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THE CARDIOVASCULAR HORMONE ANP INTERFERES WITH LPS-INDUCED EARLY INFLAMMATORY PATHWAYS *IN VITRO* AND *IN VIVO*

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1 INTRODUCTION

1.1 BACKGROUND AND AIM OF THE STUDY

Homeostasis is an essential aspect of the mammalian immune system. Under normal conditions, many bacteria live in coexistence with the human body. Especially the skin, the gastrointestinal tract or the external urogenital organs contain bacteria, not causing any harm to the organism. But, pathogenic as well as commensal microorganisms or their constituents, such as lipopolysaccharide (LPS), evoke an immune response after passing the barrier between the external and internal environment of the body. Sepsis, severe sepsis and septic shock describe a complex clinical syndrome, which results from an exaggerated host response to microbial infection. Sepsis develops when the initial, appropriate host response becomes amplified and then deregulated. When an organ system begins to fail because of sepsis, the sepsis is considered severe, once occurred, leading to approximately 50% mortality. Therefore, sepsis is the leading cause of mortality in intensive care units. An understanding of the mechanisms involved in sepsis and septic shock is essential to design new therapeutic strategies, to prevent sepsis, and thus improve the outcome after microbial infection.

The atrial natriuretic peptide (ANP) is a peptide hormone mainly secreted by the cardiac atria, in response to atrial stretch, reflecting increased intravascular volume. After the discovery of the atrial natriuretic peptide, the hormone was thought to mainly possess cardiovascular effects, in summary, regulation of blood pressure and plasma volume expansion. Interestingly, atrial natriuretic peptide receptors were found to be expressed in diverse tissues besides the cardiovascular and renal system, such as thymus, spleen, lymph nodes as well as isolated macrophages. The ANP and ANP-receptor expression is regulated by various immunomodulatory agents. Therefore, a great effort has already been done to study the coherence of ANP and the immune system.

For a long time, increased plasma levels of atrial natriuretic peptide have only thought to be elevated in patients with congestive heart failure. Recently, ANP has attracted interest as a new marker in the field of sepsis. Plasma levels of ANP and its precursor protein pro-ANP have been demonstrated to be highly increased in septic patients. For this reason, endogenous ANP might not only be considered a marker but also a regulatory mediator in inflammation and sepsis. With regard to severe inflammatory processes during endotoxemia, TNF-α has been demonstrated to be one of the leading pro-inflammatory cytokines.

During the last years, in our laboratory a lot of work has been done to investigate the interrelation of ANP and the immune system. Following LPS stimulation, we demonstrated that ANP is able to prevent TNF-α expression in macrophages *in vitro*. Furthermore, the atrial natriuretic peptide has quite recently been shown to protect from LPS-induced septic shock *in vivo*. The influence of ANP leads to a strong decrease in serum TNF-α levels and finally promotes survival of infected mice. Therefore, aim of the present work was to elucidate the ANP mediated protective mechanisms involved in LPS-induced septic shock. First, we aimed to answer the following question.

- How do different organs contribute to decreased serum TNF-α levels in LPS-induced sepsis in mice?

The liver is the central organ in whole body metabolism and plays a pivotal role in natural immune response of the host to infection. Especially resident liver macrophages are activated by several bacterial stimuli and produce various cytokines, such as TNF-α. Therefore, the liver seems to be one of the most interesting organs in LPS-induced septic shock, pointing to several questions.

- In which way does ANP preconditioning influence the inflammatory processes in the liver during LPS-induced septic-shock?

- Which cell type within the liver is mainly involved in inflammatory processes?

The liver constantly contains a rich blood supply, consistent with its important metabolic and immunomodulatory functions. In previous studies we demonstrated that ANP prevents LPS-induced TNF-α expression in whole human blood. Hence, we wanted to investigate the influence of ANP on LPS-induced TNF-α expression in different blood leukocytes.

- Does primarily LPS-induced TNF-α release from macrophages evoke proximate organ answers?

- How does ANP preconditioning interfere within this LPS-induced TNF-α expression in leukocytes?

1.2 NATRIURETIC PEPTIDES

The basic natriuretic peptide (NP) family comprises atrial, brain and C-type natriuretic peptide. These peptides mainly mediate natriuretic, diuretic, vasorelaxant and mitogenic responses. Atrial natriuretic peptide (ANP) is the first hormone, which has been described for the natriuretic peptide family ([44] de Bold, 1981). In 1988 urodilatin was isolated from the human urine. This peptide being synthesized in kidney tubules belongs to the A-type natriuretic peptides, representing a differentially processed molecular form ([51] Forssmann, 2001; [160] Schulz-Knappe, 1988). Besides the A-type natriuretic peptides, two more members, B-type or brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP) have been identified. BNP, which shares a highly homologous structure with ANP has first been isolated from the porcine brain, and therefore, named “brain natriuretic peptide” ([168] Sudoh, 1988). Following BNP discovery, a second natriuretic peptide has been isolated from the porcine brain, and in accordance to ANP and BNP it has been called CNP, C-type natriuretic peptide ([169] Sudoh, 1990). Although, as seen in Figure 1:1, ANP, BNP and CNP share a highly homologous structure, they differ in their site of synthesis and show preferences for specific cell surface receptors. Therefore, natriuretic peptides most likely influence different biological functions ([148] Pandey, 2005). Besides these three well known peptide hormones two more groups of natriuretic like peptides have been discovered, to date. The first group, *Dendroaspis* natriuretic peptides (DNPs) has been identified in 1992. DNPs, which are structurally homologous to other natriuretic peptides, have been isolated from the venom of the green mamba, *Dendroaspis angusticeps* ([162] Schweitz, 1992). First studies indicate, that DNP or DNP related peptides might also be present in the human body ([153] Richards, 2002). The second group, TNPs, taipan venom natriuretic peptides including TNP-a, TNP-b and TNP-c, has been discovered in the venom of the inland taipan, *Oxyuranus microlepidotus* ([53] Fry, 2005).

