Serum Amyloid A (SAA):
Proinflammatory functions and their regulation by serum lipoproteins

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Academic dissertation
To be presented for public examination with the permission of the Faculty of Biological and Environmental Sciences of the University of Helsinki in Biocenter 2, Auditorium 1041, Viikinkaari 5, Helsinki, on the 30th November 2012 at 12 o'clock

Helsinki 2012

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ISBN 978-952-10-8370-9 (paperback)
ISBN 978-952-10-8371-6 (PDF; http://ethesis.helsinki.fi)

Helsinki University Print
Helsinki 2012

‘I could tell you my adventures — beginning from this morning,’
said Alice a little timidly: ‘but it’s no use going back to yesterday,
because I was a different person then.’
‘Explain all that,’ said the Mock Turtle.
‘No, no! The adventures first,’ said the Gryphon in an impatient tone:
‘explanations take such a dreadful time.’

(Lewis Carroll: Alice’s Adventures in Wonderland)

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List of Original Publications

This thesis is based on the following original publications, which are referred to as Roman numerals in the text:

I. **Niemi K**, Baumann MH, Kovanen PT and Eklund KK (**2006**): Serum amyloid A (SAA) activates human mast cells which leads into degradation of SAA and generation of an amyloidogenic SAA fragment. *Biochimica et Biophysica Acta: Molecular Basis of Disease*, 1762(4):424-30.

II. **Niemi K**, Teirilä L, Lappalainen J, Rajamäki K, Baumann MH, Öörni K, Wolff H, Kovanen PT, Matikainen S and Eklund KK (**2011**): Serum amyloid A activates the NLRP3 inflammasome via $P2X_7$ receptor and a cathepsin B-sensitive pathway. *Journal of Immunology*, 186(11):6119-28.

III. **Niemi K**, Nurmi K, Öörni K, Kareinen I, Kovanen PT and Eklund KK (2012): Native and oxidized lipoproteins regulate the Serum Amyloid A-induced IL-1ß secretion in human macrophages. *Article in revision*.

In addition, some unpublished data are presented.
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Katri Niemi's contribution to the articles:

I. Participated in designing the experiments, conducted all experiments, analyzed the data and participated in writing and editing the manuscript

II. Participated in designing the experiments, conducted all experiments excluding the mouse macrophage part of the study and most western blot and some individual mRNA analyses, analyzed the data and participated in writing and editing the manuscript

III. Participated in designing the experiments, conducted all *in vitro* experiments, analyzed the data, wrote the manuscript draft and participated in editing the manuscript

Abbreviations

aa	amino acid
AA	amyloid A
ABCA1	ATP(adenosine-triphosphate)-binding cassette transporter 1
ACAT	acyl-CoA cholesterol acyl-transferase
AEF	amyloid enhancing factor
AP-1	activating protein 1
AP-HDL	acute-phase high-density lipoprotein
ApoA-I	apolipoprotein AI
ApoB-100	apolipoprotein B-100
APR	acute-phase response
APP	acute-phase protein
ASC	apoptosis-associated speck-like protein containing a CARD
ATP	adenosine 5′-triphosphate
BMM	bone marrow-derived macrophage
CAPS	cryopyrin-associated periodic syndromes
CARD	caspase activation and recruitment domain
CD	cluster of differentiation/designation
CRP	C-reactive protein
DAMP	danger-associated molecular pattern
EC	endothelial cell
ECM	extracellular matrix
FPRL1	formyl-peptide receptor-like 1
GM-CSF	granulocyte-macrophage colony-stimulating factor
HDL	high-density lipoprotein
HMC-1	human mast cell 1
HO-1	heme oxygenase 1
HS	heparan sulfate
IFN-γ	interferon gamma
IgE	immunoglobulin E
IL	interleukin
IP-10	interferon gamma-induced protein
IRF	interferon response factor
LDL	low-density lipoprotein
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MC	mast cell
MCP-1	monocyte chemoattractant protein-1

M-CSF	macrophage colony-stimulating factor
mmLDL	minimally modified low-density lipoprotein
MMP	matrix metalloproteinase
MyD88	myeloid differentiation primary response gene 88
nCEH	neutral cholesterol ester hydrolase
NF-κB	nuclear factor kappaB
NLR	nucleotide-binding domain leucine-rich repeat containing receptor
NLRP	NLR with a pyrin domain
NO	nitric oxide
ox	oxidized
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cell
PM	peritoneal macrophage
PMA	phorbol-12-myristate-13-acetate
PRR	pattern-recognition receptor
PTX	pertussis toxin
PTX3	pentraxin 3
RA	rheumatoid arthritis
RAGE	receptor for advanced glycation end-products
RANTES	regulated upon Activation, Normal T-cell Expressed, and Secreted
ROS	reactive oxygen species
SAA	serum amyloid A
SCF	stem cell factor
SMC	smooth muscle cell
$sPLA_2$	secretory phospholipase A2
SR	scavenger receptor
TEM	transmission electron microscopy
TGF	transforming growth factor
TH	T helper
TLR	Toll-like receptor
TNF	tumor necrosis factor
VCAM-1	vascular cell adhesion molecule 1
VLDL	very low-density lipoprotein

Abstract

The immune response is operated by two integrated systems, the adaptive and innate immune responses. Innate immunity includes both cellular and soluble components. The cellular part consists of host cells at the front line of defence – macrophages, monocytes, dendritic cells, neutrophils, endothelial cells and mast cells – that express receptors capable of recognizing common pathogen constituents, hence called pattern-recognition receptors, PRRs. Several cooperating PRR families, for example Toll-like receptors (TLRs) and receptors with nucleotide-binding domain leucine-rich repeats (NLRs), have been identified. They recognize two different classes of structures, pathogen-associated molecular patterns and non-microbial, danger-associated molecular patterns. The soluble component of the innate immune system includes an arsenal of acute-phase proteins, the expression of which is induced during the acute-phase response (APR), an immediate systemic reaction triggered by a local or systemic abnormal condition, such as tissue injury, infection or trauma. In addition, the innate immune response is driven by numerous proinflammatory cytokines and mediators, most notably interleukin (IL-) 1ß. The activity of IL-1ß is tightly controlled; the induction of gene expression and the activation of pro-IL-1ß require separate stimuli. IL-1ß maturation takes place in cytosolic protein platforms called inflammasomes, of which NLRP3 is the most characterized.

The major acute-phase proteins in human are C-reactive protein and serum amyloid A (SAA). In response to an inflammatory stimulus, the SAA concentration in plasma can increase up to 1000-fold. SAA circulates in association with high-density lipoprotein and is, thus, suggested to play a role in lipid metabolism and transport. In addition, SAA possesses strong cytokine-like and proinflammatory properties. A pathogenic role for SAA has most clearly been implicated in AA amyloidosis, a systemic protein misfolding disease that can complicate chronic inflammatory conditions. Current evidence indicates that SAA is also as an active mediator in cardiovascular diseases.

The aim of the study was to elucidate the interaction between SAA and two types of innate immune system cells, human mast cells and macrophages, and the consequences of this interaction in the pathogenesis of AA amyloidosis and atherosclerosis, as well as the regulation of SAA in inflammation. It was demonstrated that SAA is a potent activator of mast cells and macrophages, as indicated by a dose-dependent production of key proinflammatory cytokines, IL-1ß and tumor necrosis factor α, in both cell types. In mast cells, this activation led to the degradation of SAA by the mast cell-derived protease tryptase and to the formation of amyloid-like structures, suggesting a pathogenic role for mast cells in AA amyloidosis. The secretion of IL-1ß was studied in more detail in human macrophages, in which SAA was found to be able to induce both the gene expression of *IL1B*, via TLR2 and TLR4, and the activation of the NLRP3 inflammasome, resulting in the secretion of mature IL-1ß. The activation of NLRP3

involved the ATP-receptor $P2X_7$ and cathepsin B activity. Native serum lipoproteins were shown to inhibit the activity of SAA and this inhibition was further enhanced by lipoprotein oxidation. Besides the expression of *IL1B*, oxidized low-density lipoprotein (oxLDL) inhibited also the activation of the NLRP3 inflammasome. A decrease in the SAA-induced IL-1β production was observed also *in vivo*, suggesting that oxLDL, although possessing many pathological features, may represent a novel and significant regulator of SAA activity in inflamed tissues, including atherosclerotic lesions. All together, the findings of this study stress the significance of SAA in the pathogenesis of inflammatory diseases, such as atherosclerosis, and provide new insights into mechanisms leading to AA amyloidogenesis.

I Introduction

The immune response is operated by two integrated systems, the adaptive and innate immune responses. The innate immune system constitutes the first line of defence in response to various stimuli, which can be either foreign or host-derived, and which are recognized and responded to in a generic, non-specific manner. In contrast to the adaptive immune system and the generation of antibodies, the actions of the innate immune system do not confer any long-lasting or protective immunity. For a long time, the role of innate immunity was somewhat overlooked and also contested, culminating in the debate over the "dirty little secret" of immunology: the inability of vaccines to stimulate the adaptive immune response unless so-called adjuvants are used to evoke the innate immune system (Janeway 1992). During the last decade, the innate immunity has been under extensive study (Pelka and Latz 2011). The acute-phase response (APR) represents an integral part of the innate immune system (Cray *et al.* 2009). It can be described as an immediate systemic reaction triggered by a local or systemic abnormal condition such as tissue injury, infection or trauma. Typical characteristics include fever, changes in vascular permeability and importantly, induction of several proteins known as acute-phase proteins (APPs). The major APPs in human are C-reactive protein (CRP) and serum amyloid A (SAA) (Gabay and Kushner 1999).

In response to an inflammatory stimulus, the SAA concentration in plasma can increase up to 1000-fold. SAA circulates in association with high-density lipoprotein (HDL), which is why it has been suggested to play a role in lipid metabolism and transport. In addition, SAA has been implicated in host defence as well as in both the promotion and the attenuation of inflammation. However, the true physiological function of SAA is still under debate (Kisilevsky and Manley 2012). The pathological potential of SAA is well recognized in AA amyloidosis, a systemic protein misfolding disease that can complicate chronic inflammatory conditions such as rheumatoid arthritis (RA). However, a number of recent studies have stressed the proinflammatory properties of SAA, and the SAA research has quickly expanded beyond the scope of AA amyloidosis and RA. Current evidence now indicates SAA not only as a biomarker of inflammation but also as an active mediator in a number of pathological conditions including cancer (Malle *et al.* 2009) as well as cardiovascular diseases and associated preceeding conditions, such as obesity and type II diabetes (Herder *et al.* 2006, Yang *et al.* 2006, King *et al.* 2011).

The aim of this thesis was to investigate the interplay between SAA and two types of key innate immune system cells, mast cells and macrophages, and the consequences of this interaction on the pathogenesis of AA amyloidosis, atherosclerosis and inflammation in general. Potential mechanisms for the regulation of SAA were also studied.

II. Review of the Literature

1. The acute-phase response and innate immune system

The stimulated innate immune system initiates the inflammatory response by inducing the production of proinflammatory cytokines, notably interleukin (IL-) 1ß and tumor necrosis factor (TNF-) α, as well as several chemokines, adhesion molecules and other stimulants. This results in the recruitment of immune cells to the site of infection. The response can also be evoked by various non-microbial substances, which gives rise to sterile inflammation (Chen and Nunez 2010). Innate immunity includes both cellular and soluble components. The cellular component consists of host cells with ready-made receptors capable of recognizing common pathogen constituents, hence called pattern-recognition receptors (PRRs). The soluble component includes an arsenal of acute-phase proteins (APPs), the expression of which is induced during the acute-phase response (APR), and the complement system, which functions as a bridge between adaptive and innate immunity. Indeed, despite the division, the two immune systems are tightly connected. If the innate immune system is not capable of eliminating the pathogen, the adaptive immune system is activated through a process known as antigen presentation. Macrophages and particularly dendritic cells can function as antigen-presenting cells (Gordon and Taylor 2005). Furthermore, cytokines and growth-factors secreted from polarized subtypes of helper T cells (T_h), the key players of the adaptive immune system, mediate cell differentiation and inflammatory cascades related to innate immunity (Libby 2002).

1.1 Cells of the innate immune system

The cellular component of the innate immune system includes cells from two different origins. Macrophages, dendritic cells, mast cells, neutrophils, basophils and eosinophils originate from common myeloid progenitor cells in the bone marrow, whereas natural killer cells represent the lymphoid lineage in human hematopoiesis. Macrophages, dendritic cells and neutrophils have many similarities, the prime example of which is their ability to engulf foreign particles, bacteria or fragments of dying cells. Macrophages and dendritic cells are derived from circulating monocytes and they differentiate in tissues, whereas neutrophils mature already in the bone marrow and are abundant in blood and absent in healthy tissues. Neutrophils, together with the closely related basophils and eosinophils, are further

characterized by the so-called respiratory burst, an oxygen-consuming metabolic pathway that aims to eliminate the phagocytosed microbes. This is achieved by the rapid production and release of toxic substances, such as oxidized halogens and oxidizing radicals, into the phagosome (Babior 1984, Nathan 2006). Mast cells are derived from the common progenitor cells that are released as such from the bone marrow and the differentiation and maturation into mast cells takes place in vascularized tissues (Galli *et al.* 2005). Mast cells and macrophages that are used in this study are presented in more detail below.

1.1.1 Mast cells

Mast cells are derived from the myeloid progenitor cells that migrate into connective or mucosal tissues, differentiate, mature and ultimately reside in this local environment (Galli *et al.* 2005, Galli and Tsai 2008). With tissue macrophages and dendritic cells, mast cells represent the first cells of the immune system to interact with environmental antigens, allergens, toxins and pathogens. That is why they are distributed particularly near surfaces that are exposed to the environment, such as the skin, the lungs and the gastrointestinal tract. Mast cell differentiation and maturation is mediated principally by stem-cell factor (SCF) and its receptor c-kit, but locally secreted growth factors such as transforming growth factor β1 (TGF-β1), IL-6, T_h2-related cytokines IL-3, IL-4 and IL-9, and nerve growth factor contribute as well (Galli *et al.* 1993, Galli *et al.* 2005, Ryan *et al.* 2007). The most distinctive feature of differentiated mast cells is their ability to secrete biologically active proinflammatory, anti-inflammatory and immunosuppressive mediators upon activation. These mediators are stored in cytoplasmic granules (preformed mediators) or synthesized on demand (Schwartz and Austen 1984, Marshall and Jawdat 2004). The original name of the cells, Mastzellen, derived from the German word “Mästung”, “fattening”, was given by Paul Ehrlich, and it refers to this granular and “well-fed” appearance of mast cells (Ehrlich 1879). The preformed mediators include (1) the mast cell proteases chymase, tryptase, cathepsin G and carboxypeptidase A; (2) the proteoglycans heparin and chondroitin sulfates A and E; (3) histamine; and (4) several cytokines, chemokines and growth factors, such as TNF-α, IL-8, monocyte chemoattractant proteins 1, 3 and 4 (MCP-1, 3 and 4), Regulated upon Activation, Normal T-cell Expressed and Secreted (RANTES), vascular endothelial growth factor, basic fibroblast growth factor and TGF-β (Stevens *et al.* 1988, Gordon and Galli 1990, Welle 1997, Marshall and Jawdat 2004). Besides this, mast cells can generate numerous lipid mediators and additional cytokines and chemokines *de novo*, including IL-1α, IL-1β, IL-6, IL-18, TNF-α, granulocyte-macrophage colony-stimulating factor (GM-CSF) and several T_h1 and T_h2-related cytokines, among others (Marshall and Jawdat 2004).

The immunological activation of mast cells that is seen principally in allergy is mediated by the most widely known mast cell activator, immunoglobulin E (IgE).

Mast cells express high-affinity receptors for IgE, FcεRI, on their cell membranes (Rao and Brown 2008). Mast cell activation, which is initiated by the interaction of IgE and FcεRI through the Fc portion of IgE and by the bridging of two or three IgE-FcεRI complexes, results in the release of mast cell mediators. In the case of bacterial infection the activation can also proceed independent of IgE. Here, mast cells are activated via PRRs such as toll-like receptors (TLRs, see below), or complement receptors (Heib *et al.* 2007, Heib *et al.* 2008). In addition, the synergistic effect of FcεRI and TLRs has been demonstrated in mice (Nigo *et al.* 2006). Mast cells participate in host defence also by phagocytosing and killing bacteria (Malaviya *et al.* 1994) and by secreting antimicrobial substances and NO (Rao and Brown 2008).

Mast cells are not a homogenous cell population but instead differ from each other in terms of their mediator content, ultrastructure, size and shape, receptor expression, sensitivity and pharmacological responsiveness (Bradding 2009). Traditionally human mast cells are divided into two subtypes according to their protease content: those containing predominantly tryptase, MC_T, and those containing both tryptase and chymase as well as cathepsin G and carboxypeptidase A, MC_{TC} (Irani *et al.* 1986, Schechter *et al.* 1990, Irani *et al.* 1991). The MC_T type is usually found at mucosal surfaces while the MC_{TC} type occupies different types of connective tissue (Irani *et al.* 1986). The mast cell phenotype can change, though, in response to changes in the microenvironment of the cells. For example, the presence of IL-4, IL-6, IL-1β, TGF-β1 or lipopolysaccharide (LPS) together with SCF has been shown to enhance chymase expression in the MC_T phenotype *in vitro* (Galli *et al.* 2011).

1.1.2 Macrophages

Macrophages, the name of which originates from Greek (makros meaning "large" and phagein "to eat"; "big eaters"), are derived from circulating monocytes that migrate into tissues in the steady state or in response to inflammation (Gordon and Taylor 2005). They are long-lived cells found throughout the body but they are particularly abundant in the lungs, the gastrointestinal tract, the liver, the spleen and connective tissue. Monocyte differentiation into tissue macrophages proceeds in response to the local growth factor and cytokine environment (explained in more detail in chapter 3.2.3). Mature tissue-resident macrophages can be further classified into several subpopulations based on their anatomical location and functional phenotype: microglia in the brain, alveolar macrophages in the lung, histiocytes in the connective tissue, Kupffer cells in the liver, osteoclasts in the bone, as well as general inflammatory macrophages or tumour-associated macrophages (Gordon and Taylor 2005, Lawrence and Natoli 2011). Inflammatory macrophages are traditionally divided into two polarized phenotypes: M1 denoting

classically activated and M2 alternatively activated macrophages. M1 polarization is promoted by the T_h1 cell or natural killer cell-derived interferon-γ (IFN-γ) in concert with TNF-α or by LPS or GM-CSF (Gordon 2003, O'Shea and Murray 2008, Mosser and Edwards 2008, Krausgruber *et al.* 2011, van Tits *et al.* 2011). M1 macrophages exhibit proinflammatory activities and they are involved in host defence and antitumor immunity; they express various PRRs and are capable of producing proinflammatory cytokines, reactive-oxygen species (ROS) and nitric oxide (NO). The M2 phenotype, on the other hand, is promoted by IL-4 and IL-13 originating from T_h2 cells or by macrophage colony-stimulating factor (M-CSF), and macrophages of this phenotype are implicated in immune suppression and wound healing. For this reason, they are sometimes called wound-healing macrophages. A third phenotype, regulatory macrophages, has also been described. Regulatory macrophages are differentiated in response to anti-inflammatory IL-10, and they also produce it upon activation. They resemble the M2 macrophages in many ways (Mosser and Edwards 2008).

Nevertheless, inflammatory macrophage phenotypes do not represent stably differentiated subsets. Instead, they appear to undergo dynamic transitions among the functional phenotypes in response to their microenvironment. For example, prolonged exposure to LPS can lead to a state of responsiveness, known as endotoxin tolerance, which induces a switch of the gene expression pattern from the proinflammatory M1 phenotype to the anti-inflammatory M2 phenotype (Biswas and Lopez-Collazo 2009, Foster and Medzhitov 2009). Also, when M2 macrophages loaded with oxidized low-density lipoprotein (oxLDL) are exposed to LPS, they exhibit increased production of proinflammatory cytokines typical of the M1 phenotype (van Tits *et al.* 2011).

1.2 The acute-phase response

Inflammation is an ancient process, the function of which is to locally limit the spread of invading microbes and to participate in the resolution of infection and the repair of damaged tissues. The acute-phase response, also known as systemic inflammation, is an integral part of the innate immune system and observed across all animal species (Cray *et al.* 2009). It can be described as an immediate systemic reaction triggered by a local or systemic abnormal condition such as tissue injury, infection or trauma. The local effects of the acute-phase response are initiated by tissue macrophages and mast cells that recognize the disturbance mainly via PRRs. The recognition leads to the activation of additional macrophages and also of blood monocytes at the site of the stimulus. When activated, macrophages release various inflammatory mediators from the IL-1 and TNF-α cytokine families, the primary

proinflammatory cytokines. These mediators function on three levels. First, they induce the release of secondary cytokines from local stromal cells, which attracts neutrophils to the site. Neutrophils that are normally restricted to the circulation are now able to enter the tissue via selective extravasation through the activated endothelium of the blood vessel (Pober and Sessa 2007). Secondly, the primary cytokines up-regulate the production and release of neutrophils and monocytes from the bone marrow, thus enhancing the accumulation of inflammatory cells. The third effect, which is the phenomenon that best characterizes the systemic effects of the APR, involves rapid changes in the hepatic and extrahepatic production of acute-phase proteins (APPs). They are defined as proteins whose plasma concentration either increases or decreases by at least 25 % during inflammation (Gabay and Kushner 1999) and which can function as both mediators and inhibitors of inflammation at multiple possible sites. Generally, the rise in the plasma concentration of different APPs facilitates host defence by improving the recognition of microbes, by enhancing the mobilization of leukocytes into the circulation and by increasing blood flow to the sites of infection or injury. Positive APPs can be further classified as major, moderate or minor APPs depending on the magnitude of increase during the APR. Traditionally, major APPs increase 10- to 100-fold (or more), moderate APPs 2- to 10-fold and minor APPs only slightly. Moderate and mild increases are usually seen during chronic inflammation.

The major APPs in humans are serum amyloid A (SAA) and C-reactive protein (CRP). CRP was the first identified APP (Tillett and Francis 1930) and it is still the most routinely used marker of inflammation in clinical work, despite the fact that in many conditions SAA could actually serve as a more sensitive indicator (Malle and De Beer 1996, Hartmann *et al.* 1997, Yamada *et al.* 1999, Cunnane 2001). Positive APPs also include several members of the complement system, and other APPs, the expression of which is enhanced by SAA: secretory phospholipase A2 ($sPLA_2$) and pentraxin 3 (PTX3) (Gabay and Kushner 1999, Sullivan *et al.* 2010, Dong *et al.* 2011a, Satomura *et al.* 2012). Importantly, there are also antiproteases, such as α1-antitrypsin, and proteins related to coagulation or fibrinolysis, such as fibrinogen, among the positive APPs. Their role is to protect the host tissue against collateral damage at the affected site and to limit local hemorrhage, respectively (Manley *et al.* 2006). Such mechanisms are needed as some of the APR effectors, such as toxic substances or ROS released by neutrophils, cannot discriminate between foreign and self (Nathan 2006).

Normally the APR lasts only a few days and is ideally followed by the resolution and repair phase. Infiltrated neutrophils undergo apoptosis at the site of inflammation and they are rapidly phagocytosed by macrophages, evoking no further proinflammatory response (Fadok *et al.* 1998). This is mediated mainly by anti-inflammatory lipoxins, a family of lipoxygenase-derived eicosanoids, which inhibit

neutrophil recruitment and promote that of macrophages (O'Meara and Brady 1997, Mitchell *et al.* 2002, Serhan and Savill 2005). However, if phagocytosis is delayed, apoptotic neutrophils may undergo secondary necrosis triggering a new cycle of proinflammatory response by macrophages (Taylor *et al.* 2000). Furthermore, SAA can oppose the function of lipoxins and dampen their protective signalling (Bozinovski *et al.* 2012). A prolonged inflammatory reaction of this type can contribute to the development of chronic inflammatory states, tissue damage and disease.

1.3 Pattern and danger recognition

For years, innate immunity was regarded as a mechanism that simply discriminates "self" (e.g. host proteins) from "non-self" (e.g. microorganisms) and mediates the responses accordingly. However, a gradually increasing amount of data revealed that the mechanism was probably much more complex and that innate immunity might be more of a prerequisite rather than a mere addition to the adaptive immune system (Janeway 1992). It was suggested that the innate immune system recognizes substances in the context of "danger" (Matzinger 2002). These danger signals can be derived not only from bacterial, viral or fungal sources but also from self, marking events such as cellular stress or injury or cell death. The signals fall into two classes, pathogen-associated molecular patterns (PAMPs) and non-microbial, danger-associated molecular patterns (DAMPs) (Gay and Gangloff 2007, Martinon *et al.* 2009). They are sensed by specific PRRs, which are expressed by cells at the front line of defence: macrophages, monocytes, dendritic cells, neutrophils, endothelial cells (ECs) and mast cells. Our innate immune system consists of at least four cooperating PRR families: (1) Toll-like receptors (TLRs), (2) receptors with nucleotide-binding domain and leucine-rich repeats (NLRs), (3) retinoic acid inducible gene I-like receptors and (4) C-type lectin receptors. TLRs are located either on the outer cell surface or inside endosomes (Barton and Medzhitov 2003) whereas the other three reside in the cytoplasm. NLRs differ from the other PRRs in the sense that the final outcome of the NLR stimulation is the proteolytic activation of proinflammatory cytokines rather than the modulation of gene expression. TLRs and NLRs will be discussed in more detail below.

