Review

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Acute-phase protein synthesis: a key feature of innate immune functions of the liver

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Abstract: The expression of acute-phase proteins (APP's) maintains homeostasis and tissue repair, but also represents a central component of the organism's defense strategy, especially in the context of innate immunity. Accordingly, an inflammatory response is accompanied by significant changes in the serum protein composition, an aspect that is also used diagnostically. As the main site of APP synthesis the liver is constantly exposed to antigens or pathogens via blood flow, but also to systemic inflammatory signals originating either from the splanchnic area or from the circulation. Under both homeostatic and acute-phase response (APR) conditions the composition of APP's is determined by the pattern of regulatory mediators derived from the systemic circulation or from local cell populations, especially liver macrophages. The key regulators mentioned here most frequently are IL-1 β , IL-6 and TNF- α . In addition to a variety of molecular mediators described mainly on the basis of *in vitro* studies, recent data emphasize the *in vivo* relevance of cellular key effectors as well as molecular key mediators and protein modifications for the regulation and function of APP's. These are aspects, on which the present review is primarily focused.

Keywords: acute-phase response; alpha-1-acid-glycoprotein; alpha-2-macroglobulin; hepatocyte; inflammation; serum amyloid A.

Introduction

The electrophoretic separation of serum proteins has an established place worldwide in clinical diagnostics in both human and veterinary medicine (Alper 1974; Moore and Avery 2019). It enables to detect deviations in the concentration and composition of serum proteins from the constellations found under physiological conditions at a glance. Thus, typical shifts in the concentrations and composition of certain serum proteins occur during inflammatory reactions. These changes, together with clinical symptoms such as fever and exhaustion, are also summarized under the term acute-phase response (APR). The shifts observable in serum electrophoresis under inflammatory conditions are based on the altered production of various serum components and are also summarized under the terms negative (decreasing concentrations) or positive (increasing concentrations) acute-phase proteins (APP's) (Bode et al. 2012a; Gabay and Kushner 1999). These changes are triggered by injuries or tissue damage due to surgery, trauma, ischemia, or intoxication, but also by infections, tumors, or other causes of inflammation (Peracaula et al. 2010) including pathological activation of immune or inflammatory responses in the context of autoimmune or autoinflammatory diseases. As a consequence of the disruption of homeostasis altered production of intercellular communication signals including chemokines, growth factors, interleukins (IL's), interferons (IFN's) and members of the tumor necrosis factor (TNF)/TNF-receptor (TNFR) superfamily occurs in various cells. This results in the activation of a cascade of signaling events that culminate in the up- or down-regulation of APP production when reaching the systemic level as evidenced by typical changes in the various protein fractions observable by electrophoretic separation of serum proteins (Figure 1). In this context, the changes detected in serum electrophoresis and thus the underlying altered composition of APP's differ depending on the cause of inflammation and, above all, on the time course of the inflammatory reaction. So, for example an acute inflammatory reaction leads primarily to a down-regulation of the albumin concentration and to an increase of proteins that represent the α_1 - and α_2 -fraction. In contrast,

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a chronic inflammatory reaction is accompanied by a reduced albumin production and leads primarily to an increase of proteins that are contained in the α_2 -fraction as well as the γ -fraction (O'Connell et al. 2005; Werner 1969). This is well documented for all mammals studied so far, such as mice, rats, cats, dogs and horses, and plays perhaps an even more important role in veterinary diagnostics than in human medicine. In this regard, the observable changes between the species studied are largely comparable (Eckersall and Bell 2010; Moore and Avery 2019; Witkowska-Pilasiewicz et al. 2019) and appear to be highly conserved within evolution (Magor and Magor 2001; Pepys and Baltz 1983). For example, induction of an inflammatory response in mice by treatment with the bacterial component lipopolysaccharide (LPS) leads to a decrease in albumin and α_1 -fraction and an increase in α_2 -, β -, and γ -fraction (Cray et al. 2010) and thus to changes that can also be observed in humans in response to comparable inflammatory responses. Therefore, serum levels of APP's in mammals across all species are useful indicators of ongoing inflammation as well as its severity. Thus, the up-regulation of APP's such as serum amyloid A (SAA) and the C-reactive protein (CRP), which are considered to be the major APP's, is a component of all inflammatory diseases (Gabay and Kushner 1999; Uhlar and Whitehead 1999) and in particular the concentration of CRP directly correlates with their severity in most cases. This is also true with respect to the current pandemic, as serum concentrations of these proteins are also sensitive serological surrogate parameters for assessing the severity of a virus-related disease such as COVID-19 (Chua et al. 2020; Liu et al. 2020). Also, the serum concentrations of ferritin (FT) and other APP's are sensitive indicators in this specific context (Huang et al. 2020; Mudatsir et al. 2020; Xie et al. 2021).

However, while for the clinician changes in the serum APP composition are primarily relevant for assessing the severity and, in some cases, the prognosis of an ongoing inflammatory response or response to therapy, their actual significance goes far beyond this. Thus, the various APP's influence the systemic inflammatory process in a variety of ways and represent key factors for the containment, control and elimination of pathogens or for the release of noxious agents as well as for the initiation of repair processes. For most of these proteins the liver, more specifically the hepatocyte, is the leading or even the only site of their synthesis in the organism. In this regard, the relevance of the liver, in particular the hepatocyte, for the production of APP's is most impressively demonstrated in studies of mice with combined deletion of signal transducer and activator of transcription 3 (STAT3) and the nuclear factor of κ -light-chain enhancer of activated

B cells (NF κ B) subunit ν -rel avian reticuloendotheliosis viral oncogene homolog A (RelA) in hepatocytes. In these mice, there is a near complete arrest of APP production in response to various infectious, inflammatory, and noxious stimuli, including pneumococcal pneumonia (Quinton et al. 2012). Notably, it becomes clear from further studies that the transcription factors STAT3 and NF κ B in the hepatocyte each have distinct roles in the regulation of the APR (Ahyi et al. 2013; Hilliard et al. 2015; Kim et al. 2019).