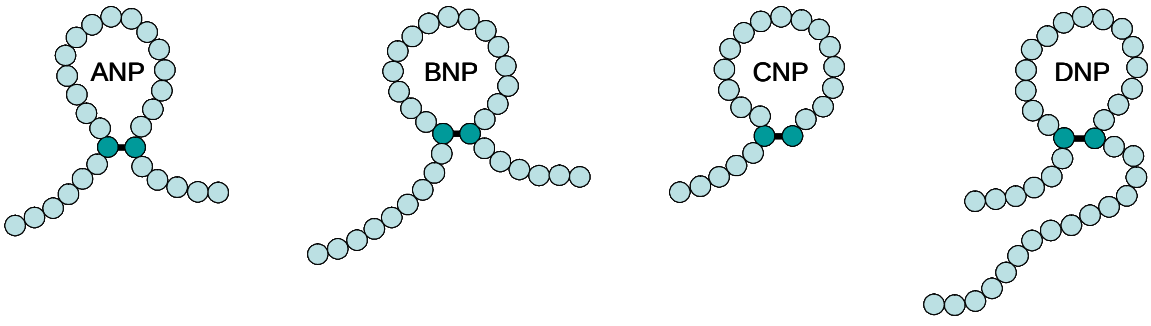


Figure 1:1 *ANP, BNP, CNP and DNP constitute the main members of the natriuretic peptide family.*

1.2.1 NATRIURETIC PEPTIDE RECEPTORS

Natriuretic peptides mediate their effects by interacting with their corresponding high-affinity receptors on the surface of target cells. The existence of different natriuretic peptides proves the complexity of their role in regulating diverse biological functions. This complexity further seems to be increased by the availability of different natriuretic peptide specific receptor proteins. In mammalian tissue, three different natriuretic peptide receptors have been identified to date (Figure 1:2) ([148] Pandey, 2005):

- natriuretic peptide receptor A = NPR-A = A-type receptor
- natriuretic peptide receptor B = NPR-B = B-type receptor
- natriuretic peptide receptor C = NPR-C = C-type receptor
  = clearance receptor

The hormonal activities of ANP and BNP are mainly mediated by the A-type natriuretic receptor. This receptor generally exhibits ligand selectivity for ANP and BNP, but hardly for CNP, and mediates most of the known actions of the natriuretic peptides. The closely related B-type receptor binds all natriuretic peptides, with main preference for CNP. Both receptors, NPR-A and NPR-B are coupled to guanylate-cyclase (GCase) and generate cyclic-guanosine-monophosphate (cGMP) in response to hormone binding. An increase in intracellular cGMP mediates hormonal activities through cGMP regulated effector molecules. NPR-A as well as NPR-B belong to a large family of receptors, that share a common overall molecular configuration ([113] Levin, 1998; [134] Misono, 2002; [173] Tremblay, 2002). The C-type natriuretic receptor lacks the GCase domain. Interestingly, NPR-C binds all natriuretic peptides with equal affinity. After binding of natriuretic peptides, NPR-C triggers removal of excess of these peptides from the circulation. It has been reported, that binding of CNP to NPR-C inhibits adenylate-cyclase (ACase) activity and activates phosphatidylinositol turnover. But to date, NPR-C has not directly been linked to any of the known hormonal effects of the natriuretic peptides ([136] Misono, 2005).

Natriuretic peptide receptors are distributed in different mammalian tissues, for example the cardiovascular system, the kidney, thymus and the spleen ([105] Kurihara, 1986; [139] Napier, 1984; [182] Vollmar, 2005). We are primarily interested in signaling pathways involved in LPS-induced inflammation in liver. Therefore, it is of main interest that NP-receptors are generally expressed in rodent and in human liver cells ([183] Vollmar, 1997; [194] Yip, 1985).

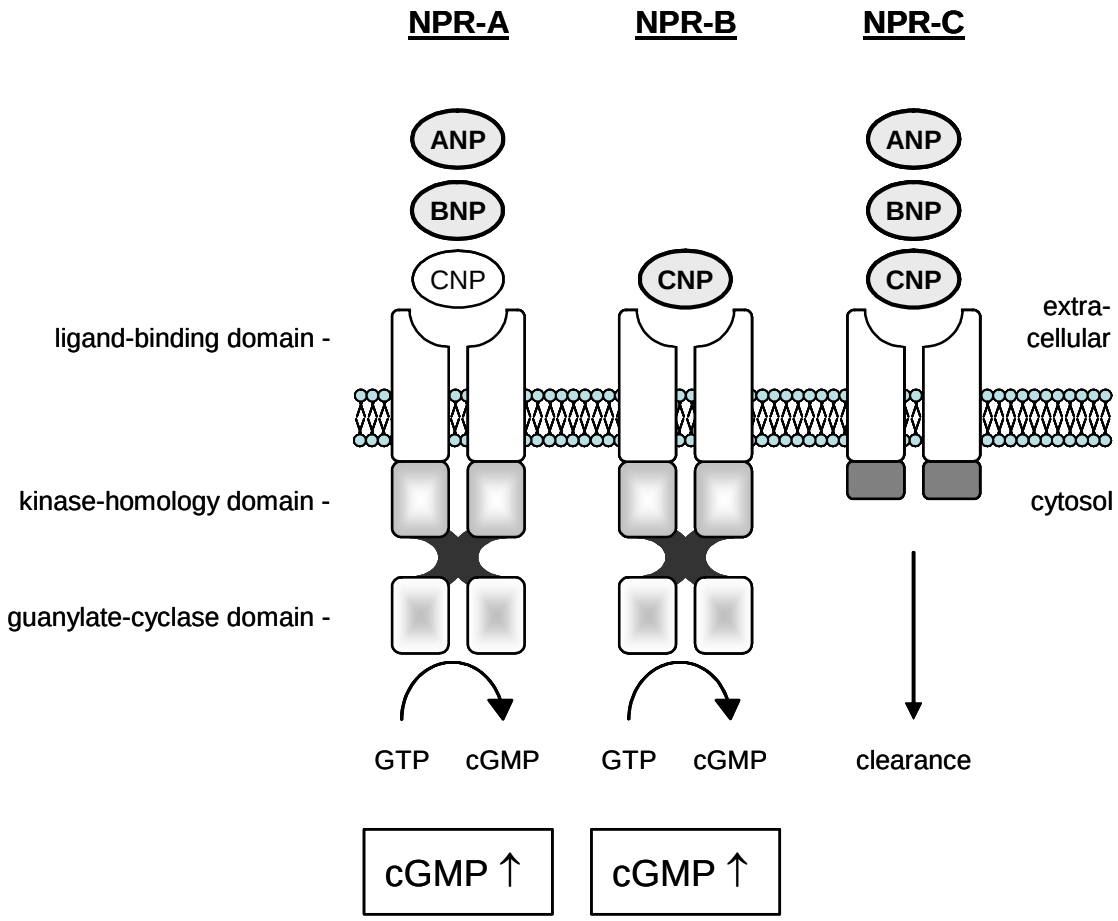


Figure 1:2 *Structure and binding specificities for natriuretic peptide receptors.* After extracellular binding at the NPR-A or NPR-B, cGMP is elevated in the cytosol. The C-type natriuretic peptide receptor is able to clear natriuretic peptides and exerts other biological effects. NPR-A preferably binds ANP and BNP, whereas NPR-B binds CNP. The C-type natriuretic peptide receptor does not show any preferences and equally binds ANP, BNP and CNP.