1.3.1 Toll-like receptors

TLRs are named for their similarity to *Toll*, a gene first identified and sequenced in *Drosophila melanogaster* (Anderson *et al.* 1985, Hashimoto *et al.* 1988). Soon after the revelation that Toll can trigger the innate immune response (Lemaitre *et al.* 1996), a group of direct homologs of Toll were identified in vertebrates. These are now known as Toll-like receptors (Rock *et al.* 1998). To date, 11 human

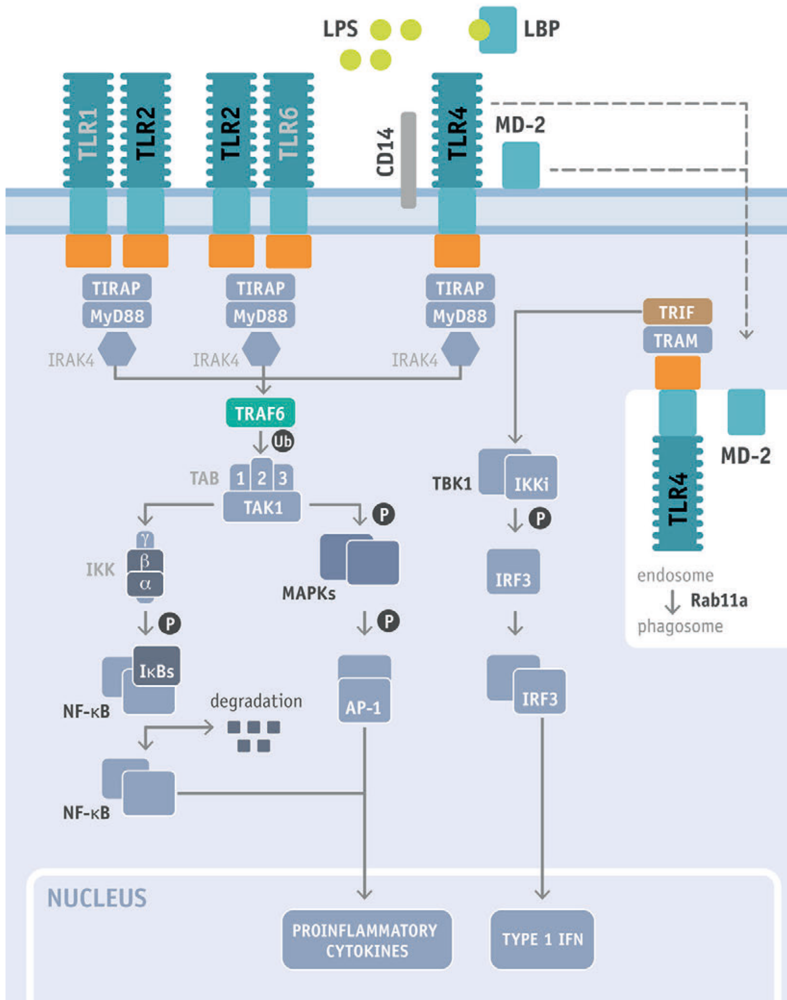

Figure 1. Signalling pathways triggered by Toll-like receptors 2 and 4. Only constitutive (and not inducible) TLR heterodimers are presented. Some steps are omitted for clarity. Data are derived from Akira 2006, Gay and Gangloff 2007, Kagan *et al.* 2008, Kawai and Akira 2011 and references mentioned in the text. AP-1, activating protein 1; CD14, cluster of differentiation 14; IFN, interferon; IκB, inhibitor of nuclear factor kappa B; IKK, IκB kinase kinase; IRAK, interleukin-1 receptor-associated kinase; IRF3, interferon response factor 3; LBP, lipopolysaccharide-binding protein; LPS, lipopolysaccharide; MAPK, mitogen activated protein kinase; MD-2, a secreted glycoprotein; MyD88, myeloid differentiation primary response protein 88; NF-κB, nuclear factor kappa B; P, phosphorylation; TAB, TAK-binding protein; TAK1, transforming growth factor-b-activated protein kinase 1; TBK, TRAF family member-associated NF-κB activator-binding kinase; TIRAP, Toll/IL-1R domain-containing adaptor protein; TRAF, tumor necrosis factor receptor-associated factor; TRAM, TRIF-related adaptor protein; TRIF, TLR/IL-1 receptor domain-containing adaptor protein inducing interferon-ß; Ub, ubiquitination

TLRs have been characterized (Hanke and Kielian 2011). TLRs 1, 2, 4-6, 10 and 11 are expressed and localized at the cell surface and TLRs 3 and 7-9 in intracellular vesicles, although cell type-specific variation has been detected (Kawai and Akira 2011). All TLRs are type I transmembrane receptors that together with IL-1 and IL-18 receptors belong to the TLR/IL-1 receptor superfamily (Akira 2006). All members of this family contain a C-terminal cytoplasmic TLR/IL-1 receptor (TIR) homology domain that is essential for signalling (Bowie and O'Neill 2000). Besides this, TLRs are typified by extracellular N-terminal leucine-rich repeats (LRR) implicated in ligand recognition.

Upon stimulation, TLRs interact with specific adaptor proteins that also contain a TIR domain. To date, five adaptor proteins have been identified: (1) myeloid differentiation factor 88 (MyD88), (2) Toll/IL-1R domain-containing adaptor protein (TIRAP; known also as MyD88 adaptor-like, MAL), (3) TIR-domain-containing adaptor inducing interferon-β (TRIF), (4) TRIF-related adaptor molecule (TRAM) and (5) SAM and ARM-containing protein (SARM) (McGettrick and O'Neill 2004). Different TLRs utilize different adaptor combinations to trigger the subsequent signalling pathways. MyD88 mediates the signalling from most TLRs, with the exception of TLR3 (Jiang *et al.* 2003), and initiates a common pathway that activates the inhibitory κB kinase complex and mitogen-activated protein kinases (MAPKs) leading to the activation of the nuclear transcription factors nuclear factor kappa B (NF-κB) and activating protein 1 (AP-1), respectively (Janeway and Medzhitov 2002, O'Neill and Bowie 2007). The activation of these transcription factors induces the expression of numerous inflammatory mediators such as cytokines, chemokines and adhesion molecules. TLR2 and TLR4 require both TIRAP and MyD88 (Vogel *et al.* 2003) to transduce the signal to NF-κB and AP-1. In addition to this, TLR4 can be translocated into Rab11a-positive endosomes and further to bacteria-containing phagosomes (Husebye *et al.* 2010) where it employs TRAM and TRIF to activate a special TRIF-dependent pathway. This results in the activation of interferon response factor 3 (IRF3) (Kagan *et al.* 2008), which regulates the expression of type I interferons and chemokines such as interferon gamma-induced protein (IP-10), RANTES and IFN-inducible T-cell a-chemoattractant (Hacker *et al.* 2006). In addition, inflammatory monocytes exhibit a unique MyD88-dependent activation of type I IFN that requires internalization of TLR2 (Kawai and Akira 2011).

To date, the most profoundly characterized TLRs are TLR2 and TLR4. TLR2 possesses the widest ligand repertoire of all TLRs, including bacterial lipoproteins and lipopeptides, fungal wall components and viral products. It associates with a co-receptor CD14 (Cleveland *et al.* 1996), besides which it forms a heterodimer with either TLR1 or TLR6, which further widens its ligand repertoire (Ozinsky *et al.* 2000, Triantafilou *et al.* 2006). In particular, TLR2-TLR6 recognizes mycoplasmic diacyl

lipopeptides and TLR2-TLR1 triacyl lipopeptides (Gay and Gangloff 2007). TLR2-TLR6 heterodimer can also associate with the scavenger receptor CD36 (Triantafilou *et al.* 2006). TLR4 recognizes LPS, a gram-negative cell wall component, and bacterial toxins as well as viral glycoproteins (Gay and Gangloff 2007). LPS recognition by TLR4 is accomplished in concert with the circulating LPS-binding protein (LBP) and the CD14 receptor (Wright *et al.* 1990), besides which constitutive interaction with the co-receptor MD-2 is required (Schromm *et al.* 2001, Re and Strominger 2002). There is evidence that TLR4 forms a heterodimer with TLR6, which further associates with CD36 upon CD36 stimulation (Stewart *et al.* 2010). Interestingly, both TLR2 and TLR4 interact not only with pathogen-derived particles but also with the large group of endogenous ligands that are likely to be found at sites of inflammation. These include extracellular matrix (ECM) components (TLR4), the APP fibrinogen (TLR4), very low-density lipoprotein (VLDL) apolipoprotein apoCIII (TLR2), minimally modified LDL (mmLDL) (TLR2 and 4), oxidized phospholipids (TLR2 and 4), stress-inducible heat shock proteins (TLR2 and 4) and amyloid-β fibrils (TLR2 and 4) (Walton *et al.* 2003, Chavez-Sanchez *et al.* 2010, Lundberg and Hansson 2010). The signalling pathways induced by TLR2 and TLR4 are illustrated in Figure 1.

1.3.2 Nucleotide-binding domain leucine-rich repeat containing receptors

To date, 23 human NLRs have been identified. All of them display a tripartite structure containing a C-terminal LRR domain, a central nucleotide-binding domain (NBD) and a variable N-terminal effector domain. LRR domains are believed to function as ligand recognizers, similar to the LRRs in TLRs, but no clear ligand-binding has been demonstrated for NLRs yet. The NBD domain consists of NACHT (domain present in NAIP, CIITA, HET-E, and TP-1), the domain that is common to all NLRs, and often of an additional NAD domain (NACHT-associated domain). The effector domains are required for signal transduction. There are four different types of these domains: acidic transactivation domain, pyrin domain, caspase recruitment domain (CARD) and baculoviral inhibitory repeat (BIR)-like domains. They have been used as determinants by which NLRs are distinguished into the following subfamilies: NLRA (NLRs with an acidic activation domain), NLRB (NLR with a BIR domain), NLRC (NLRs with a CARD domain), NLRP (NLRs with a pyrin domain), and NLRX (NLR family with no strong homology to the N-terminal domain of any other NLR subfamily member), as per updated nomenclature by Ting *et al* (Ting *et al.* 2008). The NLRP subfamily is the largest one containing 14 members, whereas NLRC contains 5 and all the rest only one each (Ting *et al.* 2008, Tschopp *et al.* 2003). Most NLRCs and all members of the NLRP subfamily are implicated in the posttranslational activation of inflammatory

caspases and the subsequent activation of cytokines from the IL-1 family. This is accomplished via the assembly and activation of inflammasomes, high molecular weight self-oligomerizing multiprotein complexes that reside in the cytosol (Martinon *et al.* 2002). Four prototypes of inflammasomes are known: NLRP1, NLRP3, IPAF (or NLRC4) and AIM2 (Faustin *et al.* 2007, Bauernfeind *et al.* 2009, Franchi *et al.* 2009, Franchi and Nunez 2010). NLRP3 (see below) is the most intensively studied, and it is also the most important in the scope of this study.

1.4 Inflammasome assembly

1.4.1 Interleukin 1ß

The IL-1 family (IL-1F) of cytokines plays a critical role in the host response to infection, mediating a variety of functions from the induction of APPs to the alteration of metabolism and the regulation of fever and lymphocyte activation (Glaccum *et al.* 1997). The family consists of 11 members with three major forms of IL-1: IL-1α, IL-1β and the IL-1 receptor antagonist (IL-1Ra), which are encoded by separate but related genes (Rock *et al.* 2010). IL-1α and IL-1β share similarities in terms of biological activity and receptor specificity (Dinarello *et al.* 1986, Dower *et al.* 1986, Arend *et al.* 2008); they stimulate the same receptor IL-1R, while IL-1Ra acts as its competitive antagonist. Both IL-1α and IL-1β are expressed in the cytoplasm as 31 kDa precursor proteins. The expression of the IL-1α precursor is constitutive in primary cells and many cell lines, and it is also biologically active (Hacham *et al.* 2002, Hurgin *et al.* 2007). Pro-IL-1α can be cleaved by plasma membrane-associated calpain into mature IL-1α, after which it is either released or retained within or associated with the cell. Pro-IL-1α can also localize in the nucleus and act as an autocrine growth factor (Arend *et al.* 2008).

IL-1β is a key inflammatory cytokine implicated in several stages of inflammation (Gabay *et al.* 2010), and its activity is controlled at transcriptional, translational, maturation and secretion levels (Dinarello 2009). The expression of pro-IL-1β, unlike that of IL-1α or other proinflammatory interleukins, is inducible and dependent on stimuli from other cytokines or TLRs and on the subsequent activation of NF-κB and AP-1 pathways (Mariathasan and Monack 2007, Franchi *et al.* 2009). In addition, IL-1β can induce its own expression via the activation of IL-1R and MyD88 (Dinarello *et al.* 1987). Pro-IL-1β is biologically inactive, and its cleavage into a mature protein is catalyzed by cysteine protease caspase-1 (formerly known as IL-1β converting enzyme, ICE) (Thornberry *et al.* 1992) within the cytosolic inflammasome complexes in a tightly regulated manner (see below). Alternatively, pro-IL-1β can be released from the cell and cleaved by extracellular proteases (Fantuzzi *et al.* 1997, Coeshott *et al.* 1999).

IL-1β does not contain a secretory signal sequence. Instead of being

transported through the classical endoplasmic reticulum/Golgi pathway, it is processed and secreted via a non-classical secretory system, i.e. unconventional secretion. To date, four detailed models for this secretion have been proposed. The first two depend on Ca^{2+} influx that is induced by the stimulation of the ATP receptor $P2X_7$ (Di Virgilio *et al.* 2001). In the model proposed originally by Andrei *et al.*, pro-IL-1β and pro-caspase-1 are targeted to special secretory lysosomes for IL-1β maturation and are then released by exocytosis (Andrei *et al.* 1999, Andrei *et al.* 2004). The second model, also Ca^{2+}-dependent, suggests that IL-1β maturation and release proceed within microvesicles that are derived from blebs of the plasma membrane (MacKenzie *et al.* 2001). However, the significance of Ca^{2+} influx seems to be uncertain as contradicting findings have been reported (Walev *et al.* 2000, Qu *et al.* 2007). Indeed, Qu *et al.* have described a Ca^{2+}-independent model in which IL-1β and inflammasome components are released within exosomes following the entrapment of pro-IL-1β and inflammasome complexes inside recycling endosomes and the formation of multivesicular bodies (Qu *et al.* 2007). Besides these, there is also preliminary evidence for a direct, yet uncharacterized, mechanism that is suggested to transport cytosolic IL-1β together with inflammasome components through the cell membrane (Brough and Rothwell 2007).

1.4.2 The NLRP3 inflammasome

The assembly of the NLRP3 inflammasome requires cytosolic overexpression of its components; the induction of both *NLRP3* and *IL1B* is a prerequisite for the activation of NLRP3 and the release of IL-1β (O'Connor *et al.* 2003, Mariathasan *et al.* 2007, Bauernfeind *et al.* 2009, Bauernfeind *et al.* 2011). Upon activation, NLRP3 receptors oligomerize and bind to ASC, apoptosis-associated speck-like protein containing a CARD (caspase activation and recruitment domain). NLRP3 oligomerization proceeds via NACHT domains and requires the binding of ATP (Duncan *et al.* 2007). ASC acts as a bridging molecule between NLRP3 and pro-caspase-1; ASC contains an N-terminal pyrin domain, which interacts with the pyrin domain of NLRP3, and a C-terminal CARD that binds pro-caspase-1. The structure of NLRP3 is presented in Figure 2. Caspases are cysteine proteases implicated in the initiation or execution of cellular programs leading to inflammation (proinflammatory caspases) or cell death (proapoptotic caspases). Similar to other caspases, caspase-1 is synthesized as an inactive zymogen (pro-caspase-1) that is activated via controlled dimerization within the inflammasomes. Subsequently, the activated caspase-1 cleaves pro-IL-1β into its mature form (Martinon *et al.* 2002). Caspase-1 can also activate other IL-1 family members, such as IL-18 and IL-33 (Keller *et al.* 2008).

The NLRP3 inflammasome is activated by a number of endogenous and exogenous activators. Host-derived activators ensuing sterile inflammation are

associated with danger of some type, such as cell damage or cell death. They include ATP (Pelegrin *et al.* 2006, Mariathasan *et al.* 2006), monosodium uric acid crystals (MSU), calcium pyrophosphate dihydrate (Martinon *et al.* 2006), amyloid-β fibrils, islet amyloid polypeptide oligomers and mutated superoxide dismutase-1 (Halle *et al.* 2008, Masters *et al.* 2010, Meissner *et al.* 2010), cholesterol crystals (Duewell *et al.* 2010, Rajamäki *et al.* 2010) and ECM components hyaluronan (Yamasaki *et al.* 2009) and biglycan (Babelova *et al.* 2009). Currently, there is no evidence for direct ligand binding to NLRP3. Instead, NLRP3 appears to act as a sensor of intracellular changes and mislocalization; the inflammasome pathway is activated when normally extracellular particles, such as crystals or protein aggregates, are sensed intracellularly or when intracellular components, such as ATP, are released into the extracellular milieu, as indicators of cell death. Foreign activators causing sterile inflammation known to date include different kinds of crystalline or particulate material, such as silica (Hornung *et al.* 2008, Dostert *et al.* 2008), asbestos (Dostert *et al.* 2008), metal alloy particles (Caicedo *et al.* 2009), alum and particulate vaccine adjuvants (Li *et al.* 2008, Sharp *et al.* 2009), as well as UV radiation (Feldmeyer *et al.* 2007, Watanabe *et al.* 2007) and certain antibiotics (Allam *et al.* 2011).

As for non-sterile inflammation, several bacteria, viruses and fungi have been implicated in the activation of NLRP3 (Davis *et al.* 2011, Menu and Vince

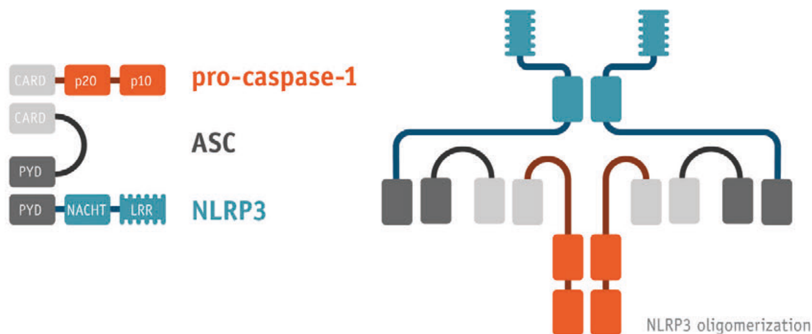

Figure 2. The structure of the NLRP3 inflammasome. The unoligomerized NLRP3 complex is depicted on the left. NLRP3 oligomerization is driven by the NACHT domains and results in the clustering of the caspase-1 domains. Caspase-1 activation is achieved by the removal of CARD and the autocleavage at CARD/p20 and p20/p10, leading to the formation of the active caspase-1 p10/p20 tetramer. CARD, caspase recruitment domain; LRR, leucine-rich repeat; NACHT, nucleotide-binding and oligomerization domain; PYD, pyrin domain.

2011). Of special interest and the most characterized in terms of their mechanism are probably the bacteria-derived pore-forming toxins, such as hemolysins from *Staphylococcus aureus* (Munoz-Planillo *et al.* 2009), listerialysin O from *Listeria*

monocytogenes (Meixenberger *et al.* 2010), nigericin from *Streptomyces hygroscopicus* (Mariathasan *et al.* 2006), pneumolysin from *Streptococcus pneumoniae* (McNeela *et al.* 2010), toxin A from *Clostridium difficile* (Ng *et al.* 2010), tetanolysin O from *Clostridium tetani* (Chu *et al.* 2009) and aerolysin from *Aeromonas hydrophila* (Gurcel *et al.* 2006). Various roles have also been demonstrated for flagellin from *Salmonella typhimurium*, malarial hemozoin crystals, β-glucan from *Candida albicans* as well as adenoviral DNA and RNA from influenza virus (Muruve *et al.* 2008, Allen *et al.* 2009, Shio *et al.* 2009, Kumar *et al.* 2009, Broz *et al.* 2010).

To date, three distinct models, which may not be exclusive, have been proposed for the activation of NLRP3: the potassium-efflux-inducing pathway, the lysosomal disintegration pathway and the ROS pathway (outlined in Figure 3). The first model is driven by ATP, which can be released from cells for various reasons including trauma, oxidants and pathogens (Burnstock *et al.* 2006). Also, LPS and other PRR agonists have been shown to trigger the release of ATP from mononuclear phagocytes (Ferrari *et al.* 1997, Piccini *et al.* 2008). Extracellular ATP stimulates the ATP-gated ion channel $P2X_7$, which then triggers the efflux of potassium, induces the recruitment of the pannexin-1 membrane pore and results in the activation of NLRP3 (Pelegrin *et al.* 2006, Mariathasan *et al.* 2006, Petrilli *et al.* 2007, Kanneganti *et al.* 2007). The role of pannexin-1 has since been questioned, however (Qu *et al.* 2011). Decreased intracellular K^+ has been shown to promote the assembly of NLRP3, whereas high extracellular K^+ prevents the efflux and inhibits the activation of NLRP3 in response to numerous NLRP3 activators (Franchi *et al.* 2007, Dostert *et al.* 2008, Rajamäki *et al.* 2010). Also, in an experimental setting where cell integrity was distrupted, inflammasome assembly could be inhibited by K^+ levels that mimicked the normal K^+ level in the cytosol (Kahlenberg and Dubyak 2004, Petrilli *et al.* 2007). However, the exact mechanism by which the intracellular K^+ concentration regulates NLRP3 is not clear. It is also possible that the pore formation allows small DAMPs and PAMPs to enter the cytosol and to activate NLRP3 directly (Kanneganti *et al.* 2007).

The second model implicates phagocytosed material, e.g. crystals, crystalline material, particles and protein aggregates, in the activation of NLRP3. Internalization of these activators by phagocytic cells initiates a cascade in which lysosomal swelling or destabilization results in the leakage of lysosomal contents into the cytoplasm. The released material appears to mediate the activation of NLRP3 and this activation is induced especially by cathepsin B, which is activated by the acidification of phagosomes (Hornung *et al.* 2008, Sharp *et al.* 2009). The mechanism behind this activation is not known, but it has been suggested that cathepsin B might generate a ligand for NLRP3 (Rock *et al.* 2010). Another possibility is that NLRP3 somehow senses the lysosome rupture itself as NLRP3-dependent

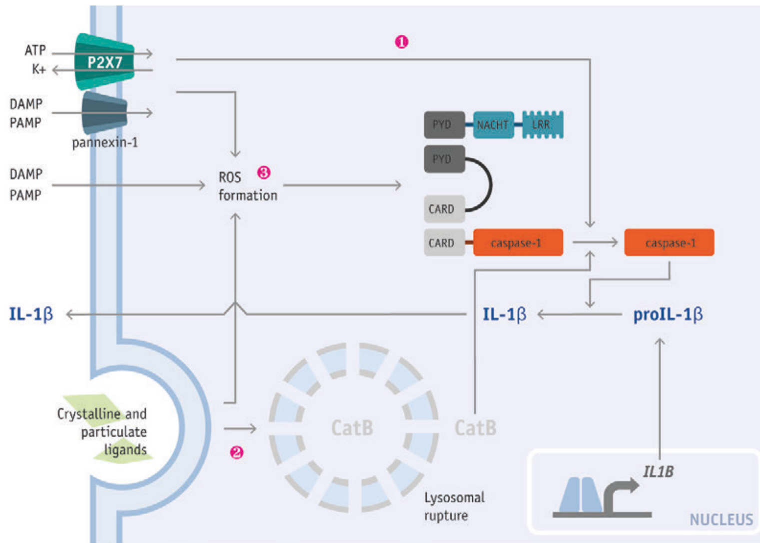

Figure 3. Proposed models for the activation of NLRP3. Three major models have been proposed for the activation of the NLRP3 inflammasome. For clarity, only the unoligomerized NLRP3 inflammasome complex is depicted. (1) Extracellular ATP stimulates the ATP-gated ion channel $P2X_7$, which then triggers the efflux of potassium and induces the recruitment of the pannexin-1 membrane pore. A decreased intracellular K^+ concentration or extracellular NLRP3 agonists entering the cytosol via pannexin-1 may activate the NLRP3 inflammasome. (2) Internalization of crystalline or particulate material initiates a cascade in which lysosomal swelling or destabilization results in the leakage of lysosomal contents into the cytoplasm. The released material, especially cathepsin B, mediates the activation of NLRP3, possibly by generating a direct NLRP3 ligand. (3) All DAMPs and PAMPs, including those implicated in the other activation models, induce ROS production directly or indirectly. ROS is presumed to originate from the activity of one or several NADPH oxidases or from mitochondria, and it triggers the assembly and activation of NLRP3. Data are derived from the references in the text. ATP, adenosine triphosphate; CARD, caspase recruitment domain; CatB, cathepsin B; DAMP, danger-associated molecular pattern; IL, interleukin; LRR, leucine-rich repeat; NACHT, nucleotide-binding and oligomerization domain; PYD, pyrin domain.

IL-1β release can also be achieved by rupturing empty endocytic vesicles (Hornung *et al.* 2008). In addition, the potassium ionophore nigericin, a potent activator of caspase-1, has been shown to promote the release of cathepsin B from the lysosomes (Hentze *et al.* 2003, Dostert *et al.* 2008).

The third model depicts a common pathway for the NLRP3 activators: the generation of ROS. All DAMPs and PAMPs tested, including ATP and phagocytosed material, induce ROS production directly or indirectly and the blocking of ROS diminishes inflammasome activation (Cruz *et al.* 2007, Schröder and Tschopp 2010). ROS production can also be triggered by so-called frustrated phagocytosis in which a particle is too large for endocytosis and remains trapped on the cell surface (Dostert *et al.* 2008, O'Neill 2008). ROS may also promote lysosomal

rupture or, in turn, the production of ROS could be affected by the released lysosomal proteases. ROS is presumed to originate from the activity of one or several NADPH oxidases (Dostert *et al.* 2008) or from mitochondria (Zhou *et al.* 2011). However, some controversy regarding the ROS model does exist: LPS alone can induce ROS (Emre *et al.* 2007) but is not able to activate the inflammasome in macrophages (Ferrari *et al.* 2006). Also, cells collected from patients with defective NADPH activity and thus an inability to produce ROS via NADPH still demonstrated inflammasome activation and the release of IL-1β (van de Veerdonk *et al.* 2010). Furthermore, in superoxide dismutase deficiency, the caspase-1 activity decreases despite the increasing ROS levels (Meissner *et al.* 2008).

1.5 Clinical implications of the activation of NLRP3

IL-1β plays an important role in host defence as part of the innate immune response. However, its dysregulation and excess activity underlies a growing number of diseases. These IL-1β activation disorders, or inflammasomopathies, include several types of conditions: autoinflammatory diseases, autoimmune diseases and other diseases with an inflammatory component and/or linkage to inflammasomes, either direct or indirect (Stojanov and Kastner 2005, Menu and Vince 2011, Shaw *et al.* 2011).