Therefore, in addition to its importance in the metabolism of carbohydrates, lipids and proteins and in the detoxification or disposal of xenobiotics the liver is also a central regulator of inflammatory responses and thus an important immunoregulatory organ. This is particularly true, among other reasons, because APP's produced by the liver bind to cytokines and thereby modulate their activity. Furthermore, they are involved in the control of leukocyte adhesion and extravasation, but also for macrophage reprogramming. For example, fibrinogen- γ (FGG) binds to the integrin receptor α M, also known as cluster of differentiation (CD) 11b, on leukocytes (Flick et al. 2004; Ugarova et al. 1998) and is important for the local induction of cytokines such as IL-1 β , IL-6, and TNF- α *in vivo* (Flick et al. 2007). Moreover, independent of its role in facilitating the clearance of cellular debris and bacteria by phagocytic cells, CRP may interact with certain receptors like the fragment crystallizable γ receptors (FC γ R's) I and IIa in a phosphocholine-complexed form, thereby enhancing the inflammatory response of macrophages (Newling et al. 2019). Other APP's directly bind cytokines and act as protein transporters or stabilizers in the serum. In addition, some APP's play a role as soluble pathogen-associated molecular pattern (PAMP)-recognition receptors (PRR's) or block cytokine secretion for example by preventing their proteolytic processing via inhibition of the involved protease. This has been demonstrated for α_1 -anti-trypsin (AAT) that is able to impede the TNF- α -converting enzyme a disintegrin and metalloprotease 17 (ADAM17), which is essential for TNF- α secretion (Guttman et al. 2015). Accordingly, apart from the liver's relevance in the induction of immunological tolerance as well as its function in adaptive immunity such as the local accumulation of cytotoxic T lymphocytes (CTL's) in so-called intrahepatic myeloid-cell aggregates for T cell population expansion (iMATE's) during viral infections (Huang et al. 2013), the liver also plays an essential function in innate immunity.

However, although hepatocytes and thus the liver are by far the most important source of APP's, it must be emphasized that they are not the only site of APP production. Certain APP's, for example pentraxins, are also

Figure 1: Schematic summary of the regulation of hepatic acute-phase protein synthesis by inflammatory mediators and the involvement of intercellular communication. Different challenges such as an infection, organ injury, inflammation, a tumor or a response towards tissue damage lead to the activation of different inflammatory cell-types including organ-infiltrating monocytes (MC) and tissue-resident macrophages like Kupffer cells (KC). Thereby, monocytes and macrophages are a primary source of different inflammatory mediators like chemokines, growth factors, interferons and interleukins. The expression of these molecules creates a local inflammatory milieu that enables an intercellular communication between parenchymal cells (PC), respectively hepatocytes, and non-parenchymal cells of the liver (HSC: Hepatic stellate cells; KC: Kupffer cells; SEC: Sinusoidal endothelial cells). Consequently, this communication process initiates the production of acute-phase proteins (APP's) in hepatocytes, which are secreted into the circulation. The serum concentrations of APP's can be analyzed by electrophoresis that is a part of clinical diagnoses. The resulting chart (blue color) reflects the abundance of different APP fractions as depicted (α_1 , α_2 , β_1 , β_2 , γ), which changes upon challenge as demonstrated for the case of an acute inflammation (brown color).

detectable in macrophages or dendritic cells or in tissues such as lung or kidney (Deban et al. 2011; Ercetin et al. 2019; He et al. 2007; Malle and De Beer 1996; Roka et al. 2019). Here, it should be noted that, to date, it is unclear whether nonhepatic production of APP's occurs independently of the initiating cause or whether it is related to the initiating cause or disease.

With regard to the regulation of APP synthesis the central localization of the liver, its specific architecture and its cellular composition play an important role, since the production of these proteins by hepatocytes occurs in response to systemic and/or locally delivered intercellular communication signals, in particular a number of cytokines. These are not, or only to a small extent, produced by hepatocytes themselves, but by some other cell populations, mainly by cells of innate immunity, especially macrophages. Among these, the liver harbors the largest pool of tissue-resident macrophages in the organism. In addition to macrophages, myeloid cells and innate lymphoid cells are also very abundant in the liver. These aspects also emphasize the special importance of the liver for innate immunity. Although all these functions of the liver and liver-derived factors have long been known, recent developments have further emphasized the importance of this organ in the regulation of both adaptive and innate immunity and have shed new light on cellular regulators. The immunological significance of the liver has been the subject of comprehensive reviews (Crispe 2009; Gao et al. 2008; Tacke et al. 2009). Therefore, the present review aims to elucidate the role of the liver as a major source of acute-phase reactants, which are important components of the innate immune response and thus, the importance of the liver as a central entity in the regulation of inflammatory processes and innate immunity.

