REVIEW ARTICLE

Regulation of serum amyloid A protein expression during the acute-phase response

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The acute-phase (AP) serum amyloid A proteins (A-SAA) are multifunctional apolipoproteins which are involved in cholesterol transport and metabolism, and in modulating numerous immunological responses during inflammation and the AP response to infection, trauma or stress. During the AP response the hepatic biosynthesis of A-SAA is up-regulated by pro-inflammatory cytokines, and circulating concentrations can increase by up to 1000-fold. Chronically elevated A-SAA concentrations are a prerequisite for the pathogenesis of secondary amyloidosis, a progressive and fatal disease characterized by the deposition in major organs of insoluble plaques composed principally of proteolytically cleaved A-SAA, and may also contribute to physiological processes that lead to atherosclerosis. There is therefore a requirement for both positive and negative control mechanisms that permit the rapid induction of A-SAA expression

until it has fulfilled its host-protective function(s) and subsequently ensure that its expression can be rapidly returned to baseline. These mechanisms include modulation of promoter activity involving, for example, the inducer nuclear factor κ B (NF- κ B) and its inhibitor I κ B, up-regulatory transcription factors of the nuclear factor for interleukin-6 (NF-IL6) family and transcriptional repressors such as yin and yang 1 (YY1). Post-transcriptional modulation involving changes in mRNA stability and translation efficiency permit further up- and down-regulatory control of A-SAA protein synthesis to be achieved. In the later stages of the AP response, A-SAA expression is effectively down-regulated via the increased production of cytokine antagonists such as the interleukin-1 receptor antagonist (IL-1Ra) and of soluble cytokine receptors, resulting in less signal transduction driven by pro-inflammatory cytokines.

INTRODUCTION

The physiological response to infections and injuries involves local inflammation and the initiation of events leading to a wide-ranging systemic response. Inflammatory cells, e.g. macrophages and neutrophils, accumulated at the site of challenge produce a wide range of mediators, including the pro-inflammatory cytokines/interleukins (ILs) IL-1 (IL-1 α and IL-1 β), IL-6, tumour necrosis factor α (TNF- α), interferon γ and growth promoters such as transforming growth factor β , which induce a number of responses (Scheme 1; [1]): (i) they induce expression of selectins (carbohydrate-binding membrane proteins) on local endothelial cells, thereby promoting recruitment of inflammatory cells from the bloodstream [2–4]; (ii) they activate these recruited cells for optimal antibacterial activity, e.g. increased expression of receptors, such as complement receptors, increased cell metabolism and protein expression; and (iii) they induce further production of cytokines by other cells. The cytokines also, via the central nervous system, lymphatic fluids and the blood circulation, initiate a wide range of systemic responses including fever, increased bone-marrow-derived-cell production and differentiation, metabolic changes in several organs and a dramatic alteration in the protein-synthetic profile of the liver (Scheme 1). The above changes are collectively called the ‘acute-

phase’ (AP) response, the purpose of which is to provide optimal conditions for counteracting the underlying challenge and for the restoration of homeostasis.

During the AP response the hepatic production of a number of serum proteins (AP proteins), which are essential for a number of defence systems, increases. The apolipoproteins serum amyloid A 1 (SAA1) and serum amyloid A 2 (SAA2), collectively referred to as ‘AP SAAs’ (A-SAAs) are ‘major’ AP proteins in that their serum concentrations can increase by as much as a 1000-fold [5]. Other major AP proteins are the pentraxins, C-reactive protein in man and serum amyloid P component in mice [5]. More modestly induced are the complement components C1s, C2, factor B, C3, C4, C5, C9, C4b-binding protein, C1 inhibitor and mannose-binding protein, which have all been reported to increase upon AP stimulation, with factor B and C3 the most significantly changed in terms of absolute increase in amount [5–8]; the coagulation factors fibrinogen, prothrombin, Factor VIII and plasminogen; the proteinase inhibitors heparin cofactor II, plasminogen activator inhibitor I, α_1 -antitrypsin, α_1 -antiplasmin and α_1 -antichymotrypsin; and the transport proteins α_1 -acid glycoprotein, haptoglobin, haemopexin, caeruloplasmin and ferritin. For these AP proteins the rise in serum concentrations range up to three times the initial concentration, thereby defining them as minor or intermediate positive AP proteins.

Abbreviations used: Apo-A1, apolipoprotein A 1; A-SAA, acute-phase serum amyloid A protein (human SAA1 and SAA2); AP, acute phase; Bcl-3, B-cell lymphocytic-leukaemia proto-oncogene 3 product; C/EBP, CCAAT/enhancer-binding protein; CAT, chloramphenicol acetyltransferase; GR, glucocorticoid receptor; HDL, high-density lipoprotein; IL, interleukin; IL-1R, IL-1 receptor; IL-1Ra, IL-1 receptor antagonist; iCL-1Ra, intracellular IL-1 receptor antagonist; I κ B, inhibitor of nuclear factor κ B (NF- κ B); IKK, I κ B kinase; IRAK, IL-1R-associated kinase; LPS, lipopolysaccharide; NF-IL6, nuclear transcription factor for IL-6 (identical with C/EBP β); PMNs, polymorphonuclear cells; sIL-1Ra, secreted IL-1 receptor antagonist; TNF- α , tumour necrosis factor- α ; sTNF-R, soluble tumour-necrosis-factor receptor; TRAF6, TNF-R-associated factor 6; UTR, untranslated region; YY1, yin and yang 1 transcription factor

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Scheme 1 Activation of the AP response

The cytokine-mediated network linking the inflammatory site and target organs/tissues is shown. Following exposure to the stimulating agent, cytokines produced by macrophages act on neighbouring cells, e.g. leucocytes (monocytes, neutrophils and lymphocytes) and endothelial cells, in the vicinity of the inflamed site. These cells themselves become activated and express additional cytokines and receptors. Endothelial cells express selectins (●) which recruit circulating leucocytes and platelets from the bloodstream. Cytokines released to the circulation induce the hepatic AP response, which involves the increased synthesis of AP proteins e.g. A-SAA, C-reactive protein and complement components. Stimulation of the central nervous system by cytokines induces fever and the synthesis of glucocorticoids by the adrenal cortex. Glucocorticoids enhance the hepatic AP response and at the same time feed back to down-regulate the local inflammation (open arrow) and its systemic inflammatory consequences.

Albumin, prealbumin, transferrin, apolipoprotein A 1 (Apo-A1) and 2, properdin, α_2 -HS glycoprotein, inter- α -trypsin inhibitor and histidine-rich glycoprotein are all considered negative AP proteins because their serum levels decrease during the AP response [5,8].

There are four SAA genes in the human genome, of which two are AP-hyperinducible (*SAA1* and *SAA2*, which encode the A-SAAs), one is a pseudogene (*SAA3*) and one is expressed constitutively (*SAA4*, which encodes C-SAA). The *SAA1* and *SAA2* genes are believed to be co-ordinately regulated and are arranged 'head-to-head' in a gene cluster which also contains *SAA3* and *SAA4* on chromosome 11p15.1 [5]. The A-SAA and C-SAA proteins contain 104 and 112 amino acid residues respectively [5]. Although the precise functions of these proteins are still a matter of some debate, the fact that no individual lacking these proteins or the capacity to up-regulate them has been described, together with their massive induction during the AP response, indicate that they are of fundamental physiological importance following infection or trauma.