Inherited cryopyrin-associated periodic syndromes (CAPS), cryopyrin being an earlier name for NLRP3, include three chronic inflammatory diseases: familial cold autoinflammatory syndrome, Muckle–Wells syndrome and neonatal onset multi-systemic inflammatory disease / chronic infantile neurological cutaneous articular syndrome (NOMID/CINCA). These syndromes are most typically caused by gain-of-function -mutations in the NACHT domain of NLRP3 (Aganna *et al.* 2002, Agostini *et al.* 2004, Shinkai *et al.* 2008), and they exhibit overactivation of NLRP3 and accumulation of IL-1β. Common characterics include recurrent rash and fevers, joint pain, fatigue, deafness, disabilities of the central nervous system, vision loss and joint and bone deformation (Shaw *et al.* 2011). Furthermore, one fourth of the MWS patients and up to 50% of the NOMID/CINCA patients develop AA amyloidosis in concert with elevated SAA levels and renal impairment (Aganna *et al.* 2002, Menu and Vince 2011). CAPS have been successfully treated with targeted IL-1β therapy (Hawkins *et al.* 2004, Lachmann *et al.* 2009). Besides CAPS, the dysregulation of NLRP3 due to a mutation in the inflammasome-related genes has been demonstrated in the pathogenesis of familial Mediterranean fever (FMF) and the pyogenic arthritis, pyoderma gangrenosum and acne syndrome (Chae *et al.* 2003, Shoham *et al.* 2003, Chae *et al.* 2006). Complex inflammatory conditions such as gout and

pseudogout, type II diabetes and occupation-related disorders, such as silicosis and asbestosis (Maedler *et al.* 2002, Martinon *et al.* 2006, Dostert *et al.* 2008, Masters *et al.* 2010), on the other hand, show abnormal inflammasome activation, which is not due to genetic mutations but to a chronic exposure to particulate activators (McDermott and Tschopp 2007, Ng *et al.* 2010). Atherosclerosis and Alzheimer's disease can also be included in this group as cholesterol crystals and amyloid-β fibrils, respectively, have been identified as NLRP3 activators (Halle *et al.* 2008, Duewell *et al.* 2010, Rajamäki *et al.* 2010). Gout and type II diabetes also respond to IL-1 blocking therapy (Larsen *et al.* 2007, So *et al.* 2007) and so do several other diseases such as TNF-α-receptor-associated periodic syndrome and hyper IgD syndrome, in which, however, the involvement of NLRP3 has not yet been demonstrated (Masters *et al.* 2009).

The role of inflammasomes in the pathogenesis of autoimmune diseases is still unclear. This group includes different types of complex diseases, such as RA, multiple sclerosis (the autoimmune status of which, however, has also been questioned; Corthals 2011), type I diabetes, systemic lupus erythematosus and celiac disease. IL-1β and IL-18 are known to shape adaptive immune responses in several ways (Sims and Smith 2010), and at least one of these cytokines contributes to the pathogenesis of each of the above-mentioned autoimmune diseases, excluding type I diabetes (Calvani *et al.* 2005, Voronov *et al.* 2006, Lotito *et al.* 2007, Acosta-Rodriguez *et al.* 2007, Garrote *et al.* 2008, Goldbach-Mansky 2009). Thus, it is possible that at least one of the inflammasomes is involved in these diseases.

2. Serum amyloid A (SAA)

Serum amyloid A (SAA) is a plasma protein that was first identified as a component of systemic amyloid deposits detected in patients with persistent inflammation (Benditt *et al.* 1971, Levin *et al.* 1973). Later it was found to function as an apolipoprotein of HDL, the concentration of which can increase up to 1000-fold in response to inflammation or injury as a part of the acute-phase response (APR) (Benditt and Eriksen 1977). SAA in mice was identified in 1971 (Isersky *et al.* 1971). SAA genes fall into two groups: those induced during the APR by proinflammatory mediators (A-SAA) and those expressed constitutively (C-SAA) (Whitehead *et al.* 1992). While the expression of C-SAA has been documented only in humans and mice (Whitehead *et al.* 1992, De Beer *et al.* 1994), the A-SAA genes are highly conserved throughout evolution, found not only in eutherian mammals (Uhlar *et al.* 1994) and marsupials (Uhlar *et al.* 1996) but also in other vertebrates, such as birds (Guo *et al.* 1996, Kovacs *et al.* 2005) and fish (Jensen *et al.* 1997). The current nomenclature of SAA genes and proteins is as suggested in 1999 by Sipe (Sipe 1999).

2.1 The SAA gene family

The human SAA gene family comprises four genes, *SAA1-4*, that are all located in the chromosome 11p15.1 (Sellar *et al.* 1994a, Sellar *et al.* 1994b) and share a four-exon three-intron organization typical of apolipoproteins (Steel and Whitehead 1994). *SAA1* and *SAA2* encode the acute-phase proteins SAA1 and SAA2 (A-SAA), respectively, and *SAA4* encodes a constitutional protein of HDL (C-SAA) (Steel *et al.* 1993). *SAA3* is a pseudogene with no corresponding protein product (Kluve-Beckerman *et al.* 1991, Sellar and Whitehead 1993). Due to allele polymorphism, there are five isoforms of SAA1 (*SAA1.1, SAA1.2, SAA1.3, SAA1.4* and *SAA1.5*) and two of SAA2 (*SAA2.1* and *SAA2.2*) (Uhlar and Whitehead 1999). In mice, five SAA genes, located in chromosome 7p, have been described (Lowell *et al.* 1986, De Beer *et al.* 1994, Butler and Whitehead 1996). Mouse *Saa1.1* and *Saa2.1* encode A-SAA proteins, and they are evolutionary homologs to human SAA1 and SAA2. Mouse *Saa3*, unlike human *SAA3*, is an expressed A-SAA gene, but it differs from *Saa1* and *Saa2* both in terms of sequence and the location of expression (Meek and Benditt 1986, Meek *et al.* 1989). Mouse *Saa4* is a constitutively expressed homolog to human *SAA4*, and *Saa-ps1* is a pseudogene

like *SAA3* (Lowell *et al.* 1986, De Beer *et al.* 1994). Besides these, the inbred CE/J mice, a mouse strain reported to be unusually resistant to amyloidosis, expresses a single isoform, *Saa2.2* (De Beer *et al.* 1993, Sipe *et al.* 1993). In this review, mouse SAA genes and proteins are referred to as *Saa* and Saa, respectively, to distinguish them from their human counterparts, *SAA* and SAA.

The amino acid similarities are relatively low, ~50%, between A-SAA and C-SAA proteins in both humans and mice (De Beer *et al.* 1994, Whitehead *et al.* 1992), implying that the constitutive SAA represents a separate branch in the SAA family (Uhlar and Whitehead 1999). Among the A-SAA proteins the amino acid sequence differences are minimal; SAA1 and SAA2 share a 92% homology in humans and a 91% homology in mice (Yamamoto and Migita 1985, Uhlar *et al.* 1994). However, these differences seem to have an impact on the function and pathogenicity of the proteins as will be discussed later.

2.2 The expression of SAA

During the APR the serum level of circulating SAA protein can increase 1000-fold compared with the baseline (Benditt and Eriksen 1977), reaching concentrations as high as 1 mg/ml. For this, the liver directs a significant proportion of its biosynthetic capability into producing SAA; in mice up to 2.5% of the total hepatic protein synthesis may be comprised of SAA production during the APR (Morrow *et al.* 1981). The SAA concentration starts to increase 3-6 hours after the inflammatory stimulus, peaking on the third day and returning to the baseline after day 4 (Malle and De Beer 1996, Yamada *et al.* 1999). SAA has a relatively short half-life of one day (Tape and Kisilevsky 1990) but the capacity of the liver to catabolize SAA has been shown to decrease during the APR (Gollaher and Bausserman 1990).

The main inducers of A-SAA production are IL-1β, IL-6 and TNF-α, which bind to their designated hepatic receptors. The role of IL-6 seems to be the most critical because it functions in synergy with the other SAA-inducing proinflammatory cytokines (Uhlar and Whitehead 1999, Hagihara *et al.* 2004). The signalling from IL-1β and TNF-α activates NF-κB, while IL-6 activates the NF-IL6 (also known as CCAAT/enhancer-binding protein, C/EBP) pathway (Jensen and Whitehead 1998). The transcription factor SAA-activating factor 1 (SAF-1) is also involved (Ray *et al.* 2006). In addition to cytokines, LPS and oxLDL have been shown to induce SAA expression in human hepatocytes and in the human THP-1 monocytic cell line as well as *in vivo* in mice (Liao *et al.* 1994, Ray *et al.* 1999, Migita *et al.* 2004).

Besides the liver, both SAA mRNA and SAA protein have been detected in the epithelial components of a wide array of other tissues, such as the tissues of the

intestine, lung, kidney, skin, tonsil, prostate, breast, thyroid and pancreas (Urieli-Shoval *et al.* 1998, Vreugdenhil *et al.* 1999). Adipose tissue seems to be a significant source of SAA under non-acute-phase conditions (Sjöholm *et al.* 2005, Yang *et al.* 2006). SAA mRNA and/or protein have also been detected in histologically abnormal tissues including atherosclerotic lesions (Meek *et al.* 1994, Yamada *et al.* 1996), Alzheimer's disease brain (Liang *et al.* 1997), inflamed RA synovial tissue (Kumon *et al.* 1999, O'Hara *et al.* 2000, O'Hara *et al.* 2004), irradiated bone marrow (Goltry *et al.* 1998) and cancer cell lines of hepatic and non-hepatic origin (Thorn *et al.* 2003, Gutfeld *et al.* 2006, Kovacevic *et al.* 2006, Kovacevic *et al.* 2008, Malle *et al.* 2009) as well as in tumours (Urieli-Shoval *et al.* 2010). In addition, various cell types have been implicated in the expression of SAA, including epithelial cells, fibroblasts, lymphocytes, ECs, monocytes/macrophages, smooth muscle cells (SMCs) and adipocytes (Meek *et al.* 1994, Steel and Whitehead 1994, Urieli-Shoval *et al.* 1998, Kumon *et al.* 2002a, Yang *et al.* 2006). It should also be noted that the regulation of SAA transcription outside the liver varies depending on the cell type. In SMCs, for example, SAA expression is induced by glucocorticoids instead of proinflammatory cytokines (Kumon *et al.* 2002a).

2.3 The molecular characteristics of SAA proteins

Human SAA proteins consist of 104 (SAA1 and SAA2) or 112 (SAA4) amino acids (aa), which are preceeded by 18-aa signal peptides in primary translation products. SAA proteins are 12-14 kDa in size. The AA fragments most typically deposited in amyloid fibrils correspond to the first 76 amino acids of SAA (Skogen *et al.* 1983), although variations have been documented (Levin *et al.* 1973, Westermark 1982, Westermark *et al.* 1987). Despite the important physiological and pathological implications proposed for SAA, there is relatively little data on its structure, which is mostly due to the low solubility of the native lipid-free isoforms. Early secondary structure predictions suggested that SAA is a typical globular protein, the structure of which consists of both α-helices and β-pleated sheets and contains putative calcium and lipid-binding sites (Turnell *et al.* 1986). There are three hydrophobic regions spanning amino acids 1-27, 40-63 and 79-94 on SAA (Turnell *et al.* 1986). It has been estimated that at least one third of SAA is helical (McCubbin *et al.* 1988, Meeker and Sack 1998) and that it forms a helical bundle (Stevens 2004). Lipid-free SAA has also been reported to aggregate in solutions and to form a hexameric channel in lipid bilayers (Hirakura *et al.* 2002, Kinkley *et al.* 2006).

Besides the calcium binding site (aa 48-51), several other binding regions on SAA have been identified. The hydrophobic, amphipathic N-terminus (aa 1-11) has been implicated in lipid binding (Turnell *et al.* 1986), based on data achieved by

degradation studies, mutagenesis or usage of SAA fragments or antibodies against different epitopes (Husebekk *et al.* 1987, Malle *et al.* 1995, Patel *et al.* 1996, Malle *et al.* 1998, Ohta *et al.* 2009). Indeed, amphipathic α-helix formation has been suggested to be a general requirement for the binding of apolipoproteins to lipids (Segrest *et al.* 1992). The N-terminus, and the first 10-15 aa in particular, has also been shown to be the major determinant for amyloid formation (Patel *et al.* 1996, Westermark *et al.* 1992, see also chapter 3.1.3). The area spanning aa 24-42 contains elements that resemble the cell-binding domains of two cell adhesive glycoproteins of the ECM: laminin and fibronectin (Kawahara *et al.* 1989, Preciado-Patt *et al.* 1994, Preciado-Patt *et al.* 1996a, Ancsin and Kisilevsky 1997). Besides this, a binding site for another ECM component, heparan sulfate (HS) as well as for its structural derivative, heparin, has been located at the C-terminus between aa 77 and 103 (Ancsin and Kisilevsky 1999). However, this applies to neutral pH only as in acidic pH HS/heparin binds to aa 17-49 (Elimova *et al.* 2009). The functional domains of SAA are summarized in Table 1.

SAA residues	Binding site/motif	Proposed functions	References
1-11/18	binding site for HDL/lipid/ cholesterol	cholesterol transport and metabolism, foam cell formation	Turnell *et al.* 1986, Kisilevsky & Subrahmanyan 1992, Liang *et al.* 1995
1-15	amyloidogenic determinant	amyloid formation	Westermark *et al.* 1992
1-20 (1	ACAT-inhibiting region	cholesterol efflux	Kisilevsky & Tam *et al.* 2003
17-49	HS/heparin binding site (low pH)	HDL-SAA remodeling, amyloid formation	Elimova *et al.* 2009
24-76	laminin binding site	amyloid formation	Ancsin & Kisilevsky 1997
29-42	YIGSR and RGD-like adhesion motifs	cell adhesion	Linke *et al.* 1991, Preciado-Patt *et al.* 1994
48-51	calcium binding sequence GPGG	amyloid formation?	Turnell *et al.* 1986
74-103 (1	CEH-activating region	cholesterol efflux	Kisilevsky & Tam *et al.* 2003
77-103/4	neutrophil and HS/heparin binding	anti-inflammatory activity, amyloid formation	Preciado-Patt *et al.* 1996a, Ancsin & Kisilevsky 1999

Table 1. The functional domains in human SAA protein
ACAT, acyl-CoA cholesterol acyl-transferase; HS, heparan sulfate; CEH, cholesterol ester hydrolase.
(1 study conducted using mouse SAA2.1, more details in chapter 2.5.1

2.4 The functions of SAA

To date, SAA has been linked to several physiological functions, along with the identification of SAA-binding receptors, although none of them has so far been widely accepted (Kisilevsky and Manley 2012). The majority of SAA associates and circulates with HDL_3 (Benditt and Eriksen 1977, Skogen *et al.* 1979, Bausserman *et al.* 1980),

replacing apolipoprotein A-I (apoA-I), the normal HDL apolipoprotein, during the APR (Coetzee *et al.* 1986). This applies to A-SAA only as C-SAA, which is only minimally induced during the APR, associates with both normal HDL and acute-phase-HDL, AP-HDL (Whitehead *et al.* 1992). Current evidence implicates SAA as an apolipoprotein and an acute-phase reactant with various immune- and lipid-related functions.

2.4.1 Receptors for SAA

The early studies on SAA interactions already demonstrated specific binding sites for SAA on macrophages (Kisilevsky and Subrahmanyan 1992), the number of which increases during the APR. This interaction leads to endocytosis of SAA, and it seems to be dependent on HS and to involve the specific HS binding site on SAA (Röcken and Kisilevsky 1998, Ancsin and Kisilevsky 1999). HS as well as a low pH and physiological calcium concentrations have been suggested to promote the dissociation of SAA from HDL and the subsequent cellular uptake of SAA (Tam *et al.* 2008), although the whole complex can be internalized as well. In terms of the mechanism, HDL and SAA-containing acute-phase (AP-) HDL appear to be taken up in a similar manner (Röcken and Kisilevsky 1998). Also, when mouse macrophages are subjected to purified lipid-free SAA, SAA is endocytosed, possibly via clathrin-mediated endocytosis, and directed to the endosomal-lysosomal pathway for degradation (Kluve-Beckerman *et al.* 1999, Kluve-Beckerman *et al.* 2001). More recent studies have suggested that only the SAA 2.1 isoform is internalized, and, when it is presented as a SAA-HDL complex, SAA, or fragments thereof, it may actually proceed to the nucleus, after which it is promptly returned to the cytoplasm and exocytosed (Kinkley *et al.* 2006).

As for signal transduction, several cell-surface receptors on different cell types have been identified as potential SAA receptors. The fact that SAA can bind to more than one receptor and induce the activation of several signalling pathways makes it a powerful proinflammatory mediator. However, it is intriguing and not yet understood on a detailed molecular level how SAA can interact with such a variety of structurally diverse receptors. The receptors implicated in SAA signalling and/or uptake are reviewed below and illustrated in Figure 4.

FPRL1

N-formyl peptide receptor 2 (FPR2), also known as formyl peptide receptor-like 1 (FPRL1) or lipoxin A4 receptor (LXA4R), is a seven-transmembrane Gi protein-coupled receptor encoded by the *FPR2* gene. The expression of FPRL1 has been documented in a wide range of cell types, and several ligands, including bacterial and mitochondrial peptides, the lipid metabolite lipoxin A4, chemokine variants and amyloidogenic proteins, have been suggested (Migeotte *et al.* 2006). The physiological functions of FRPL1 include chemotaxis that results in the

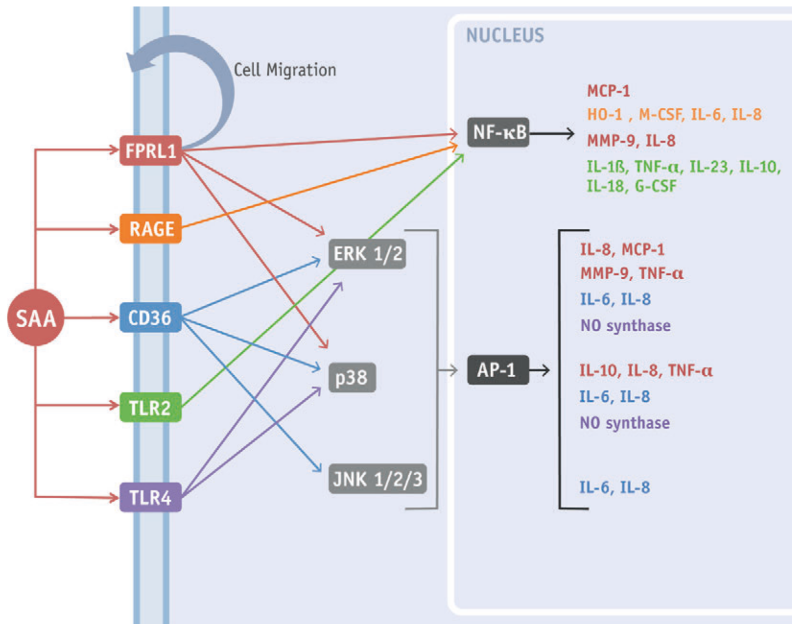

Figure 4. Proposed SAA receptors and subsequent signalling pathways. The data are derived from the references mentioned in the text. AP-1, activating protein 1; CD36, cluster of differentiation 36; ERK, extracellular signal-regulated kinase; FPRL1, formyl peptide receptor-like 1; G-CSF, granulocyte colony-stimulating factor; HO-1, heme oxygenase 1; IL, interleukin; JNK, c-Jun N-terminal kinase; MCP-1, monocyte chemoattractant protein 1; MMP, matrix metalloproteinase; NF-κB, nuclear factor kappa B; NO, nitric oxide; RAGE, receptor for advanced glycation end-products; TLR, Toll-like receptor; TNF, tumor necrosis factor.

migration and accumulation of neutrophils and monocytes *in vivo* and, depending on the ligand, the mediation of both anti- and proinflammatory effects. Indeed, SAA has been shown to act as a chemotactic ligand for FPRL1 and to induce phagocyte migration (Su *et al.* 1999) as well as the release of chemokine MCP-1 and the induction of matrix metalloproteinase (MMP-) 9 in human monocytes and human umbilical cord endothelial cells (Lee *et al.* 2005, 2008, 2009, 2010). In neutrophils, SAA induces a FPRL1-dependent calcium release and the activation of NF-κB, MAPKs and AP-1, leading to the secretion of IL-8 (He *et al.* 2003). In monocytes, it induces the activation of p38 and the secretion of both anti- and proinflammatory cytokines, IL-10 and TNF-α, respectively (Lee *et al.* 2006).

RAGE

The receptor for advanced glycation end-products (RAGE) has a single transmembrane domain and it belongs to the immunoglobulin superfamily that has been connected to diverse pathologies from atherosclerosis to Alzheimer's disease. Rather than single polypeptides, RAGE recognizes families of ligands, including

advanced glycation end products (AGEs), amyloid fibrils of different origins, amphoterins and S100/calgranulins, implying that the interaction is dependent on conformational determinants. The expression of RAGE is up-regulated by its ligands, which are present in the diseased tissues, and the RAGE-ligand interaction has been indicated as a propagation factor for the cellular perturbation in chronic disorders (Schmidt *et al.* 2000). Both soluble and fibrillar SAA have been implicated in RAGE activation (Yan *et al.* 2000, Okamoto *et al.* 2008). RAGE expressed on BV-2 transformed mononuclear phagocytes has been shown to bind amyloid A fibrils and soluble amyloidogenic mouse Saa1.1 protein, but not Saa2.1 or Saa2.2. The binding leads to NF-κB activation and the expression of heme oxygenase-1 (HO-1) and M-CSF (Yan *et al.* 2000). SAA induces IL-6 and IL-8 via RAGE as well as the activation of NF-κB also in synovial fibroblasts (Okamoto *et al.* 2008).

Class B scavenger receptors

Class B scavenger receptors are a group of receptors implicated mainly in lipid transport and pattern recognition in innate immunity. The family consists of CD36, first indentified as a receptor for oxLDL (Endemann *et al.* 1993), scavenger receptor class B type I (SR-BI) (Acton *et al.* 1994), LIMP II (Vega *et al.* 1991) and the human homologue CD36-LIMPII analogous 1 (CLA-1) (Calvo and Vega 1993). CLA-1 and SR-BI were originally indicated as HDL receptors but have later also been shown to bind to native and modified LDL (Acton *et al.* 1994), native VLDL (Krieger 1999), LPS (Vishnyakova *et al.* 2003), amyloid fibrils (Husemann *et al.* 2001), and amphipathic peptides possessing one or more amphipathic α-helices (Bocharov *et al.* 2004, Baranova *et al.* 2005). CLA-1/SR-BI expression has been detected in the liver, ovary and adrenal gland (Landschulz *et al.* 1996) as well as in atherosclerotic lesions (Chinetti *et al.* 2000). CD36 has a ligand repertoire somewhat similar to that of CLA-1/SR-BI, except that it does not bind acetylated or extensively oxidized LDL but rather binds moderately oxidized LDL, i.e. mmLDL (Endemann *et al.* 1993). Besides this, CD36 participates in signal transduction by associating with a heterodimer formed either by TLRs 2 and 6 (Triantafilou *et al.* 2006) or TLRs 4 and 6 (Stewart *et al.* 2010) or by regulating JNK signalling and the cascade of foam cell formation (Rahaman *et al.* 2006). SR-B1, CLA-1 and CD36 have all been suggested to participate in the internalization of SAA (Baranova *et al.* 2005, Cai *et al.* 2005, Baranova *et al.* 2010). For example, both lipid-free SAA and SAA-HDL are bound and internalized by SR-BI on hepatocytes, while the uptake of HDL is inhibited (Cai *et al.* 2005). CLA-1 and CD36 also participate in downstream SAA signalling resulting in the activation of AP-1 and the production of IL-6 and IL-8 (Baranova *et al.* 2005, Baranova *et al.* 2010).

TLRs

The biology and specificity of TLRs were reviewed in chapter 1.3.1. In terms of the functions of SAA, signalling via both TLR2 and TLR4 has been demonstrated. The interaction with TLR2 results in the activation of NF-κB and the release of TNF-α, IL-23, IL-10 and granulocyte colony-stimulating factor (G-CSF) from mouse macrophages and in the release of TNF-α, IL-10 and IL-18 from human macrophages derived from patients with sarcoidosis (He *et al.* 2009, Chen *et al.* 2010). SAA signalling through TLR4 causes an ERK1/2 and p38 MAPK-mediated induction of NO synthase, which leads to an increased production of NO (Sandri *et al.* 2008).

2.4.2 The immune-related functions of SAA

SAA possesses various cytokine-like and proinflammatory properties. The release of proinflammatory cytokines, such as IL-1α, IL-1β, IL-6, IL-8 and TNF-α, in response to either lipid-free SAA or HDL-bound SAA has been documented in different cell types. These include monocytes (Patel *et al.* 1998, Lee *et al.* 2006, Franco *et al.* 2011), macrophages (Song *et al.* 2009, Baranova *et al.* 2010, Ather *et al.* 2011), neutrophils (Furlaneto and Campa 2000, He *et al.* 2003), lymphocytes (Song *et al.* 2009), fibroblasts (Okamoto *et al.* 2008) and adipocytes (Faty *et al.* 2012). Besides cytokines, SAA induces the production of G-CSF in mouse macrophages and the release of chemokines, such as MCP-1, from monocytes, peripheral blood mononuclear cells (PBMCs), human umbilical cord endothelial cells and adipocytes (Lee *et al.* 2008, Lee *et al.* 2009, Song *et al.* 2009, He *et al.* 2009, Lee *et al.* 2010, Faty *et al.* 2012) and the release of liver activation regulated chemokine (LARC, CCL20) from synoviacytes and mononuclear cells (Sandri *et al.* 2008, Migita *et al.* 2009). In addition, SAA in its lipid-free form possesses chemoattractant properties and is able to induce the migration, adhesion and tissue filtration of inflammatory cells (Badolato *et al.* 1994, Badolato *et al.* 1995, Xu *et al.* 1995, Preciado-Patt *et al.* 1996a, Olsson *et al.* 1999, Su *et al.* 1999, Dong *et al.* 2011b). This can accelerate local inflammation and may also play a role in chronic inflammatory diseases, such as atherosclerosis (see chapter 3.2). Furthermore, SAA can contribute to inflammation-associated thrombosis by inducing the expression of tissue factor (TF) in PBMCs (Cai *et al.* 2007). Also, ROS production in neutrophils is increased in response to SAA (Björkman *et al.* 2008). During the APR, SAA enhances the expression of other APPs, such as $sPLA_2$ and pentraxin 3 (PTX3) (Sullivan *et al.* 2010, Dong *et al.* 2011a), although contradictory data do exist (Tietge *et al.* 2002). In addition, SAA participates in host defence by aiding in the clearance of microbes; SAA binds to Gram-negative bacteria via outer membrane protein A (OmpA) and can act as an opsonin for macrophages and neutrophils (Hari-Dass *et al.* 2005, Shah *et al.* 2006). Indeed, it has been suggested that the epithelial expression of SAA in the

intestine reduces the bacterial load and functions as a protective factor against colitis (Eckhardt *et al.* 2010).