Differential functions of acute-phase proteins

As aforementioned APP's have a wide range of functions that can either promote pro-inflammatory conditions or rather support anti-inflammatory actions and repair processes. For some APP's these differential effects are further influenced or controlled by their molecular modifications. Under homeostatic conditions the liver continuously expresses detectable levels of APP's that "patrol" through the body. Their functions comprise the removal of dead or dying cells (e.g., CRP, serum amyloid P [SAP]), the detection and elimination of bacteria, fungi and toxins (e.g., CRP, SAP, pentraxin 3 [PTX3], complement component 3

[C3]), the regulation of tissue regeneration as well as of blood clotting (e.g., fibrinogen [FG]), and of iron homeostasis (e.g., hepcidin antimicrobial peptide [HAMP], FT). In challenges such as in the context of infections, cancer, tissue injury or necrosis, APP's exert important immunomodulatory functions as mentioned above as well as functions in the context of repair processes. A brief overview of the spectrum of action of selected APP's is provided in the Supplementary information (Supplementary Table 1). In this context, some APP's perform multiple and in some cases opposing functions in the context of the APR. In this respect, SAA should be mentioned, as it can perform bilateral functions in the regulation of inflammatory processes within the APR. On the one hand, SAA supports inflammation by upregulating its two subtypes 1 and 2 very rapidly up to 1,000-fold during inflammatory processes and by binding mainly to low-density lipoprotein (LDL) and high-density lipoprotein (HDL) in serum. This makes SAA a leading component of HDL3, reducing the anti-inflammatory and anti-oxidant properties of HDL (Tolle et al. 2012; Weichhart et al. 2012). In addition, SAA promotes the recruitment of neutrophils and mononuclear cells by inducing a Th17 lymphocyte response. These cell-types can damage tissues by releasing collagenases and oxidases or by expressing pro-inflammatory mediators including IL-1 β (Abouelasrar Salama et al. 2020; Badolato et al. 1994, Connolly et al. 2012; Migita et al. 2014). On the other hand, SAA also has protective functions by inactivating bacterial toxins and exerting anti-fungal or anti-viral effects. Moreover, high SAA levels protect against a secondary inflammatory stimulus (Cheng et al. 2018; Gong et al. 2019; Lavie et al. 2006; Renckens et al. 2006; Rose et al. 2012). SAA also induces an M2b-like polarization of macrophages and indirectly induces proliferation of regulatory T cells. An effect that tends to promote anti-inflammatory mechanisms (Nguyen et al. 2011; Wang et al. 2017). Notably, the huge range of different functions of SAA is mediated by a variety of structurally diverse receptors including formyl peptide receptor 2 (FPR2), scavenger receptor class B type I and II (SR-BI/II), CD36 and receptor for advanced glycation endproducts (RAGE) (Baranova et al. 2005, 2017; Cai et al. 2005, 2007; Liang et al. 2000). Also, an involvement of SAA in the activation of Toll-like receptors (TLR) 2 and 4 and the consecutive induction of the expression of various pro-inflammatory cytokines in the context of inflammatory processes have been described (Abouelasrar Salama et al. 2020). Although interesting, this observation is controversial because these observations are based on recombinant proteins, which may have been contaminated by bacterial components during the manufacturing process.

An important aspect that has become increasingly clear in the last decade is that the function of some APP's is shaped by molecular modifications. One example on which this has been studied in more detail is the α 1-acid-glycoprotein (AGP), also known as orosomucoid (ORM). This protein acts as a carrier of basic and neutrally charged lipophilic compounds, such as steroids and protease inhibitors, as well as drugs, thus affecting their pharmacokinetics and pharmacodynamics (Barrail-Tran et al. 2010; Ofotokun et al. 2011). Under pathophysiological conditions this protein further induces the expression of pro-inflammatory cytokines such as TNF- α , IL-1 β , IL-6, or IL-12 by leukocytes (Hochepied et al. 2003). At the same time, however, anti-inflammatory effects have also been described, as AGP induces the secretion of soluble TNFR and IL-1 receptor antagonist (IL1RA) (Tilg et al. 1993; Van Molle et al. 1997) or inhibits neutrophil chemotaxis, migration, and superoxide production (Laine et al. 1990; Mestriner et al. 2007; Spiller et al. 2012) and lymphocyte proliferation (Elg et al. 1997). Furthermore, it inhibits macrophage activation (Gemelli et al. 2013). Recent studies provided evidence that these diverse and partly opposite or contradictory biological functions of AGP depend on its glycosylation pattern (Ceciliani and Lecchi 2019). In humans, 12 to 20 different glycosylation forms have been detected to date with qualitative changes occurring during the inflammatory process (Luo et al. 2015). The correlation between the status of protein glycosylation and the course and duration of an inflammatory response have led to the implementation of these molecular modifications as diagnostic and prognostic markers for various cancers (Hashimoto et al. 2004) or for acute-on-chronic liver failure in chronic HBV infection (Ren et al. 2010).

The function of APP's can also be regulated by protein cleavage, resulting in a change in the molecular conformation of the respective APP and thus in its function, as has been well demonstrated for α ₂-macroglobulin (A2M), among others. This APP exerts immunomodulatory effects in two different ways. First, it inactivates a variety of proteases including asparagine, cysteine and serine proteases or metalloproteinases through steric inhibition. For example, A2M inhibits coagulation or fibrinolysis by inhibiting the serine protease activity of thrombin and plasmin and kallikrein, respectively (de Boer et al. 1993). On the other hand, A2M also functions as a carrier protein by binding numerous growth factors and cytokines, such as platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), transforming growth factor β (TGF- β), insulin and IL-1 β . In this context, the binding activity of A2M is controlled by proteolytic cleavage, which converts

