scavenger receptors expressed in macrophages are different from LDL receptors. Their activation is not regulated by intracellular cholesterol pools.[114] The antagonist of SR-BI, BLT-1, produced a significant inhibition of rhSAA-stimulated nitrite production in murine macrophages. These results strongly indicate that rhSAA stimulates the production of nitrite via SR-BI.

A potential cytotoxicity of the antagonist BLT-1 is not responsible for this inhibitory effect. According to instruction of the manifacturer (Merck Millipore, Darmstadt, Germany), the recommended concentration of BLT-1 is 100 µmol/L. MTT assay revealed that higher concentration of BLT-1 (50 and 100 µmol/L) decreased viability of murine macrophages, but the concentration of 10 µmol/L did not. Unfortunately, there is no selective agonist of SR-BI up to now. Thus, the results cannot be confirmed by using a SR-BI agonist.

4.4.3 rhSAA-induced nitrite production in RAW264.7 cells via both TLR2 and TLR4

This study, also showed that rhSAA induces nitrite production in dependence on both TLR2 and TLR4 signaling pathways in murine macrophages. TLRs are able to bridge the innate and adaptive immune systems. Activated TLRs lead to activation of innate immune responses, meanwhile, induction of adaptive immune responses in response to exogenous noxious materials.[115]

The human TLRs are grouped in five subfamilies: TLR2, TLR3, TLR4, TLR5, and TLR9 according to the genomic structure, chromosomal localization, and amino acid sequences.[116] In 1998, the first ligand of TLR4 was found, LPS, a component of cell wall derived from Gram-negative bacteria.[117] However, mammalian TLR4 primarily recognize the LPS binding protein (LBP), not the LPS directly.[117] LPS also recognize the TLR2. Besides LPS, other microbial components like parasites and viruses, Gram-positive bacteria and so on, also recognize the TLR2.[118]

The present study demonstrated that rhSAA-induced and LPS-induced nitrite production was significantly inhibited by a TLR4 antagonist (Cli-095) in murine macrophages.

rhSAA-induced nitrite production was completely abolished by 10 µg/ml of Cli-095 in the murine cell line used. These results strongly indicate that rhSAA mediates the production of nitrite via TLR4. These results are in agreement with a previous study,[18] which showed that rhSAA is an endogenous ligand of TLR4 and that it induces nitrite production via TLR4 in mouse monocytes. In addition to TLR4, TLR2 also plays a role in the rhSAA-induced nitrite production. In the present study, the antagonist (OxPAPC) of both TLR2 and TLR4 signaling significantly inhibited rhSAA-induced nitrite production. This effects was dose-dependent. To further establish a correlation between TLR2 and rhSAA-induced nitrite production, HKSA, the selected agonist of TLR2, was tested and shown to induce nitrite production significantly. Therefore, there is a causal relationship between rhSAA and TLR2/4-dependent nitrite production in murine macrophages.

4.4.4 FPR2 does not possess a significant role in rhSAA-stimulated nitrite production in RAW264.7 cells

Given that FPRs are also expressed in macrophages, additional studies showed that rhSAA induces cytokine secretion in THP-1-derived macrophage-like cells via FPR2.[105] The speculation arose that rhSAA could also regulate nitrite production in macrophages through FPR2.

FPR includes three subtypes, FPR, FPRL1 and FPRL2, in humans and other primates.[119] Besides their expression on phagocytic cells, human neutrophils also express FPR, but only subtype FPR and FPRL1, not FPRL2.[120] These receptors can be activated by a variety of natural ligands, such as endogenous peptides, exogenous bacterial and virus.[121] The FPR family is related with defence against exogenous pathogens, and many studies indicated that FPRs are critical for the regulation of immune responses.[105]

Thus, experiments to check whether FPR2 is involved in rhSAA-induced nitrite production in mouse macrophages were performed. Our data showed that WRW4, the antagonist of FPR2, could not inhibit rhSAA-stimulated nitrite production. Furthermore, MMK-1, selective agonist of FPR2, failed to induce nitrite production in murine macrophages. These results strongly suggest that FPR2 does not possess a significant role in rhSAA-stimulated nitrite production in murine macrophages. Up to now, there are no studies investigating this receptor subtype in macrophages when studying NO.

4.4.5 Summary of signaling pathways of rhSAA-induced nitrite production in RAW264.7 cells

Taken together, the present findings indicate that the acute-phase protein rhSAA induces nitrite production in murine macrophages via TLR2/4 and SR-BI. These results support a potential hypothesis that TLR2/4 and SR-BI can be recognized as endogenous "danger signals" and these receptors are critical for the pathogenesis of several inflammatory diseases, such as atherosclerosis, sepsis and CKD. Meanwhile, these results can help explain NO production with sterile inflammation *in vivo*.