Interestingly, SAA seems to participate in the modulation of anti-inflammatory activities as well. SAA induces the release of anti-inflammatory cytokines, particularly IL-10, from monocytes and PBMCs (Lee *et al.* 2006, Song *et al.* 2009) and inhibits platelet aggregation (Zimlichman *et al.* 1990). Furthermore, SAA has been implicated in the reduction of the local inflammatory response to *Acinetobacter baumannii* pneumonia in mice (Renckens *et al.* 2006). The reason for this effect is not clear, but it is speculated that preceeding high levels of SAA may down-regulate neutrophil responsiveness to secondary inflammatory stimuli (Renckens *et al.* 2006). There is also evidence that SAA binds to neutrophils via its C-terminal binding site and inhibits the respiratory burst (Linke *et al.* 1991, Preciado-Patt *et al.* 1996b). More recently, SAA has been shown to induce a microenvironment that promotes the expansion of regulatory T cells (Treg) at sites of infection or injury (Nguyen *et al.* 2011). This property also connects SAA to the regulation of the adaptive immune response. In addition, SAA has been shown to promote the development of the T_h17 type of immune response in a mouse model of asthma in an indirect way by increasing the expression of IL-1β (Ather *et al.* 2011), which has been implicated in the conversion of Treg into IL-17-producing T_h17 cells (Chung *et al.* 2009).

2.5 SAA in association with lipoproteins

Lipoproteins, the transporters of lipids in the blood, can be divided into different classes according to their density. In principle, dense particles have a high protein content, whereas less dense particles contain more triglycerides. The four classes from the most dense to the least dense are HDL, LDL, intermediate density lipoprotein (IDL), and VLDL. The ApoB-100-containing lipoproteins LDL, IDL and VLDL are considered atherogenic, while HDL and its major apolipoprotein apoA-I appear to play a protective role against atherosclerosis (Barter *et al.* 2004, Lewis and Rader 2005). This is mostly due to the involvement of HDL in reverse cholesterol transport (RCT), the process in which excess cholesterol from peripheral tissues and cells is transported to the liver for excretion (Lewis and Rader 2005). In addition, HDL particles possess antioxidant properties, which have a direct impact on LDL oxidation; apoA-I is capable of removing hydroperoxides from LDL (Navab *et al.* 2000a, Navab *et al.* 2000b), besides which HDL carries enzymes, such as paraoxonase-1, which can destroy such oxidant molecules (Navab *et al.* 2001, Mackness *et al.* 2004). HDL also reduces the accumulation of inflammatory cells by inhibiting the oxLDL-stimulated MCP-1 and the cytokine-induced expression of adhesion molecules (Barter *et al.* 2004, Calabresi *et al.* 1997).

SAA associates and circulates normally with HDL_3, utilizing its N-terminal lipid binding site (Turnell *et al.* 1986). The proinflammatory features of SAA, such as cytokine induction, have been shown to decrease in the presence of HDL or when SAA is bound to HDL (Furlaneto and Campa 2000, Baranova *et al.* 2010 Franco *et al.* 2011). The baseline level of lipid-free SAA is relatively low. Nevertheless, the distribution of SAA among different lipoprotein classes and the lipid-free fraction is not constant (Marhaug *et al.* 1982, Cabana *et al.* 2004). In human patients with stable coronary artery disease, almost one fourth of the circulating SAA is associated with LDL or VLDL, and the LDL-SAA complex has been indicated as a potent marker associated with an increased risk of a future cardiac event, its sensitivity exceeding that of SAA or CRP alone (Ogasawara *et al.* 2004). More recently, the biomarker value of the complex was also implicated in the metabolic syndrome (Kotani *et al.* 2009). Studies on several mouse models of obesity and/or atherosclerosis actually suggest that a high-fat diet may promote the redistribution of SAA to the apoB-100-containing lipoproteins (Lewis *et al.* 2004, Subramanian *et al.* 2008, King *et al.* 2010). It has also been speculated that SAA binds to non-HDL lipoproteins or remains lipid-free when the serum level of SAA exceeds the capacity of HDL to bind SAA (Cabana *et al.* 2004, Bausserman *et al.* 1987). This could indeed be the case during a strong APR when the levels of HDL decrease while significant amounts of SAA are produced also outside the liver. SAA can also bind cholesterol directly, and it has been suggested that SAA may modulate the flux of cholesterol between cells and lipoproteins during the APR (Liang and Sipe 1995, Liang *et al.* 1996).

2.5.1 Reverse cholesterol transport

In macrophages, any internalized cholesterol that is not utilized for membrane homeostasis or other cellular functions is esterified by acyl-CoA cholesterol acyl-transferase (ACAT). Cholesteryl esters (CEs) are the predominant form of intracellular storage of cholesterol, although a continuous cycle of de-esterification by neutral cholesterol ester hydrolase (nCEH) and re-esterification by ACAT does take place. Under normal conditions apoA-I constitutes 70-100% of the apolipoprotein content of HDL and it also represents the starting material for HDL synthesis and maturation. ApoA-I is secreted by the liver or the intestine as a lipid-poor apoA-I, which then obtains phospholipids and cholesterol via efflux from the liver, or from chylomicrons or VLDL in a lipoprotein lipase-mediated process. The end product is a nascent phospholipid-rich, cholesterol-poor HDL particle also known as preβ-HDL. Preβ-HDL and lipid-poor apoA-I are the initial and key acceptors of cellular cholesterol (Lewis and Rader 2005).

In the reverse cholesterol transport (RCT) pathway, the CEs stored in peripheral cells are first de-esterified by nCEH, after which the free cholesterol, accompanied by phospholipids, is transferred to extracellular lipid-poor apoA-I or

preβ-HDL. The interaction between the amphipathic helical apolipoprotein and cellular cholesterol leading to the cholesterol efflux is mediated by ATP-binding cassette transporter proteins, such as ABCA1, also known as cholesterol efflux regulatory protein, and ABCG1 (Denis *et al.* 2004, Vedhachalam *et al.* 2007). Cholesterol efflux can also proceed via SR-B1 (De la Llera-Moya *et al.* 1999). In the circulation, the acquired cholesterol in preβ-HDL is esterified by lecithin-cholesterol acyl-transferase (LCAT), generating a mature, spherical α-HDL particle with a CE-rich core (Daniels *et al.* 2009). α-HDL can be divided into two subclasses differentiated by density and size, the smaller and denser HDL_3 and the bigger HDL_2, the latter of which is formed as a result of the continuous accumulation of CEs. In the end, HDL_2 binds to SR-B1 on hepatocytes, while CEs are selectively taken up by the liver or alternatively, the whole HDL particle is internalized (Tulenko and Sumner 2002, Robichaud *et al.* 2009). The hepatic uptake can also be indirect and proceed via the CE transfer protein (CETP) that promotes the transfer of CEs to LDL, IDL, VLDL and chylomicrons in exchange for triglycerides (Stein and Stein 2005).

Reverse cholesterol transport during the APR

During the APR, the HDL particle faces many changes. Firstly, the plasma concentrations of HDL cholesterol and apoA-I decrease rapidly (Coetzee *et al.* 1986, Khovidhunkit *et al.* 2004), the reason behind which is not, however, completely understood. SAA-containing AP-HDL is cleared faster than normal HDL (Salazar *et al.* 2000), but it is unlikely that the mere presence of SAA on HDL is the cause as HDL levels seem to decrease before the increase in the concentration of SAA (Ly *et al.* 1995). Secondly, the composition and physico-chemical properties of HDL change dramatically (Pruzanski *et al.* 2000, Cabana *et al.* 1999, Abe-Dohmae *et al.* 2006, Hu *et al.* 2008). SAA becomes the major apolipoprotein on HDL, comprising up to 80% of its apolipoprotein content (Marhaug and Husby 1982, Coetzee *et al.* 1986, Husebekk *et al.* 1987). The newly-formed AP-HDL has a higher density and larger particle size than normal HDL (Clifton *et al.* 1985, Coetzee *et al.* 1986, Abe-Dohmae *et al.* 2006), although at least in mice the increase in size is not related to the presence of SAA but rather to an increase in surface phospholipids (De Beer *et al.* 2010). SAA-containing AP-HDL can form via two pathways: the displacement of apoA-I by SAA from the HDL particles or the biogenesis of HDL driven by SAA. ApoA-I displacement by SAA, as demonstrated *in vitro*, can reach a displacement rate as high as 87% (Coetzee *et al.* 1986). The *de novo* synthesis of AP-HDL seems to resemble the synthesis initiated by apoA-I; both syntheses depend on ABCA1 activity and require amphiphilic α-helical segments as the key conformation (Abe-Dohmae *et al.* 2006, Hu *et al.* 2008). However, it is still unclear how similar the SAA-HDL generated by apoA-I displacement and the SAA-HDL obtained via

biogenesis are in terms of function, if at all. Also, the proportions of these two types in plasma are currently unknown (Cabana *et al.* 1999, Abe-Dohmae *et al.* 2006).

Lipid metabolism and the properties of HDL are also affected during the APR and inflammation. Proinflammatory cytokines have been shown to inhibit LCAT in the circulation (Ly *et al.* 1995, Skretting *et al.* 1995) and also the expression of ABCA1 (Yin *et al.* 2010). Furthermore, during systemic inflammation the anti-inflammatory and antioxidant properties of HDL may be overcome by an excess of accumulated oxidants. In that case, HDL can turn into 'proinflammatory HDL', HDL particles with limited capabilities to promote RCT and to inhibit the oxidation of LDL (Navab *et al.* 2006, McMahon *et al.* 2006, Skaggs *et al.* 2012). The impairment of RCT in response to inflammation has indeed been demonstrated *in vivo* in mice and in humans (McGillicuddy *et al.* 2009, Malik *et al.* 2011, De la Llera Moya *et al.* 2012). Furthermore, a recent study reported an inverse correlation between SAA enrichment on HDL and the anti-inflammatory capacities of HDL (Tölle *et al.* 2012). This is supported by earlier findings suggesting that SAA promotes the selective CE uptake by peripheral cells (Artl *et al.* 2000) and the reduction of cholesterol efflux and RCT (Banka *et al.* 1995, Artl *et al.* 2000, Cai *et al.* 2005, Annema *et al.* 2010), under the condition that SAA consititutes more than 50% of the HDL protein (Banka *et al.* 1995). However, there is a fair amount of controversy regarding the impact of SAA on lipid metabolism. It has been shown that the presence of SAA on HDL reduces the affinity of HDL for normal hepatocytes and increases that for macrophages, PBMCs and ECs (Kisilevsky and Subrahmanyan 1992, Hayat and Raynes 2000). Accordingly, the number of AP-HDL binding sites increases on macrophages and decreases on hepatocytes (Kisilevsky and Subrahmanyan 1992), suggesting that the net effect is a shift in HDL cholesterol carrying capacity towards the macrophage. Later on, the presence of SAA in AP-HDL was shown to enhance the mobilization and efflux of intracellular cholesterol both in tissue cultures (Tam *et al.* 2002, Stonik *et al.* 2004, van der Westhuyzen *et al.* 2005) and *in vivo* (Tam *et al.* 2002, Kisilevsky and Tam 2003). Furthermore, mouse Saa2.1 can modulate the intracellular cholesterol balance so that it shifts from CEs to free cholesterol. This is mediated via two specific regions identified on Saa2.1; the N-terminal region acts as an inhibitor for ACAT and the C-terminus as an enhancer for nCEH. It has been demonstrated that liposomal formulations containing either the whole Saa2.1 protein or peptides spanning the above-mentioned activities enhance RCT from cholesterol-laden macrophages (Tam *et al.* 2002, Kisilevsky and Tam 2003). Importantly, these peptides have been shown to reverse the development of atherosclerotic lesions in hyperlipidemic mice (Tam *et al.* 2005). Also, HS, bound by SAA, has been implicated in AP-HDL remodeling that results in increased cholesterol efflux from macrophages (Tam *et al.* 2008). In summary, Kisilevsky *et*

al. have proposed that the role of SAA during acute inflammation may in fact be to recycle cholesterol from damaged cells and tissues; SAA would first target the HDL-SAA complex to macrophages, detach from HDL and enter the cell, inside which it would promote the efflux of cholesterol to HDL by regulating the activities of ACAT and nCEH (Kisilevsky and Manley 2012).

3. SAA in pathological conditions

The involvement of SAA has been established in several pathologies. The prime example is AA amyloidosis, a systemic protein misfolding disease that develops secondary to a prolonged inflammatory condition, most commonly RA (Hazenberg *et al.* 2004). In the pathogenesis of RA, SAA has been shown to induce the expression of adhesion molecules, angiogenesis and matrix degradation via the activation of NF-κB (Mullan *et al.* 2006). Another example is the group of cardiovascular diseases, in which SAA can potentially function as an independent risk predictor (Jousilahti *et al.* 2001, Johnson *et al.* 2004) or as an active mediator in their pathogenesis (Lewis *et al.* 2004, Dong *et al.* 2011b). SAA has also been implicated in many conditions associated with cardiovascular diseases, or their risk, such as obesity, insulin resistance, type 2 diabetes and the metabolic syndrome (Faty *et al.* 2012, Zhao *et al.* 2010, Yang *et al.* 2006, Herder *et al.* 2006, Karlsson *et al.* 2004, Kotani *et al.* 2009). Furthermore, it has been proposed that SAA may function as a direct link between obesity and atherosclerosis (Yang *et al.* 2006). The role of SAA in AA amyloidosis and atherosclerosis will be discussed below.

Growing evidence also indicates a connection between SAA and cancer. There is a close association between malignant transformations and chronic inflammation, particularly via the synthesis and secretion of proinflammatory cytokines; tumour promotion and progression involve several signal transduction pathways that are activated by proinflammatory cytokines (Malle *et al.* 2009). The expression of SAA has been detected in cancer cell lines and also in different types of tumours (Thorn *et al.* 2003, Gutfeld *et al.* 2006, Kovacevic *et al.* 2006, Kovacevic *et al.* 2008, Urieli-Shoval *et al.* 2010, Cocco *et al.* 2010). Elevated SAA levels correlate with tumour grading (Weinstein *et al.* 1984, Liu *et al.* 2007) and many studies indicate SAA as a potential serum biomarker in the monitoring of the recurrence or status of the disease or treatment response (Howard *et al.* 2003, Cocco *et al.* 2009, Fischer *et al.* 2012). Several proinflammatory properties of SAA, such as the ability to induce cell adhesion/migration and the production of matrix metalloproteinases (MMPs), are also compatible with the mechanisms related to tumour invasion and metastasis and suggest that SAA could be involved in tumour pathogenesis (Gutfeld *et al.* 2006).

3.1 AA amyloidosis

The German pathologist Rudolph Virchow, who conducted his studies in the 1850s, is widely considered to have discovered amyloid and invented the name, although a number of other scientists were most likely also involved (Aterman 1976). Based on

its staining features, the new substance was first thought to be cellulose and hence it was named amyloid after the Greek word ‘amylon’, which means starch or cellulose.

Amyloidosis is a clinical disorder caused by the extracellular deposition of insoluble fibrils in tissues and/or organs. These fibrils originate from aggregations of misfolded proteins that are normally soluble. Amyloid fibril formation in humans has been demonstrated for 27 different proteins and 9 of them have also been studied in animals (Sipe *et al.* 2010). Local amyloidoses, such as certain forms of AL (amyloid light chain) amyloidosis or Alzheimer’s disease, affect only one organ or tissue, whereas in systemic amyloidoses, such as AA amyloidosis or AGel amyloidosis (Finnish-type amyloidosis), the deposits can be found in any internal organ, or in all of them, as well as in connective tissue and blood vessel walls. Amyloidoses can be further divided into hereditary and acquired forms.

3.1.1 The prevalence and clinical characteristics of AA amyloidosis

AA amyloidosis or secondary amyloidosis is the most common form of systemic amyloidosis (Buxbaum 1996), except in the US where the frequency of AL amyloidosis is higher. AA amyloidosis occurs secondary to chronic inflammation, the origin of which can be infectious or non-infectious, or to other conditions, including hereditary periodic fever or neoplasms, such as Hodgkin’s disease and renal cell carcinoma. The disease is characterized by the systemic deposition of extracellular SAA-derived fibrils in tissues and organs. The spleen and the liver are the first organs to be affected but even a remarkable amyloid load in these organs can remain asymptomatic (Hazenberg and van Rijswijk 1994, Hawkins 2002). Next, the deposits start to appear in the kidneys, the adrenal gland, the liver, the gastrointestinal tract, the intestine, the peripheral nervous system, the skin and the respiratory system (Lachmann *et al.* 2007). The only exception is the brain, which remains mainly unaffected, although cerebrovascular involvement has been reported (Schroder and Linke 1999). Cardiac involvement is also rare (Lachmann *et al.* 2007). The renal deposition of AA fibrils causes proteinuria in up to 95% of cases (Hazenberg and van Rijswijk 1994, Dember 2006, Ferrario and Rastaldi 2006a, Ferrario and Rastaldi 2006b, Pinney and Hawkins 2012), and the diagnosis is typically made after the appearance of the renal symptoms. From the viewpoint of prognosis, this is often too late as around 10% of patients have already reached end-stage renal failure at the time of diagnosis (Pinney and Hawkins 2012). If the inflammatory conditions persist, renal dysfunction and renal failure, requiring dialysis and renal transplantation, will follow.

The absolute prevalence of AA amyloidosis is difficult to confirm as the disease occurs secondary to other diseases and as there is also great variation among individuals regarding the extent of amyloid deposition and the severity of the disease. In some patients AA amyloidosis can remain asymptomatic and thus

also undiagnosed unless an autopsy is performed. Recent studies have estimated that only 25-50% of the patients with histological findings of amyloid have clinically apparent amyloidosis (Wakhlu *et al.* 2003, Sanmarti *et al.* 2004, Koivuniemi *et al.* 2008). An early autopsy study conducted in the Netherlands estimated the total prevalence of AA amyloidosis to be as low as 1 in 100 000 (Janssen *et al.* 1986). Later on, estimations have mainly been based on the prevalence of the primary diseases and the portion of these patients also affected by AA amyloidosis. It should also be noted that patients with the same primary disease can be affected at different rates. The most common underlying disease for AA amyloidosis in industrialized countries is RA, which is proposed to be the primary disease in 56% of cases of AA amyloidosis (Hazenberg and van Rijswijk 1994). On the other hand, the prevalence of AA amyloidosis in RA varies greatly depending on the country, and estimates between 3 and 30% have been suggested (Kobayashi *et al.* 1996, Gomez-Casanovas *et al.* 2001, El Mansoury *et al.* 2002, Wakhlu *et al.* 2003, Koivuniemi *et al.* 2008). Generally, environmental factors, such as food and lifestyle, as well as drug treatment, the type of the primary disease and also genetic factors most likely play a role in the pathogenesis of AA amyloidosis and might explain the differences.

When the amyloid deposition develops into renal amyloidosis, the prognosis for both the patient and renal survival becomes poor. In one study the renal survival rate after 5 years was 30% and the patient survival rate after 10 years 20% (Sasatomi *et al.* 2007). In another study, which followed the clinical outcome of patients with RA and AA amyloidosis receiving hemodialysis, 50% of the patients died within less than a year (Kuroda *et al.* 2006). The typical causes of death for patients with RA and renal AA amyloidosis are infections, renal failure and cardiovascular disease (David *et al.* 1993, Sasatomi *et al.* 2007).

3.1.2 The pathological and physical characteristics of AA amyloidosis

Amyloidosis is caused by the misfolding of proteins into β-sheet aggregated structures. This conformation is stabilized by intermolecular interactions, leading to the formation of different amyloid species, such as oligomers, protofibrils and fibrils, which in the end accumulate as amyloid deposits in affected tissues. Amyloid is primarily recognized by staining with a dye called Congo Red, and Congo Red positive amyloid exhibits a distinctive apple-green birefringence when examined under polarized light. In addition, amyloid deposits from diverse origins share a strikingly similar fibrillar ultrastructure. On electron microscopy, individual amyloid fibrils are typically straight, unbranched filament bundles, which have a diameter of 10 nm and a length of several micrometers. On X-ray diffraction analysis, they show a typical cross-β structure with their β-sheets organized parallel and their β-strands arranged perpendicular to the fibre long

axis (Pauling and Corey 1951, Sunde *et al.* 1997). The mature amyloid fibrils in AA amyloidosis also contain a serum amyloid P (SAP) component and glycosaminoglycans (GAGs), such as chondroitin sulfate, dermatan sulfate, HS and heparin (Magnus *et al.* 1994, Inoue *et al.* 1998, Inoue and Kisilevsky 1999), as presented in Figure 5. The mature fibril is fairly resistant to proteolysis (Tennent *et al.* 1995).

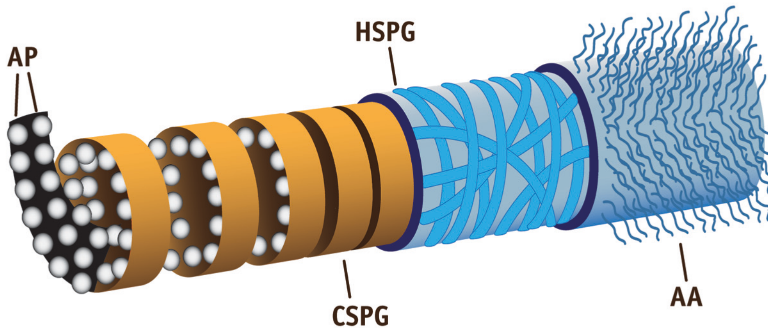

Figure 5. A schematic drawing of an AA amyloid fibril. Modified from Inoue and Kisilevsky 1999. AA, amyloid A; AP, amyloid P; CSPG, chondroitin sulfate proteoglycan; HSPG, heparan sulfate proteoglycan.

Amyloid fibril formation follows a nucleated growth mechanism, which is a two-step reaction consisting of a slow nucleation step, the so-called lag phase, and of a subsequent rapid exponential growth phase (Naiki and Nakakuki 1996, Serio *et al.* 2000). It is assumed that the lag phase is the time required for the formation of the “nucleus” or “seed”. After this, monomers or oligomers associate with the nucleus, which leads to rapid fibril growth (Chiti and Dobson 2006). The nucleation step seems to play a key role in determining the kinetics of the fibril formation and it can be shortened by several substances, including preformed fibrils and amyloid-enhancing factor (AEF, see below), or by introducing mutations (Uversky and Fink 2002). The different amyloid species are illustrated in Figure 6.

The proposed nucleated growth mechanism is crucial in light of what is currently understood about the toxicity and function of amyloid preforms. Although it is clear that the physical disruption in the tissue architecture caused by the amyloid deposits can be detrimental (Pepys 2001), more recent data indicate amyloidogenic precursors such as soluble oligomers, protofibrils and other folding aggregates as the most potent mediators of cell damage and cell toxicity. This “corrected” amyloid hypothesis shows an analogue to prion proteins; it has been verified that the oligomers and small aggregates, rather than fibrils, are the prion species most damaging to neurons (Fontaine and Brown 2009). However, the

mechanism by which these pre-fibrillar species cause cell toxicity is not completely understood. The channel formation capability of the protofibrils may play a role in their effects (Kagan and Thundimadathil 2010). This is outlined in the amyloid pore hypothesis, which suggests that protofibrils and soluble oligomers target cell membranes (Lashuel *et al.* 2002, Hirakura *et al.* 2002) and that the membrane permeabilization by these species is a common component of the toxicity (Lashuel

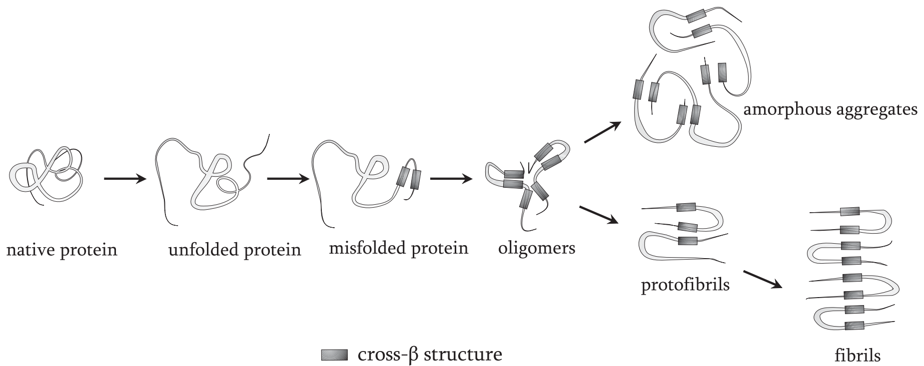

Figure 6. Protein misfolding and aggregation. Printed from Herczenik & Gebbink 2008 with permission.

2005). Toxicity can also be at least to some extent due to the misfolded state of the protofibril, which can enable abnormal interactions and interrupt larger cellular systems, causing oxidative stress or sequestration of some essential proteins, which could then lead to apoptosis or other forms of cell death (Chiti and Dobson 2006). Lastly, misfolded proteins and aggregates can trigger inflammation (Masters and O'Neill 2011). Three types of amyloidogenic or misfolded proteins have been suggested to activate the NLRP3 inflammasome, leading to the secretion of potentially cytotoxic IL-1β: amyloid-β fibrils in microglia in Alzheimer's disease, soluble islet amyloid polypeptide oligomers in macrophages in type 2 diabetes and the mutated, misfolded superoxide dismutase 1 in microglia in amyotrophic lateral sclerosis (Halle *et al.* 2008, Masters *et al.* 2010, Meissner *et al.* 2010). Also, it has been proposed that the lipid composition of the cell membrane can modulate the toxicity of oligomers (Evangelisti 2012).

3.1.3 Factors influencing AA fibril formation

SAA is the precursor protein for the AA protein present in the amyloid deposits in AA amyloidosis. Two steps are required for the transformation from SAA to AA: C-terminal cleavage and the adoption of β-sheet configuration. The exact mechanism and order of the events leading to the transformation are still not fully

understood. The main amyloid A component found in the amyloid fibrils corresponds to the first 76 amino acids of the full-length SAA protein, but the stretches can also be shorter or longer (21-89 aa) or sometimes span the whole protein (Levin *et al.* 1973, Westermark 1982, Westermark *et al.* 1987, Prelli *et al.* 1991, Kisilevsky and Young 1994). The N-terminus usually remains intact or lacks at most one or two aa (Röcken and Shakespeare 2002). It is not completely clear whether SAA dissociates from HDL before or after the cleavage or whether SAA is first cleaved and then deposited as fibrils. However, the proven presence of full-length SAA in the deposits and also the fact that degradation of SAA *in vivo* is hindered in the presence of HDL (Bausserman and Herbert 1984), suggest that the dissociation from HDL takes place first. Furthermore, as most full-length SAA isoforms can form fibrils *in vitro*, it may be that SAA first transforms into fibrils and is cleaved after that (Wang *et al.* 2002). In addition to the experimental and transgenic mouse models of AA amyloidosis (Kindy and De Beer 1999, Solomon *et al.* 1999), AA amyloidosis has been induced in several cell types including mouse and human peritoneal cells, human peripheral blood monocytes and J774 macrophages (Kluve-Beckerman *et al.* 1999, Kisilevsky *et al.* 2004, Magy *et al.* 2007).