the molecular conformation of A2M from an inactive native form to a form activated by proteases. Thereby, the non-cleaved homotetrameric form preferentially binds molecules such as PDGF, nerve growth factor (NGF) or IL-6 without affecting their biological activity and protects these factors from proteolytic degradation (Garber et al. 2000). In contrast, cleavage of A2M by proteases and the conformational change associated with this leads to the exposure of a binding site for low-density lipoprotein receptor-related protein 1 (LRP1), also known as the A2M receptor. As a consequence, molecules bound to A2M under these conditions, such as IL-1 β , IL-4, IL-10, TGF- β , and TNF- α , exhibit reduced biological activity and are rapidly removed from circulation by LRP1 receptor-mediated endocytosis of A2M (Garber et al. 2000). However, recently it was discovered that matrix metalloproteinase 9 (MMP9) trimers, but not monomers, can bind as hitchhikers to proteolytically cleaved A2M without affecting the protease function of MMP9 and also without internalization of the molecular complex by LRP1. This suggests that the influence of proteolytically activated A2M on the function of bound molecules is even more complex and may critically affect their fate (Serifova et al. 2020). Another distinctive feature of A2M is the splitting of its native homotetramer form into two dimers under the influence of hypochlorite, which is increasingly released in the context of inflammation, oxidative damage, endothelial cell dysfunction or apoptosis. Compared to the homotetramer these dimers bind TNF- α , IL-2, and IL-6 with higher affinity and show a significantly increased chaperone activity, but also a higher affinity to LRP1 (Wu et al. 1997, 1998; Wyatt et al. 2014). Overall, these findings impressively demonstrate that protease activity in a local environment as well as the level of hypochlorite concentration lead to comprehensive changes in intercellular communication by influencing the conformation and thus the function of A2M. The multitude of functions of A2M, such as the regulation of fibrinolysis and coagulation as well as the modulation of immune and inflammatory responses also plays a relevant role in diseases. For example, A2M is involved in the formation of immunoglobulin G (IgG) aggregates in chronic lymphocytic leukemia (Naseraldeen et al. 2020). Furthermore, a protective role of A2M in children with severe course of COVID-19 is discussed (Schramm et al. 2020; Seitz et al. 2021).

These examples impressively demonstrate that APP's sometimes not only have one function, but that their function is comprehensively regulated by molecular modifications, such as the degree of glycosylation or by conformational changes resulting from proteolytic cleavage. The resulting variability of the function of certain

APP's depending on environmental conditions also highlights that the complexity with which the network of different APP's is involved in the regulation of physiological as well as pathophysiological processes is only rudimentarily understood. This is all the more true since the qualitative as well as quantitative composition of APP's is also subject to complex control, e.g., by protein degradation or endocytosis, but also by regulation of their expression. The latter is subject to equally complex control by intercellular signaling networks on the one hand as well as by intracellular signal transduction on the other hand.

Soluble mediators that regulate acute-phase protein synthesis

When challenged by e.g., bacteria or activated by bacterial components such as LPS a variety of signaling molecules are released by monocytes, macrophages or other antigen-presenting cells. This includes the expression of inflammatory mediators like IL-1-type and IL-6-type cytokines as well as TNF- α . Since the regulation of hepatocyte APP synthesis by intercellular communication signals has been extensively addressed in previous reviews, we refrain from presenting this topic in detail. However, after an extensive literature search, we summarized the major regulators of APP synthesis in hepatocytes in the form of a color-coded heat map (Figure 2). This heat map shows that especially the cytokines IL-1 β and IL-6 are consistently described as central regulators of APP synthesis. Their importance for APP production in the liver is substantiated by the observation that following an *in vivo* injection with bacteria like *Escherichia coli* or *Streptococcus pneumoniae* the production of APP's like SAA1, SAP, and lipopolysaccharide-binding protein (LBP) occurs IL-6 receptor (IL6R)-dependently and also requires TNFR/IL1R-mediated signaling. Interestingly, upon *S. pneumoniae* injection synthesis of IL-1 β , IL-6 and TNF- α was only observed in the lung, not in the liver whereas upon *E. coli* injection these cytokines were expressed in both organs. However, APP concentrations in both organs were in the same level independent from the pathogen injected (Quinton et al. 2009). This supports the long-standing assumption that, depending on the cause of disease, APP synthesis in the liver may also be controlled by another organ via the delivery of intercellular communication signals into the blood flow. As both pathogens were applied by the same route, this observation also supports a degree of organ tropism of the pathogens, since infection with

S. pneumoniae leads to cytokine synthesis only in the lung but not in the liver.

In addition, cytokine-induced synthesis of APP's can also be counter-regulated by APP's that affect or alter cytokine activity and bioavailability. As already described above, the proteolytic activated form of A2M binds and inactivates a number of secreted intercellular communication signals in serum. This ultimately results in inhibition of the synthesis of APP's that are induced in their expression by corresponding cytokines. In addition, IL-1 receptor antagonist (IL1RA) represents an APP that inhibits the expression of SAA, SAP, CRP, AGP, and C3 *in vivo* by neutralizing the stimulatory effects of IL-1-like cytokines (Grehan et al. 1997).

Consistent with what has been discussed above, these results reemphasize the complexity of the regulatory network through which an APR is controlled. Thus, the release of inflammatory cytokines generally begins at the site of infection or injury and shows some organ-tropism of distinct pathogens. However, a systemic APR occurs only when a local cytokine production is amplified and leads to an increase in the concentration of corresponding cytokines in the bloodstream. Although a number of studies have shown that non-hepatocytic cells are also capable of producing at least some APP's, they do not appear to be able to generate a system-wide APR. At least to the extent that the results from genetically modified mice have more general cross-species validity, it can be concluded that APP production in the context of a systemic APR occurs primarily in the liver (Quinton et al. 2012). This in turn also implies a system-wide regulatory relevance of hepatocyte APP synthesis. This assumption is at least emphasized by the observations that an excessive inflammatory response occurs in animals with a hepatocyte-specific blockade of STAT3 activation or in animals with a hepatocyte-specific deletion of STAT3 (Hilliard et al. 2015; Sakamori et al. 2007; Sander et al. 2010).

