Chapter 6

The Invariant Peptide Clusters of Serum Amyloid A Are Humoral Checkpoints for Vital Innate Functions as Probed by Monoclonal Antibodies, Including in Sepsis: Induction by Febrile Temperatures and Path of Discoveries

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Additional information is available at the end of the chapter

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Abstract

Serum amyloid A (SAA) is the most prominent acute-phase protein in vertebrates and its role in innate immunity has been reviewed. SAA functions are located on special regions of SAA, which are highly conserved in all vertebrates. 1. The discovery of the acute-phase nature of SAA before its existence was known by experimental murine AA amyloidosis induced by septic conditions. 2. Identification of the amyloid substance and its precursor. 3. SAA changes its conformation and antigenic presentation when it is separated from HDL during the acute phase. Febrile temperatures activate SAA through the separation from HDL. There is a temperature-specific gradual separation of SAA isotypes or groups of isotypes from HDL. 4. Generic monoclonal AA antibodies mc4 and mc29 assist in elucidating selected SAAs' vital functions (as in defense, platelet functions, female propagation and others). 5. In a murine sepsis model, the monoclonals mc4 and mc29 can cause early death while the intact SAA can prevent this. Through this, a checkpoint ("stop and go") for survival was discovered. Generic monoclonals can also identify the life-saving structures of SAA's vital functions and those of other acute-phase proteins. This principle is essential for the production of novel drugs against sepsis and other innate-related diseases. 6. Some remarks follow.

Keywords: amyloidosis, serum amyloid A (SAA), febrile temperatures, inflammations, sepsis, invariant checkpoints, generic monoclonal antibodies, innate immunity, therapeutic options

1. The acute-phase nature of an enigmatic amyloid precursor in early experiments

1.1. Microscopic morphologic evidence

The task of my earliest work in pathology was to find out whether there was a soluble serum precursor for amyloidosis (This spacious scientific field has been reviewed pain part recently) [1, 2]. In experimental murine amyloidosis induced by septic conditions [3], morphologically significant signs are visible in **Figure 1** showing large amounts of hepatic amyloid (white areas), now recognized as amyloid A (AA). This amyloid was first deposited at the sites where the blood stream entered from the triangle of Glisson into the liver capillaries. The entire branches of blood vessels entering the liver parenchyma (left large vessel) are decorated with amyloid and, on the other hand, the branches that allow the blood to leave the liver are devoid of amyloid (right large vessel). This implies that the amyloidogenic protein is being deposited immediately when it reaches the liver upon assumed changing conditions (still unknown) by which amyloid is deposited from a soluble precursor. With time, the deposits grow by

Figure 1. Photomicrograph of murine AA amyloidosis. Tissue section showing hepatic capillary amyloid deposits (white areas) induced by septic multi-microbial exposure after 25 days. The amyloid is deposited under pressure visible as the rough liver surface (at the top), which is usually sleek. Formalin-fixation, 4–6 μm paraffin section, HE-staining, magnification 24.1× [4].

apposition toward the central liver vein (darker areas) until most of the liver is transformed into amyloid in a fatal amyloidosis. This behavior requires a consistent, steady and very fast transformation of an assumed precursor to amyloid by entering the liver capillaries. The amyloid is deposited under pressure and visible as the rough liver surface (at the top), which is usually sleek.

1.2. Relative parabiotic barrier

When the assumed precursor is present in blood, as suggested by morphologic evidence, it should cross the anastomosis between artificial Siamese twins (parabiosis) with one partner induced to develop amyloidosis through septic conditions. However, this transmission does not always occur. The septic partner developed amyloid in 92.5% of pairs and the untreated partner in only 13.4%, and the latter was statistically not different to that seen in control pairs without any treatment [4]. The failure of crossing the anastomosis was excluded by ^{51}Cr-tagged erythrocytes [5]. Since the anastomosis was fully permeable, this type of parabiotic barrier was not an absolute one but a relative one caused by a short half-life of the agent that was removed from the bloodstream before it could cross the permeable anastomosis. The results of quantitation of the blood flow by ^{51}Cr erythrocytes, including a mathematical model of the exchange rate, shows that half of the blood was exchanged between the partners in 22.3 min. Therefore, a short half-life far below 22.3 min indicates a protein with a rapid clearance in minutes or even seconds, a property that is addressed today as an *acute-phase nature*, but still enigmatic precursor [5].

1.3. The clearance of SAA reported by other groups

The half-life of SAA1 and SAA2 in plasma of normal mice was reported for SAA-HDL as a T1/2 of 75–80 min and both isotypes were similar. However, when trace amounts of SAA were given, they were rapidly cleared [6]. Another report measured the clearance of the complex SAA-HDL for SAA1 T1/2 of 75 min and SAA2 T1/2 of 30 min, respectively. The clearance was delayed when both isotypes were bound to high-density lipoprotein (HDL) [7]. Both reports did not measure SAA under acute-phase conditions (APC). However, the report of Hoffman and Benditt [6] found a rapid clearance with trace amounts of SAA devoid of HDL and confirmed our data that are performed under a septic acute-phase condition that was to be observed when SAA was separated from HDL (see below).

2. Identification of the amyloid substance and isolation of its serum precursor

Amyloids of different clinical settings (also in animals, see **Figure 1**) represent characteristic fibrils under electron microscopy [8]. Therefore, for chemical identification of the amyloid, these fibrils had to be extracted in pure form followed by chromatographic isolation of the major amyloid protein for its chemical analysis by amino acid sequence analysis. The method of isolation of the pure amyloid fibrils was pioneered by Pras et al. [9]. The first amino acid sequence of an amyloid protein was published by Glenner et al. [10], which was derived from

a monoclonal immunoglobulin κ-light chain and was named ALκ. The first sequence identifying the chemical nature of inflammation-induced amyloid in monkey and human amyloid was published by Benditt et al. [11], which was named amyloid A (AA). The first anti-AA antibodies were prepared in rabbits where a serum protein in patients suffering from inflammations was detected immunochemically. This protein had an α_1-electrophoretic mobility and was in serum approximately 180 KDa by calibrated gel filtration [12] and thus ready to monitor the isolation of the soluble with anti-AA reactive precursor. This isolation of serum protein began in summer 1972 and was monitored with another rabbit anti-AA antibody. Its chromatographic separation from serum yielded a native 200 ± 20 kDa AA reactive protein, which was further chromatographically isolated in 5 M guanidine-HCl. The AA reactive protein had an α_2-electrophoretic mobility and a molecular size of 12.5 kDa. Since this new protein had the same N-terminal amino acid sequence as AA, it was named serum amyloid A (SAA) [13]. Since SAA was larger than AA, a limited proteolytic cleavage had to be presumed in order for the former to generate AA. During the isolation of SAA and its purification to one size by gel filtration, by isoelectric focusing, however, eight SAA bands of different isoelectric point named A-H were identified with anti-AA antibodies (with AAE as the major SAA species for the planned radioimmunoassay), thus indicating the first signs of a polymorphism of SAA [13]. In addition, in plasma, SAA is bound to HDL [14].