The 10-15 aa at the N-terminus of SAA have been identified as the major determinant of amyloid formation. Either the replacement of a single amino acid (Gly8->Asp8) or the deletion of the whole stretch diminishes or inhibits amyloid fibril formation *in vitro* (Westermark *et al.* 1992, Patel *et al.* 1996). Consistent with this, the constitutively expressed and non-amyloid-related C-SAA as well as mouse Saa isotypes Saa2.1 and Saa2.2 lack glycine at the corresponding position (Watson *et al.* 1992). In mice, only Saa1.1 can be found in amyloid deposits (Yamamoto and Migita 1985; note: Saa1.1 was called Saa2 at the time of this publication), while Saa2.1 is principally absent. Saa2.2, on the other hand, is the sole Saa isoform expressed in the amyloid-resistant CE/J mouse (De Beer *et al.* 1993, Sipe *et al.* 1993). It is not clear, though, whether the absence of Saa2.2 in the deposits is due to the inability to form fibrils per se or to some other factors (Wang *et al.* 2002, Patke *et al.* 2012). The central region of SAA may also play a role in amyloid formation since SAA1 and its isoforms are predominantly found in AA deposits even though the N-termini of human SAA1 and SAA2 are identical (Liepnieks *et al.* 1995).

The involvement of macrophages

According to the current understanding, the impaired proteolytic digestion of SAA may be the major contributor to the development of AA amyloidosis. Macrophages that promptly internalize SAA have been implicated in the process (Magy *et al.* 2007, Kluve-Beckerman *et al.* 2001, Kluve-Beckerman *et al.* 2002). Under normal conditions SAA is directed to the endosomal-lysosomal pathway and degraded. However, as studies conducted in the mouse macrophage cell culture

model of AA amyloidosis suggest, in the presence of consistently elevated SAA concentrations the degradation is incomplete and SAA intermediates can aggregate into fibrils. Amyloid is detected first in intracellular vesicles and later also extracellularly. Changes in lysomal morphology can be seen, which implies that the exocytosis is mediated by lysosome-derived vesicles (Kluve-Beckerman *et al.* 2001, Kluve-Beckerman *et al.* 2002, Magy *et al.* 2007). SAA has also been shown to colocalize with lysosomal cysteine proteases, called cathepsins, in the intracellular milieu (Röcken *et al.* 2006). Indeed, several macrophage or monocyte-derived enzymes have been reported to degrade SAA *in vitro*. These include serum serine proteases such as thrombin, kallikrein and plasmin; metalloproteinases such as collagenase, stromelysin and elastase (Uhlar and Whitehead 1999); and several cathepsins (B, D, G, K and L) (Yamada *et al.* 1995a, Yamada *et al.* 1995b, Elliott-Bryant *et al.* 1998, Röcken *et al.* 2005, Röcken *et al.* 2006). Cathepsin B can produce the 76-aa AA protein most commonly found in AA deposits (Yamada *et al.* 1995b) as well as a significant portion of other AA proteins reported to date (Röcken *et al.* 2005). However, since the inhibition, or knock-out, of cathepsin B does not inhibit the amyloid formation in human cell cultures or *in vivo* in mice (Röcken *et al.* 2006, van der Hilst *et al.* 2009), it may be that cathepsin B only mediates AA peptide formation without affecting the actual amyloid load. Cathepsin L, on the other hand, has been identified as a potential amyloid-promoting protease. Furthermore, it has been suggested to function as a proteolytic activator of SAA as it releases the C-terminal region of SAA implicated in the nCEH enhancement (Röcken *et al.* 2006, Kisilevsky and Tam 2003, see also chapter 2.5.1). Elastase and cathepsins D and K digest SAA mainly in its amyloidogenic N-terminus and do not form AA proteins (Westermark *et al.* 1992, Yamada *et al.* 1995a, Yamada *et al.* 1995b, Patel *et al.* 1996, Röcken *et al.* 2005, Röcken *et al.* 2006, van der Hilst *et al.* 2009). Thus, they are likely to have a protective or retarding role in amyloidogenesis. Indeed, inhibiting cathepsin D in the mouse model of amyloidosis has been shown to significantly increase the amyloid deposition (van der Hilst 2011). MMPs 1-3 are capable of degrading both SAA and AA fibrils (Stix *et al.* 2001). The MMP digestion of SAA leaves the N-terminus intact and although AA proteins corresponding to those generated by MMPs have not yet been detected in amyloid deposits (Röcken *et al.* 2005), MMPs are thought to contribute to the pathogenesis of AA amyloidosis. MMPs are present in AA amyloid deposits and SAA1 itself can induce their expression in mononuclear phagocytes and synovial fibroblasts (Migita *et al.* 1998, O'Hara *et al.* 2004, Lee *et al.* 2005). Furthermore, there are differences among SAA isoforms in their susceptibility to proteolysis by MMPs (see below).

As for receptor-linked factors, RAGE has been implicated in amyloid accumulation in tissues. The expression of RAGE is increased in AA amyloidosis,

besides which RAGE mediates the NF-κB activation in response to AA fibrils and mouse Saa1.1 in cells originating from monocytes (Yan *et al.* 2000). Importantly, in the mouse model of AA amyloidosis the blockage of RAGE decreases cellular stress and splenic accumulation of amyloid (Yan *et al.* 2000). The activation of inflammatory cells by amyloid fibrils can also represent a general pathway in which induced proinflammatory mediators function as a source of the toxic effects observed in amyloidosis.

Genetic factors

Polymorphisms in the *SAA1* genes have been identified as risk factors for AA amyloidosis. Both SAA1 and SAA2 can be found in amyloid deposits, but SAA1 clearly predominates (Liepnieks *et al.* 1995, Yamada *et al.* 1999). Two single nucleotide polymorphisms (SNPs) at exon 3 create three different isotypes of *SAA1: SAA 1.1* (Val52-Ala57), *SAA 1.3* (Ala52-Ala57) and *SAA 1.5* (Ala52-Val57) (Moriguchi *et al.* 2001). The frequencies of these genotypes vary remarkably among different countries and/or races, and both *SAA1.1* and *SAA1.3* have been found to increase the risk for AA amyloidosis. Caucasians have a 76% frequency for *SAA1.1* (Moriguchi *et al.* 1999), and Caucasian patients with FMF and the *SAA1.1/1.1* genotype carry a 3- to 7-fold risk for AA amyloidosis (Cazeneuve *et al.* 2000, Gershoni-Baruch *et al.* 2003, Altiok *et al.* 2003, Yilmaz *et al.* 2003). In contrast, in the Japanese population the combination of RA and the *SAA1.3* allele (frequency 40%), but not *SAA1.1*, constitutes a risk factor of amyloidosis (Moriguchi *et al.* 1999, Baba *et al.* 1995). RA patients with the 1.3/1.3 genotype have been estimated to have a risk 8 times higher than the control group, and they also seem to develop AA amyloidosis sooner after the onset of RA (Moriguchi *et al.* 2001). In addition, *SAA1.4*, another *SAA1* isoform, has been proposed to be linked to the uniquely high prevalence of AA amyloidosis in some areas of Papua New Guinea (Westermark *et al.* 1996, Westermark and Westermark 2008). However, the differences among the various alleles and/or ethnic groups and their vulnerabilities to AA amyloidosis are still mainly unexplained. Differences in protein secondary structure are hardly the cause considering that SAA1.1 and SAA1.3, for example, diverge in one single amino acid only (Val52-Ala52). Indeed, SNP -13T/C, which is located in the 5'-flanking region of *SAA1* and associated with increased transcriptional activity, has been suggested to serve as a link among the different alleles; the *SAA1* -13T allele is tightly associated with *SAA1.1* in Caucasians and with *SAA1.3* in the Japanese population (Moriguchi *et al.* 2001, Moriguchi *et al.* 2005), thus indicating -13T positivity as a connecting factor. Another explanation for the differing vulnerabilities to AA amyloidosis arises from the studies in which SAA1.1 in comparison with SAA1.5 has been shown to be more susceptible to proteolysis by MMP-1 (van der Hilst *et al.* 2008).

Transmission and amyloid-enhancing factors in AA amyloidosis

Amyloid-enhancing factor (AEF) can be described as transferable amyloidogenic activity present in the splenic extracts prepared from amyloidogenic mice (Werdelin and Ranlov 1966, Axelrad *et al.* 1982). AEF has been shown to contain small AA fibrils. When AEF is administered to mice together with an inflammation-stimulating agent, it functions as a seed for fibril formation, and the lag phase of amyloidoisis can be dramatically shortened from one month to one week (Werdelin and Ranlov 1966, Lundmark *et al.* 2002). Similarly, transferring peripheral blood monocytes from amyloidotic mice into healthy ones induces extensive amyloidosis in the recipients (Sponarova *et al.* 2008). AEF speeds up the amyloid deposition also in the cell culture models of AA amyloidosis (Kluve-Beckerman *et al.* 2001, Magy *et al.* 2007). Premade AA fibrils and, interestingly, also synthetic amyloid-like fibrils and other amyloid proteins have been shown to function in a similar way (Brissette *et al.* 1989, Ganowiak *et al.* 1994, Johan *et al.* 1998, Larsson *et al.* 2011), indicating that cross-seeding is possible. Furthermore, even non-pathogenic fibrillar proteins, such as *Escherichia coli* curlin, silk fibers and yeast Sup35p, are capable of triggering AA amyloid formation in the mouse model of AA amyloidosis (Lundmark *et al.* 2005). All these findings might explain how environmental or dietary factors (inhaled or ingested substances, for example) contribute to fibril formation. Indeed, AA amyloidosis, at least in mice, is considered a transmissible disease that shows clear analogy to prions. In prion diseases the normal prion protein PrP^{C} is transformed into an abnormal, "infectous" PrP^{Sc}, which in turn starts to induce a similar transformation in other PrP^{C} proteins (Prusiner 1982). Prions are transmitted by different routes. As for the experiments on the mouse models of AA amyloidosis, AEF or AA fibrils are normally administered intravenously or intraperitoneally. However, oral transmission via drinking water as well as goose-derived foie gras, has also been reported (Lundmark *et al.* 2002, Solomon *et al.* 2007).

Another group of potent amyloid-enhancing factors consists of HS and its derivative heparin, which are found in mature amyloid fibrils, in the ECM and on cell surfaces (Bellotti and Chiti 2008, Motamedi-Shad *et al.* 2012). HS has been shown to bind Saa1.1 *in vitro* and to accelerate AA fibril formation by increasing the β-sheet content of Saa (McCubbin *et al.* 1988). In terms of the mechanism, it has been shown that GAGs have a higher affinity for oligomeric amyloidogenic proteins than for their monomeric forms. Binding increases the density of negative charges on the GAG surface, which in turn promotes protein aggregation (Castillo *et al.* 1998, Suk *et al.* 2006). Besides SAA and HS, a similar effect has also been reported for the interaction between HS/heparin and several other amyloidogenic proteins (McLaurin *et al.* 1999, McLaughlin *et al.* 2006, Suk *et al.* 2006).

3.2 SAA and atherosclerosis

SAA has been implicated in several stages of atherosclerosis (King *et al.* 2011). The plasma levels of SAA, and also of the SAA-LDL complex, correlate with the risk of cardiovascular disease (Jousilahti *et al.* 2001, Johnson *et al.* 2004, Ogasawara *et al.* 2004). Furthermore, in hyperlipidemic mice, the SAA concentration correlates directly with the size of atherosclerotic lesions, independent of the cholesterol concentration in plasma (Lewis *et al.* 2004, Dong *et al.* 2011b). SAA protein can be found in atherosclerotic lesions, resulting from infiltration or local expression by ECs, foam cells, SMCs, adventitial macrophages and adipocytes (Meek *et al.* 1994). In the lesions, SAA colocalizes with apo B, apoA-I and proteoglycans (Lewis *et al.* 2004, Yamada *et al.* 1996, O'Brien *et al.* 2005). SAA can accelerate inflammation in the arterial wall by attracting inflammatory cells to the site and by inducing the expression of cytokines, chemokines and other mediators. In addition, SAA might contribute to lipid accumulation by promoting both lipoprotein retention and internalization as well as to lesion destabilization by inducing the expression of ECM degrading enzymes. These features are discussed in more detail in the next three sections and summarized in Figure 7.

3.2.1 The clinical and pathological characteristics of atherosclerosis

Atherosclerosis is the most common form of cardiovascular disease (CVD). With its variable clinical manifestations, CVD is the principal cause of death in the US, Europe and most of Asia, killing nearly 20 million people in the world annually. In Europe, CVD accounts for over 4.3 million deaths per year and represents 48% of all deaths (Lopez *et al.* 2006). Despite the available drug therapies, such as statin treatment (Baigent *et al.* 2005), 70% of the clinical events cannot be prevented, and 10% of them occur in seemingly healthy individuals (Greenland *et al.* 2003).

Atherosclerosis is a chronic inflammatory disease of the intima, the innermost layer of the arterial wall. It can be characterized by a progressive accumulation of both cholesterol and inflammatory cells – macrophages, T cells and mast cells – within the intima and by the subsequent formation of atherosclerotic lesions. The lesions mainly affect large and medium-sized muscular arteries (Fuster *et al.* 2005). The development of the disease begins already in the first decade of life with the formation of so-called fatty streaks, the earliest type of lesions (Stary *et al.* 1994, Napoli *et al.* 1997). The fatty streaks are composed of macrophage-derived foam cells (see below) and T cells, and they generally contain very little extracellular lipid. At this stage, the disease is asymptomatic and will not become clinically overt for many years. If a consistent accumulation of lipids continues, it will lead to the thickening of the intima and the formation of a lesion. An advanced atherosclerotic lesion is composed of modified lipids, mast cells, foam cells and a lipid-rich necrotic core that

is covered by a fibrous cap. The fibrous cap is a rigid structure consisting of inflamed SMCs, which have migrated from the media (the middle layer of the arterial wall), and of components of the ECM, such as collagen and proteoglycans. Depending on the vulnerability of the lesion, which is principally determined by the thickness of the fibrous cap, the complications of atherosclerosis range from hindered blood flow to acute occlusion, which is caused by a lesion rupture and/or thrombus, and can lead to myocardial infarction or stroke (Lusis 2000). Besides the architecture of the plaque, ECM degradation by macrophage- or mast cell-derived proteases as well as calcification play a role in lesion destabilization.

3.2.2 Lipid accumulation in atherosclerosis

In advanced atherosclerotic lesions, lipids can be found in two forms: either as intracellular lipid droplets inside foam cells or as extracellular lipid deposits that form the lipid core of the lesion.

Lipoprotein retention

The extracellular lipid in the lesions is generated via the retention of lipoproteins from the circulation or via lipid release from dying foam cells, and it is mainly stored as lipid droplets (Kruth 1997), besides which the crystal-form cholesterol may also be present (Abela *et al.* 2009, Duewell *et al.* 2010). The retention is initiated by the filtration of lipoproteins through a layer of ECs into the intima (Steinberg *et al.* 1985). This occurs via transcytosis, which is a non-selective endocytic pathway utilizing pinocytic vesicles, or by passing between the ECs (Kao *et al.* 1995). Once in the intima, the lipoproteins are bound and trapped by the proteoglycans of the ECM that exhibit high specificity towards the apoB and apoE fractions of the lipoproteins (Hurt-Camejo *et al.* 1990). Then, the trapped lipoproteins, principally LDL, are subjected to various modifications, such as proteolytic or lipolytic processing or oxidation (Öörni *et al.* 2000). The members of the $sPLA_2$ family, which exhibit variable lipolytic activities, contribute to the modification and retention of LDL in particular (Jonsson-Rylander *et al.* 2008, Öörni and Kovanen 2009). The modified LDL particles are recognized and can be taken up by the local inflammatory cells; furthermore, modified LDL particles become prone to aggregation and fusion, contributing to the formation of the lipid core (Pentikäinen *et al.* 2000, Hakala *et al.* 2001, Öörni *et al.* 2005). This cascade, outlined in the response-to-retention hypothesis (Williams and Tabas 1995, Williams and Tabas 1998), indicates the proteoglycan-mediated lipoprotein retention as one of the initial stages of atherosclerosis.

Nearly all lipoproteins that are small enough (less than 70 nm in diameter) to be transported inside the pinocytic vesicles can enter the intima via the same transcytotic pathway as LDL (Kruth 2001). Due to the lack of apoB and low amounts

of apoE, however, HDL particles are not retained in the ECM as efficiently. Instead, they move through the intima, acquire cholesterol from foam cells, and enter the lymphatic system within the media (Nanjee *et al.* 2001). In the intima, HDL particles can also inhibit the oxidation of the proteoglycan-bound LDL (Barter *et al.* 2004). However, apoA-I can be found in atherosclerotic lesions, colocalized with apoB and proteoglycans in both humans and mice (O'Brien *et al.* 1998, Kunjathoor *et al.* 2002, O'Brien *et al.* 2005), indicating retention. SAA carries binding sites for proteoglycans (Ancsin and Kisilevsky 1999, Elimova *et al.* 2009) and could, thus, promote the retention of AP-HDL. Indeed, the role of SAA as a mediator of the HDL-proteoglycan interaction is supported by a number of studies (O'Brien *et al.* 2005, Wilson *et al.* 2008, Chiba *et al.* 2011). Similarly, the presence of SAA on LDL (Ogasawara *et al.* 2004) can promote LDL retention by increasing the number of proteoglycan binding sites on the particle. SAA also induces the synthesis of proteoglycans in SMCs, biglycan in particular, via the induction of endogenous TGF-β (Little *et al.* 2002, Wilson *et al.* 2008). Although all proteoglycans can bind LDL and many of them have been implicated in atherogenesis, biglycan is the proteoglycan most often found to be colocalized with apoB (O'Brien *et al.* 1998, Nakashima *et al.* 2007). In addition, SAA can modulate the expression and release of $sPLA_2$-IIA from SMCs and also the enzyme activity of $sPLA_2$ (Pruzanski *et al.* 1995, Sullivan *et al.* 2010).

Pathways for LDL internalization and foam cell formation

The filtration of inflammatory cells into the intima is another key event in the initiation of atherosclerosis. Once inside the intima, monocytes differentiate into tissue macrophages in response to the local cytokine environment and start to express scavenger receptors that enable them to internalize modified lipoproteins. The expression of TLRs also changes; an increased expression of TLR1, TLR2 and TLR4 on macrophages and ECs can be detected in atherosclerotic intima compared with normal intima (Xu *et al.* 2001, Edfeldt *et al.* 2002).

Macrophages acquire intracellular lipid via two kinds of pathways: receptor-mediated and receptor-independent pathways. The uptake of native LDL proceeds mainly via the LDL-receptor (LDLR). However, due to the downregulation of LDLR in the presence of excess cholesterol (the LDL concentration in the intima can be two times higher than in the circulation), differentiated macrophages in the intima express LDLR very poorly (Hoff *et al.* 1978, Brown and Goldstein 1986). Oxidized and acetylated LDL particles, on the other hand, are internalized via scavenger receptors (SRs), the expression of which is not regulated by the cholesterol concentration (Goldstein *et al.* 1979, Hoff *et al.* 1990, Steinberg 1997, Schrijvers *et al.* 2007). For this reason, modified LDL has for long been considered the main source of the lipid in foam cells (Kruth 2001), and the scavenger receptor class AI/

II (SR-AI/II) and CD36 (from the SR-B family) have been indicated as the principal receptors in foam cell formation (Suzuki *et al.* 1997, Kunjathoor *et al.* 2002, Rahaman *et al.* 2006). Recent data, however, underscore the role of receptor-independent and non-selective pathways, such as fluid-phase pinocytosis, in lipid accumulation and suggest that native LDL may similarly serve as source material for foam cells (Kruth 2011).

Medial SMCs that have migrated into the intima can also internalize lipids and become foam cells (Llorente-Cortes *et al.* 1998). As in macrophages, the expression of LDLR is downregulated by excess LDL, and, thus, the contribution of native LDL to foam cell formation is minimal. Instead, lipids in SMC foam cells are mainly derived from aggregated LDL that is taken up by LDLR-related protein 1 (Llorente-Cortes *et al.* 2000). Curiously, SAA has been shown to be chemotactic for aortic SMCs (Kumon *et al.* 2002b) and also to increase the uptake of cholesterol into these cells *in vitro* (Liang *et al.* 1996), suggesting a direct role for SAA in foam cell formation.

Under normal conditions the internalized lipoproteins are degraded by lysosomal enzymes, such as cathepsins and lipases, inside the lysosomes. OxLDL, though, is partly resistant to lysosomal enzymes (Jessup and Kritharides 2000) and can also reduce their activity (Hoppe *et al.* 1994, O'Neil *et al.* 2003). Subsequently the protein fractions are secreted out of the cell, and the cholesterol, if not directed to cellular membranes, is re-esterified by ACAT and stored in cytosolic lipid droplets (Brown and Goldstein 1986), as mentioned in chapter 2.5.1. If the uptake of large amounts of lipoproteins continues, exceeding the limits the RCT pathway, macrophages turn into foam cells; the lipid droplets can occupy most of the cytoplasm, and the cells have a foamy appearance (Pasquinelli *et al.* 1989). Foam cells eventually die by apoptosis (Akishima *et al.* 2005), contributing to the formation of the lipid core.

3.2.3 Inflammation and SAA in atherosclerosis

Despite the wide acceptance of the response-to-retention hypothesis (Williams and Tabas 1995, Williams and Tabas 1998) and the causative role of cholesterol in atherosclerosis, many factors also challenge this paradigm. One half of all heart attacks and strokes occur among individuals without hypercholesterolemia, and extensive experimental evidence suggests that inflammation is actually a key contributor to all stages of the disease (Libby *et al.* 2009, Libby 2012). The inflammatory aspect of atherosclerosis has gained more and more attention during the last 10 years, initiated by the landmark article "Atherosclerosis – An Inflammatory Disease" by Russell Ross in 1999 (Ross 1999). Prior to this, Ross and his colleagues had already proposed a model stressing the importance of endothelial dysfunction in the development of atherosclerosis (Ross 1993). Indeed, even in healthy arteries some regions are more prone to

atherosclerosis than others. These are typically branches or curvatures where laminar blood flow might become disturbed or oscillated. A mere reduction in shear stress can lead to the constitutive activation of NF-κB in the endothelium (Hajra *et al.* 2000). Furthermore, studies conducted on rabbits and homozygous apoE-deficient mice demonstrate that exposure to a hypercholesterolemic diet can result in the upregulated expression of adhesion molecules in the lesion-prone areas prior to any detectable lesion formation or overlying very early lesions (Cybulsky and Gimbrone 1991, Li *et al.* 1993, Nakashima *et al.* 1998, Iiyama *et al.* 1999). The role of proinflammatory cytokines and other mediators in atherosclerosis is highlighted by the observation that mice deficient in IL-1β, IL-17A, TNF-α, M-CSF or MCP-1 exhibit less severe atherosclerosis (Smith *et al.* 1995, Gu *et al.* 1998, Kirii *et al.* 2003, Ohta *et al.* 2005, Smith *et al.* 2010). Importantly, several PRRs, particularly TLR1, TLR2 and TLR4, are expressed at higher levels in ECs, macrophages and SMCs in the atherosclerotic intima than in normal intima (Xu *et al.* 2001, Edfeldt *et al.* 2002, Vink *et al.* 2002, Otsui *et al.* 2007).

Also, continuously high levels of proinflammatory cytokines in the circulation can have an impact on the endothelium and disturb its normal homeostatic functions (Sattar *et al.* 2003). This partly explains why chronic systemic inflammatory diseases, such as RA, systemic lupus erythematosus and psoriatic arthritis are connected to a significantly elevated risk of cardiovascular disease (Myasoedova and Gabriel 2010, Skaggs *et al.* 2012). In fact, cardiovascular disease is the major cause of death in RA (Avina-Zubieta *et al.* 2008). Systemic inflammation can promote the development of atherosclerosis also by inducing changes in the size and density of LDL (Khovidhunkit *et al.* 2004) and by enhancing the oxidation of LDL by macrophages and ECs (Maziere *et al.* 1994). The involvement of the innate immune system is further highlighted by the fact that APPs, such as CRP and other pentraxins as well as SAA, have been indicated as both risk predictors (Morrow *et al.* 2000, Jousilahti *et al.* 2001, Johnson *et al.* 2004, Tsimikas *et al.* 2006) and active mediators of atherosclerosis (Ridker *et al.* 2002). In hyperlipidemic mice, the SAA concentration has been shown to correlate directly with the size of atherosclerotic lesions, independent of the cholesterol concentration in plasma (Lewis *et al.* 2004, Dong *et al.* 2011b), indicating SAA as an atherosclerotic factor.

Cell recruitment and inflammatory cascades in atherosclerosis

Inflammation in atherosclerotic lesions represents a complex set of interactions between inflammatory cells, ECs and SMCs, connected by a number of mediators and inducers at different levels. The inflammatory cascade is initiated by endothelial dysfunction (Gimbrone *et al.* 2000), a condition involving oxidative stress, the generation of ROS and a subsequent reduction in the endothelial production of NO. NO is an important regulator of vascular tone,

platelet and leukocyte interactions and vascular SMC proliferation (Ignarro *et al.* 1987, Azuma *et al.* 1986, Kubes *et al.* 1991, Tanner *et al.* 2000). Oxidative stress and endothelial dysfunction can result from several factors including high levels of LDL, free radicals caused by smoking, hypertension, disturbed blood flow and infectious agents (Ross 1999, Libby *et al.* 2009). In chronic systemic inflammatory conditions, the circulating proinflammatory cytokines (IL-1α, IL-1β, TNF-α and IFNγ) can also directly affect endothelial function (Sattar *et al.* 2003); TNF-α, for example, reduces the bioavailability of NO (Yoshizumi *et al.* 1993) and increases endothelial permeability (Petrache *et al.* 2003). Upon activation, ECs start to produce adhesion molecules that promote the adhesion of monocytes, T cells and mast cells to the endothelium. The deposition and oxidation of LDL in the subendothelial space can also promote the expression of adhesion molecules (Kume *et al.* 1992, Watson *et al.* 1997) and proinflammatory cytokines in ECs (Leitinger 2003, Matsuura *et al.* 2006).