Following AP induction, A-SAA associates with high-density lipoprotein fraction 3 (HDL₃), replacing Apo-A1 as the predominant apoprotein on this class of HDL particle [9,10]. Remodelling with A-SAA appears to enable the HDL₃ particle to modulate cholesterol efflux from monocytes (macrophages), thereby facilitating the uptake and removal of cholesterol at inflammatory sites [11–13]. In a mouse model of sterile abscess, A-SAA levels in plasma peak at the same time as plasma cholesterol levels, and cholesterol injected directly into the abscess

is redirected to the plasma, supporting the hypothesis that A-SAA modulates reverse cholesterol transport to optimize the clearance of cholesterol from dying cells at inflamed sites [14]. Studies designed to establish whether the A-SAA-HDL₃ particle has an increased affinity for neutrophils and macrophages and a decreased affinity for hepatocytes, a change which has been speculated to help target the A-SAA-HDL₃ to inflamed sites, have produced conflicting results [10,13,15]. A-SAA also enhances the activity of secretory phospholipase, which cleaves triacylglycerols into glycerol and fatty acids [16] on the HDL₃ particle; this property correlates with the observation that plasma AP HDL₃ has a 10% decreased phospholipid content compared with normal HDL₃ [17]. The presence of A-SAA decreases the HDL₃ associated activity of lecithin-cholesterol acyltransferase (phosphatidylcholine-sterol O-acyltransferase), thereby decreasing the esterification of cholesterol [18]. The circulating HDL₃ particle is transported to the liver, where cholesterol is taken up by hepatocytes. This uptake is enhanced when phospholipids are cleaved and cholesterol is not esterified, both of which effect a redistribution of cholesterol from the core of the HDL₃ particle to the surface [19]. The uptake of free cholesterol by hepatocytes is also actively enhanced by the presence of A-SAA [11].

Purified A-SAAs have been reported to have inhibitory effects on several aspects of the immune response, including fever, prostaglandin E₂ production, platelet activation, oxidative burst and antibody production [20–24]. Free A-SAA can also mediate chemotaxis for monocytes, polymorphonuclear leucocytes and

T-cells, but has no such effect when associated with HDL [25–28]. The capacity of A-SAA to act as an autocrine inducer of collagenase has also been reported [29].

Extensive local inflammation may cause, and/or contribute to, the symptoms of disorders like inflammatory bowel disease, rheumatoid arthritis and systemic lupus erythematosus [1,30,31]. In a small proportion of patients with chronic inflammatory conditions and episodic infectious diseases the long-term maintenance of some aspects of the AP response may cause concentrations of A-SAA to be chronically elevated. Continuous high expression of A-SAA is a prerequisite for the development of secondary, or reactive, amyloidosis, a progressive and fatal condition in which AA, a proteolytic fragment of A-SAA, is deposited as insoluble plaques in major organs [8]. A-SAA is synthesized extrahepatically by macrophages, endothelial and smooth-muscle cells and is found in large amounts in atherosclerotic lesions. When A-SAA's modulations of platelet activation and cholesterol metabolism are considered, the possibility that A-SAA is involved in the pathogenesis/pathology of atherosclerotic lesions and plaques is reinforced [16,24,32,33]. Because of these negative effects there is a need for the AP response and inflammation to be closely regulated and resolved as soon as there is no longer a need for their positive protective properties. Some of the mechanisms whereby this is achieved will be described below. Why these control mechanisms are sometimes not sufficient, and why diseases such as those listed above occur, is not fully understood. Exposure to allergens or chemicals, and physical and mental stress, can initiate or promote inflammation and the AP response and may contribute to the development of some of the above diseases. Also, immune deficiencies and defects in the normal control mechanisms may sometimes be responsible.

INITIATION OF RESPONSE

The hepatic AP response is induced by a wide range of mediators, including IL-1, IL-6, TNF- α , leukaemia inhibitory factor, transforming growth factor β , interferon γ , IL-11, oncostatin, ciliary neurotrophic factor and retinoic acid, whereas others such as insulin and okadaic acid have inhibitory effects [1,5]. Glucocorticoids have dual actions: they enhance the effects of other mediators on hepatocytes [1,5] and at the same time down-regulate the production of, for example, TNF- α and IL-1 by macrophages and polymorphonuclear cells (PMNs; see under 'Glucocorticoids' for a description). The above mediators act by binding to their respective receptors on the cell surface. After binding they are internalized and/or transmit signals into the cells to effect a number of different intracellular regulatory mechanisms, e.g. activation and/or *de novo* synthesis of transcription factors and changes in processing and stability of RNA, translation efficiency and protein secretion.

A-SAA gene transcription is mainly induced by IL-1 and TNF- α . IL-6 has little effect by itself, but can synergize with IL-1 and TNF- α to generate a massive induction of the A-SAA promoters. Glucocorticoids also enhance the effect mediated by cytokines (see [5] for references). In addition, A-SAA may also be induced at the post-transcriptional level.

Transcriptional regulation

One of the effects of the signals transduced from the IL-1, TNF- α and IL-6 receptors is induction of a series of transcription factors, either by activation of resident pools of inactive factors in the cytoplasm and/or by increased factor biosynthesis (Scheme 2; [34–43]). Increased transcription of the A-SAA genes during the AP response has been demonstrated both *in vivo* and *in vitro*

[44–47], and the human A-SAA promoter has been studied using chloramphenicol acetyltransferase (CAT) constructs. Three regulatory elements, two positive [a nuclear factor κ B (NF- κ B) site located at –91 to –82 and an NF-IL6 (nuclear factor for IL-6) site at –184 to –171] and one negative site (located at –635 to –626) have been described [48–50].

CCAAT/enhancer binding proteins (C/EBPs) are a family of transcription factors which are regulated in a complex manner that is not yet fully understood. C/EBPs are involved in cell differentiation, during which they are themselves expressed differentially. C/EBP α is a constitutive transcription factor whose own transcription is down-regulated in hepatocytes during the AP response. The activity of both NF-IL6 and NF-IL6 β , respectively identical with C/EBP β and C/EBP δ (NF-IL6 and NF-IL6 β are the names used, in general, for the human analogues of C/EBP β and C/EBP δ respectively in other species) can be induced by the endotoxin lipopolysaccharide (LPS), IL-1 and IL-6 (Scheme 2, top). Increased NF-IL6 activity is, depending on tissue type, achieved by increased transcription and phosphorylation, whereas increased NF-IL6 β activity is achieved mainly by transcriptional up-regulation. All three C/EBPs are expressed in hepatocytes and most other tissues ([36–38] and references cited therein). The use of alternative translation start sites in the C/EBP α and NF-IL6 mRNAs give rise to isoforms that have different binding specificities and different transcription activator and repressor functions. The relative expression of the isoforms changes during the AP response by a start-site-selection mechanism that remains poorly understood ([51,52] and references cited therein).

NF- κ B, which is one of the main factors involved in the induction of gene transcription during inflammation and the AP response, was originally identified as a B-cell-specific transcription factor that binds to the promoter of the κ light chain of immunoglobulin [53]. Later it was discovered that NF- κ B is widely used (probably) by all cell types to regulate the expression of many different genes and that it is not a single factor, but in fact comprises a family of related proteins with sequence similarity to the avian-retrovirus-Rev-T oncogene product v-Rel (reviewed in [54]). The NF- κ B family (also called the Rel family) includes p50 and p52, which are the N-terminal proteolytic products of the precursors p105 and p100 respectively; p50 and p52 dimers both bind avidly to DNA, whereas the precursors are inactive. The NF- κ B family also includes p65 (RelA), RelB, c-Rel, the viral v-Rel, the *Drosophila* proteins Dorsal and Daf [54] and possibly a novel p55 recently identified in mouse B-cells [55]. These proteins have strong transcription transactivating features and they contain a nuclear localization signal. Homo- and hetero-dimers can form between any of the Rel proteins, giving rise to protein complexes with different DNA-binding sites and activities [54]. Most of the dimers, of which p50–p65 is probably the most common, are retained in the cytoplasm in an inactive form because the nuclear localization signal is masked either by the uncleaved p105 or p100 [56] or by I κ B (inhibitor of NF- κ B) proteins [57,58].