3. SAA-HDL and febrile temperatures

3.1. Temperature-induced structural changes of SAA in serum

When examining a patient's acute-phase serum (APS) with elevated SAA in immunodiffusion (ID) at different temperatures and different times using a polyclonal AA antiserum in comparison with isolated control AA, this resulted in the three different precipitation patterns presented in **Figure 2**. In (a), one recognizes a line of identity of AA with all four patients' sera as if the SAA (probably SAA1 and SAA2) reaction were done with pure SAA. At 4°C in (b), however, there is no reaction with SAA-HDL in serum. This is due to the hiding of the AA-reactive parts of SAA through HDL. However, when the temperature was switched to room temperature after the reaction in (b) at 4°C the SAA containing serum resulted in a strong line after releasing the SAA from HDL in (c) as seen in (a). However, different from the pattern in (a), the precipitation line of AA-anti-AA is somewhat independent of the SAA-anti-AA line, thus indicating that the homologous AA-anti-AA line seems to be more stable than the SAA-anti-AA line. These results show that SAA-HDL is stable in full APS at 4°C where the AA-reactive sites are covered by HDL. When at room temperature (in ID buffer), where SAA is released from HDL and is now accessible to antibodies for precipitation, it is reactive. Therefore, the separation of SAA from HDL is temperature dependent [15]. These results became only fully explainable through Section 3.2, where the separation of SAA from HDL became clear [14].

In addition, we prepared recombinant SAA2 and, when added to normal human serum, it was possible to repeat exactly that behavior reported in **Figure 2**. This shows that SAA alone can reproduce this phenomenon [16].

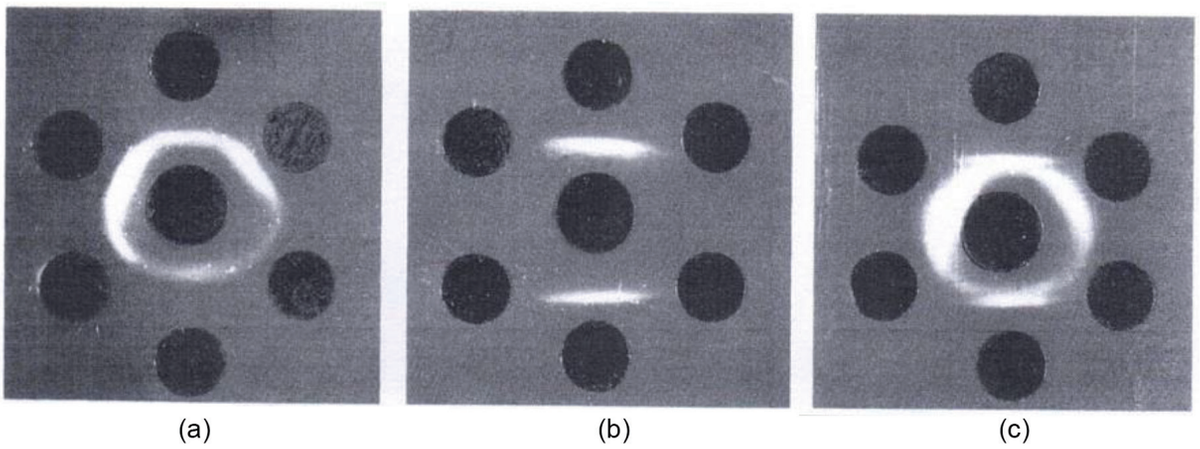

Figure 2. Immunochemical comparison of SAA-HDL, SAA and AA. Immunodiffusion (ID) at different temperatures [14, 15]. The ID was performed in 1.5% Seakem agarose in 0.03 M barbital buffer, pH 8.6 with the same reagents in each of the three plates à 6 wells ((a)-(c)). Top and bottom well contained AA (0.1 mg/ml), the middle well contained polyclonal rabbit anti-AA antibodies undiluted and the 4 side wells contained elevated SAA-HDL containing APS from 4 patients at 1/10 diluted. Plate (a) after diffusion over night at room temperature, plate (b) at 4°C over night and plate (c) first at 4°C over night as plate B at 4°C followed by room temperature for 6 h similar to plate (a).

3.2. The molecular size of the SAA and SAA-HDL at different febrile temperatures

Temperature-dependent molecular weight determination of AA-antigenic proteins of acute-phase serum (APS) has been performed using an ACA-34 gel filtration column in PBS with the enzyme inhibitor phenylmethylsulfonylfloride (PMSF) under various temperatures as shown in **Figure 3**. The size grading was done by the serum proteins IgM, IgG, albumin and, in addition, cytochrome C and the salt marker N-ε-DNP-lysine. The proteins were identified by way of the size position in the column by immunodiffusion as SAA-HDL at a size of ca. 180–200 kDa or SAA at 12.5 kDa. The different temperatures were kept with a temperature-controlled glass jacket, that is at 37°C in column run A, at 38°C in B, at 40°C in C and at 42°C in D. E was run as D, but without enzyme protection by PMSF, thus showing some degradation of SAA [18].

At a normal body temperature of 37°C, AA-containing proteins are at a single position as that of the SAA-HDL stable complex in A (fractions 34–37). However, already at 38°C, the stable complex SAA-HDL begins to dissociate as shown in **Figure 3**, run B. AA antigenic proteins appear at three positions, that is first of all at the void volume at fractions 19–20 (which has not been further analyzed, but could be related to aggregated SAA and/or its derivatives), secondly at the position of the stable SAA-HDL complex at fractions 34–36 and thirdly at the position of the HDL-free SAA at 53–56, as determined by the antigenic differentiation as seen in **Figure 2**. This size differentiation may also indicate functional heterogeneity, as the different affinities of SAA to HDL. This dissociation begins at 38°C and progresses with diminution of the SAA-HDL complex until run C. SAA-HDL disappeared at a "threshold of life" in run D at 42°C and above where the SAA species was maximized and the broadest was seen at fraction 53–56. This shows a temperature-induced gradual dissociation of SAA from HDL at the different febrile temperatures, which was shown here in vitro. This may also occur under systemic and local, acute-phase conditions, with the release of different SAA isotypes at different temperatures, for functions to be discovered. Finally, the SAA monomers released at different temperatures differ

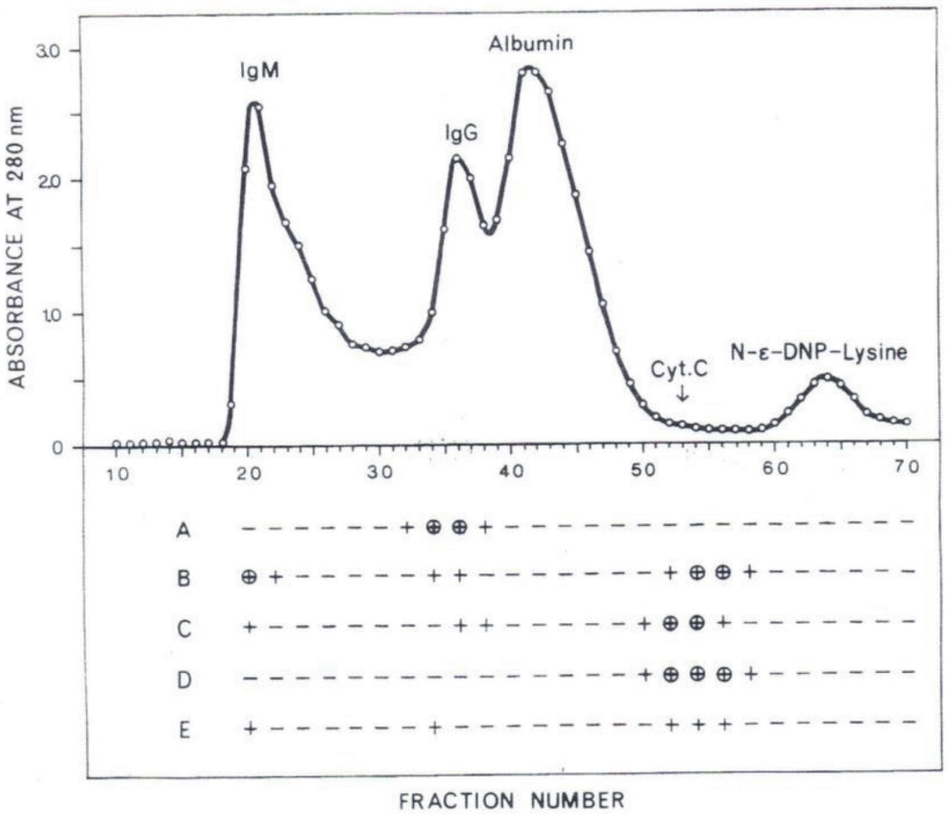

Figure 3. Size separation of SAA-HDL at febrile temperatures. SAA-HDL in a patient's acute-phase serum with a common cold was separated in A at 37°C, B at 38°C, C at 40°C and D at 42°C by gel filtration. All individual fractions (20–70) were examined and semi-quantified by ID using polyclonal rabbit anti-AA antibodies [15, 17].