4.5 Mechanisms of rhSAA-induced cytokine secretion in THP-1-derived macrophage-like cells

Macrophages are not only a pivotal player of the innate and adaptive immune systems, but they also are the major source of many pro-inflammatory cytokines. Macrophages are able to synthesize and secrete large amounts of cytokines in reaction to exogenous or endogenous stimulation.[107] Most functions and cell surface markers of macrophages can be regulated by these cytokines.[108] In addition, chemokines, such as MCP-1, contribute to recruiting the monocytes into inflammatory tissues.[108]

4.5.1 Cytokine secretion in THP-1-derived macrophage-like cells upon rhSAA treatment in a dose-dependent manner

The present study has shown that rhSAA induced MCP-1 and IL-6 secretion in THP-1-derived macrophage-like cells in a dose-dependent manner. This is consistent with previous studies,[107] which reported that rhSAA induces MCP-1 and IL-6 production in human macrophages. The results strongly suggest that SAA has a powerful effect on THP-1-derived macrophage-like cells to produce pro-inflammatory cytokines.

Concentration of rhSAA as low as 1 μg/mL dramatically increased IL-6 secretion, and 5 μg/mL of rhSAA significantly induced MCP-1 secretion in THP-1-derived macrophage-like cells. With increased concentration of rhSAA, the secretion was more intense. In comparison with IL-6, the THP-1-derived macrophage-like cells secreted a higher basal level of MCP-1. However, IL-6 secretion by macrophages seemed more responsive to rhSAA than to MCP-1 secretion.

Both MCP-1 and IL-6 play important roles in inflammatory diseases, including atherosclerosis and sepsis. In addition, reports in the literature have shown that SAA induces MCP-1 and IL-6 in human VSMCs.[2, 122] Thus, this potent effect of SAA may be important in the pathogenesis of inflammatory diseases, such as atherosclerosis, sepsis and CKD. For example, elevated SAA levels predict the risk of cardiovascular events.[12] Our previous study showed that a high level SAA can reduce the HDL's anti-inflammatory properties during disease conditions.[2]

4.5.2 rhSAA-induced MCP-1 secretion in THP-1-derived macrophage-like cells via TLR4; rhSAA-induced IL-6 secretion in THP-1-derived macrophage-like cells via both TLR2 and TLR4

Since rhSAA induced pro-inflammatory cytokines secretion in macrophages, it is necessary to explore its potential signaling pathways.

Based on different cellular locations and distributions, 11 TLRs have been identified in humans.[123] In addition, it was well accepted that TLR1, 2, 4, 6 and 10 recognize extracellular pathogens because these TLRs are expressed on the cell surface; whereas TLR3, 7, 8 and 9 react specifically with DNA or RNA derived from intracellular pathogens, due to these TLRs located in cell endosomes.[124]

The present study showed that OxPAPC and Cli-095 could reduce rhSAA-induced MCP-1 and IL-6 secretion significantly, indicating that TLR2/4 may play important roles in rhSAA-induced secretion of MCP-1 and IL-6. To determine whether both TLR2 and TLR4 are involved in the signaling pathway, the selected agonist of TLR2, HKSA, was used and was shown to have an effect on IL-6 production in THP-1-derived macrophage-like cells, but not on the secretion of MCP-1. The selected agonist of TLR4, LPS, could induce both MCP-1 and IL-6 secretion in THP-1-derived macrophage-like cells. These results strongly indicate that rhSAA induced MCP-1 secretion in THP-1-derived macrophage-like cells via TLR4, and induced IL-6 secretion in THP-1-derived macrophage-like cells via both TLR2 and TLR4.

4.5.3 FPR2 and SR-BI are not responsive to rhSAA-induced cytokine secretion in THP-1-derived macrophage-like cells

Previously, Ha Young Lee et al.[105] reported that rhSAA induces MCP-1 production via FPR2-mediated signaling in human monocytes. Our previous study showed that rhSAA induced MCP-1 secretion in VSMCs via FPR2.[2] These findings are intriguing. However, in the current study, WRW4, the selected antagonist of FPR2, failed to inhibit the rhSAA-induced cytokines secretion in THP-1-derived macrophage-like cells. Furthermore, we found no statistically significant increases in cytokine secretion by THP-1-derived macrophage-like cells treated with MMK-1. These results demonstrate that FPR2 is not responsible for the rhSAA-induced cytokines secretion in THP-1-derived macrophage-like cells. Investigations on the effects of several FPR2 ligands on cytokine secretion are required in different cell types.