Pathways and transcriptional regulators controlling acute-phase protein expression

Several effector molecules of intracellular signaling important for cytokine-regulated APP gene expression in hepatocytes have long been known and are already well described. However, the precise mechanisms by which these molecules control their target gene expression *in vivo*

Figure 2: Inducers and repressors of hepatic acute-phase protein synthesis. Based upon literature search in the context of this manuscript the different inducers or repressors of acute-phase protein (APP) expression in parenchymal cells of the liver or in hepatocyte cell lines were listed in the top row and compared to the target genes/proteins depicted in the left column. The heat map color reflects the frequency of citations. Therefore, the orange color indicates citations that describe mediators (depicted in the top row) with inducing characteristics on APP's, whereas the blue color reflects citations that indicate repressive features of mediators. The underlying observations were mainly based on *in vitro* studies. Abbreviations: α2-macroglobulin (A2M), α1-antitrypsin (AAT), α1-cysteine proteinase inhibitor (ACPI), α1-antichymotrypsin (ACT), α1-acid-glycoprotein (AGP), aryl hydrocarbon receptor (AHR), activator protein 1 (AP1), complement factor 1 inhibitor (C1INH), complement factor 3 (C3), complement factor 4-binding protein (C4BP), soluble cluster of differentiation 14 (sCD14), CCAAT/enhancer-binding protein (C/EBP), ciliary neurotrophic factor (CNTF), cAMP responsive element-binding protein H (CREBH), C-reactive protein (CRP), cardiotrophin-1 (CT-1), epidermal growth factor (EGF), fibrinogen (FG), fibronectin (FN), granulocyte-colony-stimulating factor (G-CSF), hepcidin antimicrobial peptide (HAMP), hepatocyte growth factor (HGF), hepatocyte nuclear factor (HNF), haptoglobin (HP), interferon (IFN), interleukin (IL), soluble interleukin 1 receptor antagonist (sIL1RA), interferon responsive factor (IRF), lipopolysaccharide-binding protein (LBP), leukemia inhibitory factor (LIF), liver receptor homolog (LRH), nuclear factor of κ light polypeptide gene enhancer in B cells (NFκB), octamer transcription factor (OCT), oncostatin M (OSM), peroxisome proliferator activated receptor γ coactivator (PGC), peroxisome proliferator activated receptor (PPAR), pancreatic secretory trypsin inhibitor (PSTI), serum amyloid A (SAA), serum amyloid A-activating factor (SAF), serum amyloid P (SAP), specificity protein (SP), signal transducer and activator of transcription (STAT), transforming growth factor (TGF), tumor necrosis factor (TNF), transferrin (TRF). The underlying references are listed in the Supplementary information (Supplementary Table 2).

are incompletely understood. This is partly due to the fact that most studies on this topic have been performed in hepatoma cell lines or in isolated primary hepatocytes *in vitro*. In addition, one has to take into account that *in vivo* hepatocytes are not a homogenous population within the liver lobule. Depending on its localization within the porto-central axis the hepatocyte exerts different functions (Ben-Moshe and Itzkovitz 2019; Gebhardt 1992). So far, it has not been clarified whether there are specific hepatocyte populations that particularly express APP's or whether the expression of individual APP's varies locally. Moreover,

most of the respective studies only used isolated cytokines, which also does not correspond to the physiological situation, in which cytokines or TLR-ligands substantially influence the signal transduction of other cytokines. For example, this is well documented for the interference of IL-1β or TNF-α each with IL-6 (Albrecht et al. 2007; Bode et al. 2003) or the influence of LPS or TNF-α on the signal transduction of IL-6 (Bode et al. 1999). Furthermore, these studies often do not adequately address the role of intermediary factors, which are required for the activation of certain signaling events. For example, LPS-induced IL-10

production in macrophages and consecutive activation of STAT3 requires activation of the type I IFN receptor (Chang et al. 2007; Ehltting et al. 2011). To date, the major transcription factors involved in the regulation of APP gene expression in hepatocytes are considered to be STAT3, NFκB and nucleotide sequence CCAAT/enhancer-binding proteins (C/EBP's) (Figure 2) (Bode et al. 2012a). Especially with regard to STAT3 and NFκB, this is also supported by the studies on genetically modified animals already detailed above. It is important to emphasize that STAT3, formerly known as acute-phase response factor (APRF), was first discovered in studies analyzing the control of APP gene expression in the liver (Wegenka et al. 1993). In the context of APR the signal transducing subunit glycoprotein with 130 kDa in size (gp130) of the IL-6 receptor complex plays a central role for the activation of STAT3 and for subsequent synthesis of APP's in hepatocytes. Notably, gp130 functions as a signal-transducing receptor subunit not only for IL-6 but for a number of cytokines belonging to the IL-6-type cytokine family. However, for complete disruption of cytokine-induced APP synthesis, inactivation of STAT3 is not sufficient (Alonzi et al. 2004), but additionally requires the deletion of the RelA subunit of the NFκB complex in the hepatocyte (Quinton et al. 2012). These data corroborate the long-known fact that, in addition to cytokines like IL-6, oncostatin M (OSM) or leukemia inhibitory factor (LIF) that mediate their biological functions primarily through activation of STAT3, cytokines like IL-1β, or TNF-α that predominantly mediate their signaling through activation of NFκB also play critical roles in the network through which APR is controlled. In this regard, for a number of APP's a gene promoter-specific crosstalk of these two transcription factors appears to play an important role. Here, NFκB promotes STAT3 binding at some enhancer elements while it disrupts STAT3 binding at other sites (Goldstein et al. 2017). This observation sheds new light on the hypothesis, generally accepted to date, that cytokines such as IL-1β differentially control IL-6-induced gene expression of APP's in hepatocytes. Thus, in these cells IL-1β and IL-6 synergistically regulate the expression of APP's such as CRP or HAMP, whereas IL-1β suppresses IL-6-induced expression of other APP's such as A2M or FGG (Bode et al. 2012a). With regard to the latter, an inhibitory or competitive interaction of STAT3 and NFκB at overlapping binding motifs in the promoter region of corresponding APP's has been discussed for some time (Albrecht et al. 2007; Bode et al. 2001a; Zhang and Fuller 1997). As already mentioned above, activation of STAT3 in