1.2.2 ATRIAL NATRIURETIC PEPTIDE STRUCTURE AND FUNCTION

In 1981, the atrial natriuretic peptide has first been described by *de Bold et al*. ([44] de Bold, 1981). As shown in Figure 1:3, the atrial natriuretic peptide is a circular peptide hormone based on 28 amino acids, containing a disulfide bond between two cysteine residues (cysteine 7 to cysteine 23). The resulting 17 amino acid ring structure is highly conserved among natriuretic peptides and essential for its biological activity ([135] Misono, 1984). The biosynthesis of ANP is typical for different peptide hormones. Pre-pro-ANP, a 151 amino acid pre-pro-hormone is generated, which is cleaved at the N-terminus, resulting in a 126 amino acid storage form, called pro-ANP. This peptide contains its biological active form in its carboxyl-terminus region.

Following stimulation, pro-ANP is cleaved into an amino-terminal fragment and its biological active peptide, ANP ([80] Inagami, 1989; [148] Pandey, 2005). Finally, the atrial natriuretic peptide is released into circulation from granules of the heart atrium *via exocytosis* ([140] Newman, 1991).

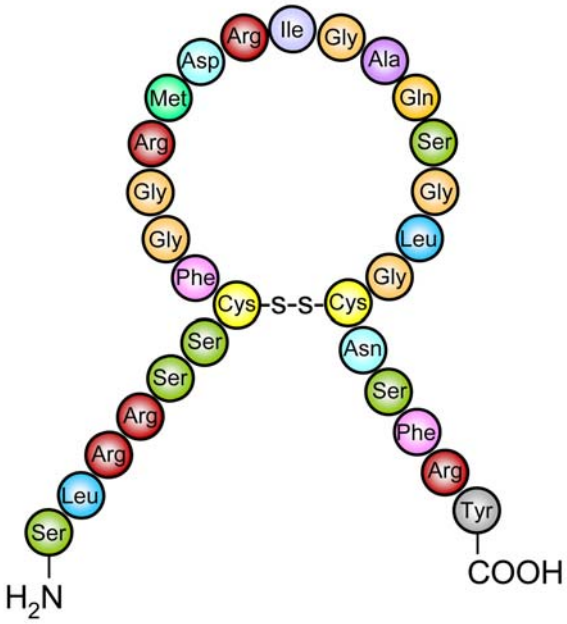


Figure 1:3 *Structure and amino-acid sequence of the human atrial natriuretic peptide*. The peptide consists of a highly conserved 17 amino acid ring-structure. This element is quite homologous among all natriuretic peptides and seems to be essential for its biological activity.

ANP elicits a variety of responses, which are basically directed toward the reduction of blood pressure and volume homeostasis. Therefore, the primary stimulus for ANP secretion is exerted by increased atrial-wall tension, due to elevation of venous blood pressure ([80] Inagami, 1989; [113] Levin, 1998; [148] Pandey, 2005; [170] Suzuki, 2001). After secretion into the plasma, atrial natriuretic peptide promotes diuresis and natriuresis, following reduction of extracellular fluid volume. This effect is provoked by direct interacting of ANP with tubular actions in the kidney ([113] Levin, 1998). ANP further mediates vasodilatation and inhibition of the renin-angiotensin-aldosterone system ([170] Suzuki, 2001). Interestingly, besides atrial distension a number of additional stimuli have been identified to induce ANP secretion from the heart, such as angiotensin-II and different cytokines. Surprisingly, several immunomodulatory agents are able to induce ANP expression, pointing to important functions of atrial natriuretic peptide in the immune system ([182] Vollmar, 2005).

1.2.3 ANP AND THE IMMUNE SYSTEM

First evidence for the link between the cardiovascular hormone, ANP, and the immune system has been found by detecting atrial natriuretic peptide besides the cardiovascular system in lymphoid organs ([184] Vollmar, 1990). Upon these investigations, it was discovered that ANP is not only distributed, but also produced by cells of the immune system, such as macrophages ([185] Vollmar, 1994). The corresponding atrial natriuretic peptide receptors are located in cells belonging to the innate, as well as the adaptive immune system ([182] Vollmar, 2005). In 1993, cGMP coupled effects of ANP on phagocytotic activity have primarily been described in macrophages ([94] Kiemer, 2002a; [127] Mattana, 1993). Additionally, ANP treatment of resident liver macrophages, also denoted as Kupffer cells, has been shown to significantly increase phagocytotic activity *via* the A-type natriuretic peptide receptor ([94] Kiemer, 2002a; [127] Mattana, 1993). However, ANP does not only influence cellular defense mechanisms of macrophages but also interferes with signaling pathways induced by various pro-inflammatory stimuli. As subsequently described, to date, ANP is known to exhibit several anti-inflammatory properties. These properties were investigated *in vitro*, such as in macrophages and endothelial cells, as well as *in vivo*, for instance in mice ([91] Keller, 2005a; [96] Kiemer, 2002b; [182] Vollmar, 2005).

1.2.3.1 INFLUENCE OF ANP ON LPS-INDUCED INFLAMMATORY PROCESSES

Following LPS-stimulation, macrophages produce large amounts of nitric-oxide (NO) *via* inducible NO-synthase (iNOS), which is an important mediator of host response. On the transcriptional level, ANP has been shown to markedly inhibit the LPS-mediated activation of the transcription factor NF-κB, which is crucial for the transcriptional activation of iNOS. Consequently ANP prevented the formation of NO. Moreover, it has been investigated that ANP mediated iNOS regulation does not just depend on transcriptional but also on post-transcriptional processes in murine macrophages ([97] Kiemer, 1997; [98] Kiemer, 1998).

Furthermore, *in vivo*, ANP has been demonstrated to affect the COX_2 system by significantly attenuating the thromboxane-B_2 and prostaglandine-E_2 (PGE_2) production in LPS treated mice. Continuatively, in murine macrophages, ANP was shown to influence the PGE_2 production predominantly *via* the C-type natriuretic peptide receptor and cAMP, involving reduced COX_2-protein and COX_2-mRNA level ([96] Kiemer, 2002b).