in size. SAA in B is somewhat smaller than SAA in C. In addition, both appear at 42°C in D together as a broad combination of the two SAAs in B and D. In conclusion, SAA separated from HDL at 38°C in B has a lower affinity to HDL and is smaller, and SAA with a higher affinity for HDL is larger. Different isotypes and sizes of SAA are known [1, 2, 13]. The acute-phase SAAs, aSAA1 and aSAA2, are each 12.5 kDa with 104 amino acids and the constitutive SAA (cSAA), which is 14 kDa and has 112 amino acids. Since SAA1 has the lowest affinity for HDL and is the most amyloidogenic SAA, it could have separated from HDL in run at 38°C in B already, while SAA4, which is somewhat larger than the aSAAs, could be a component in the C. These indications can be solidified using isoelectric focusing or SAA-isotype-specific antibodies [1, 2].

3.3. Gradual dissociation of SAA-HDL during a continuous temperature gradient

While these experiments above were done stepwise, one by one, a more precise dissociation of the SAA-HDL separation was performed by electrophoresis in 1.5% agarose across a continuous temperature gradient in a single flat gel, as shown in **Figure 4**. The two sides between the agarose gel were kept at a constant temperature of 15°C in T1 and of 65°C in T2 [17].

The results in **Figure 4** show two horizontal bands of samples of one patient in the form of dots across the temperature gradient. The SAA-HDL band of α_1-electrophoretic mobility is marginally stained due to the concealing of the AA-antigenic determinants of SAA within

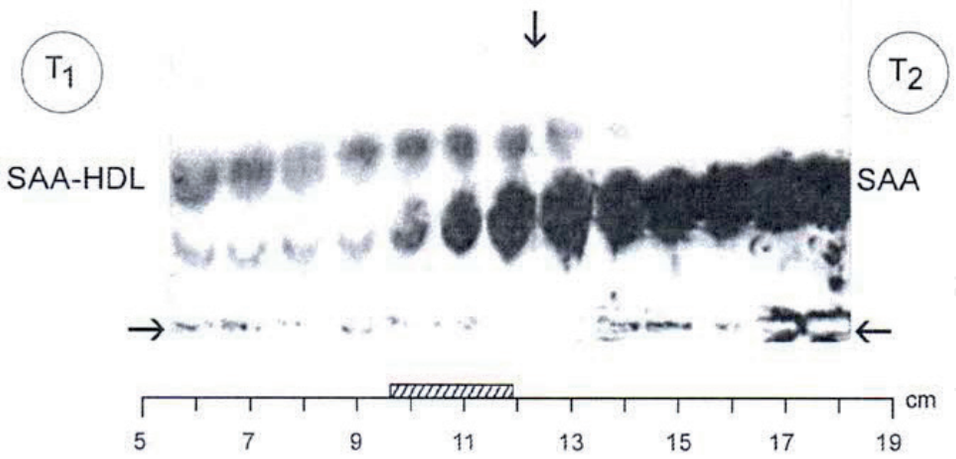

Figure 4. Electrophoresis of SAA-HDL across a continuous temperature gradient in agarose [18]. An APS was applied (see horizontal arrow) in 13 identical samples containing SAA-HDL across a temperature gradient. The small cross-hatched bar on the cm ruler denotes 37°C at the left margin and 42°C at the right one. Electrophoresis was done in 1.5% agarose (Seakem ME) in 25 mm barbital buffer, pH 8.6 followed by a standard Western blot developed with a mixture of the monoclonal anti-AA antibodies [19] mc1 + mc4 + mc29 at 1 + 1 + 1, 1/100 (see **Table 1**). One recognizes SAA-HDL at the left-side site marking the α_1-electrophoretic mobility (upper band). This band fades beginning from 38°C to 42°C, while the SAA freed from HDL (as in **Figure 3**) starts to appear at 38°C at the α_2-electrophoretic mobility with the very strong exposure of formerly concealed antigenic AA determinants detected after the strongest exposure appears at febrile temperatures between 38°C and 42°C (see cross-hatched bar). See vertical arrow at 42°C- 43°C.

the SAA-HDL complex at low temperatures. The hiding of the antigenic determinants disappeared gradually from 38°C (the 5th sample) until it is completely above 42°C with the appearance of large amounts of SAA (intensive staining of the band with α_2-mobility). By gradually increasing the temperature, the SAA release increases gradually while the SAA-HDL fades away up to the extreme exposure at 42°C and beyond, in agreement with the stepwise separation of SAA from HDL shown in **Figure 3**. Also important during this gradual temperature-induced separation from HDL seems to be that the morphology of the dots is different. They changed by their shape in the longitudinal direction, which is consistent with the fact that SAA is not uniform and consists of a group of homologous, but chemically different, SAAs demonstrating different isoelectric points (13 reviewed by [1, 2]). Another observation concerns the AA-antigenic species below 37°C in the first four samples. These slow, arc-like uniform samples could represent an SAA species, which is always active as a monomer regardless of temperature. This species seems to be less acidic. It could be a type of SAA species for the general protection under normal condition. In sample 5, this "arc SAA" is overlaid by a more acidic SAA released from the acute-phase proteins (APPs) SAA1 or SAA2, thus changing the spots to a more longitudinal pattern. With increasing temperature, the SAA spots become thicker and increase more in the longitudinal direction. This "arc SAA" needs to be analyzed since it does not seem to be part of the intact SAA-HDL complex (we did not check for SAA4). Finally, far above a temperature of 42°C, the SAA species seems to be stable. Parallel to the gradual release of SAA, at the same time, the SAA-HDL complex while losing SAA is gaining more negative charges with increased temperatures. Moreover, the trailing of SAA in samples 5–8 possibly indicates the gradual separation of differently charged SAA species.

3.4. Activation of the SAA by separation from HDL under febrile temperatures and consequences

Taken together [15, 17, 18, 21], it is clear that the mechanism of separation of SAA from HDL in vitro is also strictly regulated in vivo by body temperatures above 37°C. Therefore, this is a key mechanism that can be induced and activated basically by two different manifestations. The most common is the orthologic APR activation [33] of SAA. This occurs with a maximal SAA concentration of up to 1000 times within a day as a systemic "biochemical thunderstorm" with a myriad of activating and inhibiting events simultaneously, which are not understood in detail today [1, 2]. During these events, the cause of the APR will be eradicated and the APR becomes curative. With this beneficial outcome, the normal immune homeostasis returns in a foreseeable future. However, when the APR cannot overcome its initial cause, it will become a pathologic APR [33] with a "persistent biochemical thunderstorm" and lacking a self-driven cure. The consequences can be summarized in an exhaustion of the resources of the organism and decline of the metabolic activity through a multitude of clinically challenging conditions exemplified by severe viral and bacterial chronic inflammations, systemic inflammatory response syndrome (SIRS) or uncontrolled chronic infections, sepsis and septic shock [1, 2]. Moreover, when the infection remains limited, a local APR will take care of it.