hepatocytes and STAT3-dependent gene expression play a central role in the control of a systemic inflammatory response (Sakamori et al. 2007; Sander et al. 2010). It has been impressively demonstrated that hepatocyte-specific STAT3 activation is essential for preventing a systemic hyper-inflammatory response as well as mortality in sepsis, in part by limiting an excessive activation of immune cells (Sakamori et al. 2007). This has been consistently demonstrated in subsequent studies. In a model of APR induced by LPS as well as in a model of polymicrobial sepsis by cecal ligation and puncture (CLP) blockade of gp130/STAT3-mediated signaling in hepatocytes and consequent synthesis of APP's leads to impaired survival and significant increase in serum concentrations of inflammatory cytokines such as IL-6, TNF-α, and IFN-γ (Sander et al. 2010). In this context, myeloid-derived suppressor cells (MDSC's) appear to play a role as they represent cellular responders to APP's and have an immunosuppressive C/EBPβ-dependent phenotype (McPeak et al. 2017; Sander et al. 2010). Similarly, NFκB/RelA, also known as subunit protein with 65 kDa in size (p65), in hepatocytes plays a regulatory role in the context of systemic inflammatory processes as it balances APP expression and provides hepatoprotection in sepsis or pneumonia by attenuating TNF-α-mediated immunotoxicity. In addition, it also maintains survival (Kim et al. 2019). Of note, survival upon LPS treatment is at least in part mediated by the phosphorylation of a single serine site 536 (Ser536) of subunit p65. Mutation of this site results in reduced survival rates, but also increased levels of SAA and cytokines such as IL-1β, IL-6, and TNF-α. So, it has been postulated that this serine site rather inhibits NFκB-dependent signaling to prevent harmful inflammation (Pradere et al. 2016). This *in vivo* observation was surprising as previous *in vitro* studies suggested an opposing role of Ser536 with beneficial effects for NFκB/p65 function and an enhancement of its transcriptional activity (Yang et al. 2003). Thus, the *in vivo* data available, to date, suggest that LPS-induced phosphorylation of p65-Ser536 is a fine regulator of NFκB signaling and limits exaggerated consequences. In this context, however, it has to be noted that this observation is from mice with constitutive expression of the mutant variant of p65 and does not represent the cell type-specific relevance of phosphorylation of subunit p65 at Ser536.

With regard to the APR it is important to consider that STAT3 and NFκB are not only critical regulators of gene expression in hepatocytes, but also in myeloid cell populations such as macrophages, where they control cytokine

synthesis in response to a variety of different inflammatory triggers including the APR triggered by exposure to LPS. In the latter case the current data suggest that STAT3 and NF κ B exert anti-inflammatory functions by inhibiting the production of IL-1 β , IL-6, and TNF- α , thereby counteracting an excessive systemic inflammatory response. This view is also supported by *in vivo* studies using LPS-treated mice, in which STAT3 or NF κ B/p65 was specifically deleted in myeloid cell populations (Takeda et al. 1999; Vanoni et al. 2017). In this context, the well-documented fact that IL-10 mediates a substantial part of its anti-inflammatory effects via activation of STAT3 plays an important role (Takeda et al. 1999; Williams et al. 2004). IL-10 acts almost exclusively on immune cells and its expression is controlled at the level of transcription by STAT3 and NF κ B (Benkhart et al. 2000; Liu et al. 2006). Furthermore, the stability of the IL-10 transcript is maintained and controlled by the mitogen-activated protein kinase (MAPK) with 38 kDa in size (p38^{MAPK})-mediated activation of the MAPK-activated protein kinase 2 (MK2) (Ehltling et al. 2011, 2016). Interestingly, MK2 regulates gene expression not only at the level of transcript stability but also at the level of nuclear translocation of NF κ B and activation of interferon regulatory factor 3 (IRF3). This observation highlights that MK2 controls the regulation of gene expression at multiple levels. Thus, MK2 activation stabilizes the IL-10 transcript and is able to simultaneously control target gene expression at the transcriptional level as demonstrated for type I IFN- β , which is also a prerequisite for the induction of sustained IL-10 expression. The necessity of type I IFN-mediated activation of the IFN- α/β receptor (IFNAR) for the induction of IL-10 expression is evident from studies on IFNAR-deficient macrophages, in which LPS-induced expression of IL-10 as well as the consequent activation of STAT3 is absent (Bode et al. 2012b; Ehltling et al. 2011). This regulatory mechanism is not only relevant for the induction of IL-10 expression in response to bacterial components such as LPS, but also in the context of infections with viruses such as cytomegalovirus (CMV) (Ehltling et al. 2016). However, IL-10 is not the only target gene of MK2 as this kinase likewise regulates the expression of several other cytokines in the context of LPS treatment or CMV infection (Ehltling et al. 2016; Hitti et al. 2006; Neiningner et al. 2002). These data demonstrate that MK2 is crucial for the regulation of gene expression in macrophages and thus for the regulation of those cytokines that are mainly responsible for the initiation, but also for the resolution of a systemic inflammatory response. The relevance of MK2 for