Following activation, ANP inhibits TNF-α expression in LPS-stimulated bone marrow-derived macrophages as well as in LPS-stimulated human whole blood. These findings are consistent with diminished TNF-α mRNA levels ([95] Kiemer, 2000; [99] Kiemer, 2001). Interestingly, in LPS treated isolated Kupffer cells, ANP reduces TNF-α expression. This reduction of TNF-α secretion in ANP-preconditioned, LPS-stimulated Kupffer cells is accompanied by an increased amount of cell associated TNF-α. Even though, LPS-induced TNF-α mRNA expression was not affected. Therefore, atrial natriuretic peptide supposedly influences translational as well as post-translational processing of TNF-α ([94] Kiemer, 2002a).

1.2.3.2 INFLUENCE OF ANP ON TNF-α-INDUCED INFLAMMATORY PROCESSES

The ability of ANP to inhibit induction of inflammatory mediators, such as iNOS, COX_2 and TNF-α, represents important aspects supporting its anti-inflammatory properties. To elevate complexity, atrial natriuretic peptide does not only decrease expression of the pro-inflammatory cytokine TNF-α, but also interferes with TNF-α mediated signaling pathways in endothelial cells.

Recently, it has been demonstrated that pretreatment of endothelial cells with atrial natriuretic peptide strongly reduces TNF-α induced expression of mRNA and cell surface protein levels, such as E-selectin and ICAM-1. These effects in turn are associated with inhibition of TNF-α-induced activation of the transcription factor NF-κB ([101] Kiemer, 2002d). TNF-α is also known to induce vascular permeability. In our laboratory, ANP has been demonstrated to abrogate TNF-α induced changes in vascular permeability associated with stress-fiber formation and actin polymerization, involving cGMP and p38 MAPK dependent signaling pathways ([100] Kiemer, 2002c).

1.2.3.3 CYTOPROTECTIVE EFFECTS OF ANP

Besides its protective potential in different inflammatory mechanisms, ANP also contributes to various cytoprotective processes. Thus, atrial natriuretic peptide has frequently been shown to protect against ischemia/reperfusion injury in different organs, such as in liver. The production of cytoprotective proteins, like heat shock proteins, by ANP might account for these beneficial actions ([17] Bilzer, 1994; [57] Gerwig, 2003; [182] Vollmar, 2005).

1.3 SEPSIS, SEVERE SEPSIS AND SEPTIC SHOCK

A very essential aspect of the mammalian immune system is to maintain homeostasis. Usually, many bacteria live in coexistence with the human body. Especially the skin, the gastrointestinal tract and part of the urogenital organs contain bacteria, not causing any harm to the organism. The presence of these microorganisms is not a threat to the body, since these organs play a pivotal role in connecting the sterile “internal environment” with the “external environment”. However, pathogenic as well as commensal microorganisms or their constituents, evoke a strong immune response after passing the barrier between the external and internal environment of the body ([174] van Amersfoort, 2003). Sepsis develops, when the initial infection becomes amplified and dysregulated. Interestingly, sepsis does not define a certain state but rather describes a complex clinical syndrome, resulting from an overwhelming host response (Table 1:1). Therefore, sepsis is an infection-induced syndrome defined as the presence of two or more of the following features of systemic inflammation:

- hyperthermia/fever or hypothermia
- leucokytosis or leucopenia
- tachycardia (> 90 beats/minute) or tachypnea (> 20 breath/minute).

When an organ system begins to fail, the sepsis is considered to be severe ([189] Wheeler, 1999). Hepatic failure is a common start of septic decompensation, thus followed by multiple organ failure, often resulting in septic shock ([32] Ceydeli, 2003). The overall mortality due to sepsis is approximately 30%, rising up to 40% in the elderly and becomes elevated up to 50% or greater in patients suffering from severe sepsis and septic shock ([4] Alberti, 2002; [35] Cohen, 2002). If four or five organs fail, the mortality will be even greater than 90%. The knowledge about the molecular mechanisms of sepsis rapidly increased over the past decades. Unfortunately, the therapeutic approaches used to treat sepsis have not become very successful, to date ([141] Nguyen, 2003).

SIRS systemic inflammatory response syndrome	sepsis	severe sepsis	septic shock
severe injury in the absence of infection	systemic inflammatory response to an infection	organ failure because of sepsis	severe sepsis combined with massive hypotension
	mortality approximately 30%	mortality approximately 50%	mortality 50% up to 90%

Table 1:1 *The different faces of sepsis.*

1.3.1 THE INFLAMMATORY CASCADE

Multiple organ failure is the ultimate cause of death in patients with sepsis. Usually, patients will first develop a single organ dysfunction, which commonly results in failure of further organ systems. Primarily, the septic machinery is highly influenced by extensive cross-talk between different cascades of inflammatory mediators. Upon activation, these cascades affect various pro- and anti-coagulant pathways, finally leading to the clinical septic syndrome (Figure 1:4) ([83] Johnson, 1996; [84] Johnson, 1998).

Sepsis occurs due to a microbial infection mainly with bacteria or bacterial components, such as lipopolysaccharide (LPS), a cell wall component of gram-negative bacteria. The initial host-microbial interaction involves a strong activation of the innate immune system, leading to coordination of the host-defense response. Once activated, mononuclear cells, like macrophages/monocytes, immediately release classic pro-inflammatory cytokines, such as TNF-α and IL-1 ([172] Tom van der Poll, 1999). Investigations on the kinetic of cytokine release have shown that the first pro-inflammatory cytokine appearing in human circulation is supposed to be TNF-α. Amounts of TNF-α in plasma are soon detectable and peak 90 minutes after microbial-infection ([47] Dinarello, 1997; [175] Van Der, 1997). Following the initial response, a secondary level of the inflammatory cascade is activated, involving various cytokines, lipid mediators, reactive oxygen species and cell adhesion molecules. The latter results in leukocyte-endothelial cell interaction initiating inflammatory cell migration into tissues ([188] Weigand, 2004). Together, these mediators are playing a key role by forming a complex network of secondary responses to microbial-infection ([35] Cohen, 2002).