The I κ B family includes I κ B α , I κ B β , B-cell lymphocyte-leukaemia proto-oncogene 3 product (Bcl-3) and the *Drosophila* Cactus, which are all encoded by distinct genes. The family also includes I κ B γ , which arises either from alternative splicing of the p105 pre-mRNA or from the use of an alternative transcription start site in the p105 gene, and I κ B δ , which, because of sequence and structural similarities to I κ B γ and p105 has been suggested to arise from p100 (reviewed in [59,60]). I κ B ϵ is a recently discovered novel I κ B protein that resembles I κ B α and I κ B β in both sequence and function [61]. I κ BR and I κ BL are two recently cloned, and possibly novel, members of the I κ B family

Scheme 2 Activation of transcription factors which mediate increased transcription of A-SAA genes during the AP response

Signals transmitted (open arrows) from the agonist occupied IL-6 receptor (IL-6R) and/or the IL-1 receptor type I (IL-1RI) results in phosphorylation of the transcription factor NF-IL6, which then translocates to the nucleus, where it mediates transcription of AP genes, including the NF-IL6 β gene. *De novo*-synthesized NF-IL6 β enters the nucleus and further contributes to transcription of AP genes such as the A-SAA genes *SAA1* and *SAA2*. The transcription factor NF- κ B resides in the cytoplasm, associated with its inhibitor I κ B. Signal transduction (open arrows) from either the IL-1RI or the tumour necrosis factor receptor (TNF-R) activates the I κ B kinase signalsome, which phosphorylates I κ B (P-I κ B), and this becomes degraded by the ubiquitin–proteasome pathway. The newly liberated NF- κ B is translocated to the nucleus, where it promotes transcription of AP genes, including the A-SAA genes.

[62,63]. Often the uncleaved p105 and p100 are also considered to be I κ B family members. Each individual I κ B protein can inhibit a subgroup of the different Rel protein complexes [59,61].

Binding of IL-1 to the IL-1 receptor type I (IL-1RI) induces the assembly of a signalling complex comprising IL-1RI itself, the IL-1R-associated protein, the adapter protein MyD88, and the two IL-1R-associated kinases (IRAKs) IRAK-1 and IRAK-2. The IRAKs then interact with TNF-R-associated factor 6 (TRAF6), which in turn acts on the kinase NIK. TNF signalling is achieved via a similar pathway involving the initial trimerisation of the TNF-R, association of TRADD, RIP and TRAF2 and the subsequent activation of NIK. NIK activates the mitogen-activated protein kinase ('MAP kinase') pathway ([64–67] and references cited therein), which leads to activation of the I κ B kinase (IKK) 'signalsome' containing the recently identified serine kinases I κ B kinase-1 and I κ B kinase-2 (IKK-1 and IKK-2) [68–71]. The I κ B kinases phosphorylate I κ B (Scheme 2), an event that targets the I κ B to the ubiquitin–proteasome pathway, where it becomes degraded while still associated with NF- κ B [72–75]. I κ B α is generally degraded more quickly than I κ B β and I κ B ϵ , which results in fast and slow types of response respectively [61,76–81]. It has recently been demonstrated that p65 is phosphorylated by protein kinase A, which is associated with the NF- κ B–I κ B complex and becomes activated at the time of I κ B degradation [82]. NF- κ B is subsequently translocated to the nucleus (Scheme 2) and initiates or up-regulates the transcription of its target genes. The processing of the phosphorylated p105 precursor to p50 is also mediated by the ubiquitin–proteasome pathway [72]. Levels of p105, p100, p65 and c-Rel mRNAs are all increased by TNF- α treatment, probably via engagement of NF- κ B-binding sites which have been identified in the p105 promoter and which may also be present in the promoters of other genes [83–85].

In the hepatoma cell line HepG2, the two positive elements in the *SAA2* gene, the NF- κ B site located at –91 to –82 and the NF-IL6 site at –184 to –171, are both necessary for the full induction that can be mediated by either IL-1 or IL-6 alone to be achieved and for the synergistic effect generated by both cytokines used in combination. IL-1 induces a NF- κ B-like nuclear protein, the binding of which to the NF- κ B site is apparent in band-shift assays after only 15 min, but no longer evident after 2 h. The kinetics of this binding is not significantly changed when cells are co-stimulated with IL-6 [48–50]. Previous studies using methylation interference footprinting had indicated that the above factor has binding characteristics similar to those of p50 $_2$ [49]. In addition, the binding of a constitutive nuclear protein to this site appears to be enhanced by mutating it into a non-NF- κ B target, for which NF- κ B competes less effectively [50]. After treatment with IL-6 alone, multiple factors bind to the NF-IL6 site and generate a footprint that is similar in pattern and intensity to that observed on treatment with IL-1 and IL-6 together. The binding of these factors to the NF-IL6 target sequence is still apparent after 16 h. Binding can be inhibited by polyclonal antibodies recognizing NF-IL6 and NF-IL6 β , indicating that the factors are indeed NF-IL6 or related to NF-IL6. The nature of the synergy between IL-1 and IL-6 is not fully understood; however, Betts et al. [50] hypothesized that it could be due to co-operation between the bound factors via protein interactions that are analogous to those that occur in other systems [86,87], although they could not exclude the possibility that the 32 bp of exon 1 of the SAA gene that is present in the CAT-construct used in their study could contribute to mRNA stability and/or translational efficiency. Furthermore, the nuclear factors involved, their subunit composition and/or their putative post-transcriptional modifications were not fully defined [50]. Studies in the epithelial (cervical carcinoma) cell line HeLa have

shown that the NF- κ B site is functional when the cells are stimulated with the phorbol ester PMA, which activates protein kinases and thereby NF- κ B [48]. In monocytes a novel transcription factor termed SAA activating sequence factor [88], which is considered to be IL-6-dependent and was first identified in rabbits [89], is responsible for the induction of SAA expression by LPS. The binding site of this factor at residues -230 to -252 overlaps with a Sp1-binding site at -239 to -244 and is in close proximity to a NF- κ B binding site at -254 to -262 [88]. Further studies of this factor will help to precisely define the up- and down-regulation of A-SAA expression in both hepatic and extra-hepatic tissues.

NF- κ B and the C/EBPs have also been shown to regulate A-SAA gene transcription in mouse, rabbit and rat [90–98]. Heteromeric protein complexes between C/EBP and the NF- κ B subunit p65 (relA) in rabbit bind with different affinities to the C/EBP and NF- κ B binding sites and are strong transactivators of A-SAA gene transcription following LPS treatment [95]. The involvement of an additional novel constitutive transcription factor has been demonstrated in mouse. This factor binds next to a C/EBP binding site and is necessary for maximum transactivation by C/EBP δ [91]. In rat the transcription factor yin and yang 1 (YY1) represses A-SAA transcription by binding to a site overlapping with the NF- κ B-binding site [98] (see under 'Inhibitors of transcription factors' below for a discussion of a possible involvement of this transcription factor in the regulation of human A-SAA expression).

Post-transcriptional regulation

The total transcription of A-SAA RNA in mice challenged with the endotoxin LPS increases at least 300-fold, with each of the A-SAA genes (three A-SAA genes in mice; *SAA1*, *SAA2* and *SAA3*) being transcribed at similar levels and peaking by 3 h. Total levels of mRNA increase 2000-fold and peak at 12 h [45]. Total amounts of *SAA1* and *SAA2* mRNA remain at this high level until 36 h, by which time the level of *SAA3* mRNA has already decreased to 20% of its maximum. These results were interpreted as indicating that not only transcriptional, but also post-transcriptional, mechanisms, such as mRNA stability, are involved in the induction of A-SAA during the AP [45].

Tissue-culture experiments with the human hepatoma cell line PLC/PRF/5 have shown that A-SAA mRNA (*SAA1* and *SAA2*) levels peak at 24 h after treatment with monocyte-conditioned medium and the synthetic glucocorticoid dexamethasone and at 36 h after treatment with IL-1, IL-6 and dexamethasone. These peaks are followed by marked decreases in A-SAA mRNA levels. However, when cells are treated at peak times with actinomycin D to inhibit transcription, the A-SAA mRNA levels do not decline, but remain elevated, suggesting that the A-SAA mRNA becomes stabilized [99]. Mathematical modelling of the kinetics of A-SAA transcription and mRNA accumulation in similar experiments performed in the human hepatoma cell line Hep 3B indicated that the observed changes in transcription rates could not account for the dramatic accumulation in mRNA levels during the early stages of the AP response and that post-transcriptional regulation, probably involving changes in mRNA stability, must occur [46,47].