The functions of the four human isotypes, SAA1, SAA2, (SAA3 in humans is only transcribed in some cells) and SAA4 have not been fully analyzed. They have arisen through gene duplications, thus indicating important individual functions either alone or in combination. As described before, the human acute-phase A-SAA has two very similar isotypes, A-SAA1 and A-SAA2, in the APR mostly synthesized in the liver and expressed in most body cells (see below) and the constitutive C-SAA4 and some allotypes in SAA1 and SAA2. For a review of the SAA heterogeneity and its known functions, see the reviews [1, 2].

Another discovery was the discontinuous separation of SAA from HDL described above at different temperatures, meaning that not all SAA molecules are being separated from HDL at a single temperature except for the temperature of 42°C (**Figures 3** and **4**). In fact, these figures show that the separation of SAA is spreading out over the whole febrile temperature range starting from 38°C to 42°C and above. In addition, based on these observations in **Figure 4**, it is possible that SAA isotypes and allotypes are separated from HDL at different febrile temperatures and thereafter fulfill their different functions locally or systematically as individual SAA species as is also to be derived from **Figure 3**. Another indication for the differential release of the SAA species can be detected in **Figure 4** in the different shapes of the protein blots of the SAAs devoid of HDL, thus indicating possible SAAs with distinct isoelectric points (IP). In **Figure 4**, there are free dots before 37°C named (for convenience) "arc SAA," the least acidic SAA. The SAA species released from HDL after 37°C ("38 SAA") are probably the more acidic ones. In this sense, the dot changes also occur later on 39°C-, 40°C-released SAA, etc. Analyzing the spots for the identity of the various SAA species could show whether these indications did discover a mechanism by which the different SAA species can be released from HDL and thereby are being activated at specific temperatures alone or with other SAAs for special purposes, which need to be analyzed. These points may also be of therapeutical interest. This proposed temperature selection of SAA isotypes could specify the needed APR function for a specific purpose. The increase of the organism's temperature is being induced

by the organism as a response to various stimuli, exemplified by bacterial invasion. It could represent some sort of a "gear shift" for providing a graded response in order to release special SAAs to provide adequate amounts, which are necessary "tools" for survival. This could occur in concert with other agents including other APPs and cytokines of the APR network. The possible therapeutical manipulation of the body's temperature ("the gear shift") in vivo needs the precise analysis of this phenomenon in vivo first.

4. Application of antibodies

4.1. Polyclonal and monoclonal antibodies prepared against AA and SAA

In a collaborative study, each of the eight species-specific polyclonal AA antibodies against eight species (including humans) was immunohistochemically tested against the AA amyloids of eleven different species, including those of humans. The results showed a strong reactivity only with the homologous species and with only some cross-reaction with a related species. The reactivity was in general species specific, but a universal generic AA antibody could not be obtained in these eight polyclonal antibodies [37].

The next step was to produce murine monoclonal antibodies against AA and SAA [20]. Their value and merit have been documented by the inventors Köhler and Milstein [38]. Monoclonal antibodies are represented by one amino acid sequence and have the value of a chemical reagent. We selected 20 stable clones (see **Table 1**), which were epitope mapped [31] and immunohistochemically tested on AA amyloids in 10 different mammals, many humans and 9 different birds. Some cross-reactivity with some monoclonals was detected. Most of the 19 AA amyloids tested could be identified with the two monoclonals mc4 and mc29, showing that most of these AA amyloids have some peptides in common and these antibodies recognize the same or very similar epitopes of AA in different species. In addition, antibodies of all clones were tested for binding with 15 synthetic SAA peptides in only 4 clones the epitope could be identified. These included the known clones mc4 and mc29 (see above), and the two new ones, mc1 and mc20 (see **Table 1**). In APS, two different charge variants of SAA have been detected with these monoclonals [22].

The cause of the failing reactivity of most of the synthetic peptides with most of the monoclonals may be due to the presence of more discontinuous epitopes. This could also be deduced from the fact that SAA shows multiple short peptides that alternate between the invariable (red) and the variable (white) peptides, as shown in **Figure 5** (see also below).

Moreover, since mc21 was negative with the linear peptides, but reacted very strongly with AA amyloid in tissues, it was epitope mapped differently. It was mapped with endoproteinase Asp-N-generated peptides from a pure and partially amino acid-sequenced human AA (KIR) protein of 8.4 kDa. Of the 11 distinct peptides separated by RP-HPLC, mc21 reacted only with a single peptide, which was aa 33–42 of SAA [32]. This peptide is almost identical with the largest invariant peptide of SAA (see **Figure 5**). Two other monoclonals mc9 and mc13 did not show any reaction with these 11 distinct HPLC peaks [32] although they were reactive with AA in tissue sections. Here again, in linear SAA peptides, the discontinuous epitopes of SAA may not be preserved.

For notes	Clone no	Internal lab K-Nr.	Isotype	Quality			References (selected)
				Epitope and SAA peptides	Usage IHC	Usage EM	
	mc 1*	17	IgG 2a κ	5–16	+++	+++	[17, 20–28]
				7–15			
	mc 2				++		[22]
	mc 3	33			+		
	mc 4	34	IgG 1 κ	19–31	+++	+++	[17, 20–25, 29, 30]
	mc 8	38, 57	IgG 3 κ	25–76	++	0	[20, 22, 25]
	mc 9	39, 41, 53	IgG 1 κ		+++	0	[20, 22, 25, 31]
	mc 12	40, 42	IgG 2b κ	25–76	++	0	[20, 22, 25, 31]
	mc 13	43, 54	IgG 1 κ		+++	++	[20, 22, 24, 25]
	mc 15	45			+		
	mc 17	47			+		
	mc 20	50, 28	IgG 2a κ	60–75	++	+++	[17, 20–22, 25, 31]
		60		25–76			
	mc 21	65	IgG 1 κ	33-42	+++		[32]
	mc 22	70	IgG 2b κ		+	0	[25]
	mc 23	63	IgG 1 κ		+		
	mc 25	55, 124, 125, 126			++		
	mc 27	77, 127	IgM κ		++		
	mc 28	58, 128			+++	+	
	mc 29	129	IgG 1 κ	28–40	+++	+++	[17, 21, 24–31, 33–36]
				25–76			
	mc 30	130	IgG 1 κ		+++		
	mc 31	131	IgG 1 κ		+	0	[25]

Explanations: IHC, immunohistochemistry; EM, immunoelectron histochemistry.*Available from Dako, Denmark.

Table 1. Monoclonal AA and SAA antibodies [20].

Therefore, another strategy for the epitope mapping has been worked out that is the cooperative precipitation with either the antigens AA or SAA in 1.5% agarose gel. Applied were various combinations of two different monoclonals on one antigen, respectively. A precipitation showed that the two given monoclonals react with two epitopes. This approach resulted in precipitations and the epitope could be estimated roughly in some of the antibodies (unpublished). This has been expected since all the AA/SAA antibodies were selected by reactivity with amyloid in tissues. Finally, precipitation with SAA but not with AA pointed to a monoclonal against the SL peptide (see **Figure 5**, aa 77–104). Similarly, SAA isotype-specific monoclonals could have been selected by a similar approach.