Cytokines are also important in inducing a pro-coagulant effect in sepsis. Following microbial-infection, mononuclear and endothelial cells are activated, resulting in expression of tissue factor (TF) by these cells. Thereon, tissue factor activates numerous proteolytic cascades, leading to conversion of pro-thrombin to thrombin, which generates fibrin from fibrinogen. At the same time anti-coagulant cascades are impaired because of high plasma levels of plasminogen-activator inhibitor (PAI), thus preventing the generation of plasmin from plasminogen. Under normal conditions, fibrin is broken down by plasmin, ensuing homeostasis in coagulation. Together, these effects not only enhance production but also reduce removal of fibrin. This consequently leads to disproportion of pro-coagulant cascades and anti-coagulant mechanisms ([35] Cohen, 2002; [172] Tom van der Poll, 1999).

Additionally, down-regulation of three anti-coagulant proteins; anti-thrombin (AT), activated protein C (APC) and tissue factor pathway inhibitor (TFPI), contribute to the pro-coagulant condition during sepsis. Interestingly, these proteins not only influence coagulation but also exert important anti-inflammatory properties. These properties include inhibition of activation of the transcription factor NF-κB, thus decreasing TNF-α release by monocytes ([144] Okajima, 2001; [195] Yuksel, 2002). All these findings indicate a strong interplay between mediators of the inflammatory and coagulant cascade.

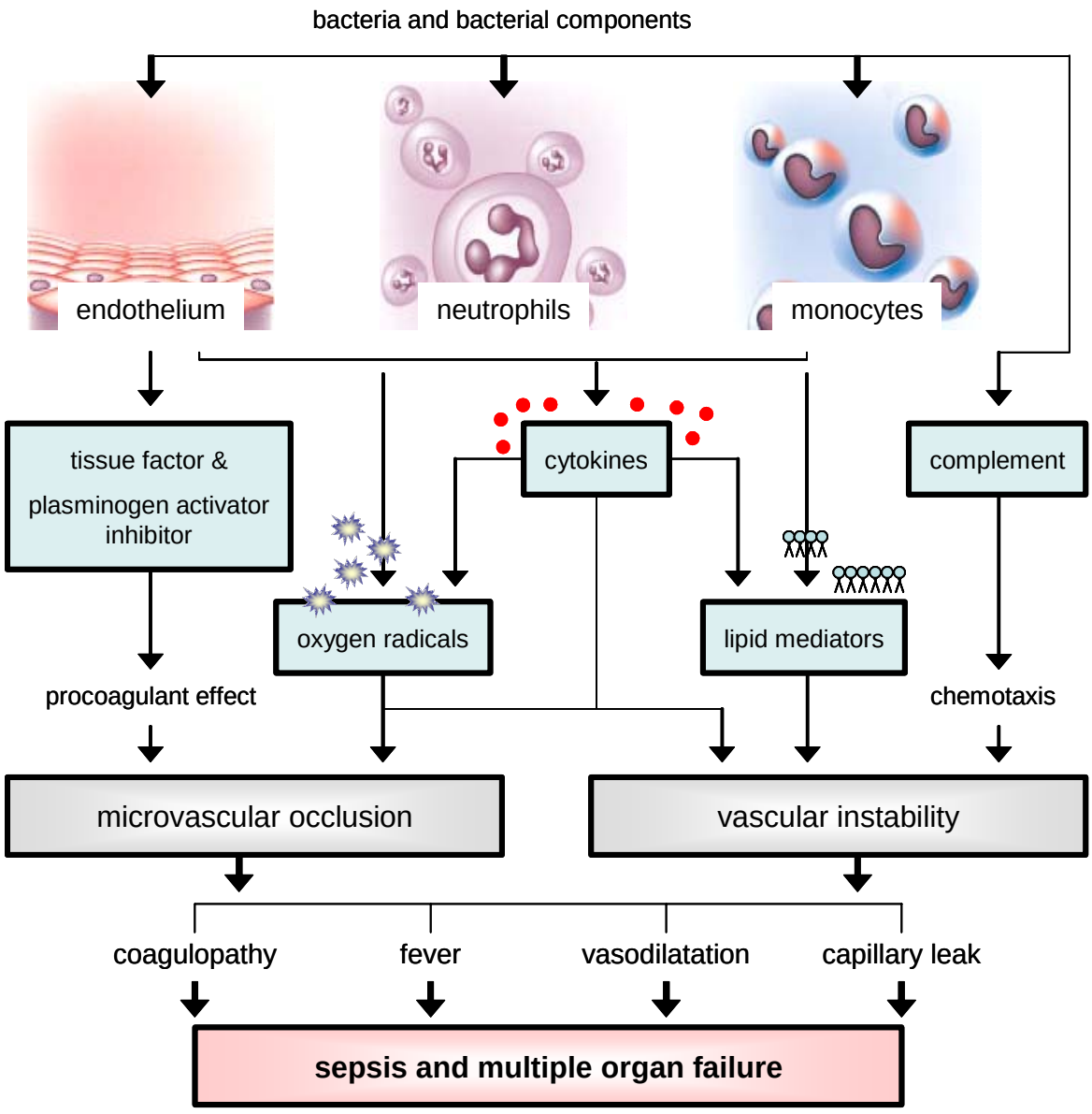


Figure 1:4 *The pathogenetic network in septic shock (adapted from ([35] Cohen, 2002)*. LPS and various microbial components activate multiple inflammatory cascades that contribute to the clinical syndrome of sepsis. Finally, the combination of impaired peripheral vascular tone and micro-vascular occlusion leads to severe tissue hypo-perfusion, resulting in organ failure.

1.3.2 SIGNALING MECHANISMS INVOLVED IN SEPSIS

As described previously, bacteria and bacterial components, fungi, viruses and parasites are recognized by the innate immune system. All of them might elicit strong pro-inflammatory responses, which can result in a sepsis syndrome (1.3.1). Bacterial components include lipopolysaccharide (LPS) from gram-negative bacteria and lipoteichoic acid (LTA) derived from gram-positive bacteria. LPS and LTA are main building parameters of the outer bacterial cell walls. Even though, all microorganisms and their products are able to induce severe inflammation, gram-negative bacteria-derived LPS is supposed to be the most common cause for sepsis to date ([5] Alexander, 2001; [125] Marshall, 2005).

1.3.2.1 LPS SIGNALING

The microbial motifs, such as LPS, that are recognized by the immune system are termed pathogen-associated molecular patterns (PAMPs). These motifs are not characterized by specific binding sites but rather comprise various typical patterns. These patterns are identified by toll-like receptors that are capable of binding various microbial motifs ([12] Beutler, 2003).