There are four major mechanisms whereby mRNA may be degraded (reviewed in [100]). Most often mRNA degradation is initiated by de-adenylation: when the poly(A) tail has been reduced to approx. 26–60 bases, the 5'-cap is removed by cleavage within the first few bases of the transcript which permits its rapid digestion by 5'-3'-exonucleases. The de-adenylated mRNA may also be digested by 3'-5'-exonucleases without decapping, but

the exact mechanism whereby this occurs, and its relative importance in mRNA degradation, is not known [100]. 'Failure' transcripts containing nonsense codons, unspliced introns or extended 3'-untranslated regions (3'-UTR) are known to be degraded by 5'-3'-exonucleases after decapping without prior de-adenylation, and certain mRNAs are degraded by what appear to be specific endonucleases that recognize consensus sequences in their 3'-UTRs (see [100] for references).

Of the four mechanisms, the de-adenylation-dependent decapping pathway of mRNA degradation is believed to be the most important [100]. Any mechanism that modifies any of the constituent steps (i.e. de-adenylation, decapping and/or exonuclease) may potentially regulate the stability of a particular mRNA. Although intermediates of both endonuclease and 3'-5'-exonuclease activity have been observed, intermediates of 5'-3' degradation are rarely seen (see [100] for references.). *In vivo* decapped, and possibly partially 5'-3'-degraded RNA intermediates, have only been demonstrated in mouse liver RNA using a 'circular PCR' method in which ligation of the 5' and 3' mRNA ends is followed by PCR across the ligated site [101]. This work underlines the strong potential of PCR methods for investigating the molecular regulation of various classes of RNA. The difficulties in observing 5'-3' digestion intermediates have been attributed to the speed of the process, which suggests that 5'-3' degradation is much faster than the preceding steps and therefore cannot be rate-limiting [100].

Changes in poly(A) tail length have been observed in both the A-SAA and α_1 -acid glycoprotein mRNAs upon AP stimulation [44,99,102,103]. Shortly after AP induction, the A-SAA and α_1 -acid glycoprotein mRNAs shift in size from 650 and 860 bases respectively to 750 and 1300 bases owing to the existence of longer poly(A) tails [44,102,104]. Later in the AP response, when the poly(A) tail becomes progressively shorter, mRNA species can be observed in all sizes between the above ranges [44,99,102,104]. Using circular PCR it has been demonstrated that, prior to stimulation, the poly(A) tails of 5'-capped A-SAA mRNA species are 20–300 residues long, whereas at 8 h and 48 h post-AP induction the poly(A) tails are 100–300 and 20–70 residues long respectively. In contrast, decapped mRNAs have poly(A) tails of only 20–60 residues at all time points [101].

Generation of the poly(A) tail is usually via fast processive synthesis, which is switched off when the tail reaches approx. 250 bases and is limited by an unknown length-control mechanism [105]. Northern-blot analysis of nuclear α_1 -acid glycoprotein pre-mRNA has demonstrated that the early change in poly(A) tail length is a nuclear process with a clear temporary shift from the shorter species to the longer species with no intermediates [102], but the mechanism of how some mRNAs are specifically targeted to receive longer poly(A) tails has not been determined. Whether A-SAA mRNA is subject to a different type of processing during the AP response is unknown, but it has been suggested that the change in poly(A) tail length could be compatible with the short transcriptional burst during the AP response [101], and certainly the increase in A-SAA poly(A) tail length is not as dramatic as that of α_1 -acid glycoprotein mRNA. It is generally considered that the poly(A) tail contributes to mRNA stability and therefore it would be expected that the longer A-SAA mRNA species would be more stable. However, evidence from tissue-culture studies of A-SAA expression in PLC/PRF/5 hepatoma cells has established that the stabilities of both the 'long' and the later partially deadenylated mRNA species are intrinsically similar (with half-lives of more than 24 h) [99]. Unfortunately these studies could not detect the early pre-stimulus 'short' A-SAA mRNA [99], which may have a different half-life, as suggested by mathematical modelling [46,47]. It could be speculated that the

possible differences in stability could be due to regulatory elements in the 5'-UTR, 3'-UTR and/or coding sequence of the A-SAA mRNA. For example, a destabilizing factor may be absent or be degraded immediately after initiation of the AP response, thereby allowing the intrinsic stability of the A-SAA mRNA to be manifest; later in the AP response such a factor may be synthesized *de novo* and actively destabilize the A-SAA mRNA.

A-SAA mRNA has been shown to associate mainly with small- and intermediate-size polyribosomes. Combined with the observation that the amount of newly synthesized A-SAA protein secreted from hepatocytes after AP stimulation is lower than would be expected from the high levels of A-SAA mRNA that are present, this suggests that A-SAA protein synthesis is subject to significant translational regulation [99] and/or post-translational regulation. However, none of the above studies were able to provide definitive evidence of such regulation.

RESOLUTION OF INFLAMMATION AND THE AP RESPONSE

As stated in the Introduction, prolongation of the AP response and its attendant inflammatory processes may have damaging consequences, and therefore effective temporal control of the AP is desirable. Whereas systems such as the blood coagulation and complement pathways include inhibitors and feed-back loops in the actual cascades, the massive induction of some AP proteins calls for tight control of their biosynthesis.

Although most of the attention in the field of AP research has been on early initiating events, increasing amounts of data on the processes underlying the resolution of inflammation and the AP response are accumulating. Many different pathways are involved, and the picture is still rather unclear. Some of the better documented pathways involve transcriptional repressors, glucocorticoids, receptor antagonists and decoys, and cytokines which either directly or indirectly mediate down-regulation of biosynthesis. Many of these regulatory mechanisms have been described in the context of inflammation, but are likely to apply to the early AP response also.

Control of transcription

Edbrooke et al. [49] have identified a negative regulatory element (SAA κ B2), located at -635 to -626 in the human A-SAA promoter, which contains a NF- κ B binding site in reverse orientation to the SAA κ B1 binding site. Mutation of this site into a non-NF- κ B-binding site results in increased constitutive and IL-1-stimulated A-SAA transcription. Located immediately 3' to this NF- κ B site is a C/EBP motif that, in footprinting studies, appears to bind (a) constitutive factor(s) which is displaced by a SAA κ B2-binding factor upon IL-1 treatment [49]. The significance of this has not been studied further.

The engagement of the transcription factor NF- κ B in the up-regulation of A-SAA gene transcription suggests that the NF- κ B/I κ B feed-back loop must be a component in the subsequent return to basal levels of gene transcription. Down-regulation of gene transcription may also be achieved through the action of repressor factors, but details of whether such factors reduce A-SAA transcription during the late phases of the AP response, and if so the mechanism(s) by which they act, still need to be elucidated.