The adhesion of monocytes, the most abundant inflammatory cells in atheroslerorotic lesions, is mainly mediated by vascular cell adhesion molecule 1 (VCAM-1). Adherent monocytes then enter the intima via diapedesis following a chemoattractant gradient formed by EC-derived chemotactic proteins such as MCP-1 and IL-8 (Libby 2002). Within the intima, monocytes differentiate into tissue macrophages in response to M-CSF and GM-CSF, which are also produced by the activated ECs, or their combination (Rajavashisth *et al.* 1990). Macrophages replicate in the intima and can become foam cells following a continuous uptake of lipids. Activated macrophages and foam cells secrete proinflammatory cytokines, such as IL-1β, IL-6, IL-8 and TNF-α, which amplify the local inflammatory response, as well as ROS, growth factors and MMPs (Østerud and Bjørklid 2003). The secreted cytokines and other mediators stimulate the migration of medial SMCs into the intima and the subsequent production of ECM proteoglycans (Lusis 2000).

T cells, like monocytes, enter the intima mainly via VCAM-1-mediated adhesion. The chemoattractant gradient is formed by a triplet of IFN-γ-inducible chemokines of the CXC family: IP-10, monokine induced by IFN-γ and IFN-inducible T-cell α-chemoattractant. T cells can be activated by different ligands, such as oxLDL, heat-shock proteins and microbial surface proteins (Rocha and Libby 2009). Upon activation, T cells can polarize into two subtypes of secretory cells, those secreting mainly proinflammatory cytokines, such as IL-1β, TNF-α and IFN-γ (T_h1 cells), or those secreting mainly anti-inflammatory cytokines, such as IL-10 and IL-4 (T_h2 cells) (Libby 2002). These cytokines may modify the expression patterns of other cell types, such as macrophages, if present. Besides T_h1 and T_h2 cells, T_h17 and Treg subtypes have been identified (Rocha and Libby 2009, Chen and Nunez 2010).

Mast cell progenitors are recruited from the circulation by eotaxin and stem

cell factor (SCF) (Miyamoto *et al.* 1997, Haley *et al.* 2000), the latter of which also mediates their differentiation into tissue mast cells (Galli *et al.* 2011). The preformed as well as *de novo* synthesized proinflammatory cytokines and growth factors (chapter 1.1.1) released from mast cells amplify the inflammation in the intima. In addition, released histamine can enhance the permeability of the ECs and thus promote an easier entry for more lipoproteins (Ma and Kovanen 1997).

SAA is potentially involved in nearly all stages of arterial inflammation. Starting from the endothelial level, SAA has been shown to increase ROS production and, similarly to TNF-α, to reduce the bioavailability of NO *in vitro*, and it could, thus, induce endothelial dysfunction (Björkman *et al.* 2008, Wang *et al.* 2008, Witting *et al.* 2011). The chemoattractant properties of SAA may enable it to mediate the migration of phagocytes, T cells, mast cells and monocytes and to promote their adhesion to the endothelium and their infiltration into the intima (Dong *et al.* 2011b, Badolato *et al.* 1994, Badolato *et al.* 1995, Xu *et al.* 1995, Preciado-Patt *et al.* 1996a, Olsson *et al.* 1999, Su *et al.* 1999). In addition, SAA promotes the release of MCP-1 from both PBMCs and ECs (Song *et al.* 2009, Lee *et al.* 2010), which would further increase the recruitment of inflammatory cells. In the intima SAA induces the release of proinflammatory cytokines, such as IL-1α, IL-1β, IL-6, IL-8 and TNF-α, from most cell types involved in the lesions: monocytes, macrophages and neutrophils (see chapter 2.4.2 for more details). Besides this, foam cells have been reported to secrete SAA (Meek *et al.* 1994). The mechanisms by which SAA has been suggested to affect the development of atherosclerosis are summarized and illustrated in Figure 7.

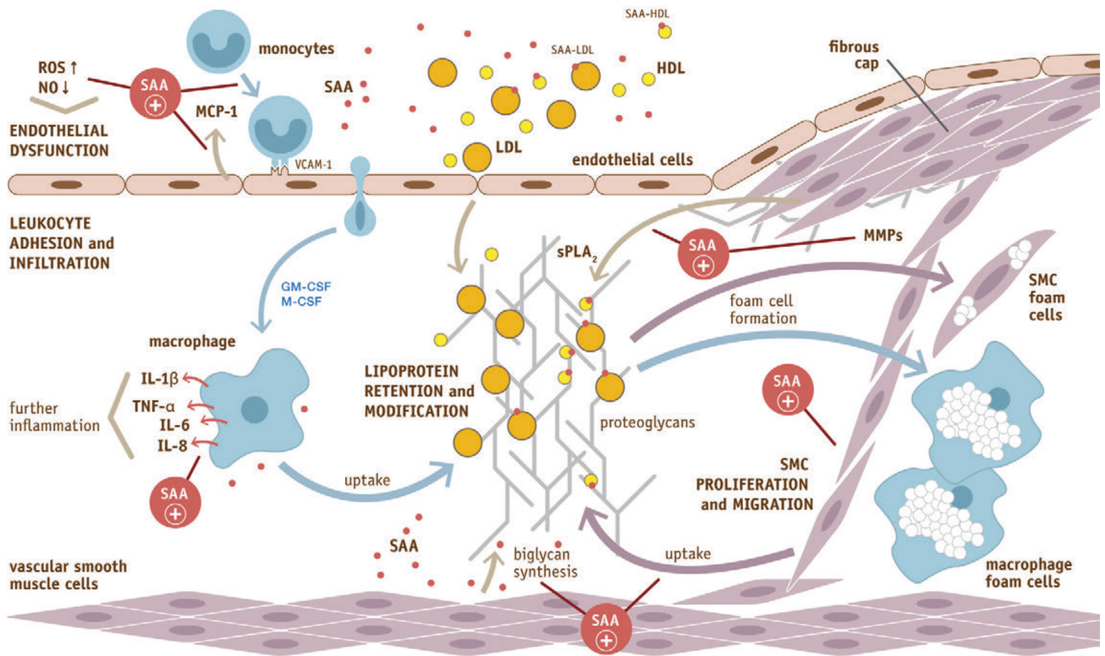

Figure 7. The involvement of SAA in the development of an atherosclerotic lesion. SAA is potentially involved in several steps in the formation of an atherosclerotic lesion. On the endothelial level, SAA promotes endothelial dysfunction and the secretion of MCP-1 and acts as a chemoattractant for leukocytes. In the intima, SAA-associated LDL and also HDL are more efficiently trapped by proteoglycans. SAA activates resident macrophages, which leads to the secretion of proinflammatory cytokines and further local inflammation. SAA also induces the synthesis of biglycan and $sPLA_2$ by SMCs, enhancing lipoprotein retention and modification, and promotes the migration of SMCs and their lipid uptake, which may promote SMC foam cell formation. SAA also induces the production of MMPs and might, thus, contribute to the destabilization of the lesion. The data are derived from King *et al.* 2011 and from the references mentioned in the text. GM-CSF, granulocyte-macrophage colony-stimulating factor; HDL, high-density lipoprotein; IFN, interferon; IL, interleukin; LDL, low-density lipoprotein; MCP-1, monocyte chemoattractant protein 1; M-CSF, macrophage colony-stimulating factor; MMP, matrix metalloproteinase; NO, nitric oxide; ROS, reactive oxygen species; SAA, serum amyloid A; SMC, smooth muscle cell; $sPLA_2$, secretory phospholipase A2; TNF, tumor necrosis factor; VCAM, vascular cell adhesion molecule.

III Aims of this Study

The aim of this study was to elucidate the interaction between Serum amyloid A (SAA) and cells of the innate immune system, to study the consequences of this interaction in terms of AA amyloidosis and atherosclerosis, and to explore the regulation of the proinflammatory activity of SAA in inflammation.

The specific aims can be outlined as follows:

1) to study whether SAA can induce the activation and degranulation of mast cells and to research the consequences of this activation, with an emphasis on amyloid formation

2) to elucidate whether SAA is able to activate human macrophages and to investigate the mechanisms underlying this activation, with an emphasis on the inflammasome function, and

3) to explore the impact of native and oxidized lipoproteins on the proinflammatory activity of SAA.

IV Materials and Methods

The methods used in this study are summarized in Table 2. Detailed descriptions can be found in the original publications and/or reference material, as indicated. Only the methods related to the unpublished data are explained in more detail below.

Method	Original publication	Reference/ source
Cell cultures and cell stimulation		
Isolation, maturation, culture and stimulation of human mast cells	I	Saito *et al*. 2006
Culture and stimulation of the HMC-1 cell line	I	Butterfield *et al*. 1988
Isolation, differentiation, culture and stimulation of human macrophages	II & III	Nakanishi *et al*. 2009
Isolation and stimulation of mouse peritoneal macrophages from wild-type and ASC-deficient mice	II	
Isolation, differentiation and stimulation of mouse bone marrow-derived macrophages from wild-type and ASC-deficient mice	II	
Culture, differentiation and stimulation of THP-1 cells	II & III	
Fluorescence imaging of cathepsin B and lysosomes in THP-1 cells	II	BIOMOL
Small interfering RNA (siRNA) experiments	II	Qiagen
Animals		
Induction of sterile peritonitis in C57BL/6J mice	III	
Enzyme-linked immunosorbent assay (ELISA) for human IL-1ß, TNF-α and histamine and for mouse IL-1ß	I, II & III	R&D Systems, IBL, BioLegend
Quantitative real time reverse transcriptase polymerase chain reaction (qRT-PCR)	II & III	
Western blotting	II	
Gel electrophoresis		
Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)	II	Laemmli *et al*. 1970
Tricine-SDS-PAGE	I	Schagger and von Jagow 1987
High-performance liquid chromatography with tandem mass spectrometry (HPLC-MS/MS)	I	
Degradation/digestion of SAA *in vitro*	I	
Transmission electron microscopy (TEM)	I	

Lipoprotein preparations		
Isolation of LDL and HDL_3 from plasma	III	Havel *et al.* 1955
Copper oxidation of LDL and HDL_3	III	
Lipid extraction from LDL	III	Folch *et al.* 1957
Preparation of lipid microemulsions	III	Ginsburg *et al.* 1982
TBARS analysis	III	Hessler *et al.* 1983
Lowry assay	III	Lowry *et al.* 1951
SAA-lipoprotein complex formation	III	
Thin layer chromatography (TLC)	III	
Statistical analyses (one-way ANOVA with post tests)	I, II & III	

Table 2 Methods used in the present study

The assessment of the distribution of SAA in lipoprotein classes

SAA (100 µg/ml) was mixed with pooled plasma collected from five healthy donors and incubated for 2 hours at +37°C. After this, lipoproteins were fractioned by sequential ultracentrifugation (Havel *et al.* 1955) and the amount of SAA in each fraction was determined by a commercial enzyme-linked immunosorbent assay (ELISA) kit for human SAA (Invitrogen).

V Results and Discussion

1. SAA activates cells of the innate immune system
(Studies I & II)

Experiments in the present study were mostly conducted using a recombinant human SAA protein, the amino acid sequence of which corresponds to the sequence of SAA 1.1 except for the addition of Met at the N-terminus, the substitution of Asp for Asn at position 60 and the substitution of His to Arg at position 71. In addition, human SAA derived from plasma was used.

1.1 SAA activates HMC-1 cells and human mast cells

The induction of cytokine production by SAA was studied using the human mast cell line (HMC-1), the only established cell line that resembles human mast cells in terms of phenotype (Butterfield *et al.* 1988, Nilsson *et al.* 1994). SAA induced a significant and dose-dependent increase in the release of TNF-α and IL-1β (Study I, figure 1). The induction of cytokines was as strong as or even higher than what was achieved by chemical activation with a combination of phorbol-12-myristate-13-acetate (PMA), which is a protein kinase C (PKC) activator, and a Ca2+ ionophore. Interestingly, using a combination of chemical activation and SAA stimulation resulted in further stimulation of TNF-α production, which suggests that the effect of SAA is not mediated via the same pathway, i.e. G protein or PKC signalling. Human albumin and heat-inactivated SAA failed to reproduce the induction, implying that the effect is specific for and dependent on the native conformation of SAA (Study I, figure 1). Although HMC-1 cells resemble human mast cells in many respects, they do not possess intact FcεRI receptors. Thus, the effect of SAA on IgE-induced degranulation was studied in human mast cells (huMCs) that were derived from cord or peripheral blood CD34+ progenitor cells. A low but dose-dependent release of histamine in the presence of SAA was observed (Study I, figure 2), confirming the degranulation in response to SAA.

Despite the common factors, such as RA, there is still very little data on the direct interaction between SAA and mast cells. The SAA-induced adhesion of mouse mast cells to ECM components (Hershkoviz *et al.* 1997) and the chemotaxis of HMC-1 cells and huMCs (Olsson *et al.* 1999) have been described. The SAA chemotaxis appears to depend on the Gi class G proteins as the effect is sensitive to pertussis-toxin (PTX) as well as to inhibitors of tyrosine kinase and PKC (Olsson *et al.* 1999). The release of IL-1β and TNF-α from HMC-1 cells and the release of histamine from huMCs described here are novel findings as no MC mediator release in response to SAA has been reported before. In contrast, Hershkowiz *et al.* concluded that SAA is, in fact, unable to activate mouse mast cells (Hershkoviz *et al.* 1997). This controversy, however, might be due to species-related differences. Furthermore, the involvement of G proteins and/or tyrosine kinases seems to characterize the SAA-induced mast cell adhesion and chemotaxis (Hershkoviz *et al.* 1997, Olsson *et al.* 1999), whereas our data suggests that the activation of mast cells by SAA is not dependent on G protein signalling. Thus, the two different types of activities would also be mediated via separate pathways.

The molecular mechanisms involved in the effect of SAA on mast cells were not elucidated further in Study I. Interestingly, NLRP3, although predominantly expressed in monocytes, granulocytes and chondrocytes (Feldmann *et al.* 2002, Manji *et al.* 2002), has more recently been implicated in the production of IL-1β by the mast cells of CAPS patients (Nakamura *et al.* 2009, Kambe *et al.* 2010). Furthermore, using mouse bone marrow-derived mast cells Nakamura *et al.* demonstrated that mast cells carrying a CAPS-associated *NLRP3* mutation produced IL-1β in a constitutive manner, while normal mast cells required two signals for the production of mature IL-1β, similarly to macrophages (Nakamura *et al.* 2009). Importantly, the combination of LPS priming and NLRP3 activation resulted in IL-1β secretion but not in mast cell degranulation (Nakamura *et al.* 2009). This indicates that the mechanisms involved in the secretion of IL-1β and in the degranulation are differentially regulated, which is logical considering that IL-1β is synthesized on demand and not stored in granules. As the release of histamine and the presence of tryptase-like activity in the cell culture medium (see chapter 3.1 in Results and discussion) were observed in the present study, one can conclude that SAA is able to trigger both degranulation and IL-1β synthesis in human mast cells. It remains to be elucidated which signalling pathways are employed. Six members of the TLR family are expressed in mast cells: TLR 1, 2, 3, 4, 6 and 9 (Rao and Brown 2008), and, as SAA has been shown to interact with TLR2 and TLR4 (Sandri *et al.* 2008, Chen *et al.* 2010, He *et al.* 2009) in monocytic cells, these receptors might mediate the effects of SAA also in mast cells.

1.2 SAA induces the release of cytokines from human macrophages

Next, the role of SAA in the production of cytokines in human monocyte-derived macrophages as well as in mouse peritoneal (PMs) and bone marrow-derived macrophages (BMMs) and monocytic THP-1 cells was studied. Monocytes and macrophages have differential requirements for inflammasome activation and the subsequent IL-1β processing (Netea *et al.* 2009). Monocytes can release IL-1β in response to TLR ligands without additional stimulation, most likely because of their autocrine production of ATP and/or constitutive activation of caspase-1 (Dinarello *et al.* 1987, Burchett *et al.* 1988, Ferrari *et al.* 1997). Macrophages, on the other hand, are not capable of releasing substantial amounts of endogenous ATP and thus require two signals for the production of IL-1β (Netea *et al.* 2009). THP-1 monocytic cells also require a priming step unless they are differentiated into macrophages in the presence of PMA; PMA-differentiated THP-1 cells show constitutive expression of pro-IL-1β (Fenton *et al.* 1988).

We found that SAA was able to induce the gene expression of *IL1B* and *TNFA*, both of which peaked at 6 hours, in human macrophages that had been differentiated from monocytes by GM-CSF (Study II, figure 1A). SAA also induced a clear and dose-dependent secretion of these proteins in human macrophages (Study II, figure 1B) as well as in mouse PMs and BMMs (Study II, figures 5AB) and PMA-differentiated THP-1 macrophages (Study II, figure 6B). Based on these observations, 3 mg/ml was selected as the standard concentration of SAA for the subsequent experiments. The maturity of the secreted IL-1β was confirmed by immunoblotting (Study II, figures 1C and 5B). To verify that the observed effect was not due to the properties of a recombinant protein, plasma-derived human SAA (Pussinen *et al.* 2001) was also tested and found capable of inducing the secretion of IL-1β (Study II, figure 1D). Furthermore, the possibility of endotoxin involvement was excluded by treating the recombinant SAA preparation with proteinase K or polymyxin B, an inactivator of endotoxins, prior to the experiments (Study II, figure 2D).

2. SAA activates inflammasome signalling in human macrophages (Study II and unpublished data)

The production of IL-1ß is under strict regulation, exemplified by the fact that two separate signals are required for the release of IL-1ß in its mature form; the first one induces the expression of *NLRP3* and *IL1B* and the second one induces the maturation of pro-IL-1ß via inflammasome activation. Some mediators have been shown to provide both of these signals. Live bacteria are the prime example; they stimulate the NF-κB pathway via PRRs, while NLRP3 is activated by bacterial toxins and/or pore-formation (Bauernfeind *et al.* 2011). Also, soluble biglycan, which can be released from the ECM during tissue injury, has been shown to induce IL-1ß release in macrophages without additional stimulants, via the concerted action of TLRs 2 and 4 and $P2X_7$ in a process that involves ROS generation (Babelova *et al.* 2009). The above results indicated that SAA can also act as such a mediator, capable of providing both signals needed for the secretion of IL-1ß.

2.1 SAA primes human macrophages via TLR2 and TLR4

First we elucidated the signal transduction pathways by which SAA primes the macrophages, i.e. induces the expression of IL1B. Previous reports suggest that SAA is involved in the signalling pathways mediated by FPRL1, class B scavenger receptors, RAGE and TLRs 2 and 4.

In the present study, we observed that both TLR2 and TLR4 play a role in the SAA-mediated expression of IL1B in human macrophages (Study II, figures 2BC). This finding was verified by using neutralizing antibodies against these receptors. The involvement of TLR4 was further verified by chemokine analysis, according to which SAA induced a clear increase in Macrophage inflammatory protein-1β, IP-10, RANTES and GM-CSF (unpublished observations, data not shown). The expression of IP-10 and RANTES is mediated by the TRIF-dependent pathway and the transcription factor IRF3 (Hacker *et al.* 2006), which are stimulated by the action of intracellular TLR4.

In contrast, neutralizing the CD36 receptor with a specific antibody had no effect in the present study (Study II, figure 2B), suggesting a role less significant than that of TLRs for CD36 in the

SAA-mediated induction of *IL1B*. It should be noted, though, that the expression of CD36 can vary markedly among different macrophage phenotypes *in vitro*, M1 (GM-CSF) macrophages showing a lower expression level of CD36 (van Tits *et al.* 2011), and there is also variation among individuals (Kashiwagi *et al.* 1995). A lack of CD36 may also be compensated by other pathways. As scavenger receptors, including CD36, form pairs with TLRs, the impact of blocking a particular SR might depend on whether or not it is associated with a TLR.

2.2 NLRP3 is the SAA-responsive inflammasome

Inflammasome activation results in the recruitment and autoproteolytic activation of caspase-1, the key enzyme in the processing of pro-IL-1β into mature IL-1β. In the present study, the involvement of caspase-1 and the inflammasome pathway in the SAA-induced secretion of IL-1β was confirmed by using a caspase-1 inhibitor, Z-YVAD-fmk, which caused a dramatic decrease in the secretion of IL-1β by SAA (Study II, figure 3A). In addition to this, SAA induced the secretion of proteolytically processed caspase-1 (Study II, figure 3B), which is in keeping with previous studies demonstrating exocytosis of caspase-1 with other inflammasome components (Andrei *et al.* 2004, Qu *et al.* 2007). Indeed, ASC isoforms were also detected in the cell culture supernatant subsequent to SAA stimulation (Study II, figure 5C). The role of ASC was further confirmed by stimulating PMs and BMMs derived from ASC-deficient (Mariathasan *et al.* 2004) and wild-type mice with SAA (Study II, figures 5AB). The most characterized inflammasome so far, NLRP3, requires ASC to function as a bridge between itself and caspase-1. However, as ASC is associated with other inflammasomes as well, we employed the siRNA technique to assess the importance of NLRP3. Silencing the gene encoding NLRP3 in PMA-differentiated THP-1 macrophages led to a significant decrease in the SAA-induced secretion of IL-1β while the secretion of TNF-α remained unaffected (Study II, figure 6B). Lastly, SAA also induced a rapid and robust increase in the expression of *NLRP3* (Study II, figure 6C), which has been shown, similarly to *IL1B* expression, to be a prerequisite for the assembly and activation of NLRP3 (Bauernfeind *et al.* 2009, Bauernfeind *et al.* 2011). SAA had no effect on the expression of *NLRP1* or *NLRP2* (Study II, figure 6C). Taken together, these findings strongly implicate NLRP3 as the SAA-responsive inflammasome.

These findings have later been confirmed by and are in keeping with studies by others that demonstrate the expression and secretion of IL-1β in response to SAA in monocytes, macrophages and PBMCs (Song *et al.* 2009, Ather *et al.* 2011, Franco *et al.* 2011), although the role of NLRP3 was discussed only by Ather *et al.* In their study they demonstrated a robust neutrophilic inflammation and release of IL-1β in mouse

lung following the instillation of recombinant human SAA. This induction was dependent on TLR2, MyD88, NLRP3 and IL-1, as verified with anakinra, an IL-1 receptor antagonist. Furthermore, Ather *et al.* showed that SAA induced the secretion of IL-1β along with IL-1α, IL-6 and IL-23 from mouse macrophages and dendritic cells. Besides this, the priming capability of SAA has been demonstrated in synovial fibroblasts, in which SAA cannot activate the inflammasome but is able to prime the cells for the MSU-induced activation of NLRP3 (Migita *et al.* 2012).

2.3 SAA activates the NLRP3 inflammasome via the $P2X_7$ receptor

To date, numerous NLRP3 activators along with different activation models have been proposed (see chapter 1.4.2 in Review of the literature). Extracellular ATP, which stimulates the ATP receptor $P2X_7$, is one of the main activators of NLRP3 (Mariathasan *et al.* 2006, Ferrari *et al.* 2006). In the present study, we used the compound KN-62 (Baraldi *et al.* 2003) as well as oxidized ATP, both of which block the $P2X_7$ receptor, and observed that SAA mediates the activation of NLRP3 via $P2X_7R$ signalling (Study II, figure 3A). Whether or not SAA stimulates the receptor directly was assessed by using apyrase, an ATP/ADP-hydrolyzing enzyme. Macrophages are not capable of releasing substantial amounts of endogenous ATP (Netea *et al.* 2009), but it is still theoretically possible that SAA, similarly to LPS, induces the release of ATP through TLR4 instead of activating $P2X_7R$ directly. Also amyloid-β peptides have been shown to trigger the release of ATP from human microglia (Sanz *et al.* 2009). However, apyrase had no effect on the SAA-mediated secretion of IL-1β (Study II, figure 3A), and no increase in the release of ATP in response to SAA was observed either. Thus, the results suggest that the SAA-mediated activation of the inflammasome is mediated by a direct interaction of SAA with $P2X_7R$ and that it is not associated with the release of ATP or ADP. This is an interesting finding considering that ATP is so far the only established physiological activator of $P2X_7$ (Ferrari *et al.* 2006), although the interaction with soluble biglycan has been discussed (Babelova *et al.* 2009). SAA has previously been reported to be involved in the antiapoptotic effects of $P2X_7$ in neutrophils but the authors concluded that in relation to these effects $P2X_7$ is not a specific receptor for SAA (Christenson *et al.* 2008).

2.4 Cathepsin B in the SAA-mediated activation of NLRP3

Cathepsin B activity has been shown to play an important role in the activation of the NRLP3 inflammasome. Models have placed it either downstream (Duncan *et al.*

2009) or upstream (Hentze *et al.* 2003, Halle *et al.* 2008, Hornung *et al.* 2008, Terada *et al.* 2009, Duewell *et al.* 2010) from the inflammasome assembly. The role of cathepsin B as an activator of caspase-1 is fairly established; the inhibition of cathepsin B blocks not only the secretion of IL-1β but also the maturation of IL-18 (Hentze *et al.* 2003). The cathepsin B-mediated activation of pro-caspase-1 has been demonstrated inside enlarged lysosomes in microglia (Terada *et al.* 2009). Cathepsin B can also maturate pro-caspase-1 in a cell-free system at acidic pH (Vancompernolle *et al.* 1998), confirming the physical importance of the interaction between the two enzymes. Importantly, phagocytosis of particulate and fibrillar material, such as silica, amyloid-β fibrils and crystalline cholesterol, has been shown to induce lysosomal destabilization and leakage of cathepsin B into the cytoplasm, which then results in the activation of the NLRP3 inflammasome (Halle *et al.* 2008, Hornung *et al.* 2008, Duewell *et al.* 2010, Rajamaki *et al.* 2010).

In the present study, we observed that the activity of cathepsin B was also required for the SAA-mediated maturation and release of IL-1β (Study II, figure 3A). This was verified by utilizing an irreversible, cell-permeable cathepsin B inhibitor, Ca074Me. The finding may suggest the involvement of the same lysosomal disintegration pathway that has been demonstrated for the above-mentioned particulate material, especially as lysosomes have been implicated also in intracellular AA amyloid fibrillogenesis and in the subsequent exocytosis of amyloid fibrils (Kluve-Beckerman *et al.* 2001, Kluve-Beckerman *et al.* 2002, Magy *et al.* 2007). The SAA concentration used in the present study, however, did not induce lysosomal destabilization or leakage of cathepsin B into the cytosol (Study II, figure 4A). Furthermore, treating macrophages with cytochalasin D had no effect on the release of IL-1β by SAA (Study II, figure 3A). These observations clearly imply that intracellular fibril formation followed by lysosomal disintegration, or phagocytosis of SAA-derived extracellular fibrils, is hardly the mechanism inducing the activation of NLRP3 in the present study.