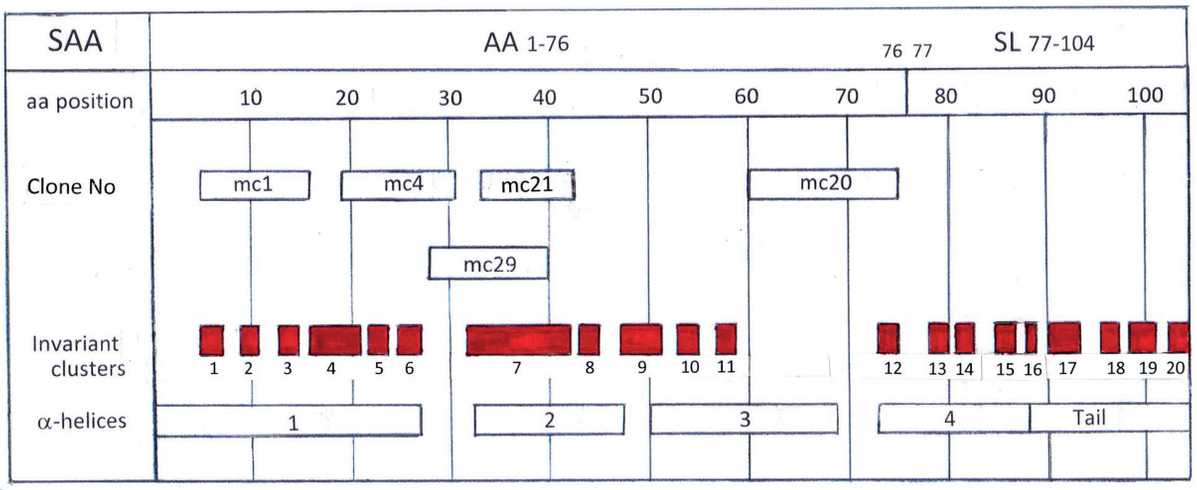

Figure 5. Structure of SAA and epitope-mapped AA monoclonal antibodies. The structure of SAA 1–104 and its fragments AA 1-76 and SL-77-104 with invariant (in red) and variant (white in between the red) peptides and epitope mapped of five monoclonal AA/SAA antibodies. There are three species-independent (mc4, mc21 and mc29), and two variant as well as species-specific (mc1 and mc20) monoclonal antibodies against AA and SAA. The positions of epitopes on the α-helical coils 1–4 and the contribution of the invariable (red) and variable peptides are also visible.

4.2. Identification of functional SAA epitopes by the monoclonals: finding their use and detecting their function

4.2.1. Diagnostic application

Monoclonals (see **Table 1**) are being applied for classification of AA amyloid in tissue sections when a recent amyloidosis was diagnosed in a patient or in an animal. Every amyloid has to be classified for therapeutic and prognostic purposes. This is exemplified in exploiting the generic monoclonals mc4 and mc29 (in Refs. [23, 24]) by either the immunohistochemical classification (IHC) on paraffin section of animal AA amyloidosis [23, 24] or by the immunoelectron microscopic classification (EM) of human AA amyloid on ultrathin sections [25].

4.2.2. The structure of SAA

The SAA1 and SAA2 proteins are presented in **Figure 5** as a continuous string with 1–104 amino acids. SAA consists of two parts, the N-terminal AA 1–76 polypeptide, which causes AA amyloidosis in humans [11] and animals under unfavorable inflammatory conditions [23, 24], and the C-terminal SL 77–104 polypeptide, whose function is stabilizing the two double coils [39, 40]. **Figure 5** was constructed using data from the USCS Genome Browser (GRCH38hg38) Assembly, as reported by 2.

The structure of SAA consists of four α-helical coils, 1–4, with 1–27, 32–47, 50–69 and 73–88 aa in length, respectively, followed by a tail after the 4th coil. These four coils are arranged in two antiparallel double coils, whereby 1 joins 3 and 2 joins 4 [39]. Each α-helix and its tail contain alternating blocks of twenty invariable peptides (**Figure 5**, in red, numbered 1–20). The variable peptides that can be species specific are the white, unstained sites in between the invariable peptides. Variables are also the peptides joining the coils, which

represent the turns. In addition, the tail is winding around these double coils for stabilization [39, 40]. This conformation of SAA with the short-distance, alternating peptides in red and white would need more exact three-dimensional analyses for identifying the proposed discontinuities of peptides based on the partial un-reactivity of the monoclonals with linear peptides (see above).

4.2.3. The invariant peptide clusters of SAA and the binding sites of the SAA monoclonal antibodies

The monoclonal antibodies can be divided into three categories by the kind of epitope onto which they bind. In (a), they bind to species-specific epitopes and could perhaps also be detected with polyclonal antibodies [37]; in (b), they bind to AA amyloid in tissue section, but not to synthetic linear peptides, and are probably reacting with discontinuous epitopes of the SAA, and in (c), they bind to species independent, i.e., the invariant peptides and epitopes of AA and SAA, which are almost identical throughout the vertebrates (reviewed in [1, 2]). These special antibodies can also be called "generic" AA/SAA antibodies. Generic SAA antibodies are mc4, mc21 and mc29 (the latter contains also an additional specificity; see later). The two monoclonals that functionally bind to variable epitopes are mc1 and mc20 and belong to a category in (a). All monoclonals and their known binding synthetic peptides are listed in **Table 1**, together with their binding to patients' and animals' AA amyloid in formalin-fixed paraffin tissue sections [20, 23, 24] and ultrathin sections for EM [25].

The binding sites of the monoclonals to SAA are shown in **Figure 5**. The invariant parts of SAA contain ancient peptide clusters preserved during their evolution from the lampreys (over 500 Mio years without hardly any changes, Wikipedia) to the mammals, including humans. Again, we as humans have the invariable peptides of SAA in common with all vertebrates and the lamprey. Therefore, these special peptides have to be of utmost importance for mechanisms related to the proteostasis of many systems. They become extremely activated when in imbalance, exemplified by injuries and inflammation or bacterial infection, and in the event that their activation cannot be resolved. This can result in a sepsis. Thus, one can assume with some likelihood that a single amino acid exchange in these 19 invariant areas must not have been accepted throughout evolution. Indications are in the literature that natural SAA behaves differently as compared to recombinant SAA or SAA with a single amino acid change or exchange [1, 2]. The importance of the proteins of the SAA family for survival can therefore hardly be overestimated and the phrase that SAA is "the hub in the interaction network" [40] can express this eminent role of the SAA.

4.2.4. Properties of the individual monoclonals against AA presented here

4.2.4.1. mc1

This monoclonal antibody mc1 (see **Table 1** and **Figure 5**) is of interest since it binds to the most N-terminally positioned human-specific epitope on SAA (aa 5–16), but only when it is devoid of HDL. Thus, SAA can be distinguished from SAA-HDL through the failure of mc1 to bind to the complex of SAA-HDL, since HLD conceals the mc1 epitope of SAA [15].