Inhibitors of transcription factors

It has been postulated that I κ B can freely diffuse into the nucleus and actively remove NF- κ B from promoter regions (Scheme 3a

[106]), and some direct evidence for this has been reported. When I κ B is overexpressed on its own, it localizes to the nucleus, but if co-overexpressed with p50 or p65 it remains in the cytoplasm [58,107,108]. Using purified I κ B α and I κ B β and electrophoretic-mobility-shift assays it has been demonstrated that, *in vitro*, NF- κ B dissociates faster from its DNA-binding site when one of these inhibitors is present. In the absence of inhibitor the half-life of the NF- κ B-DNA complex is 45 min; I κ B α , the more potent inhibitor, reduces the half-life of the complex to 3 min, compared with 7 min for I κ B β [106]. Evidence that NF- κ B is recycled has been obtained by stimulating B-cells, which normally do not have active NF- κ B, with a phorbol ester [109]. After 30 min the cells have active NF- κ B located in the nucleus and inactive NF- κ B located in the cytoplasm. However, after 8 h, almost all of the NF- κ B is inactive and located mainly in the cytoplasm, with a minor fraction in the nucleus. When stimulated cells were co-treated with cycloheximide for 8 h, some NF- κ B remained active and was present in the nucleus, although some was also present in the cytoplasm, where inactive NF- κ B could also be identified [109]. These results indicated that the inactivation of NF- κ B depends on *de novo* protein synthesis. More recent studies identifying NF- κ B binding sites in the I κ B α gene promoter which lead to *de novo* synthesis of I κ B α [83,110-116] and demonstrating prolonged NF- κ B activity in I κ B α -deficient mice [117,118] together suggest that the principal NF- κ B 'deactivating' protein is I κ B α . More definitive evidence has been obtained from TNF- α - and IL-1-activated HeLa cells, in which I κ B α can be co-purified by immunoprecipitation of NF- κ B from nuclear protein extracts. In addition, the appearance of I κ B α in nuclei is associated with decreased DNA binding activity of NF- κ B, as demonstrated in electrophoretic-mobility-shift assays, and leads to decreased NF- κ B-dependent gene transcription of luciferase reporter constructs [119]. A functional nuclear export signal [120] has been identified in I κ B α and, by injecting I κ B α into the nuclei of *Xenopus* oocytes, it has been shown that it can mediate the export of NF- κ B complexes from the nucleus, thereby terminating NF- κ B-dependent transcription [121].

Initial degradation and later *de novo* synthesis of I κ B β and I κ B ϵ has also recently been demonstrated [61,76,77,80]. Newly synthesized I κ B β may accumulate in a hypophosphorylated form [79,80] that binds to NF- κ B, but does not prevent nuclear translocation, DNA binding and transcription initiation. This hypophosphorylated I κ B β is believed to contribute to the constitutive activation of NF- κ B by preventing its association with I κ B α and the phosphorylated form of I κ B β [78]. I κ B ϵ can also be found in differently phosphorylated forms [61], but whether it functions via a similar mechanism is unknown.

To summarize, the above constitutes an effective regulatory feed-back loop: I κ B sequestering NF- κ B in the cytosol is initially phosphorylated and degraded, thereby permitting NF- κ B to enter the nucleus (Scheme 2) and bind to target NF- κ B sites in the promoters of selected genes, including that encoding I κ B α , thereby providing the signal for transcription initiation and/or up-regulation (Scheme 3). The newly synthesized I κ B α and I κ B β , if subsequently phosphorylated, will enter the nucleus (Scheme 3), switch-off NF- κ B activity by preventing reassociation with DNA, and mediate its transport back to the cytosol in an inactive form. Additional complexity is added to this loop by the induction of p105 (p50 precursor), p100 (p52 precursor), p65 and c-Rel mRNAs by TNF- α , resulting in increased levels of NF- κ B precursors/subunits. Furthermore, the possible regulated processing and degradation of the inhibitory precursor proteins p105 and p100 suggest that the network of agonists and antagonists in the system involves even more subtle complexity [83-85,122,123].

Scheme 3 Possible pathways for down-regulation of the hepatic AP expression of A-SAA

Control of A-SAA gene transcription may be achieved through the NF-κB-IκB feedback loop and/or the transcription factor YY1 (**a**). Activation of the transcription factor NF-κB results in increased transcription of the IκB gene. *De novo*-synthesized IκB enters the nucleus, where it enhances dissociation of NF-κB from its binding site in the A-SAA promoter. The possible competition with YY1 for a partially overlapping binding site in the A-SAA promoter may also contribute to the dissociation of NF-κB from its target site. Translocation of the NF-κB-IκB complex to the cytoplasm is mediated by a nuclear export signal in IκB. Re-activation of transcription factors is prevented [indicated by 'blocked' arrows ($\rightarrow$ |)] by blocking further signal transduction from the cytokine receptors (**b**). Control of signal transduction (lack of signal transduction is indicated with broken lines) is achieved through *de novo* synthesis of soluble IL-1 receptor antagonist (IL-1Ra), which binds to the IL-1 receptor type I (IL-1RI, top left) and thereby prevents IL-1 from binding. Binding of IL-1 to the type II receptor (IL-1RII) does not result in signal transduction. Proteolytic cleavage of the extracellular regions of the tumour necrosis factor receptor (TNF-R) and the IL-1RII (top right) result in soluble receptors which act as 'decoys', preventing the agonists from binding to the membrane receptors which control and co-ordinate signal transduction.

There is increasing evidence that the cascade of NF-κB activation, IκB degradation and *de novo* synthesis of IκB can vary widely according to cell type and stimulus, e.g. IL-1 induces prolonged NF-κB activation in B-cells, but only a transient activation in endothelial cells, whereas TNF-α induces transient NF-κB activity in B-cells and persistent activity in endothelial

cells [76,77]. The same studies demonstrated transient degradation of IκBα that correlates with the initial activation of NF-κB in both cell types, but highlighted differences in the degradation of IκBβ. In B-cells IκBβ is slowly degraded after IL-1 treatment and is undetectable after 24 h, whereas TNF-α has no effect on the IκBβ levels. In endothelial cells IL-1 induces a

slower and less sustained reduction in $\text{I}\kappa\text{B}\beta$ levels than the partial reduction induced by $\text{TNF-}\alpha$. Although these data suggest that degradation of $\text{I}\kappa\text{B}\alpha$ is responsible for the initial activation of $\text{NF-}\kappa\text{B}$ and that the deactivation/maintenance of $\text{NF-}\kappa\text{B}$ is regulated by $\text{I}\kappa\text{B}\beta$ [76,77], the specificities of the responses according to both cytokine agonist and target cell type indicate that great care should be taken when interpreting results of $\text{NF-}\kappa\text{B}$ activation in relation to $\text{I}\kappa\text{B}$ protein levels. Furthermore, consideration of the number of different $\text{NF-}\kappa\text{B}$ species underlines the importance of precise identification of the proteins that participate in such processes.

Repressing transcription factors

In studies of the human A-SAA promoter the transcription factor that binds to the $\text{NF-}\kappa\text{B}$ consensus site has a specificity resembling that of p50_2 [49]. This is interesting, since the two $\text{NF-}\kappa\text{B}$ variants p50_2 and p52_2 , which are capable of high-affinity binding to $\text{NF-}\kappa\text{B}$ sites, are transcription-suppressing because they lack transactivating domains [124]. There is conflicting evidence regarding the interaction of $\text{NF-}\kappa\text{B}$ complexes with the oncogene product Bcl-3. Bcl-3 associates mainly with p50_2 or p52_2 [125–132] but, if present in sufficiently high concentration, may also associate with p50-p65 heterodimers [132]. In contrast with $\text{I}\kappa\text{B}$, Bcl-3 can act as a transcription activator. Two mechanisms have been proposed. One postulates that the suppressing activity of p50_2 and p52_2 can be relieved when bound to Bcl-3, which provides a strong transactivating domain [130,131]. This appears to be incompatible with the second proposed mechanism, which postulates the dissociation of p50_2 from $\text{NF-}\kappa\text{B}$ sites upon binding to Bcl-3, thereby allowing transactivating $\text{NF-}\kappa\text{B}$ complexes access to the sites [125–129,132]. However, Fujita et al. [130] used transfection constructs of different sizes derived from p105, the precursor of p50 , to demonstrate that the product most similar to p50 could synergize with Bcl-3 in transcription activation, whereas a larger construct (p60) could not, indicating that the second mechanism may be an artefact arising from the p60 construct. Phosphatase treatment of Bcl-3 blocks its ability to dissociate p50_2 from $\text{NF-}\kappa\text{B}$ sites, suggesting that the phosphorylation status of Bcl-3 may determine its *in vivo* activity, possibly by facilitating switches between direct/indirect activator and/or inhibitor forms [130,132]. Studies using thymocyte extracts from transgenic mice that constitutively express Bcl-3 in thymocytes have demonstrated that, whereas the activity of p52_2 is unchanged, the DNA-binding activity of the endogenous p50_2 is increased dramatically. The increased binding in these extracts can be abolished by *in vitro* phosphatase treatment; however, the inducible activity of p50-p65 was unaffected [133]. Further studies of these transgenic mice and of endogenous gene expression in non-transfected cells might provide the final evidence for one or both of the above mechanisms and the significance of p50_2 and p52_2 in the regulation of A-SAA and other AP proteins.