In summary, our results suggest that the observed SAA-induced activation of the inflammasome depends on the activity of cathepsin B but that it is not mediated through fibril formation or lysosomal destabilization. Instead, in the present study, caspase-1, cathepsin B and ASC were secreted to the cell culture media in response to SAA (Study II, figures 3B, 4B and 5C). This is a logical observation in light of the findings by others showing that inflammasome components, including cathepsin B, are released in response to $P2X_7R$ stimulation, independent of cytokine release (Lopez-Castejon *et al.* 2010), or as a part of the non-classical secretion of IL-1β (Andrei *et al.* 2004, Qu *et al.* 2007). In addition, the release of cathepsin B in response to SAA has recently been observed in synovial fibroblasts, in which SAA induces the priming step (Migita *et al.* 2012). However, it is still unclear exactly how the cathepsin activity then contributes to

the SAA-mediated production of IL-1β considering that the role of a passive secreted component or marker hardly supports the results of the present study with a cathepsin B inhibitor.

It is also important to note that the cell-permeable cathepsin B inhibitor (Ca-074Me) used in this study has been shown to inhibit not only cathepsin B but also the closely related cathepsin L when tested on mouse fibroblasts (Montaser *et al.* 2002). Thus, both cathepsins could potentially contribute to the activation of NLRP3. In accordance with this, a recent study utilized a similar non-specific cathepsin inhibitor and reproduced our findings, i.e. the attenuation of the SAA-induced secretion of IL-1β in response to the inhibitor, on mouse wild-type peritoneal macrophages, but they also reported that SAA could induce the secretion of IL-1β in cathepsin B-deficient macrophages as well (Poynter 2012). This, although not discussed by Poynter, indeed supports the potential role of cathepsin L in the activation of NLRP3 and requires further study.

3. The activation of the innate immune system cells is involved in AA amyloid formation (Studies I & II)

Tissues that are either prone to develop amyloid deposits or are already affected by them also contain an abundant number of mast cells (Westermark 1971). This is particularly exemplified in renal AA or AL amyloidosis, in which the affected kidney also shows an increased density of interstitial mast cells (Toth *et al.* 2000, Danilewicz and Wagrowska-Danilewicz 2002). Thus, we hypothesized that mast cell mediators might be involved in the extracellular processing of SAA. SAA was found to be a strong activator of mast cells based on the induction of the secretion of IL-1ß and TNF-a and also of histamine (Study I, figures 1 and 2). Tryptase is the major protease stored in the granules and it can be found in all human mast cells (Schwartz *et al.* 1987). Tryptase possesses trypsin-like specificity, i.e. it cleaves its substrates at the carboxyl side of Arg or Lys residues (Hallgren and Pejler 2006). Chymase, on the other hand, resembles chymotrypsin and cleaves its substrates at the carboxyl side of aromatic or Leu residues (Powers *et al.* 1985). HuMCs used in the present study were cultured from cord blood or peripheral blood CD34+ progenitor cells as per Saito *et al.* (Saito *et al.* 2006) with modifications. In short, SCF, IL-4, IL-6 and IL-10, and/or combinations thereof, were utilized to produce mature mast cell cultures expressing both tryptase and chymase.

3.1 Mast cell proteases degrade SAA

First, the ability of mast cell proteases to degrade SAA was studied using purified human tryptase and chymase (Study I, figure 3). Tandem mass spectrometric analysis (MS/MS) of the digestion mixture revealed that tryptase generated an SAA peptide whose mass corresponded to the N-terminal fragment of SAA (residues 3-16; SFFSFLGEAFDGAR), which is the area implicated in amyloid formation (Westermark *et al.* 1992, Patel *et al.* 1996). Chymase and a mixture of chymase and tryptase degraded the N-terminus completely (Study I, figure 3B). Next, the degradation was studied using cell culture media collected from immunologically (IgE-)activated huMCs, which also contained MC granules. The degradation pattern of SAA in the medium was similar to that achieved by purified tryptase (Study I, figure 4A) and importantly, the N-terminal SAA fragment was found intact. This is surprising considering that the granules contain both proteases. However, the explanation may arise from the

fact that upon degranulation tryptase is released from the granules, whereas chymase remains bound to the heparin proteoglycans of the remnants (Kovanen 1991, Lindstedt *et al.* 2001). SAA, in turn, can bind to the heparin proteoglycan matrix via two separate binding sites (Ancsin and Kisilevsky 1999, Elimova *et al.* 2009), and, thus, the interaction between SAA and chymase might be sterically hindered. It should also be noted that *in vivo* chymase, unlike tryptase, can be inactivated by several natural inhibitors present in interstitial fluids, principally α1-antitrypsin (Schechter *et al.* 1989, Kokkonen *et al.* 1997). Interestingly, a chymotrypsin-like mast cell protease purified from rat brain has been shown to proteolyze the amyloid precursor protein in a manner that releases the area containing the amyloid-β peptide (Nelson *et al.* 1993). This supports our findings and suggests that proteases of this type might very well participate in the proteolytic processing and activation of amyloidogenic protein precursors.

We also elucidated whether SAA can induce the degranulation of mast cells, resulting in the release of chymase and tryptase and the degradation of SAA itself. Indeed, the presence of SAA in the culture medium led to a partial disappearance of full-length SAA, as analyzed by western blotting. The degradation was as efficient as what was observed after the immunological activation of huMCs (Study I, figure 4BC). In summary, these findings demonstrate that both purified mast cell proteases and human mast cell-conditioned media can degrade SAA and that SAA alone is sufficient to induce the degranulation of mast cells and the subsequent degradation of itself.

3.2 Continuously elevated SAA levels lead to amyloid formation in mast cell cultures

During prolonged inflammation *in vivo*, the levels of SAA in serum are persistently high due to the hepatic production of SAA induced by proinflammatory cytokines. In order to simulate this physiological condition in our experimental setting, we set up a 7-day experiment where SAA was added to the cell cultures in two-day intervals, ensuring an excess of SAA throughout the experiment. The cell culture media were analysed by transmission electron microscopy, which revealed an extensive formation of fibril-like structures and aggregates (Study I, figures 5AB). This finding was further supported by the fact that the N-terminal fragment of SAA could not be detected by MS analysis in the cell culture medium samples collected after day 4, suggesting that the fragment was no longer in a soluble form (Study I, data not shown).

In summary, we propose that mast cells can contribute to the formation of amyloid deposits in tissues according to the following sequence. High levels of

SAA activate mast cells, which degranulate and release neutral proteases, notably tryptase. The tryptase-generated amyloidogenic SAA fragment can then function as a local amyloid-enhancing factor and induce AA fibrillogenesis. This may be further enhanced by heparin, a component of mast cell granules as well as by mature amyloid fibrils. HS and/or its structural derivative heparin have indeed been implicated in the acceleration of AA amyloidosis (Bellotti and Chiti 2008, Motamedi-Shad *et al.* 2012). Elimova *et al.* have demonstrated that, in an acidic pH, both heparin and HS promote the aggregation of HDL-Saa as well as the dissociation of Saa from HDL (Elimova *et al.* 2009). The HS-binding regions on SAA, the N-terminal aa 17-49 (Elimova *et al.* 2009) or the C-terminal aa 77-103 (Ancsin and Kisilevsky 1999), could then facilitate the cell surface binding and the HS-mediated aggregation of Saa. In accordance with this, the inhibition of the SAA-HS interaction, either by blocking the HS binding site (Kisilevsky *et al.* 1995, Elimova *et al.* 2004) or by utilizing heparanase (Li *et al.* 2005), decreases AA fibrillogenesis *in vivo*.

3.3 Macrophage-derived cathepsin B might contribute to extracellular fibril formation

In addition to mast cells, macrophages also appear to accumulate near AA deposits *in vivo* (Kuroiwa *et al.* 2003) and they have been implicated in the processing of exogenous SAA in the cell culture models of AA amyloidosis. Amyloid formation in these macrophage models requires high SAA concentrations and most often also the presence of amyloid-enhancing factor (AEF). Not surprisingly, we observed no signs of intracellular amyloid formation or endocytosis or exocytosis of fibrils based on an analysis of lysosomal integrity (Study II, figure 4A). However, since the secretion of the active form of cathepsin B into the cell culture medium was detected (Study II, figure 4B), it can be hypothesized that the processing of SAA into amyloidogenic fragments and the formation of fibrils could also take place in the extracellular milieu. This is in accordance with a previous study that compared SAA clearance in the HepG2 cell line and in hamster peritoneal exudate cells, including macrophages, and demonstrated that SAA is degraded mostly extracellularly when incubated with peritoneal cells (Liang and Sipe 1995). Furthermore, a $P2X_7$-mediated and IL-1β-independent release of lysosomal cathepsins from human and mouse macrophages has been reported. Importantly, in that study the secreted cathepsins were proteolytically active as evidenced by the degradation of ECM collagen *in vitro* (Lopez-Castejon *et al.* 2010). In terms of pH, extracellular space is not the optimal environment for lysosomal proteases (pH 6.0 for cathepsin B), but the data from Lopez-Castejon *et al.* as well as others (Werle *et*

al. 1997, Linebaugh *et al.* 1999) imply that residual or some forms of cathepsin B activity exists also in a neutral pH. Furthermore, local acidosis is a well-known feature of inflammatory loci (Lardner 2001), and macrophages can acidify their surroundings by different mechanisms (Leake 1997). As mentioned earlier (chapter 3.1.3 in Review of the literature), several members of the cathepsin family have been implicated in the proteolytic processing of SAA. Cathepsin B, the expression of which increases during inflammation (Lah *et al.* 1995), has been shown to degrade SAA and to release the amyloidogenic N-terminus (Yamada *et al.* 1995b, Röcken *et al.* 2005). The impact of this activity on the actual formation of fibrils is not clear, though, as blocking cathepsin B does not seem to affect the amyloid load *in vitro* or *in vivo* (Röcken *et al.* 2006, van der Hilst *et al.* 2009). However, fibril formation in the extracellular fluid can be affected by several factors including components of the ECM and mediators, such as other proteases released from resident cells. Thus, the secretion of active cathepsin B from macrophages in response to SAA and the ability of cathepsin B to create the amyloidogenic SAA fragments may well represent a pathway that serves as a source of seeding material for amyloidogenesis. One can also envision a cascade involving both resident macrophages and mast cells in which cytokines secreted from both cell types as well as proteases, including tryptase and cathepsin B, would continuously activate more cells and produce more amyloidogenic AA fragments, respectively.

4. The activity of SAA is regulated by native and oxidized lipoproteins (Study III & unpublished data)

The SAA concentration used in the present study was within a normal physiological range (1-5 mg/ml). The fact that this concentration induced a strong inflammatory response in human macrophages suggests that the findings are relevant also *in vivo*. Considering that during the APR the serum level of SAA can increase up to 1000-fold, reaching a concentration of 1 mg/ml or more, it is obvious that strict regulatory mechanisms other than merely transcriptional ones must exist. One such mechanism has been suggested to be the interaction of SAA with HDL. Indeed, many pathological features of SAA, including proinflammatory and also proatherogenic activities are associated with the free protein and become blocked or at least altered in the presence of HDL. For example, pre-incubation of SAA with HDL inhibits SAA-induced events, such as endothelial dysfunction in human aortic ECs (Witting *et al.* 2011), the production of monocyte TF and cytokines in PBMCs (Cai *et al.* 2007, Song *et al.* 2009, Franco *et al.* 2011) and the expression of $sPLA_2$ in rat SMCs (Sullivan *et al.* 2010). Most *in vitro* studies concentrating on the interaction between SAA and lipoproteins have been conducted using either SAA-rich AP-HDL or preformed complexes of SAA and HDL, justified by the fact that HDL is naturally associated with SAA *in vivo*. Depending on the conditions, however, the extrahepatic expression of SAA can lead to significant local concentrations of lipid-free SAA. Thus, the utilization of free SAA and free lipoproteins, instead of preformed complexes, in the experiments can actually better mimic the physiological conditions at the site of inflammation.

4.1 The distribution of SAA among lipoprotein classes

Although HDL_3 is the major carrier of SAA in the circulation (Benditt and Eriksen 1977, Skogen *et al.* 1979, Bausserman *et al.* 1980), the distribution of SAA among the lipoprotein classes is not constant. In order to verify that all lipoproteins are indeed capable of binding to SAA *in vitro* under our experimental conditions and to establish the binding ratios for the complexes, SAA was mixed with pooled plasma, after which the plasma lipoproteins were fractioned and the amount of SAA in each fraction was assessed. As illustrated in Figure 8, approximately 85% of SAA was found to be associated with HDL (either HDL_2 or HDL_3) and 3% with LDL. The proportion of lipid-free SAA was 11%. We observed that SAA bound to HDL_3 and LDL at molar ratios of 1:1.1 (SAA:HDL_3, mol/

mol) and 1:2.7 (SAA:LDL, mol/mol), respectively. This is generally in keeping with the reported *in vivo* observations during the APR in humans (Marhaug *et al.* 1982, Marhaug and Husby 1982) and mice (Cabana *et al.* 2004) as well as under mimicking conditions *in vitro* (Cabana *et al.* 2004). Variations in the distribution of SAA are likely to result from different SAA or lipoprotein concentrations and from other factors, such as the inflammatory status. The binding of HDL to SAA *in vitro* seems to decrease as a function of SAA concentration (Baranova *et al.* 2010). The concentration of SAA used in this experiment (100 µg/ml) reflects the SAA level typically seen during a medium-to-strong APR.

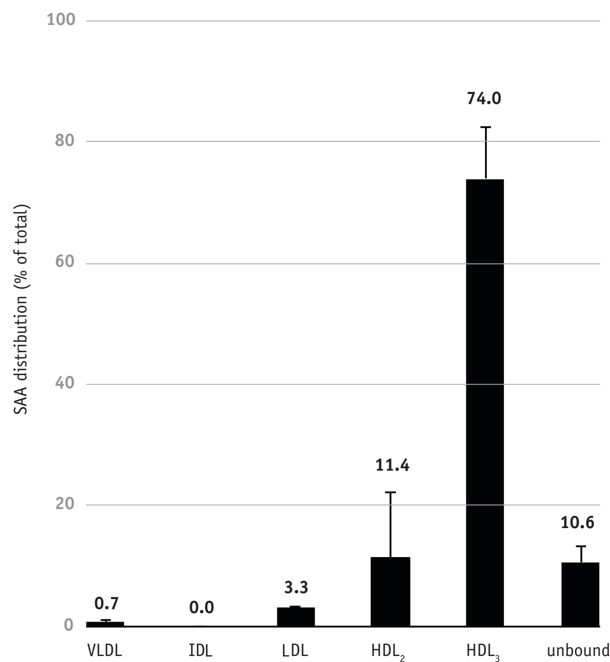

Figure 8. The distribution of SAA among lipoprotein classes *in vitro*. The data are means from two independent experiments conducted using a plasma pool of five donors. HDL, high-density lipoprotein; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; SAA, serum amyloid A; VLDL, very low-density lipoprotein.

4.2 Native and oxidized lipoproteins decrease the SAA-induced secretion of cytokines

First, the effect of native and oxidized lipoproteins on the SAA-induced expression and release of IL-1β and TNF-α in human macrophages was studied. Lipoproteins were isolated from human plasma and oxidized by Cu^{2+}. The lipid peroxidation was confirmed by TBARS analysis.

The analysis of mRNA levels revealed that the expression of *IL1B* and *TNFA* induced by SAA decreased dose-dependently in the presence of native and oxidized HDL_3 and LDL (Study III, figure 2). A significant and dose-dependent decrease was also observed in the SAA-induced secretion of IL-1β and TNF-α proteins (Study III, figures 1A and 1C). The effect was not dependent on the order of the additions (first the lipoproteins, then SAA, or vice versa) or whether or not SAA and the lipoproteins were introduced as pre-made complexes. Also, SAA-enriched AP-HDL purified from plasma was unable to induce IL-1β production (Study III, figure 1B).

While the inhibition of SAA activity by HDL_3 has been demonstrated previously, the inhibition of SAA by LDL, to our knowledge, has not been reported before. In fact, a recent study reported that LDL failed to inhibit the SAA-mediated release of TNF-α (Franco *et al.* 2011). However, that study was conducted on non-differentiated THP-1 cells and PBMCs (vs. GM-CSF macrophages and PMA-differentiated THP-1 macrophages used in our study) and also with a higher SAA concentration, which might explain the different results. The *in vivo* formation and presence of the SAA-LDL complex has been previously demonstrated (Ogasawara *et al.* 2004, Kotani *et al.* 2009). OxLDL particles have been shown to possess many proinflammatory properties, and lipoprotein oxidation is also thought to play a major role in the development of atherosclerosis (Kruth 2001). Against this background, the above findings are somewhat surprising. However, they are supported by previous studies that demonstrate that oxLDL may inhibit or delay the LPS-induced expression of *IL1B* (Hamilton *et al.* 1990, Fong *et al.* 1991, Hamilton *et al.* 1998, Mikita *et al.* 2001). It has been suggested that oxLDL promotes NF-κB dysregulation or inhibits the binding of NF-κB to DNA (Hamilton *et al.* 1998, Ohlsson *et al.* 1996, Brand *et al.* 1997).

4.3 The requirements for co-presence and lipoprotein uptake

Next we elucidated whether the co-presence of SAA and the lipoproteins is required for the inhibitory effect of these lipoproteins. When the cells were thoroughly washed following the incubation with the lipoproteins and prior to the addition of SAA, the ability of the native lipoproteins to inhibit the expression of *IL1B* and the secretion of IL-1β was completely lost (Study III, figures 3AB). In contrast, the inhibitory effect of oxLDL and $oxHDL_3$ persisted even after their removal, suggesting that the inhibition of the SAA-induced secretion of IL-1β by oxidized lipoproteins is mediated through a direct effect on macrophages. The inhibitory activity of native lipoproteins, in contrast, involves interaction with SAA, possibly resulting in an impairment of the ability of SAA to interact with its receptor(s), such as TLR2 and TLR4. This might be a consequence of complex formation between SAA and lipoproteins, which could result in steric hindrance. Recombinant SAA

added to a serum-containing cell culture medium has indeed been shown to readily associate with HDL (Magy *et al.* 2007). Alternatively, SAA could be internalized as an SAA-lipoprotein complex and become degraded, resulting in the loss of its activity. The uptake of oxidized lipoproteins could, in addition, trigger intracellular signalling, which could dampen the proinflammatory response. Lipoproteins can be internalized via receptor-mediated or receptor-independent, non-specific pathways, such as different forms of fluid-phase pinocytosis, micropinocytosis and macropinocytosis (Amyere *et al.* 2002, Swanson 2008). These are mainly processes requiring actin polymerization (Conner and Schmid 2003, Schrijvers *et al.* 2007, Kruth 2011). Thus, we next utilized cytochalasin D to explore whether the actin-dependent internalization of lipoproteins is involved in the inhibitory effect. As expected, cytochalasin D inhibited the uptake of LDL and also the uptake of pre-made LDL-SAA complexes based on the decreased intracellular content of cholesterol esters (Study III, figure IA in the online-only data supplement). On the contrary, cytochalasin D had no effect on the ability of native or oxidized lipoproteins to inhibit the SAA-induced secretion of IL-1β (Study III, figure IB in the online-only data supplement), suggesting that the internalization of lipoproteins is not required for their inhibitory effect.

4.4 Oxidized lipoproteins and lipid particles inhibit the activation of NLRP3

To assess whether the reduction of the SAA-induced secretion of IL-1β by lipoproteins is a mere consequence of the reduced expression of *IL1B*, the above-described experiments were repeated using PMA-differentiated THP-1 macrophages, which exhibit constitutive expression of *IL1B* (Fenton 1988). Interestingly, native lipoproteins had no impact on the SAA-induced release of IL-1β in THP-1 macrophages, while oxidized lipoproteins significantly inhibited it also in these cells (Study III, figure 4A). This implies that the inhibitory effect of native lipoproteins can be explained by the reduced expression of *IL1B*, whereas their oxidized forms also inhibit the inflammasome cascade and the maturation of IL-1β. To see if this inhibitory activity is specific for the SAA-induced activation of NLRP3, nigericin, a bacterial-derived pore-forming toxin and an activator of the NLRP3 inflammasome (Mariathasan *et al.* 2006), was tested. Indeed, both oxidized lipoproteins, but in particular oxLDL, also significantly inhibited the nigericin-induced secretion of IL-1β in THP-1 macrophages (Study III, figure 4B), indicating that the ability of oxidized lipoproteins to inhibit the NLRP3 inflammasome is not restricted to the SAA-induced activation.

In order to study whether the inhibitory effect of oxLDL was conferred by its

lipid or protein fraction, microemulsion particles were prepared of lipids that had been extracted from oxLDL. When introduced to human macrophages, the microemulsion particles derived from oxLDL were equally efficient in inhibiting the release of IL-1β as was intact oxLDL (Study III, figure 4C). Thus, the lipid fraction is the part mediating the inhibitory effect. Oxidized phospholipids, such as the oxidation products of 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (PAPC), have been proposed to act as inhibitors of signalling by TLR2 and 4 (Bochkov *et al.* 2002, Walton *et al.* 2003, Nonas *et al.* 2006). The phospholipid fraction of oxLDL may also inhibit the intracellular activity of cathepsin B (Hoppe *et al.* 1994, O'Neil *et al.* 2003). As the activity of cathepsin B is required for the SAA-induced activation of NLRP3, it can be speculated that this property of oxLDL could at least partly contribute to the observed reduction in the SAA-induced release of IL-1β by oxLDL.

4.5 OxLDL and oxidized lipids induce an anti-inflammatory response

Some studies have demonstrated that mmLDL and oxLDL can induce the expression of *IL1B* (Masters *et al.* 2010) and give rise to a modest release of IL-1β via activation of NLRP3 (Duewell *et al.* 2010, Jiang *et al.* 2012). The uptake of oxLDL appears to be a prerequisite for the induction of IL-1β secretion as it is sensitive to cytochalasin D (Jiang *et al.* 2012). In contrast to this, the inhibition of the SAA-induced activation of NLRP3 observed in the present study was independent of the oxLDL uptake (Study III, figure I in the online-only data supplement). This suggests that oxLDL may be involved in two opposite regulatory pathways, triggered by various mediators in the cellular microenvironment and perhaps also by the properties of the oxLDL particles themselves. For example, mmLDL, once taken up and directed to lysosomes, can form cholesterol crystals that have been indicated as NLRP3 activators (Duewell *et al.* 2010, Rajamaki *et al.* 2010). In addition, the two pathways could be temporally separated and/or involve secondary signalling cascades, such as anti-oxidative and anti-inflammatory responses. Indeed, in the present study oxLDL and $oxHDL_3$, as well as microemulsion particles prepared from the lipids of oxLDL, induced an increase in the gene expression of heme oxygenase (HO-1) that was inversely proportional to the release of IL-1β by SAA (Study III, figure 5). HO-1, also referred to as heat shock protein-32 (Hsp32), catalyzes the conversion of the heme group of hemoglobin into bilirubin, biliverdin and carbon monoxide (Siow *et al.* 1999, Takahashi *et al.* 2007). HO-1 is highly anti-inflammatory, and it can be induced by a number of physiological and pathological stimuli, including oxidative stress signals, cytokines, bacterial

compounds and growth factors (Paine *et al.* 2010). The gene expression of *HO1* is mediated by redox-dependent transcription factors, notably Nrf2 (Alam *et al.* 1999), which is translocated into the nucleus upon stimulation. Importantly, oxLDL, mmLDL as well as oxHDL have been indicated as inducers of *HO1* (Anwar *et al.* 2005, Ma *et al.* 2007, Rossmann *et al.* 2011), and HO-1 has recently been implicated in a negative feedback mechanism involved in the activation of the NLRP3 inflammasome (Nurmi, K. *et al.*, unpublished data). Thus, the induction of *HO1* by oxLDL could at least partly explain the dampening of the SAA-induced activation of NLRP3 observed in the present study. Interestingly, a similar feedback mechanism has been described for oxLDL and its ability to induce oxidative bursts in macrophages (Fischer *et al.* 2002). Fischer *et al.* observed that oxLDL induced ROS production upon first contact but was also able to reduce it via the activation of the insulin-sensitizing and anti-inflammatory peroxisome proliferator-activated receptor gamma (PPARγ) (Li *et al.* 2000) that led to desensitization of macrophages.

4.6 OxLDL inhibits the SAA-induced peritonitis

Lastly, we studied whether oxLDL can inhibit the SAA-induced production of IL-1β also *in vivo*. For this, SAA was used to induce peritonitis in mice. Previously, the induction of sterile peritonitis has been demonstrated by several substances including thioglycolate (Gilmour *et al.* 2006), yeast-derived zymosan (Perretti *et al.* 1992), casein (Iversen *et al.* 2005), high-mobility group box 1 (HMGB1) (Orlova *et al.* 2007), a combination of proteose peptone and IL-1α (Merinen *et al.* 2005) as well as MSU and octacalcium phosphate crystals (Getting *et al.* 1997, Martinon *et al.* 2006, Uratsuji *et al.* 2012, Narayan *et al.* 2011). SAA-induced peritonitis has not been described before. However, considering the proinflammatory potential of SAA it was reasonable to expect an inflammatory response upon injection of SAA into the peritoneal cavity.

SAA and oxLDL were injected into the peritonea of wild-type C57BL/6J mice. The injection of SAA increased the concentration of IL-1β in the peritoneal fluid, as determined 4 hrs after the injection. There was also an increase in the neutrophil count (Study III, figure 6). OxLDL alone did not induce these effects. Importantly, the injection of oxLDL one hour prior to SAA clearly diminished the SAA-induced concentration of IL-1β in the peritoneal fluid (Study III, figure 6). These findings strongly suggest that the inhibitory effect of oxidized lipoproteins that was observed in the preceding cell culture experiments can also occur *in vivo* and has physiological relevance. OxLDL may represent a novel and significant regulator of SAA activity in inflamed tissues, notably in atherosclerotic lesions.

5. General discussion

5.1 Methodological aspects

SAA protein

Native human SAA is fairly difficult and laborious to purify, and probably for this reason it is not commercially available. Thus, most SAA studies conducted so far, including ours, have utilized a recombinant form of human SAA, "the consensus SAA molecule", that is a mixture of SAA1 and SAA2. Alternatively, some groups have decided to work with mouse Saa proteins. It is certainly possible that recombinant SAA differs from the native SAA, especially in terms of posttranslational modifications. However, native and recombinant SAA have been used in parallel (Christenson *et al.* 2008, Linke *et al.* 1991, and the present study: Study II, figures 1D and 2C). One study has reported on functional differences between the two, noting, though, that the purification procedure of human SAA may also affect its properties (Björkman *et al.* 2010). The bacterial origin of the recombinant SAA and the possibility of endotoxin contamination might also be problematic issues. However, this is routinely controlled by including polymyxin B or a similar LPS blocker in the experimental design (Christenson *et al.* 2008, Sandri *et al.* 2008, Chen *et al.* 2010, Li *et al.* 2010, Ather *et al.* 2011).