Recombinant SAA shows the same binding to HDL and the same temperature release of SAA from HDL in vitro [21]. When separated from HDL under febrile temperatures, it can rebind again to HDL at body (or lower) temperatures. The binding is therefore reversible except when the temperature is above 41°C for some time, when it probably aggregates irreversibly [34]. The temperature-dependent mechanism has been proposed for activating the SAAs (see **Figures 2–4**). When fever is systemic, the free SAA load is part of the systemic APR. When local febrile temperatures are induced by local injuries or infection, a local APR is induced with local SAA. This can also apply for local tumors (see below). How this acts is not fully known. Where is the mc1 epitope located, considering that three invariant peptides are located within the 5–16 peptide stretch? Since the specificity is human specific, these peptides should be among the 2–3 variant areas; those are probably the white areas of the 5–16 peptide stretch; see **Figure 5**. In addition, this mc1 epitope (aa 5–16) has an overlap of 7 [5–11] of the 11 aa residues with the presumptive lipid-binding site (aa 1–11) (established by Turnell et al. [41]). Finally, mc1 binds very reliably to human (and some primate) AA in fixed paraffin sections and in ultrathin sections for EM [25], and not to SAA-HDL in serum at lower body temperatures (see above). Therefore, this murine monoclonal anti-AA mc1 has become a standard for examining human AA and SAA (available from Dako).

4.2.4.2. mc20

In tissue sections, this monoclonal (see **Table 1** and **Figure 5**) reliably binds to human AA amyloid and is being used for diagnostic purposes. It binds to the synthetic peptide aa 60–75 of SAA, which is located at the longest variable peptide stretch of SAA and located at the C-terminal half of the third α-helical coil, and, to a minor extent, at the small N-terminal part of the fourth coil, which contains the first invariable peptide no. 12. We do not know whether this mini peptide is part of the mc20 paratope.

4.2.4.3. mc4, mc21 and mc29

These antibodies demonstrated immunohistochemical, species-independent binding to most AA-type amyloids of the vertebrates (see above). They were therefore directed against the invariant peptides of SAA, which are located on the first and second α-helical coils. Their extent and their differences are depicted in **Figure 5**. The clone mc4 reacts largely with the invariant peptide no. 4–6 on coil 1. This clone binds differently as compared to mc21 and mc29, both of which bind to the 7th peptide of coil 2, the largest invariant peptide of SAA. While mc21 seems to be only reacting with peptide no. 7, the monoclonal mc29 extends to the variant joining peptide area (aa 28–32) that is between coil 1 and coil 2. This may explain the additional, partial binding of mc29 to the variable peptides. In addition, it binds to most of the animal AA amyloids tested (see below).

4.2.4.4. mc1, mc4, mc13, mc29 and mc31

This series of monoclonal antibodies has been probed and exerted to establish a monoclonal micro-ELISA for quantification of SAA [42].

5. On SAAs' functions and their identification by blocking the invariant epitopes of SAA

5.1. The multifunctional SAA family

Various functions of SAA have been reported, including the systemic and local elevation of SAA in inflammations in an APR due to the systemic and local cytokine increase. SAA is involved in very many functions as being an opsonin of Gram-negative bacteria, a chemoattractant, an inducer of chemokines and cytokines, a stimulator of angiogenesis, important in cholesterol transport and a modulator in the migration of white blood cells. SAA acts concentration dependently on polymorphonuclear cells and the degradation of SAA (by matrix degradation enzymes?), which can release the AA 1–76 fragment and can thereby induce the fatal AA amyloidosis in humans and animals. Other fragments of SAA and other APPs may, in vivo, influence this still not understood complex network of the SAA family, which has been reviewed in [1, 2, 43, 44]. Here, some of these vital functions of SAA have been identified by blocking these functions by way of monoclonal AA/SAA antibodies. At the same time, the SAA binding motives have been localized at the surface of SAA (see **Figure 5**). Alternatively, these ligands for the SAA binding motives can, in part, be blocked with the respective synthetic peptides of SAA [44, 45].

5.2. HDL binding site

The HDL binding site of SAA was identified as the peptide aa 5–17 with the monoclonal mc1 (see Section 4). The presumptive estimate by Turnell et al. [41] was aa 1–11.

5.3. Human neutrophils

Strong binding of isolated, acute-phase human SAA (and recombinant SAA2, not presented) were shown with human neutrophils [33] assuming the existence of an SAA receptor, which may have regulatory functions [1, 2]. The FMLP-induced oxidative burst of normal human neutrophils could be reduced, concentration dependently, by SAA at concentrations of 0.1 μg/ml and 1.0 μg/ml. This inhibitory reduction of SAA could be blocked by the monoclonal antibody mc29 (see **Table 1** and **Figure 5**), which binds to the synthetic peptide aa 28–40 of SAA, thus proving that this blocked area is responsible for this inhibitory effect of SAA [33]. This was the first time that a function of SAA was blocked by a monoclonal AA antibody. Moreover, at the same time, the responsible peptide of SAA was identified, which was the invariant peptide no. 7 of coil 2. The monoclonal antibody mc29 used probably also blocks the laminin-like domain (aa 29–33) and may also be participating partially with the RGD-like domain (aa 39–41). In addition, human neutrophils were exposed to full human APS at different temperatures [34]. At 41°C, the inhibition of the oxidative burst was much stronger than at 37°C, indicating the role of SAA freed from HDL and in its active state (see Section 3.4; see **Figures 3** and **4**). However, when the acute-phase serum was preheated to 41°C for 15 min and assayed at 37°C, the SAA-containing serum did not return to the 37°C value, but stayed with the increased 41°C inhibiting effect at 37°C. This indicated an irreversible structural change

of SAA (or its fragments) during high fever, which is blocking SAA's return to the reversible binding to HDL. (This febrile temperature that induced the aggregation of AA-antigenic proteins has also been noticed in vitro and documented in **Figure 3** at 38°C and 40°C). The possibly unfavorable consequences of these aggregates in humans or animals are unknown today.

5.4. Anti-inflammatory potential of SAA on neutrophils

The anti-inflammatory potential of SAA on neutrophils [33] has been confirmed for SAA at reported serum concentrations [46]. Oxidative burst, migration and the neutrophil myeloperoxidase release were also inhibited. SAA peptides (aa 1–14, 15–101 and 83–104) also contributed to this inhibitory effect. However, at higher concentrations of more than 50 μg/ml, SAA was stimulating. In addition, O_2 release was inhibited up to 0.1 μg/ml, but the O_2 release was increased above that. Thus, SAA plays a dual role, it downregulates inflammatory processes in lower concentration, but, during the full APR, the action of SAA can be promoting.

5.5. SAA functions inhibited by synthetic peptides

SAA functions can be identified by SAA-generic antibodies [33, 34, 46] but they can also be blocked by synthetic peptides of SAA [45]. This was shown through the use of a 14mer synthetic peptide (aa 29–42) of SAA. This peptide inhibited the binding of T lymphocytes and mouse M4 melanoma cells to adhesive glycoproteins of the extra cellular matrix. This SAA 14mer peptide contained the laminin-like (aa 29–33) and fibronectin-like (aa 13–15) domains of the extracellular matrix. Finally, by extending these data of the 14mer SAA peptide, by comparing to the binding of our generic antibodies mc21 and mc29, it is to be said that these antibodies bind to a similar peptide of SAA, which is the largest invariable peptide (no. 7 of coil 2) as shown in **Figure 5**.

5.6. Phagocytosis

Phagocytosis was examined on fixed bacteria by normal and stimulated blood monocytes at the SAA concentration that were inhibitory to human neutrophil activation [33]. There was no difference in phagocytosis in the presence or in the absence of SAA [47].