Another transcription factor which is involved in the tight regulation of A-SAA gene expression is YY1, which actively binds to a consensus target in the rat SAA1 promoter region [98]. The YY1-binding site is located between the positive *cis*-acting $\text{NF-}\kappa\text{B}$ - and C/EBP-binding sites and overlaps with the former. YY1 therefore competes with $\text{NF-}\kappa\text{B}$ for a partly shared promoter target. When the YY1 site in SAA1/reporter constructs is mutated to a non-YY1-binding site, there is enhanced expression of the reporter gene in both stimulated and unstimulated cells, indicating that YY1 acts as a negative regulator of SAA1 expression. Conversely, SAA1/reporter construct expression is suppressed when YY1 is overexpressed from a co-transfected

YY1 construct. YY1 is displaced by $\text{NF-}\kappa\text{B}$ and C/EBP in cells treated with cytokines, further substantiating the negative regulatory role of YY1 on the SAA1 promoter [98]. Lu et al. [98] speculated that YY1 could participate in the down-regulation of SAA1 expression during the later stages of AP response, although they could not demonstrate any changes in YY1 binding activity, or displacement of $\text{NF-}\kappa\text{B}$ and C/EBP by YY1. Antagonism between YY1 and the serum response factor has been demonstrated in studies of the activation of *c-fos* and α -actin promoters by the serum response element. The relative levels of the two factors determine the level of transcriptional activity [134,135], constituting a balanced mechanism which appears to be similar to the one suggested for rat SAA1 expression [98]. YY1 has not yet been formally identified as a component of SAA expression in species other than rat, but several sites with high sequence similarity to the YY1 sites are present in the promoters of the human A-SAA genes. Whether these sites are of functional importance is unknown, but it is noteworthy that two potential YY1 sites at –223 to –210 and –141 to –132 in the human SAA2 promoter [48] are in close proximity to the NF-IL6 and $\text{NF-}\kappa\text{B}$ sites and may interfere with binding of their cognate transcription factors if bound by YY1.

YY1 is an intriguing transcription factor because it has inherent transactivating activities as well as repressing properties. Which, if either, of these opposing activities are in effect under a given circumstance seems to depend on the promoter context and intracellular environment. YY1 activates the mouse *c-myc* promoter via a conventional 5'-upstream binding site [136], whereas, in the mouse rpl32 ribosomal protein gene YY1 enhances expression via two binding sites downstream of the transcription start site [137]. In contrast with the latter case, in the mouse rpl30 gene, which is related to rpl32, there is only one such downstream binding site and YY1 has no apparent influence on transcription [137,138]. Both of the above ribosomal protein genes lack conventional TATA boxes, and this might be a contributing factor to the use of downstream regulatory elements [137]. In the human 3'-enhancer of the immunoglobulin κ light chain, which is located downstream of the constant region, an orientation-independent YY1 site represses expression at the pre-B-cell stage, whereas in the mature B-cell this repression is abolished by an unexplained mechanism [139]. A mechanism by which repression of transcription by YY1 is relieved was defined in studies of the adenovirus-associated virus P5 promoter [140,141]. In experiments using CAT constructs driven by P5 promoters, the level of transcription was repressed by the presence of a functional YY1-binding site, when compared with the level achieved in the presence of a YY1 site which had been mutated to abolish its binding capacity. Following transfection of cells with an E1A (adenovirus protein) expression construct, transcription was greater in those that were co-transfected with the P5 reporter construct containing the wild-type YY1 site than in those co-transfected with the construct containing the mutated non-binding YY1 site, indicating that YY1 can be changed from a repressor to an activator [140]. Three mechanisms which could explain these results were suggested. YY1 may be bound to the promoter in a repressing form (Scheme 4a), which is changed by the presence of E1A to an activating form either by a modification or by direct association. Alternatively YY1 may repress due to association with an additional factor (Scheme 4b) which is released by E1A, thereby permitting YY1 to function as an activating factor [140]. Further studies demonstrating direct interaction between YY1 and E1A suggest a mechanism which is intermediate between these two (Scheme 4c; [142,143]). In both adenovirus-infected and non-infected cells YY1 is associated with p300 (a transcriptional co-activator which links trans-

Scheme 4 Transcription repression and de-repression mediated by transcription factor YY1

Transcriptional repression mediated specifically by YY1 (open arrows) may be relieved by the virus protein E1A either by a modification of YY1 (**a**, top pathway) or by associating with YY1 (**a**, bottom pathway), whereby a signal for transcriptional activation is achieved (filled arrows). Transcription may alternatively be initiated after E1A-mediated dissociation of a protein associated with YY1 (**b**) or by formation of a trimeric protein complex (**c**) of YY1, E1A and p300 (a transcriptional co-activator).

criptional activators with the basal transcription complex). Introducing mutations in E1A that disrupt binding of E1A to p300 also abolish E1A's ability to relieve repression by YY1, thereby suggesting the involvement of a heterotrimeric complex which acts as a transactivator ([141] and references cited therein). c-Myc is another protein which can modulate the activities of YY1. By directly binding to YY1, c-Myc can inhibit either transcriptional repression or activation mediated by YY1. The simplest model that explains this observation is one in which c-Myc causes YY1 to dissociate from its DNA-binding site [144]. Another component, YY1-associated factor 2, is a novel protein which binds directly to YY1 and during myogenic differentiation relieves YY1 transcriptional repression, probably by promoting its degradation [145].

Control of signal transduction

For the AP response and inflammation to be resolved, it is essential that the stimulation of target cells ceases and that their biosynthetic profile and activity are returned to normal. Several natural mediators of this process have been described. Some affect cytokine-receptor interactions to prevent further signal transduction. Others interfere with the intracellular signalling cascade. Reactive oxygen species have been implicated in the activation of NF- κ B and other transcription factors, and antioxidants such as vitamin E might be involved in controlling this (reviewed in [146]). Overexpression of IL-1 receptor-interacting protein, which only associates with the IL-1RI when the receptor is not occupied by IL-1, leads to reduced sensitivity to IL-1. This protein contains a GTPase-activating-protein-like domain and

may be involved in the inactivation of activated GTP-binding protein in the IL-1 signalling cascade by converting GTP into GDP, but its specific molecular target(s) has (have) not been identified ([64] and references cited therein). Likewise other phosphatases and GTPases are likely to be involved in the inactivation of the many kinases and possible GTP-binding proteins in the signalling cascades that follow engagement of the IL-1RI, TNF-R and IL-6R.

Antagonists

The IL-1 receptor antagonist (IL-1Ra) is, as yet, the only natural antagonist of the action of IL-1 to be defined. IL-1Ra can be detected at low levels in plasma from normal individuals and at increased levels in patients suffering from infectious diseases, certain cancers and trauma [147]. It was first identified in monocytes as three differently glycosylated secreted forms (one 18 kDa and two 22 kDa) of a single polypeptide, secreted IL-1Ra (sIL-1Ra) [148]. Human sIL-1Ra has 26/72% and 19/69% amino acid sequence identity/similarity to human IL-1 β and IL-1 α respectively [149,150] and binds to IL-1RI with an affinity equivalent to those of the two IL-1 species IL-1 α and IL-1 β [148,150]. Despite binding to IL-1RI (Scheme 3b, left), sIL-1Ra itself has no agonist activity and is therefore a classical receptor antagonist [148,150]. Although there have been some conflicting reports on the effect of sIL-1Ra on hepatoma cells and primary hepatocytes in *in vitro* studies [151–153], the demonstration *in vivo* in mice that the sIL-1Ra can completely block AP induction of SAA provides evidence that IL-1Ra is a powerful immune regulator [154].