BLT-1, the antagonist of SR-BI, also failed to inhibit the rhSAA-induced cytokines secretion in THP-1-derived macrophage-like cells in the current study. Due to the fact that there is no selected agonist of SR-BI to date, this finding could not be confirmed in further studies. Based on the results with the antagonist used in the present study, SR-BI did not seem to be involved in rhSAA-induced cytokines secretion in THP-1-derived macrophage-like cells.

In summary, the potential signaling pathways involved in the activation of monocytes/macrophages exposure to rhSAA are illustrated in Figure 21.

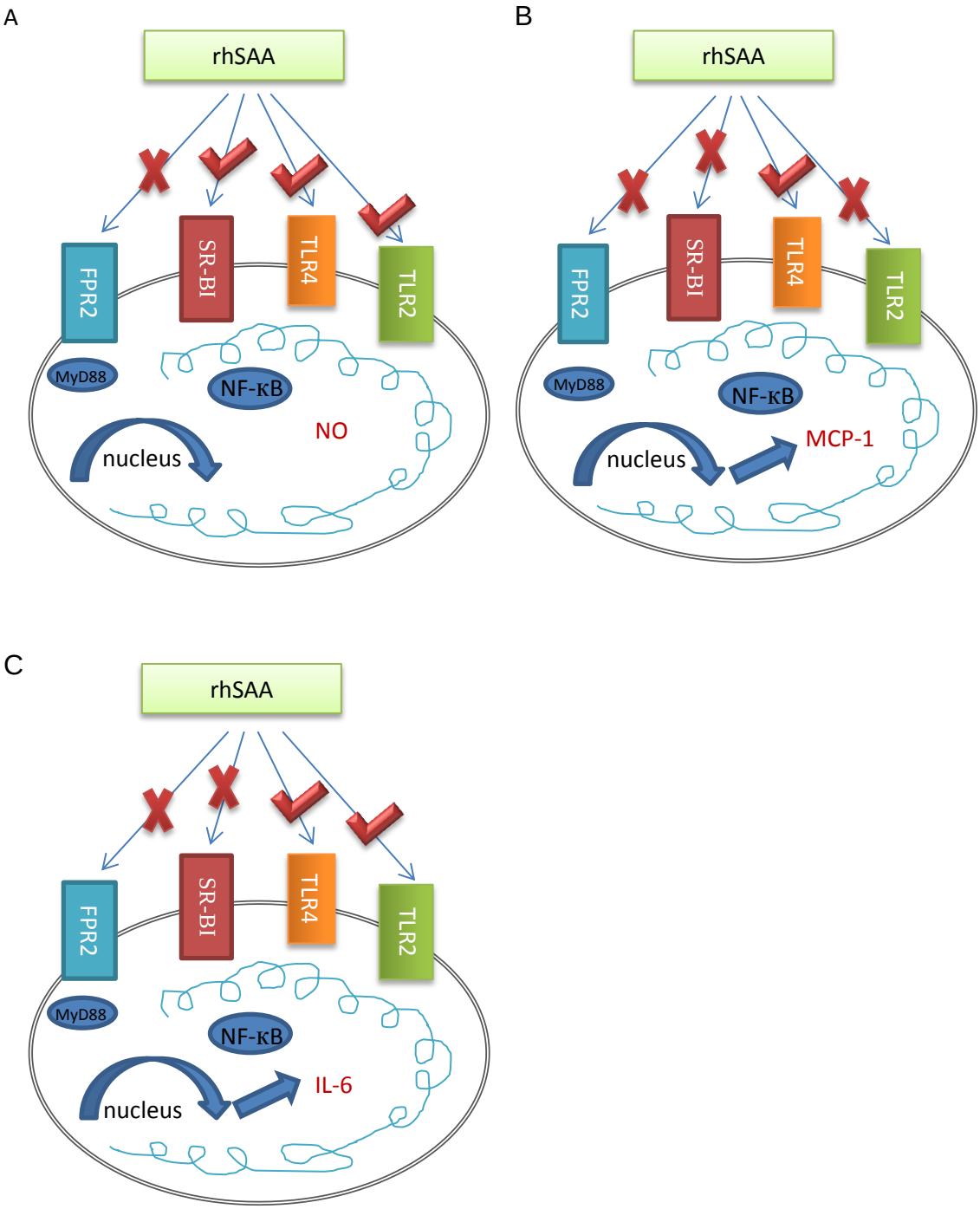

Figure 21. Signaling pathways involved in the activation of macrophage by rhSAA
A, rhSAA recognized by TLR2/4 and FPR2, induces the production of nitrite in murine macrophages. B, rhSAA recognized by TLR4, induces the secretion of MCP-1 in THP-1-derived macrophage-like cells. C, rhSAA recognized by TLR2/4, induces IL-6 secretion in THP-1-derived macrophage-like cells. The activating kinase cascades by a MyD88 or NF-κB dependent mechanism might be involved.