Toll-like receptors

The family of toll-like receptors has been discovered by identification of the drosophila protein Toll. This protein is expressed by various insects and is supposed to be essential for developmental functions ([6] Anderson, 1985). Ten years ago, studies revealed that TLRs are not only involved in embryogenesis but also participate in the innate immune response against fungal infections of Drosophila ([112] Lemaitre, 1996). In 1998, the TLR-4 was primarily identified as the lipopolysaccharide-recognition receptor, required for mice to respond to infection caused by gram-negative bacteria ([150] Poltorak, 1998). Up to now, 13 mammalian toll-like receptors have been identified, whereof ten of them have been found in humans ([13] Beutler, 2004; [146] Palsson-McDermott, 2004) . Today, in 2006, it seems to be taken for granted that Toll-like receptors are central recognition proteins during microbial infections.

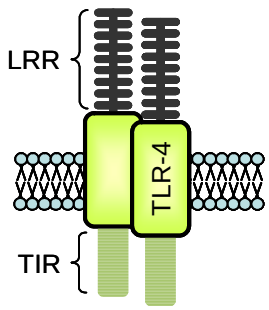


Figure 1:5 *TLR-4 schematic structure.*

Toll-like receptors (Figure 1:5) are transmembrane proteins, all of which exhibit a common extracellular leucine-rich region (known as leucine rich repeats, LRR) and a highly conserved intracellular cytoplasmic domain. This domain is structurally homologous to cytoplasmic structures located in various interleukin (IL) receptors, and therefore, termed toll/IL-1 receptor homology domain (TIR) ([165] Slack, 2000). On the basis of this homology toll-like receptors belong to the TLR/IL-1R-superfamily ([48] Dunne, 2003).

Toll-like receptor-4 mediated LPS signaling

As seen in Figure 1:6, probably the first protein involved in LPS recognition is LPS-binding protein (LBP) ([161] Schumann, 1990). LBP circulates in the bloodstream where it forms a high affinity complex with LPS, appearing either as fragments, free molecules or still bound to the outer membrane of intact bacteria. Thereby, the apparent role of LBP is to guide LPS in activating the TLR complex, by binding LPS and forming a complex with a third molecule CD14 ([192] Wright, 1990). CD14 is found, as a membrane bound and soluble form. The latter occurs in plasma, helping to convoy LPS signaling in cells lacking membrane bound CD14. Membrane bound CD14 is attached to the outer cell membrane. Interestingly it does not exhibit a transmembrane domain, handicapping CD14 to transfer LPS signaling from the cell surface into the cytosol. The toll-like receptor complex exists of TLR-4 and MD-2, an extracellular adaptor protein that seems to be essential within the LPS signaling cascade ([159] Schromm, 2001). Therefore, MD-2 is supposed to be involved in ligand recognition by the receptor and TLR-4 sensitization for LPS binding ([180] Visintin, 2001). After activation of the TLR-complex, LPS-TLR-4 signaling is initiated by the formation of a TLR-4 homodimer. Following MD-2-TLR-4 dimerization, several combinations of signaling molecules become associated with the receptor complex. Primarily, intracellular myeloid differentiation factor-88 (MyD88) is recruited to the TLR complex by its TIR domain ([131] Medzhitov, 1998). However, the TLR-4 mediated response to LPS can be divided into two main signaling pathways:

- the early phase MyD88-dependent response ([12] Beutler, 2003)
- consecutively, the delayed MyD88-independent response ([89] Kawai, 1999; [90] Kawai, 2001). Since we are mainly interested in early events during LPS-induced sepsis, we will now focus on the MyD88-dependent pathway.

The early response – the MyD88-dependent pathway

MyD88 is recruited to the TLR-complex by its own TIR domain, which is able to interact with the TIR domain of TLR-4. Hence, this allows the further attraction and activation of downstream IL-1 receptor associated kinases (IRAK) ([81] Janssens, 2003).

IRAK4 is primarily recruited to the complex, subsequently leading to association and phosphorylation of IRAK1. Now, formation of the complex results in TNF-receptor associated factor-6 (TRAF6) activation that is immediately recruited to the receptor complex ([26] Bradley, 2001). Phosphorylated IRAK1 and TRAF6 dissociate from the receptor complex, which may induce phosphorylation of mitogen-activated protein kinases (MAPK) (further described under 1.3.2.4) and activation of the transcription factor NF-κB (further described under 1.3.2.3). This finally allows the expression of different target genes, such as TNF-α ([3] Akira, 2004; [12] Beutler, 2003; [146] Palsson-McDermott, 2004). Interestingly, LPS stimulation has also been shown to result in phosphorylation of MyD88, and the formation of a PI3K-MyD88 complex, following activation of the protein kinase Akt. However, binding of NF-κB to DNA is not affected by inhibition of Akt-kinase activation during LPS-induced inflammation (see chapter 1.3.2.4) ([116] Li, 2003; [143] Ojaniemi, 2003).