5.7. Platelets and binding motives similar to SAA

Human platelet adhesion was shown to immobilize SAA and the mechanism of binding was examined [35]. Among the many receptors on platelets, the receptors for laminin and fibronectin were chosen to be examined because SAA has laminin-like and fibronectin-like motives in its sequence. Immobilized SAA binds platelets as do fibronectin and, to a lesser degree, fibrinogen. This binding of SAA to platelets was completely abolished by anti-SAA (mc29), which binds to the laminin-like motive on SAA (aa 29–33) that is part of the mc29-binding peptide. Also, a 29–42-containing peptide could inhibit the binding of platelets to SAA. In addition, an antibody against an integrin receptor also inhibited the binding as well the RGD-containing peptide GRGDSP. Also, the anti-SAA (mc29) did not inhibit the RGD-dependent binding motive to a significant extent, thus indicating that the overlap of two amino acids (aa 39–40) of the peptide

(see **Figure 5**) did not lead to an efficient paratope subsite of mc29 for the method applied. Finally, all controls were in line with the conclusion that SAA was binding to platelets via the laminin-like and fibronectin-like motives. Since the related binding motives are not chemically identical with laminin or fibronectin, they could have a lower affinity, which may be exerted differently at lower concentrations as compared with higher concentrations, i.e., during the APR.

Thus, SAA may play a role in inhibiting and modulating platelet adhesion at vascular injury sites by sharing platelet receptors with other platelet-adhesive proteins. In addition, depending on the kind of disease, the window between bleeding and thrombosis may sometimes be very narrow; how can it be widened? Finally, systemic and local thrombosis are not rare, which are life-threatening sequels of many conditions. These are related to arteriosclerosis, heart conditions, nutrition-related ailments, deranged lipid metabolism, smoking and other drugs, cancer, injuries, bacterial infections and sepsis, mostly in a more advanced age as well as in cases of vascular injury. The role of platelets is central in these and many other diseases, and the concentration-dependent role of SAA and its antidotes (humanized monoclonals and others) in vivo needs to be explored and then further developed.

5.8. Presence of SAA in human cancer and other cells

Intracellular SAA of colon tissue with cancer of progressing stages of anaplasia was examined on formalin-fixed paraffin sections from 26 patients with colon cancer (after SAA plasma levels were shown by others to be elevated in carcinomas, assuming that the elevated SAA is of hepatic origin) [26]. SAA was detected immunohistochemically by using the monoclonal antibodies mc1 and mc29 (specificity, see **Figure 5** and **Table 1**). On normal cells, no reaction or only traces were detected. However, stronger reactions were found in carcinoma cells. The staining intensity increased gradually from dysplasia to the stage of malignant neoplasia. The metastases also showed the presence of SAA, but weaker. In addition, cells, other than colon cells in these sections, also showed the presence of SAA as lymphoid cells of the intestinal wall, inflammatory cells, ganglion cells and endothelial cells. The presence of SAA has been confirmed by in situ hybridization and reverse transcriptase polymerase chain reaction (RT-PCR). The genes of SAA1 and SAA4 in the colon carcinomas were activated. Although the role of SAA in colon carcinoma is unknown, the close association of the increasing grade of malignancy with the increased SAA synthesis may indicate a role of SAA in tumorigenesis. SAA can serve as an adhesive ligand for tumor-cell homing; it induces inflammation, which may be neoplastic. It also induces migration and can be involved in metastasis, or it can be inhibitory to attachment [26].

5.9. Protein SAA enhances plasminogen activation and may contribute to tumor spread

The colon carcinoma cell line HT-29 showed plasminogen activity (PA) enhanced by SAA measured with a chromogenic substrate. This activity could be inhibited using monoclonal antibodies against SAA (mc1 + mc29). The cell line also produced endogenous SAA1 by itself, which could be augmented by exogenous SAA and also by cytokines IL-1b and IL-6. This activity was also inhibited in part by the monoclonal antibodies against SAA [36]. The concomitant overexpression and co-localization of SAA and PA in colon cancer cells raises the possibility of

a functional relationship between these two systems. The authors suggest that SAA produced in the malignant tissue may contribute to increased matrix degeneration and tumor spread [36].

5.10. Gradual increase of SAA while progressing to malignancy in ovarian epithelial tumors

Increased levels of SAA were reported in a wide range of malignancies, as well as another unspecific tumor marker, with an increase in metastatic tumors and regression when therapy is successful [26]. Here, the presence of SAA in serum (with CRP and CA-123) and expressed locally in tissues was examined and compared with different stages of tumor growth. Compared were normal ovarian tissues, benign, borderline, carcinoma and metastatic tissues of patients using immunohistochemistry with monoclonal antibodies against AA (mc1 and mc29, see **Table 1** and **Figure 5**) and in situ hybridization. In some patients (and in cell line OVCR-3), RT-PCR was applied, and SAA1 and SAA4 were detected. The result shows a continuous increase of SAA (CRP and CA-125) in serum during the gradual increase of the malignant nature of the ovarian tissue proliferation. In addition, and most important, the SAA expression in tissue increases, in the same manner, with a steep increase in the SAA-synthesizing cells from the normal cells, without (or with only a trace of) SAA, over the borderline tumors with weak expression to the maximal expression of the distinct carcinomas and metastases. Therefore, it is likely that the serum level of SAA in these malignancies may, in part, originate from the ovarian tumor itself.

The data show that the quantity of local intracellular expression of SAA correlates directly with the grade of malignancy of the ovarian epithelial neoplasias and runs in parallel with the serum value of SAA, CRP and CA-125. Therefore, SAA may have a role in ovarian tumorigenesis [27].

5.11. SAA in the female reproductive system

Ovarian reproduction includes a kind of inflammatory process [28]. Therefore, the cellular expression and localization of SAA in all stages of follicular development was examined in in vitro fertilization (IVF) patients applying nonradioactive in situ hybridization and immunochemistry with the monoclonal anti-AA (mc1 and mc29) antibodies. In parallel, SAA of follicular fluids and SAA in serum were examined using micro-ELISA. Expression of SAA mRNA was found in all follicular cells (granulosa, thecal and luteal) of all stages of development, from primordial, primary and secondary follicles to corpora lutea and even in oocytes.

The concentration of SAA in serum and in the matched follicular fluid was very closely associated ($R^2 = 0.80$), although both values could vary considerably by a factor of ca. 30× for blood SAA and by 100× for the follicular fluid. In addition, elevated follicular SAA values have a strong correlation with the patients' body mass index. Values over 30 are associated with a reduced pregnancy rate. Taken together, SAA is locally produced by all follicular cells and is a constituent of the follicular fluid. Therefore, it has a role in ovarian development and in the rate of pregnancy, which is reduced when SAA values are too high in overweight female patients with a BMI of over 30.

Finally, since human ovarian epithelial tissues reproduce SAA during reproduction (see above), the neoplastic degenerated cells in ovarian carcinoma continue their SAA synthesis [26].