4.6 Conclusion

SAA is an acute phase protein, produced mainly by the liver in reaction to stimulation by inflammatory cytokines, such as IL-6, IL-1, and TNF.[125] Its level increases in numerous inflammatory diseases including atherosclerosis, CKD and sepsis, and elevated SAA levels predict adverse outcomes.[126, 127]

Monocytes/macrophages are an important component of innate immune and adaptive immune system. Upon stimulation by local inflammatory responses or exogenous noxious materials, monocytes/macrophages secrete a number of pro-inflammatory mediators. Furthermore, monocyte-derived macrophages are more responsive than monocytes after SAA exposure.[107] Immune system regulation is a very complex signaling network. When macrophages are exposed to SAA, the production of pro-inflammatory mediators increases, such as NO, MCP-1, IL-6, etc.[18, 107] These pro-inflammatory mediators have corresponding physiological functions. For instance, MCP-1 is able to recruit circulating monocytes into inflammatory tissues; IL-6 and NO are typical pro-inflammatory mediators. These pro-inflammatory mediators together with other factors induced by SAA, including TNF-α, colony stimulating factor (CSF), could lead to over-activation of macrophages upon SAA stimulation. A dense mass of such pro-inflammatory mediators produced by activated macrophages is able to cause tissue lesions. e.g., endothelial damage, shock and multiple organ dysfunction syndrome (MODS), even death. Collectively, macrophage activation must be strictly limited.

In conclusion, rhSAA can increase nitrite production in murine macrophages via TLR2/4 and SR-BI. In addition, it was shown for the first time that rhSAA stimulates nitrite production via the SR-BI-mediated pathway in the murine macrophages cell line RAW264.7. Moreover, rhSAA can increase MCP-1 secretion in THP-1-derived macrophage-like cells via TLR4, and increase IL-6 secretion via both TLR2 and TLR4. In summary, these results provide the context for the assumption that SAA bridging innate and adaptive immune systems, and determine a particular development of inflammatory diseases.

The current study has several limitations. First, the cells studies are two different cell lines and derived from two different species. It was not possible to demonstrate each effect in both cell lines, e.g., NO activation could not be induced in THP-1 cells. This point is controversy discussed in literature: as discussed before, some groups found NO

activation in THP-1 cells, while others found also no NO activation in THP-1 cells. Second, the experiments were performed in monocyte/macrophage cell lines and not in human primary macrophages, which might give a better information on the situation in the human body. Third, the data presented in this project is achieved by a pharmacological approach. Neither RNAi nor TLR knockout mice are used to confirmed the data out of the *in vitro* experiments.

Based on current results and previous findings, further work is warranted to establish animal models to determine whether SAA has the same signaling *in vivo*. Further clarification of the functions of SAA and its potential receptors is to investigate their potential therapeutic effects as immune-modulators of inflammatory diseases, such as atherosclerosis, CKD and sepsis, are worthwhile.

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6 Affidavit

I, [Yuexing Tu], certify under penalty of perjury by my own signature that I have submitted the thesis on the topic [The influence of serum amyloid A on pro-inflammatory signaling in monocytes/macrophages cell lines]. I wrote this thesis independently and without assistance from third parties, I used no other aids than the listed sources and resources.

All points based literally or in spirit on publications or presentations of other authors are, as such, in proper citations (see "uniform requirements for manuscripts (URM)" the ICMJE www.icmje.org) indicated. The sections on methodology (in particular practical work, laboratory requirements, statistical processing) and results (in particular images, graphics and tables) correspond to the URM (s.o) and are answered by me. I interest in any publications to this dissertation correspond to those that are specified in the following joint declaration with the responsible person and supervisor. All publications resulting from this thesis and which I am author correspond to the URM (see above) and I am solely responsible.

The importance of this affidavit and the criminal consequences of a false affidavit (section 156,161 of the Criminal Code) are known to me and I understand the rights and responsibilities stated therein.

Date Signature

7 Curriculum Vitae

Due to privacy reasons, my CV is not published in the e-version of the thesis.

8 Acknowledgments

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