the control of gene expression in macrophages in response to inflammatory stimuli such as LPS is also evident in a more recent study investigating the differential regulation of LPS-induced gene expression by MK2 and its homologous kinase MK3 (Ehltling et al. 2019). In light of the foregoing, it can be assumed that MK2 also plays a critical role in the regulation of APP synthesis in the context of APR because of its importance in controlling cytokine expression in macrophages. However, whereas the essential role of MK2 for the regulation of immune cell responses in the context of inflammatory processes is well studied, the relevance of p38^{MAPK} and its two effector kinases MK2 and MK3 for the control of the hepatocyte's APP synthesis is largely unknown. Here, there are only a few predominantly inhibitor-based studies in cell cultures with partly contradictory results. The different findings may be due to the concentration of the inhibitors used. This is at least suggested by the findings of a study, in which it was observed that high concentrations of the p38^{MAPK} inhibitor SB202190 lead to an increase in the transcriptional activity of a STAT3-dependent APP promoter, while even higher concentrations of the inhibitor are repressive. Here, studies using constitutively active mutants of the p38^{MAPK}-activating kinase MKK6 suggest that activation of p38^{MAPK} in a hepatocyte cell line inhibits activation of STAT3-dependent transcription via the induction of suppressor of cytokine signaling 3 (SOCS3) expression. These observations tend to support the assumption that inhibition of p38^{MAPK} in hepatocytes should result in increased expression of STAT3-dependent APP's (Bode et al. 2001a, 2001b). In this context, it is also interesting to note that systems biology studies suggest that activation of p38^{MAPK} and MK2 in hepatocytes is far more sensitive to low doses of IL-1 β than in macrophages (Kulawik et al. 2017). Not least against the background that inhibitors of MK2 are ready to be used in clinical trials (Fiore et al. 2016), clarification of the relevance of the p38^{MAPK}/MK2 pathway for hepatocyte function and in particular for APP expression using *in vivo* models is important. This could help to develop novel therapeutic approaches in the management of systemic inflammatory responses such as in the context of infectious diseases. This is underlined by the observations that inhibitors against this pathway may limit coronavirus MERS-CoV activity and that APP expression is up-regulated in coronavirus SARS-CoV-2 infected patients (Liu et al. 2020; Maeurer et al. 2016).

The above suggests that, ostensibly, only a comparatively small number of signaling molecules, such as

IL-1 β , IL-6, and TNF- α at the intercellular level and C/EBP proteins, STAT3, or NF κ B at the intracellular level, are leading regulators of hepatocytic APP synthesis. However, the regulatory network controlling the activity or expression of these molecules at both the intercellular and intracellular level is much more complex and still relatively poorly understood. This is illustrated by the interaction of p38^{MAPK}/MK2/MK3-mediated signal transduction with STAT3- and NF κ B-controlled gene expression in macrophages, which ultimately interferes extensively with the regulation of cytokine expression in APR. The extent of the complexity becomes clear when one considers that this interaction is only one example for a number of other signaling pathways, which modulate the activation of STAT3 and NF κ B both in the macrophage and in the hepatocyte.

Cell populations involved in regulation of acute-phase protein synthesis by hepatocytes

In the preceding part the function of APP's as well as the complexity of the regulatory inter- and intracellular signaling networks that control APP expression was discussed. Another level of complexity that has not been well studied so far refers to cell populations that serve here as a source of regulating signaling molecules. In most reviews effector cells of innate immunity such as monocytes or macrophages are mentioned as the main source of cytokines controlling the synthesis of APP's in hepatocytes (Bereta et al. 1989; Darlington et al. 1986; Jiang et al. 1995). In particular, reference is made to liver macrophages. Not taken into account in this context and hardly studied so far is the fact that macrophages exhibit high heterogeneity and plasticity. Their response to inflammatory stimuli and thus their contribution to the regulation of the APR could vary considerably.

The heterogeneity of macrophage populations in the liver arises in part from the particular fact that two main populations of macrophages can be roughly distinguished in the liver: recruited macrophages, which originate from the bone marrow, and tissue-resident macrophages, which in a narrower sense represent Kupffer cells (KC's). The latter are considered to originate from the fetal liver, are capable of self-renewal, and are characterized by high expression of the cell surface antigen for the monoclonal antibody F4/80 and the cell surface protein C-type lectin (Clec) 4f receptor (Davies et al. 2013; Guillems et al. 2018; Krenkel and Tacke 2017). Clec4f is important, for example,

for the rapid phagocytosis of platelets in murine liver after *in vivo* treatment with neuraminidases from Gram-positive bacteria (Jiang et al. 2021). This receptor is considered an exclusive feature for macrophages of the liver and is currently recommended to distinguish tissue-derived macrophages or KC's from recruited macrophages. However, recent studies show that the transition between these populations is not sharply drawn and is rather fluid. This is particularly so since macrophages recruited to the liver from the circulation can also take on the properties of tissue-resident macrophages under the influence of sinusoidal environmental conditions (Bonnardel et al. 2019; Scott et al. 2016). The ability of macrophages to adapt their polarization and function to the needs signaled by the environment underscores the high plasticity and thus heterogeneity of this cell population (Murray 2017). It is currently unclear whether the different macrophage populations of the liver differ in their contribution to the regulation of APP synthesis by hepatocytes. Despite individual attempts to establish a uniform nomenclature, the determination and assignment of macrophages to certain subpopulations remains poorly standardized. Thus, most studies on this topic are limited to the use of single surface markers from the CD system or combinations thereof to assign immune cells to specific populations (Austyn and Gordon 1981; Lai et al. 1998) or to describe the heterogeneity and activation state of macrophages. In this context, the marker patterns used are usually purely descriptive and do not necessarily have a direct functional relationship to the activation or differentiation state to which they are assigned. Against this background, a statement on the question of which of the macrophage populations of the liver is of leading relevance for the regulation of APP synthesis in hepatocytes is hardly reasonable at present on the basis of the available studies on this subject.

It is quite conceivable that new techniques that allow genome-wide analyses of diverse cell populations, such as single-cell RNA sequencing or high-throughput chromatin immunoprecipitation sequencing of sorted cells, will change this field. This is particularly so as they will not only allow identification of developmental stages or tissue specificity of macrophages (Bian et al. 2020; Lavin et al. 2014), but also offer the possibility of elucidating the composition of specific liver macrophage subsets and their involvement in APP production in the future.