Two intracellular isoforms of IL-1Ra (icIL-1Ra) have been identified [155,156]. These are generated by alternative splicing of a transcript which is initiated 5' of the sIL-1Ra transcription start site; the net result substitutes almost the entire signal peptide of sIL-1Ra with one or two upstream exons. The icIL-1Ra species are expressed constitutively in epithelial cells and can be induced in activated fibroblasts, PMNs and monocytes [155–158]. They inhibit a number of IL-1-mediated activities (e.g. induction of prostaglandin, IL-2 and selectin synthesis) when administered extracellularly [155,156,159]. In epithelial cells, gene-transfer studies have established that icIL-1Ra mediates a decrease in the stability of some IL-1 β -induced cytokine mRNAs [160]. icIL-1Ra is released from dying cells, such as fibroblasts killed by natural killer cells, and it has been suggested that this isoform represents a reservoir of IL-1Ra which may be released during tissue destruction to counteract IL-1 (M. Muzio, N. Polentarutti, M. Sironi, M. Introna and A. Mantovani, unpublished work).

Although sIL-1Ra is constitutively expressed, its production by most cells (e.g. monocytes, neutrophils, fibroblasts, epithelial cells and hepatocytes) is induced during inflammation and the AP response (see [147] for a review). In PMNs and hepatocytes, increased transcription driven by the sIL-1Ra promoter is controlled by NF- κ B (Scheme 3b, left) and members of the C/EBP family [147,161], and the resulting mRNA is stabilized in PMNs by transforming growth factor- β 1 and IL-10, which prolong its half-life from approx. 2 h to 3.5 h [157,162].

Receptor decoys

There are two receptors for TNF- α , termed TNF-R55 and TNF-R75 according to their molecular masses [163–170]. TNF-R55 is involved in most of the responses to TNF- α , whereas the participation of TNF-R75 is probably limited to only a few of these [171–175]. Transcription of the TNF-R genes and stability of the TNF-R mRNAs are both increased following stimulation of cells with TNF- α [176]. However, this does not lead to a corresponding increase in the number of cell-surface receptors, as there is a coincident triggering of receptor shedding (Scheme 3, right), mediated by proteolytic cleavage of their extracellular domains by a metalloproteinase-like enzyme released from activated cells, which results in the generation of the 30 and 40 kDa soluble receptors sTNF-R55 and sTNF-R75 [163,164,167–170,177]. These soluble receptors are sometimes also referred to as TNF-binding proteins, i.e. TNF-R55-BP and TNF-R75-BP (collectively TNF-BP). TNF-receptor shedding can also be stimulated by C5a, PMA, IL-10, prostaglandin E₂, and by cell-adhesion processes involving L-selectins and integrins. Such shedding has been demonstrated for neutrophils, monocytes and T-cells [178–185], although there are conflicting opinions on whether T-cells actually do shed receptors [181,183]. Soluble TNF receptors (sTNF-Rs) can still bind TNF- α after release from the membrane and are therefore considered to be part of the following feed-back loop: TNF- α binds to the membrane TNF-R, which then transmits a signal into the cell to generate more membrane-bound TNF-R and to elicit phenotypic changes to the immunological activated state appropriate to the particular cell type. Upon binding of TNF- α an additional signal is sent to a protease, which in turn cleaves off the extracellular region of TNF-R to release sTNF-R (Scheme 3b, right). This sTNF-R will limit further stimulation of the activated cell by competing with the membrane-bound receptor for unbound TNF- α agonist. In addition to being found locally at inflammatory sites, sTNF-R is also present in circulating blood and urine from healthy individuals [163,164,169] and at increased levels in these fluids in

patients with inflammatory diseases (see [181] for references), indicating that sTNF-R plays a systemic role in controlling the action of TNF- α .

A mechanism similar to the TNF- α /sTNF-R feedback loop has been demonstrated for IL-1 and its two receptors, IL-1RI and IL-1RII (Scheme 3b; reviewed in [186]). IL-1RI and IL-1RII are 80 kDa and 60 kDa transmembrane proteins respectively. Although evolutionarily and structurally related, the capacity to transduce signal is restricted to IL-1RI. IL-1RII is unable to transduce signals, as it has a truncated cytoplasmatic domain of only 29 amino acid residues, in contrast with that of IL-1RI, which is 215 amino acids residues long (see [186] for references). IL-1RII can be released from PMNs, presumably by proteolysis, as a soluble 47 kDa fragment [187,188] less than 30 s after stimulation [189] and can also be released from monocytes and macrophages as a 60 kDa fragment [190]. Soluble IL-1RII (sIL-1RII) retains its IL-1-binding activity (binding IL-1 β most efficiently [191]) in these modified forms [192]. However, it loses its affinity for IL-1Ra when released, which allows it to co-operate with the antagonist in the functional down-regulation of IL-1 [193,194]. The soluble receptor also acquires affinity for the IL-1 β precursor, suggesting a possible role in the regulation of IL-1 β processing if present in the cell [194]. sIL-1RII can be identified in normal plasma, and increased levels are observed during sepsis and inflammation [192,195,196].

Whether the above control mechanisms involving neutrophils, monocytes and lymphocytes also apply to the liver and therefore to the regulation of AP proteins such as the A-SAA is still unknown. Hepatocytes express at least one type of TNF-R and both of the IL-1R types, but their release from these cells as soluble receptors has not been established. The presence of cells of immunological origin in the liver and the presence of soluble receptors in plasma are interesting observations, as both could provide additional means of regulating the hepatic response to IL-1 and TNF- α .

Anti-inflammatory cytokines and glucocorticoids

Cytokines and glucocorticoids form a complex network of information exchange between cells, sending signals for up- and down-regulation of genes, cell proliferation, cell differentiation and providing the means to halt and reverse these processes. Many cytokines are capable of exerting both positive and negative effects, depending on which cell types they interact with and the context of the interaction. Whereas the pro-inflammatory cytokines (e.g. IL-1 and TNF- α) mostly have up-regulating effects on aspects of inflammation and the AP response, the anti-inflammatory cytokines, in general, have down-regulating effects. The latter exert their effect either by inhibiting further production of pro-inflammatory cytokines or by inducing the production of other powerful specific inhibitors. Glucocorticoids are synthesized in the adrenal cortex after induction by pro-inflammatory cytokines (e.g. IL-1) via the hypothalamus and the pituitary gland and are transported to their 'effector-sites', where they either enhance the effects of the pro-inflammatory cytokines (in the liver, as described above under 'Initiation of response') or provide a negative feed-back loop by shutting down the inflammatory response and thereby preventing the continued generation of up-regulating signals to the hypothalamus, adrenal cortex, immune system and liver (Scheme 1).

Anti-inflammatory cytokines

Down-regulation of synthesis of the pro-inflammatory cytokines IL-1, IL-6 and TNF- α by monocytes/macrophages and PMNs *in vitro* is mediated by IL-4, IL-10 and IL-13 probably by a

mechanism that leads to reduced transcription [197–202]. There is conflicting evidence regarding the influence of IL-4 and IL-10 on the activity of transcription factors such as NF- κ B and NF-IL6 and the stability of cytokine mRNAs. However, it has been suggested that, depending on the initial induction pathway used, and possibly also depending on co-operation between T-cells, monocytes and heterogeneous blood mononuclear cells, IL-4 and/or IL-10 can cause the activity of transcription factors to be reduced and the degradation of cytokine mRNAs to be increased [203–207]. In trials using intravenous injections of IL-10, the ability of whole-blood cells to produce IL-1 β and TNF- α could be reduced to 65–95% of normal shortly after injection [208]. In addition, both IL-4 and IL-13 can down-regulate the expression of CD14 (the LPS receptor) on monocytes *in vitro*, thereby preventing any cellular re-activation by endotoxin [201,209].