5.12. A role of SAA in the APR of murine septic inflammations

5.12.1. On the role of the APR and APPs in septic mice

In order to analyze the different steps necessary to overcome an infection by the hepatic APR, an experimental mouse model was applied and shown as an "anti-sepsis circle" (see **Figure 6**) [32]. Using polymicrobial sepsis induced by cecal ligation and puncture (CLP), the various actions begin with mice exposed to a bacterial overload that leads to the IL-6 induction, which is the dominant interleukin and major inductor of the APR. IL-6-deficient mice can still mount an APR, since IL-6 represents one member of a larger group of interleukins with redundant actions. The action of IL-6 is to initiate the intracellular signaling via the hepatic IL-6 receptor gp130 and further induction of STAT3, which is inevitable for developing the full hepatic APR in hepatic cells, including the synthesis of the dominant APP SAA. However, when mice with a deletion of gp130 or STAT3 are treated with CLP, the hepatic synthesis of SAA is not induced and these mice cannot mount an APR anymore and are thus defenseless, and mortality is greatly increased. The missing APR and the missing defense can be reversed by adding myeloid-derived suppressor cells (MDSCs), which are induced by a hepatic APR including SAA. SAA induces and activates the proliferation of bone marrow cells, which include MDSCs. These cells are accepted to be able to also act against the microbial infection. MDSCs are anti-inflammatory in cancer, cancer spread and metastases [27]. They home-in on different organs. In septic mice, they have been examined from spleen and increase their numbers when pg130 and STAT deficiency are overcome by an

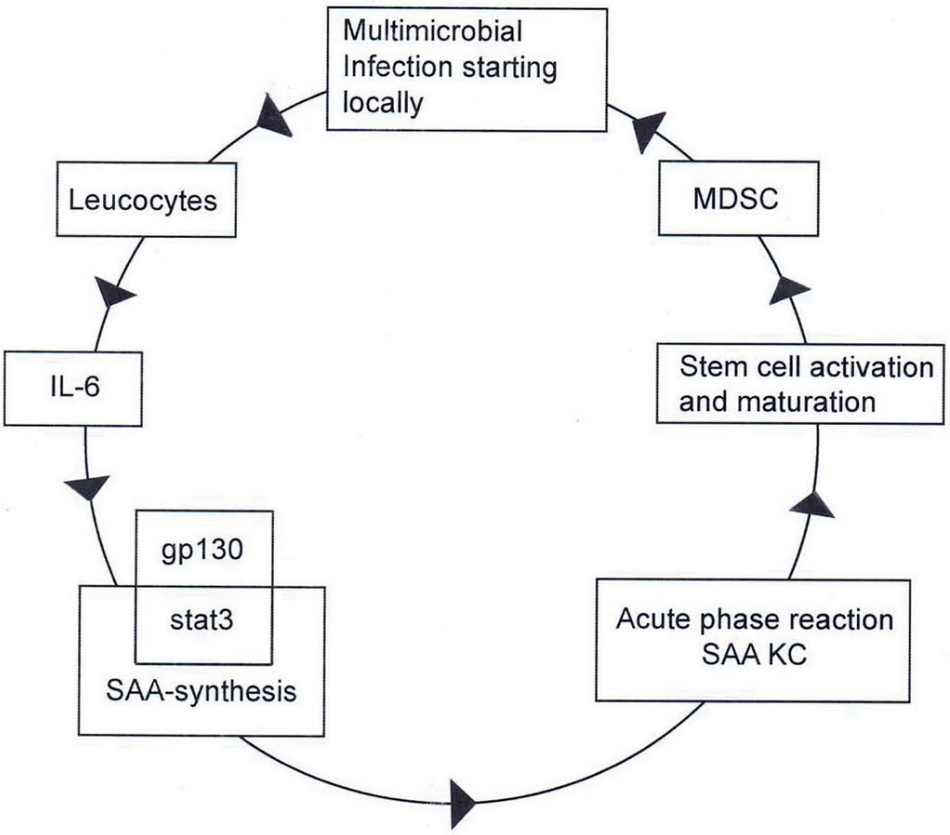

Figure 6. Closing the sepsis loop to the "anti-sepsis circle" schematically.

injection of SAA, cxc1 (KC) or SAA/KC. MDSC can be regarded as a second anti-inflammatory wave induced by SAA and the other components of the APPs when the first wave of anti-inflammatory phagocytes is beginning to wane while becoming exhausted [1, 2, 27].

When very important functions of SAA (which need to be more clarified) are being blocked by the monoclonal antibodies mc4 and mc29 in CLP mice, the essential MDSCs cannot be produced to the necessary amount and function to cope with the bacterial load so that the mice become defenseless and display a significantly accelerated death rate. This unfavorable situation can be reverted by the injection of SAA, thereby resulting in the former defense with the proliferation of MDSCs, so that the mice survived like CLP-treated mice in this sepsis model [29, 30, 48]. The APP KC has a similar, but not identical, effect. When KC was added to SAA, the recovery from the CLP fate of mice with a murine SAA inactivated by antibodies may even be slightly improved, thus indicating that SAA, although the major and dominant APP, can be assisted by KC against the bacterial load.

This demonstrates a cooperative defense of SAA and KC [29]. Cooperation can also be expected from other APPs and constituents in the APP network, including from the greater SAA family. The AA antibodies mc4 and mc29 bind to invariant and therefore very important peptides of SAA as described in detail in Section 4.2, in **Table 1** and **Figure 5**. With these antibodies, life-saving biological functions have been detected and their functions localized to invariant peptides of SAA. This approach could be extended to analyze all the invariant peptides of the SAA family. This can be regarded as a starting point for a possible therapy of a long list of such maladies as severe chronic inflammations and severe chronic infections including sepsis with (induced in vivo or recombinant) SAA isotypes (and their inhibitors as humanized generic SAA antibodies), and with other APPs and constituents of the network of the SAA family, which are able to fortify the "anti-sepsis circle" (**Figure 6**).

6. Some remarks

6.1. The septic loop became an "anti-sepsis circle" as a basis for further work

Some essential elements of the cooperative defense against the experimental multi-microbial infections became apparent as shown in **Figure 6**. The pathway from infection procedures passes, through IL-6, gp130 and STAT3, to the APR with the dominant SAA family and its network. This loop has been closed to a circle through the action of at least the SAA1 that assisted in inducing the growth of the MDSCs in the bone marrow. These cells are also shown to be essential in fighting bacterial infection. However, when gp130 or SAA was not available in this model and the "circle" was interrupted, with fatal consequences, the addition of the missing agents restored the circle with its function [29]. It should be an important goal to examine the SAA isotypes in different inflammatory states and diseases in relation to febrile temperatures (**Figures 3** and **4**) and to analyze the functions of all 20 invariant peptides (**Figure 5**) and the epitopes of the AA/SAA antibodies (**Table 1**) in order fortify it.

It is also important to define the febrile temperatures by which the individual SAAs separate from HDL (proven in vitro, **Figures 3** and **4**) and get activated to execute their function. A

novel idea could be: therapeutical hypothermia below 37°C could inactivate SAA through binding to HDL, which can be called "hypothermic deactivation of SAA." This option could be considered (after complying with the strict rules for a novel therapy) in severe inflammatory states exemplified by sepsis, septic shock, genetic hyperthermia syndromes and similar diseases summarized in SIRS (systemic inflammatory response syndrome). Inversely, a temperature-dependent conformational change of SAA at above 38°C causing SAA release from HDL can induce a "hyper-thermic activation of SAA," which could be beneficial for patients having clinical syndromes with body temperatures of 36°C and below.

6.2. Innate, humoral checkpoints for survival and application by industrial organizations

The presented view summarizes peptides of SAA that are decisive for innate humoral functions in different systems. This view can be applied to many possible possible inflammatory and infectious diseases, including sepsis. These SAA peptides provide a functional innate humoral "stop and go" mechanisms located on SAA ("SAA checkpoints") related to survival. Stop, with generic (humanized) AA/SAA monoclonal antibodies or equivalent agents, and go, with the bio-identical SAA preparations, including SAA isotypes or related peptides with special SAA functions (**Figure 5**), which need to be further explored to find out their additional role in the SAA network. This examination can also be extended to other APPs.

6.3. European Patent EP No 2368564

Due to its novelty within the field of innate immunity and the possibly far-reaching impact in medicine, in particular, in inflammatory diseases including sepsis, these discoveries by three inventors were in agreement with the two other inventors patented by the author [49, 50].

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Dedication

This chapter is dedicated to Professor Dr. Konrad Beyreuther, Heidelberg and Professor Dr. Robert Huber, Martinsried, Germany.

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