As noted above, the assumption that liver macrophages generally play a key role in the regulation of APP expression in hepatocytes is supported by a number of different studies (Bauer et al. 1984; Bereta et al. 1989; Olteanu et al. 2014; Polfliet et al. 2006; Prins et al. 2004). Furthermore, some studies suggest that not all liver

macrophages are equally involved in the regulation of APP synthesis by hepatocytes, although the data here are still very heterogeneous and inconsistent. For example, a study using a dexamethasone-coupled CD163-specific antibody suggests that in rats, CD163-positive cells play a role in regulating the expression of APP's such as A2M in response to LPS-induced APR *in vivo* (Thomsen et al. 2016). In this regard, the scavenger receptor CD163, whose best described function is the binding of hemoglobin-haptoglobin complexes, is leadingly expressed by mature tissue macrophages and has therefore been proposed as a marker for sessile tissue macrophages in rats (Fabriek et al. 2005; Polfliet et al. 2006). Although this experimental approach is relatively artificial, it is one of the few functional studies currently addressing the extent to which sessile tissue macrophages may be involved in the regulation of APP synthesis during APR. Provided that CD163 is indeed mainly expressed by mature sessile tissue macrophages, this study would support the notion that these cells play a role in the regulation of APP synthesis. In this context, however, it should be noted that CD163 is also commonly used in mice together with the mannose scavenger receptor CD206 to describe alternatively activated wound healing-type macrophages.

In another study it has been demonstrated that only a CD11b⁺/CD68⁻ subset of F4/80⁺ cells of the liver has a strong capacity for producing inflammatory cytokines, whereas the CD11b⁻/CD68⁺ subset has a more phagocytic activity. Of note, the integrin receptor CD11b is a leukocyte adhesion molecule that is mainly expressed on myeloid-derived cells including monocytes, macrophages, neutrophils and NK cells. Together with F4/80 it therefore serves as marker for macrophage populations that are recruited from the circulation. Hence, these observations suggest that recruited macrophages are the major source of cytokines driving the APR. CD68, which has been used in this study as an additional marker, is a glycoprotein highly expressed on macrophages especially under inflammatory conditions and presumably plays a role as a scavenger receptor (Chistiakov et al. 2017).

Moreover, in the human liver among a CD45⁺/CD3⁻ cell population, which comprises all nucleated hematopoietic cells excluding T lymphocytes (Alcover et al. 2018; Hermiston et al. 2003), also two different subsets have been described. These subsets are characterized as CD14^{hi}/CD32^{lo} cells displaying inflammatory and anti-microbial activities and CD14^{lo}CD32^{hi} cells with a more endocytic and immunosuppressive phenotype (Wu et al. 2020). Thereby, in humans CD14 is expressed mainly on bone marrow-derived monocytes and is therefore thought to indicate recruited macrophages. This receptor plays an important

role for the recognition of bacterial LPS and its soluble form is also expressed as an APP by hepatocytes upon inflammation (Ziegler-Heitbrock and Ulevitch 1993). CD32 acts as a receptor for immunoglobulins and pentraxins and is primarily expressed in monocytes, macrophages and neutrophils (Anania et al. 2019). Consistently, the results of these studies provide clear evidence that there are certain subpopulations of macrophages in the liver that are leading producers of inflammatory cytokines and thus are regulators of APP production. However, it remains to be established in how far those macrophages that control APP expression exclusively belong to the recruited monocyte-derived subpopulations of the liver, which is considered to mainly comprise the population of inflammatory cells, or whether tissue-resident macrophages also contribute and if so, under which conditions.

Beside macrophages also hepatic stellate cells (HSC's) are capable to express relevant cytokine amounts and induce APP's at least in co-culture with hepatocytes *in vitro* (Beringer and Miossec 2019; Thirunavukkarasu et al. 2005). However, although HSC's can produce relevant levels of cytokines *in vitro*, *in vivo* studies suggest that HSC's are more responsive to circulating cytokines and contribute less to their production in the context of APR. Rather, stimulation of HSC's in the context of APR leads to increased collagen production and thus to a worsening of any pre-existing liver fibrosis, provided that a pro-fibrinogenic stimulus is also present (Nieto et al. 2001). Furthermore, also innate lymphocytes like NK cells or $\gamma\delta$ T cells or even cells of the adaptive immune system like Th17 or Th22 lymphocytes are suggested to play a role in APP induction *in vivo* as demonstrated by injection of IL-22, an IL-10-type cytokine that is able to target hepatocytes directly and that is expressed by different types of lymphocytes in the context of microbial infection (Liang et al. 2010; Zenewicz and Flavell 2011). Therefore, induction of APP synthesis in hepatocytes appears to be not exclusively mediated by macrophages. In the course of inflammation, it is necessary to reduce the concentrations of circulating APP's in order to restore homeostasis. In this context, it has been proposed that neutrophils that are usually recruited to the liver upon inflammation play a role, because their cytokine expression pattern inhibits IL-6-induced APP synthesis in co-cultured hepatocytes when neutrophils are pre-treated with zymosan/TNF- α as demonstrated for pancreatic secretory trypsin inhibitor (PSTI) *in vitro* (Oka et al. 1993). It would be of interest, if neutrophils are capable to generally dampen hepatic APP expression or if they operate very specifically on distinct APP's. In addition, CD11b⁺/granulocyte differentiation antigen 1 (Gr1)⁺ MDSC's, which accumulate APP-dependently during later

phases of sepsis, directly inhibit inflammatory responses by suppressing innate but also adaptive immunity (McPeak et al. 2017; Sander et al. 2010). Hence, on the one hand, neutrophil-mediated suppression of APP's may temporarily maintain inflammatory processes in need of pathogen clearance at least in part by delaying APP-dependent recruitment of MDSC's. On the other hand, the intercellular communication may direct a certain APP milieu to optimize repair processes. To clarify this, further *in vivo* studies focusing on the interaction between neutrophils, hepatocytes and MDSC's need to be done.

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