Production of IL-1Ra is induced/enhanced by IL-10 in heterogeneous blood mononuclear cells [210] and PMNs [162], and by IL-4 and IL-13 in monocytes [211]. Transforming growth factor- β may also induce the biosynthesis of IL-1Ra in monocytes and PMNs [157,212,213], possibly by a mechanism involving and requiring the preceding induction of IL-1 [213]. In patients undergoing immune therapy with IL-2, circulating levels of IL-1Ra rise dramatically to a plateau of approx. 11 ng/ml, a level which is sufficient to inhibit the production of IL-1 β and TNF- α by peripheral-blood mononuclear cells *in vitro*. The circulating IL-1Ra in these patients might originate principally from peripheral-blood mononuclear cells, since it has been demonstrated that these cells produce IL-1Ra when they are exposed to IL-2 *in vitro* [214].

The production and release of soluble receptor decoys is also induced by anti-inflammatory cytokines. Patients in whom circulating IL-1Ra concentrations were increased after IL-2 immune therapy (see above) also had elevated circulating sTNF-R, the cellular origin and physiological significance of which is, however, unknown [214]. It has been demonstrated *in vitro* that monocytes slowly shed sTNF-R and that this shedding is enhanced by IL-10 [181,200]. Also heterogeneous blood mononuclear cells and fibroblasts shed sTNF-R when treated sequentially with IL-1 and IL-10 [210]. Therefore the *in vivo* effect of IL-2 may be an indirect one that acts via induction of IL-10 production.

IL-10 also down-regulates the surface expression of TNF-Rs [200], whereas IL-4 suppresses the release of sTNF-Rs from monocytes [200]. In PMNs the transcription of the IL-1RII gene is up-regulated by IL-4 and IL-13, leading to increased surface expression of IL-1RII and the consequent release of sIL-1RII, which inhibits the action of IL-1 on the same cells [215,216].

Glucocorticoids

Upon binding of glucocorticoids, the glucocorticoid receptor (GR α) is translocated to the nucleus, where it acts as a transcription factor. Positive glucocorticoid response elements have been identified in the promoter regions of some hepatic AP genes, e.g. mannan-binding lectin and α_1 -acid glycoprotein [6,217]. Possible glucocorticoid response elements with negative effects on transcription rates have been identified in the promoter region of the IL-6 gene [218]. In addition, activated GR α associates with the NF- κ B subunits p50 and p65, and these interactions prevent binding to DNA of both transcription factors, possibly by sequestering them in the cytoplasm [219–221]. Glucocorticoids have also been demonstrated to down-regulate the biosynthesis of other cytokines, such as IL1, TNF- α and interferon- β [222,223]. The down-regulation of interferon- β biosynthesis is due to glucocorticoid-sensitive destabilization of the

mRNA mediated via an AU-rich motif in its 3'-UTR [223]. It has been suggested that decreased expression of IL-1 β is due to inhibition of translation, precursor processing and secretion of the mature IL-1 β [222], whereas decreased TNF- α expression may be accomplished largely by translational repression.

GR β , which is generated by alternative splicing of the glucocorticoid receptor transcript, binds to the same DNA motifs as GR α , but has no affinity itself for glucocorticoids. By competing with glucocorticoid-associated GR α for binding to the glucocorticoid responsive elements in promoters, GR β may regulate the transcriptional activity of GR α , but the physiological consequences of this have yet to be determined [224].

The synthetic glucocorticoid dexamethasone induces the expression of surface IL-1RII and the release of sIL-1RII from B-cells and PMNs by increasing both its transcription rate and mRNA stability [187,191,215]. Dexamethasone also induces the expression of I κ B α in epithelial cells, monocytes and T-cells, the consequence of which will either be to inhibit NF- κ B activation or to down-regulate NF- κ B activity (for a description of I κ B's regulation of NF- κ B, see under 'Transcriptional regulation' and 'Inhibitors of transcription factors') depending on the combination and timing of treatments [225,226]. This is in accord with the accepted role of glucocorticoids as immune suppressors, but intuitively appears to be in conflict with their capacity to enhance aspects of the hepatic AP response, e.g. A-SAA transcription is clearly up-regulated by NF- κ B-like factors, but A-SAA expression is enhanced when hepatic cells are co-treated with glucocorticoids. One possible explanation could be that the NF- κ B species involved in up-regulating the hepatic expression of A-SAA (and other NF- κ B-inducible AP genes, the expression of which are enhanced by glucocorticoids) are regulated not by I κ B α , but by a different NF- κ B inhibitor which is insensitive to glucocorticoids. Other possible explanations are that the expression of I κ B α is not regulated by glucocorticoids in hepatocytes or that glucocorticoids have additional up-regulating effects that override, or compensate for, the reduced contribution of NF- κ B to the transcription of A-SAA.

REGULATION OF A-SAA EXPRESSION

The many mechanisms whereby the early and late stages of the AP response are controlled constitute a complex network of up- and down-regulating signals that change over time to effect the differential expression of AP proteins. The hepatic expression of the major AP protein A-SAA can be summarized as follows. After inflammatory cytokines (e.g. TNF- α , IL-1 and IL-6) are produced at the inflamed site, they bind to their hepatic receptors (respectively TNF-R55 and TNF-R75, IL-1RI and IL-6R) and transmit signals into the cell. The impact of signal transduction is enhanced by glucocorticoids released from the adrenal cortex. Soluble receptors, sTNF-R55, sTNF-R75 and sIL-1RII, are released to prevent further activation, which may otherwise prolong the response beyond the timeframe required. Signalling from the TNF-Rs and IL-1RI mediates the phosphorylation of cytoplasmic NF- κ B-I κ B complexes; I κ B becomes degraded, and activated NF- κ B is translocated to the nucleus. Signalling from the IL-6R mediates the phosphorylation of cytoplasmic NF-IL6 and its transfer to the nucleus. In the nucleus NF- κ B and NF-IL6 bind to the promoter regions of several genes, including those encoding SAA1, SAA2, I κ B, IL-1Ra and NF-IL6 β and increase their transcription rate. Subsequently a number of protein products from the newly transcribed and translated mRNAs are produced. Coincident with secretion of A-SAA, sIL-1Ra is also secreted, and this, together with the release of soluble receptors, prevents prolonged stimulation of the cell by

pro-inflammatory cytokines. Newly synthesized NF-IL6 β and I κ B enter the nucleus; I κ B inactivates NF- κ B and returns it to the cytoplasm, whereas NF-IL6 β will contribute to maintaining the increased transcription rate. Additional negative transcription factors, e.g. YY1, may reduce the A-SAA transcription rate at a later stage. Owing to its long half-life, A-SAA mRNA probably continues to accumulate for as long as there is a modest level of transcription from the A-SAA genes. At this later stage the response of the individual hepatocyte is essentially coming to an end and the A-SAA mRNA, which is still present at high concentrations, is translated less efficiently, thereby precluding the over-production and secretion of A-SAA protein.

CONCLUSION

The differential expression of inflammatory and AP proteins is achieved by their different capacities to respond to changing combinations of elements of the regulatory pathways that are activated by the various signalling cascades elicited. A-SAAs are multifunctional proteins involved in a number of immunological processes such as cholesterol transport and immune modulation, but which may also have damaging effects, e.g. amyloidosis and possibly arteriosclerosis, if overexpressed. A-SAA synthesis is regulated mainly by IL-1, TNF- α , IL-6 and glucocorticoids at both the transcriptional and post-transcriptional levels. A complex network of agonists, antagonists, feed-back loops and receptor decoys ensures that the response is optimized for counteracting the underlying immunological challenge and that the attendant negative physiological consequences of this potent protective system are minimized. However, although our understanding of the regulation of inflammation and the AP response has advanced enormously over the past decade, there remain many unanswered questions relating to the identity of the transcription factors involved in transcriptional regulation of A-SAA expression and the mechanisms of post-transcriptional regulation. Future studies of the involvement of regulatory pathways such as the NF- κ B-I κ B feed-back loop and soluble receptors will not only be important for our understanding of how A-SAA expression is regulated, but will also be essential for the development of safe and effective anti-inflammatory therapies based on manipulation of specific component pathways. Understanding the means by which the expression of A-SAA sometimes evades the normal physiological counter-inflammatory processes to remain high and participate in the pathogenesis of disease may suggest particular therapeutic strategies that could be employed to treat conditions associated with the overproduction of A-SAA.

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