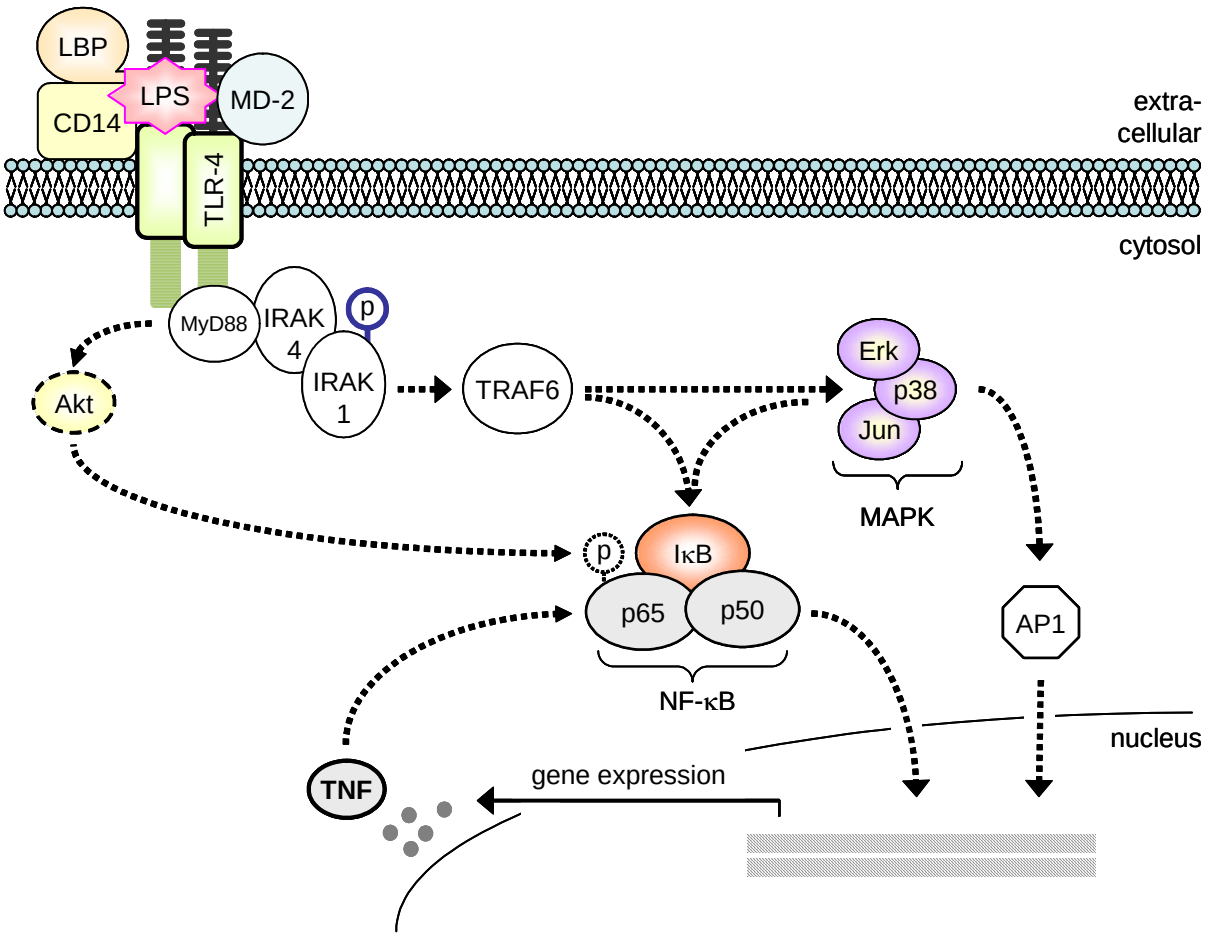


Figure 1:6 *The early phase, MyD88 dependent TLR-4 mediated LPS signaling pathways.*

1.3.2.2 TNF-α SIGNALING

Tumour necrosis factor-α (TNF-α) is one of the most commonly investigated pro-inflammatory cytokines produced by various cell types of the immune system, like monocytes/ macrophages. TNF-α is responsible for a wide range of signaling events in acute inflammation and autoimmune disorders. This leads either to inflammatory processes, necrosis, cell survival or apoptosis ([2] Aggarwal, 2003; [11] Baud, 2001). Thus, the importance of TNF-α in the pathogenesis of inflammation and sepsis has often been described ([118] Lin, 2005). Trials that involved the administration of low doses of microbial endotoxins (LPS) to healthy human volunteers resulted in elevated TNF-α plasma levels, peaking 90 minutes after LPS administration ([133] Michie, 1988; [176] van Deventer, 1990).

TNF-α processing

Tumor necrosis factor-α exists in two biologically active forms. It is initially expressed as a precursor 233-amino-acid transmembrane protein (*mem*TNF-α). Following activation, the soluble 157-amino-acid, TNF-α (*sol*TNF-α) is proteolytically liberated from the cell membrane by cleavage of its precursor protein. Thereby, *mem*TNF-α undergoes shedding by metalloproteases, particularly TNF-α-cleaving enzyme (TACE) ([55] Gearing, 1994). TACE is a membrane bound enzyme that belongs to *a d*isintegrin *a*nd *m*etalloproteases (ADAMs) family ([19] Black, 1997). Interestingly, it has been shown that treatment with a TACE inhibitor prevents mortality in LPS/D-galactosamine induced sepsis in mice ([137] Mohler, 1994). Therefore, the consequent release of *sol*TNF-α by TACE on cells of the immune system is an important factor in the pathophysiology of sepsis ([155] Robertshaw, 2005).

Signal transduction by tumor necrosis factor receptor-1

To date, there are two known TNF receptors, TNF receptor-1 (TNFR-1) and TNF receptor-2 (TNFR-2). TNF-α mainly mediates its effects by binding to its TNFR-1 ([178] Vandenabeele, 1995). Interestingly, none of the mammalian TNF receptors itself comprises any enzymatic activity. As seen in Figure 1:7, binding of a TNF-α trimer to its receptor induces receptor trimerization and several signaling proteins are recruited to the cytoplasmic domain of the receptor ([10] Banner, 1993). Following stimulation, TNFR-1 associated death domain protein (TRADD) is the first protein recruited to the receptor ([77] Hsu, 1995). At this point, TRADD subsequently recruits further effector proteins into the TNF-TNFR-TRADD-complex.

Three molecules have been shown to directly interact with the TRADD protein:

- FAS-associated death domain protein (FADD) ([33] Chinnaiyan, 1995)
- TNF receptor associated factor 2 (TRAF2)
- receptor interacting protein (RIP) ([75] Hsu, 1996a).

By now, these proteins are able to activate various signaling cascades, finally resulting in different TNF-mediated effects ([76] Hsu, 1996b). The protein FADD is very important for TNF-α induced apoptosis. This occurs by further activating a cascade of caspases, including initiator-caspase 8. TRAF2 and the serine-threonine kinase RIP are critical in pathways leading to cell survival and inflammatory processes. Thus, these proteins are mainly involved in activation of the transcription factors NF-κB and AP1. Additionally, TRAF2 and RIP are required for mitogen-activated protein kinase (MAPK) activation ([46] Devin, 2003; [71] Hehlgans, 2005; [120] Liu, 2005).