Another issue to be considered arises from the lipidic state of SAA. In fact, many of the main findings presented in this field over the years have recently been questioned (Kisilevsky and Manley 2012). Since delipidated SAA can form aggregates *in vitro* (Kinkley *et al.* 2006), Kisilevsky and Manley claim that SAA hardly exists in a lipid-free form *in vivo*, or at least not in amounts that are physiologically relevant. Thus, all the data produced using lipid-free SAA, including the majority of the data on receptor interactions, should be interpreted with extreme care. According to Kisilevsky and Manley, receptors indicated as "SAA receptors" (SR-B1, TLR2, TLR4, FPRL1, CD36) may simply be fulfilling their role as scavenger receptors as macrophages most likely internalize aggregated SAA via phagocytosis. However, the aggregation data by Kinkley *et al.* as well as the experimental data employing HDL-SAA (Kisilevsky and Manley 2012, Kinkley *et al.* 2006) have again been produced with plasma-purified SAA that has gone through a long purification and/or reconstruction process. As a result, the physiological relevance of the obtained product can also be questioned. Furthermore, the model suggested by Kisilevsky and Manley fails to explain why, then, the inhibition of actin polymerization does not affect SAA signalling as has been demonstrated (Kluve-Beckerman *et al.* 2001; Study II, figure 3A; Study III, figure I in online-only data supplement). Also, if we presume that the aggregated

SAA is internalized merely via non-specific phagocytosis, then blocking one of the proposed SAA receptors, such as TLR2 or TLR4, should have no impact because other scavenging pathways could substitute for the inhibited ones. However, this is not the case, as has been shown by using neutralizing antibodies against TLRs (Study II, figures 2BC) or macrophages from TLR2 or TLR4-deficient mice (Cheng *et al.* 2008, Sandri *et al.* 2008, He *et al.* 2009, Chen *et al.* 2010, Ather *et al.* 2011). Importantly, epitope mapping studies have suggested that the lipidic state of SAA does not affect its conformation or oligomeric form (Malle *et al.* 1995, Malle *et al.* 1998). In other words, it is possible that SAA assumes the same hexameric conformation when unbound and when in a complex with HDL. Indeed, some signalling pathways are activated in a similar fashion by the two states of SAA (Patel *et al.* 1998, Cai *et al.* 2005), whereas the exceptions might reflect the inhibitory effect of HDL that was demonstrated in the present study as well. Thus, in light of current knowledge, neither of the lipidic states of SAA, lipid-free or HDL-bound, can be considered less relevant physiologically than the other.

Lipoprotein oxidation

The modification of LDL is considered one of the key events in the pathogenesis of atherosclerosis. In contrast to some forms of modified LDL, such as acetylated LDL that is not found *in vivo*, the physiological relevance of oxLDL is supported by the presence of oxLDL in atherosclerotic lesions, or in fractions extracted from them, and the presence of oxLDL-reactive autoantibodies in the serum of both humans and animals (Steinberg 1997, Steinberg and Witztum 2010). However, the exact mechanism by which LDL is oxidized *in vivo* is not known. The incubation of LDL with ECs and also other cell types has been shown to result in the oxidative modification of LDL (Henriksen *et al.* 1981, Steinbrecher *et al.* 1984), and the suggested pathways include reactions involving metalloprotein lipoxygenase, peroxidase-mediated oxidation (with myeloperoxidase and heme) as well as oxidation mediated by ceruloplasmin and copper or by iron (Jiang *et al.* 2011). A mere presence of copper ions has been shown to oxidize LDL in a way that mimics the cell-induced oxidation of LDL in many ways (Esterbauer *et al.* 1989), and this procedure has become one of the most commonly used methods for *in vitro* LDL oxidation. Depending on the oxidation mechanism, the chemical properties of the acquired oxLDL can differ to some extent; copper oxidation may, for example, create more malondialdehyde, which can modify the apoB fraction or the net charge of the LDL particle, compared with other pathways. However, there is no exact definition for oxLDL. Instead, it can be characterized as a complex mixture of numerous chemical entities, and even in identical oxidative conditions the end product can vary from experiment to experiment, depending on the initial composition of LDL (Steinberg 1997). To date, the oxidation of HDL has drawn much less attention, but

it has been suggested to be oxidized as readily as LDL (Parthasarathy *et al.* 1990, Shuhei *et al.* 2010). Based on the available data, it is reasonable to assume that the copper-oxidized lipoproteins used in the present study resemble their physiological counterparts to a sufficient extent to draw preliminary conclusions.

5.2 The current treatment of SAA-related diseases

In AA amyloidosis, the primary treatment strategy has traditionally been the alleviation of the primary disease, which usually results in a decrease in the hepatic production of SAA. Indeed, a significant reduction in proteinuria can be detected in patients with AA amyloidosis when the underlying inflammatory disease is effectively treated (Elkayam *et al.* 2002), and maintaining the serum levels of SAA below 5 mg/ml is usually associated with the regression of amyloid deposition and the maintainance of renal function (Pinney and Hawkins 2012). Besides colchicine, which is the key therapy for FMF (Goldfinger 1972), the regression in amyloid deposition has also been achieved by immunosuppressive strategies with biological agents, such as anti-TNF therapy or treatment with IL1R antagonists (Keersmaekers *et al.* 2009, Fernandez-Nebro *et al.* 2010), or by targeting the expression of SAA directly. Kluve-Beckerman *et al.* recently demonstrated a suppression of the production of SAA by utilizing antisense oligonucleotides. In their study, the serum levels of SAA in mice were decreased by 65% relative to controls, and the amyloid load was significantly lower (Kluve-Beckerman *et al.* 2011).

The third approach to fight AA amyloid deposition is to disturb the formation or stability of amyloid fibrils. Serum amyloid P (SAP) is a universal component of all amyloid deposits (Pepys *et al.* 1996), and strategies aiming to deplete SAP have been described (Botto *et al.* 1997, Pepys *et al.* 2002, Bodin *et al.* 2010). In addition, a new type of therapy utilizing small-molecule anionic sulfonates or sulfates is under study. These HS-resembling molecules inhibit the polymerization of AA fibrils by competing with the AA fibrils on binding to GAGs. They have been shown to decrease amyloid deposition in mice (Kisilevsky *et al.* 1995) as well as to have a beneficial effect on the deterioration rate of renal function in a preliminary study with human patients (Dember *et al.* 2007, Manenti *et al.* 2008, Rumjon *et al.* 2012).

Mast cell-derived tryptase was shown to promote the formation of fibril-like structures in the present study (Study I). However, the extent of this phenomenon *in vivo* requires further investigation. More importantly, despite its numerous known pathological features, tryptase appears to play protective roles as well, for example in neurogenic inflammation or gut infections (Caughey 2011), making the targeting of tryptase a rather challenging approach.

On the other hand, the demonstration of the role of SAA as a significant inflammatory mediator, as done in the present study (Study II), has obvious implications in terms of treatment prospects. Blocking the binding of SAA to its receptors would most likely be an unfeasible approach since SAA activates a number of receptors, which are also structurally different. Instead, SAA itself represents a potential target for anti-inflammatory therapies and it is also unique in the sense that blocking its harmful effects or reducing its serum levels would have an impact not only on AA amyloidosis but also on a range of inflammatory diseases and even on atherosclerosis. As stated, several approaches can be taken to inhibit the activity of SAA. In addition to targeting IL-1 and TNF, another feasible approach could be to reduce the levels of the third key cytokine that mediates the hepatic synthesis of SAA, IL-6. Interestingly, anti-IL-6 therapy by tocilizumab, a blocker of the IL-6 receptor (Smolen and Maini 2006), has indeed shown promising results, leading to its recent approval for use in the treatment of RA. Other conditions that might respond to the same treatment include systemic-onset juvenile idiopathic arthritis, adult-onset Still's disease, Castleman's disease and Crohn's disease (Navarro-Millan *et al.* 2012). Importantly, the blocking of IL-6R has also been associated with the normalization of the serum levels of SAA and with the relief of the clinical symptoms of AA amyloidosis (Okuda and Takasugi 2006, Magro-Checa *et al.* 2011).

The fact that serum lipoproteins are capable of dimishing the proinflammatory activity of SAA (Study III) is an interesting finding and raises questions in relation to treatment. One can speculate that therapies aiming to increase the concentration of HDL (Badimon and Vilahur 2012) could then be beneficial not only for the antioxidant effects and RCT but also for the direct regulation of the inflammatory reaction. However, how the inhibitory potential of LDL or oxidized lipoproteins could be applied to practice requires further study. Also, in the present work, the impact of HDL was assessed only from the point of view of lipid-free SAA. The antioxidant and anti-inflammatory features of HDL can become impaired in AP-HDL particles (Tölle *et al.* 2012). Furthermore, SAA-containing HDL and LDL are perhaps more efficiently trapped in the proteoglycan matrix in the intima, promoting lipoprotein modification and uptake by macrophages (O'Brien *et al.* 2005, Wilson *et al.* 2008, Chiba *et al.* 2011, King *et al.* 2011). Thus, the net effect for the organism could be harmful despite the possible decrease in the activity of SAA.

VI Summary and Conclusions

The aim of this thesis was to investigate the interplay between SAA and two types of innate immune system cells, human mast cells and macrophages, and the consequences of this interplay on the pathogenesis of AA amyloidosis, atherosclerosis and inflammation in general. Potential mechanisms for the regulation of SAA were also studied. The findings presented in the publications I-III and discussed above can be summarized as follows:

1) SAA is a potent inducer of degranulation and cytokine production in human mast cells, and these features can contribute to the development and progression of AA amyloidosis. We hypothesize a pathway with the following events. High levels of SAA activate mast cells to degranulate and to release neutral proteases, notably tryptase. Tryptase releases the N-terminus of SAA, and this amyloidogenic fragment can then function as a local amyloid-enhancing factor and induce AA fibrillogenesis. This may be enhanced by heparin, a component of mast cell granules as well as by mature amyloid fibrils. The secretion of IL-1β and TNF-α from mast cells by SAA further promotes the hepatic and/or extrahepatic expression of SAA, creating a positive feedback loop. In addition to mast cells, resident macrophages may potentially contribute to AA amyloidogenesis in two ways: via extracellular processing by cathepsin B, the secretion of which is also induced by SAA, and by enhancing the hepatic expression of SAA by IL-1β and TNF-a, also induced by SAA itself. The suggested chain of events is supported by many of the findings of the present study, as illustrated in Figure 9.

2) SAA activates the NLRP3 inflammasome in human macrophages, resulting in the secretion of mature and biologically active IL-1β. No additional activator is required as SAA is able to both prime the macrophages, i.e. induce the expression of *IL1B* and *NLRP3*, and activate the inflammasome. The SAA-induced priming depends on the receptors TLR2 and TLR4, and the activation of NLRP3 is mediated via direct interaction with the ATP receptor $P2X_7$ and an increase in cathepsin B activity. However, neither lysosomal destabilization nor fibril formation is observed. Thus, the exact mechanism for the activation of NLRP3 by SAA is yet to be elucidated. Considering the relevance of the innate immune

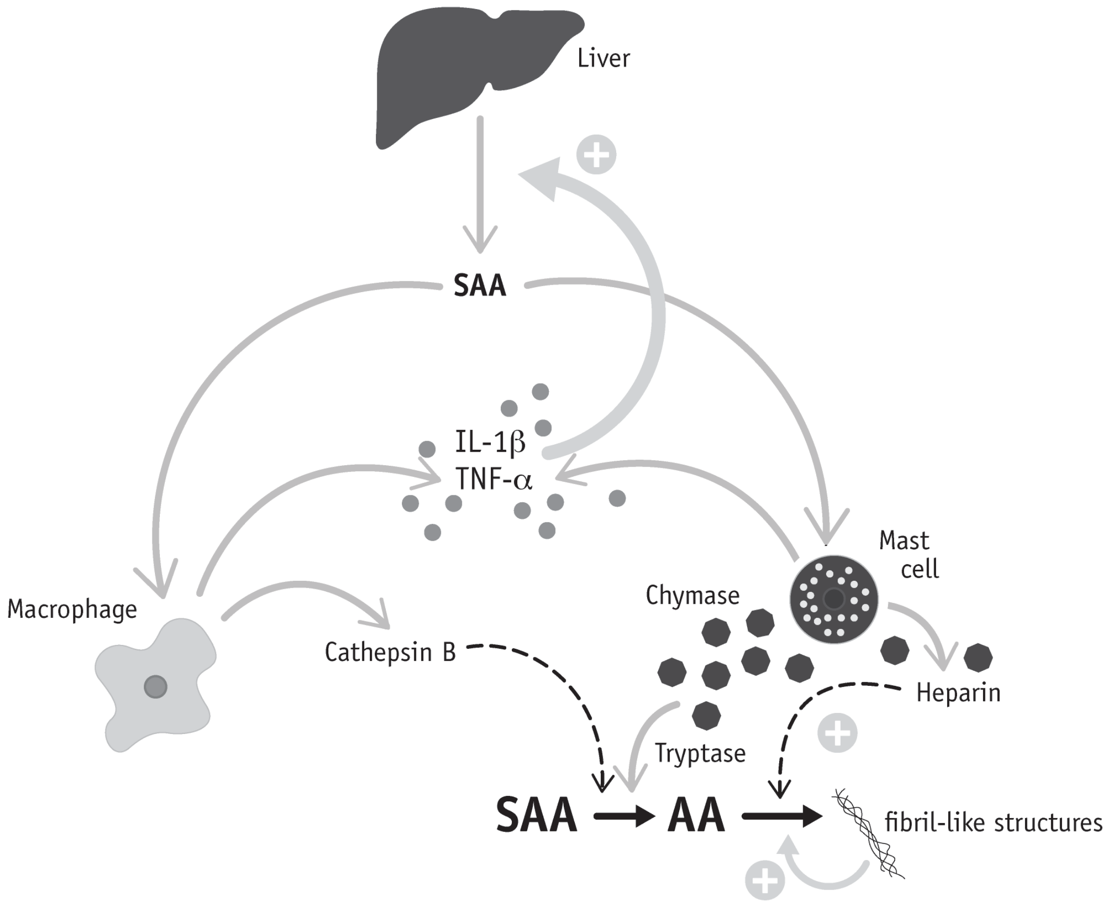

Figure 9. A schematic representation of the SAA-induced activation of human mast cells and macrophages and the proposed pathway for AA amyloidogenesis. The dashed arrows represent hypothetical pathways that were not experimentally verified in the present study. AA, amyloid A; IL, interleukin; SAA, serum amyloid A; TNF, tumor necrosis factor.

response in inflammatory diseases, including atherosclerosis, these findings could indicate SAA as a potential link between systemic and local inflammation. The findings of the present study are illustrated in Figure 10.

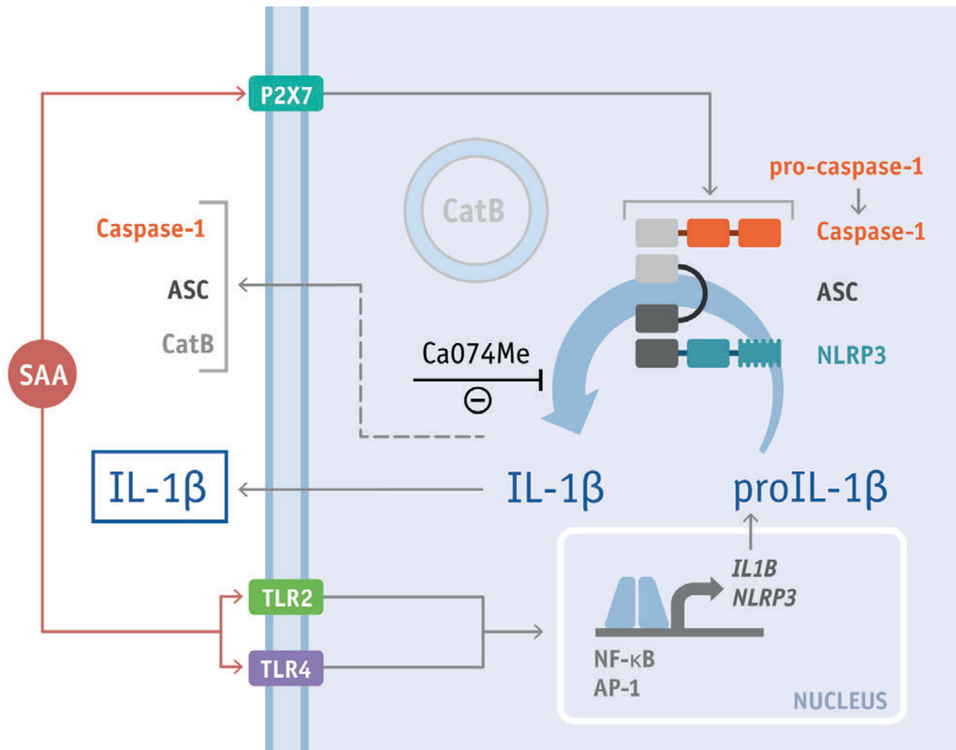

Figure 10. A schematic representation of the SAA-induced activation of NLRP3 in human macrophages. AP, activating protein; ASC, apoptosis-associated speck-like protein containing a CARD (caspase activation and recruitment domain); Ca074Me, cathepsin B inhibitor; CatB, cathepsin B; IL, interleukin; NF-κB, nuclear factor kappa B; NLRP, nucleotide-binding domain leucine-rich repeat containing receptor with a pyrin domain; SAA, serum amyloid A; TLR, Toll-like receptor.

3) The activity of SAA is regulated by serum lipoproteins. The ability of SAA to induce cytokine production in human macrophages is decreased not only by its common carrier HDL_3 but also by LDL. Furthermore, the oxidation of these lipoproteins enhances the inhibitory effect. In terms of the mechanism, divergent pathways are involved in the regulation of SAA by native and oxidized lipoproteins. Native lipoproteins most likely bind to SAA extracellularly and

prevent it from interacting with cell-surface receptors, such as TLR2 and TLR4, thus inhibiting the induction of *IL1B* by SAA. Oxidized lipoproteins also have a direct effect on macrophages, i.e they inhibit both the expression of *IL1B* and the activation of the NLRP3 inflammasome. These findings are presented in Figure 11. OxLDL inhibits the SAA-induced local inflammation also *in vivo*, as demonstrated in a mouse model of SAA-induced peritonitis. OxLDL may, thus, represent a novel and significant regulator of SAA activity in inflamed tissues, such as atherosclerotic lesions. Taken together, the presence of both native and oxidized lipoproteins in the circulation and tissues may represent an important regulatory mechanism by which the effects of the powerful proinflammatory factor SAA are regulated during inflammation.

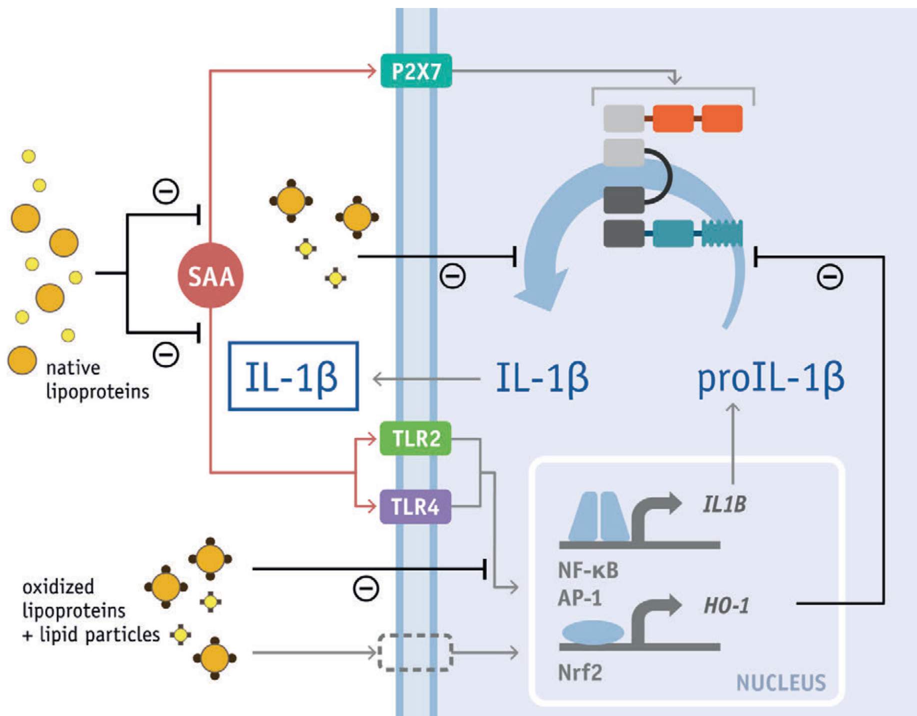

Figure 11. A schematic representation of the regulation of the SAA-induced activation of NLRP3 by serum lipoproteins in human macrophages. AP, activating protein; HO-1, heme oxygenase 1; IL, interleukin; NF-κB, nuclear factor kappa B; NLRP, nucleotide-binding domain leucine-rich repeat containing receptor with a pyrin domain; SAA, serum amyloid A; TLR, Toll-like receptor.

VII Acknowledgements

This study was carried out at the Protein Chemistry Unit of the University of Helsinki and at the Wihuri Research Institute in Helsinki, Finland. The experimental part of the project was financially supported by several sponsors: the Finnish Academy, the Päivikki and Sakari Sohlberg Foundation, the Biomedicum Helsinki Foundation, the Finnish Graduate School of Neuroscience (FGSN), the Orion-Farmos Research Foundation, the Aarne Koskelo Foundation and the Wihuri Research Institute maintained by the Jenny and Antti Wihuri Foundation. All sponsors are warmly acknowledged.

This thesis was supervised by docents Marc Baumann and Kari Eklund. I wish to thank Marc for guiding my steps in the field of proteomics and amyloid research and also for all the help outside the lab – for example, when one needs to find a new rental flat ASAP. I am most grateful to Kari for introducing me to mast cells, the most important cells in the universe, for showing me how to conduct good research in very rudimentary conditions (at Kirra!) and for believing in this project when I honestly did not. I also wish to thank professor Petri Kovanen for providing me the opportunity and facilities to continue the SAA research at Wihuri as well as for his guidance and help especially in the final stage of the thesis work.

I wish to express my gratitude to all my co-authors. I especially want to thank the "inflammasome team", Katariina Nurmi, Kristiina Rajamäki and Laura Teirilä, for sharing parts of the experimental work and docent Kati Öörni for sharing her expertise in lipoproteins and for always being so supportive and helpful. Ilona Kareinen, Jani Lappalainen and docents Sampsa Matikainen and Henrik Wolff are also warmly acknowledged.

I am very grateful to the official pre-examiners of my thesis, docents Pirkko Pussinen and Vesa Olkkonen, for their valuable comments and suggestions and for being so flexible with the changing schedules and locations as well. Docents Nisse Kalkkinen and Henrik Wolff are acknowledged for participating in my thesis follow-up group. Jutta Kölhi conducted the language revision of this thesis and I warmly thank her for her thorough and skilled work.

I wish to thank the great group of people at the Protein Chemistry Unit, many of which became "real-life" friends as well. Saara Tikka, my partner in crime in so many things, be it in eating candy (or cheesecake!) or in conquering the Grand Canyon, warmest thanks for all the support and great company over the years! You showed the way… Susanna Koskelainen is thanked for being a good and trusted friend and also for inviting me into her extended family in the role of a godmother. Docent Maciej Lalowski is acknowledged for his advice and hints concerning not only science but also Berlin; Rabah Soliymani for his kindness and patience with the mass spec analyses; Zuzana Demianová for all the great parties and company at music festivals; Reetta Rasi for the fun times during my years in Töölö; and Masahiko Shimmo for teaching me everything I know about fixing bicycles. I also

wish to thank Eemeli, Eeva, Giovanni, Janne, Joksa, Marika, Pekka and Yan Peng for the numerous enjoyable moments both inside and outside the lab.

My time at the Wihuri Research Institute was – unfortunately – much shorter overall, yet full of great memories. First and foremost, I wish to thank the supergirls Maija Atuegwu and Mari Jokinen, whose help, expertise and skills were simply priceless throughout my time there – a big big thank you! María Arraño de Kivikko, Jarmo Koponen, Hanna Lähteenmäki, Pia Martikainen and Suvi Sokolnicki are also acknowledged for their excellent technical assistance. Laura Fellman is warmly thanked for her assistance in so many practical things, such as the stressful paper handling this autumn. I also wish to thank Satu Lehti for the joyful office sharing (sometimes in very weird temperatures!), Anna Oksaharju for the "peer support" regarding the practicalities around completing a PhD as well as everybody else (and those who have not yet been mentioned) – Andrea, Hanna H, Jaakko, Kata L, Kata M, Mervi, Miriam, Pikku-Suvi, Reija, Riia, Steffi, Su, Terttu and Tiia – for making the Wihuri lab such a pleasant place to work at.

I also wish to thank all my friends near and far who, especially during this last year, were always there, ready to talk, chat, cheer, drag me out for lunch or just make me forget the SAA stuff for a while. There were times when a little "how are you?" message or a surprise phone call truly felt like the most precious thing in the world. Special thanks go to the "old gang", Heli, Lasse and Anu, who have been around since the days of first-ever bike rides, snow castles and teenage struggles. It is beautiful and also comforting to know that some things remain the same even when everything else changes. Katja, Tania, Satu, Helena and Nell are also warmly acknowledged for all the sharing and caring over the years and my "Berlin crew" for all the fun moments over brunches, dinners and beers.

I am deeply grateful to my parents Tuula and Martti for always being there for me and for supporting me with whatever choices I have made in my life. Auntie Piippi is acknowledged for being simply the best aunt one can have and for running the five-star "Hotel Kukkonen" for me while commuting from Berlin. I also wish to thank my Brazilian family and friends, especially my mother-in-law Eleanora and my sister-in-law Paula for caring and for showing interest in my work.

Lastly I wish to express my deepest and most sincere gratitude to my husband Vitório whose contribution exceeds all scales – there is absolutely no way I could have finished this project without him. My dear V, thank you for loving, supporting and believing in me, for being so unbelievably patient, for giving this thesis the great look that it has now and, above all, for always, always making me laugh. I am the luckiest.

Berlin, November 2012

Katri Niemi

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