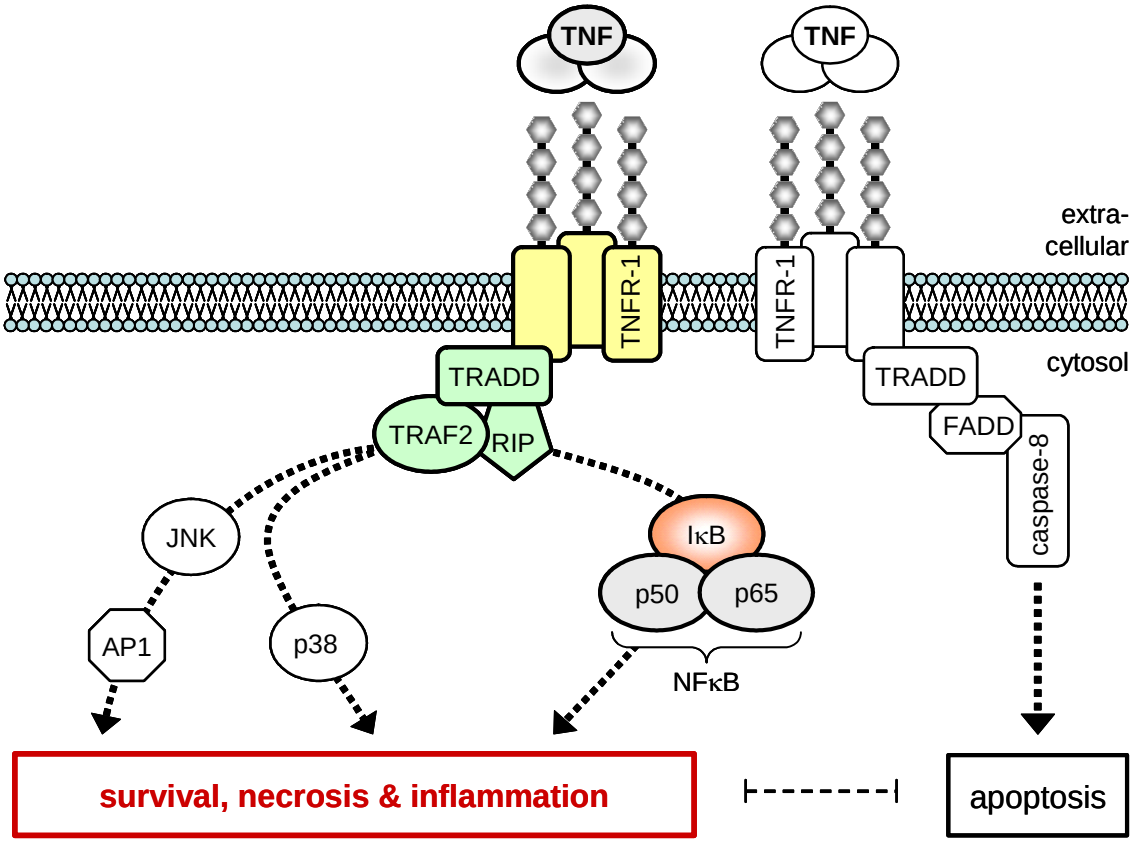


Figure 1:7 *Signal transductions pathways activated by tumor necrosis factor receptor-1 stimulation.*

The choice between life and death is often one of the main events in TNF-α-mediated regulation of the immune system. Interestingly, the pathology that is linked to TNF-α is also often associated with an inappropriate NF-κB activation.

1.3.2.3 NF-κB

As seen before, many of the LPS and TNF-α-caused effects are mediated through the activation of the transcription regulating factor, nuclear factor-κB (NF-κB). To date, over 200 genes have been shown to be regulated by NF-κB, and their products are involved in a huge variety of diseases ([2] Aggarwal, 2003). For example, NF-κB is mainly involved in modulating the expression of many immune-regulatory mediators, such as TNF-α, that participate in acute inflammatory response after microbial infection ([68] Hanada, 2002). Interestingly, NF-κB binding activity has already been studied in peripheral blood mononuclear cells (PBMC) of septic patients, whereas non-survivors could be distinguished from survivors by an increase in NF-κB binding activity. Additionally, in a murine sepsis model, intravenous gene transfer with IκBα was able to attenuate LPS-induced NF-κB binding activity and increase survival ([1] Abraham, 2003; [23] Bohrer, 1997). In account of this, NF-κB and its regulatory signaling pathways have recently become an interesting fact for intensive drug discovery ([88] Karin, 2004).

Of course, NF-κB can not function by itself, therefore a variety of proteins and mechanisms are involved in order to regulate transcription factor activity. The NF-κB (Rel) transcription factors always act as dimers. So far, five known mammalian NF-κB/Rel proteins, belonging to two classes have been identified: “class-1”: - Rel (c-Rel); p65 (RelA) and RelB
“class-2”: - p50 and p52.

“Class-1” proteins are synthesized as mature products whereas “class-2” proteins are synthesized as large precursor proteins, p105 and p100 that require proteolytic processing to produce the mature p50 and p52 NF-κB proteins ([59] Ghosh, 1998). The NF-κB protein exists in the cytoplasm in an inactive form, as a result of NF-κB association with its inhibitory proteins, IκBs. Among these proteins, the most common forms are IκBα, IκBβ and IκBε. The most established theory is that IκB proteins keep NF-κB in the cytoplasm by masking their nuclear-localization sequences located on NF-κB subunits ([78] Huxford, 1998). Nowadays, several studies have indicated that localization not only depends on IκB binding but also on balancing continuous movement between the nuclear and cytoplasmic regions ([18] Birbach, 2002). Untouched, the canonical pathway involved in NF-κB activation is still believed to be based on IκBα degradation (Figure 1:8). A crucial step in this process is the phosphorylation of IκBα at two serine residues (serine 32 and serine 36), that is mediated by IκB-kinase (IKK) complex ([179] Viatour, 2005).

The most common form of this IKK complex consists of several proteins, such as IKKα (IKK1), IKKβ (IKK2) and the NF-κB essential modulator (NEMO, IKKγ). Both, IKKα as well as IKKβ comprise kinase activity, responsible for IκBα phosphorylation, whereas the regulatory protein NEMO contains motifs known to be involved in protein-protein interactions. Answers to pro-inflammatory stimuli, as TNF-α or LPS, are largely dependent on IKKβ catalytic subunit activation ([85] Karin, 1999). Following phosphorylation IκBα is subsequently ubiquitinylated, which targets the protein for degradation by the proteasome ([86] Karin, 2000). The released NF-κB dimer translocates into the nucleus, binds DNA and activates gene transcription ([24] Bonizzi, 2004; [58] Ghosh, 2002; [87] Karin, 2002; [114] Li, 2002a). In addition to IκBα modification, the NF-κB subunits, mainly p65, are phosphorylated. This phosphorylation is mediated by protein kinases, such as Akt (further described under 1.3.2.4), which are supposed to play a very important role in regulating NF-κB transcriptional activity. To date, it is still not clarified weather phosphorylation of subunits modulates NF-κB, to either activate or repress gene expression. ([179] Viatour, 2005; [197] Zhong, 1998; [196] Zhong, 2002).

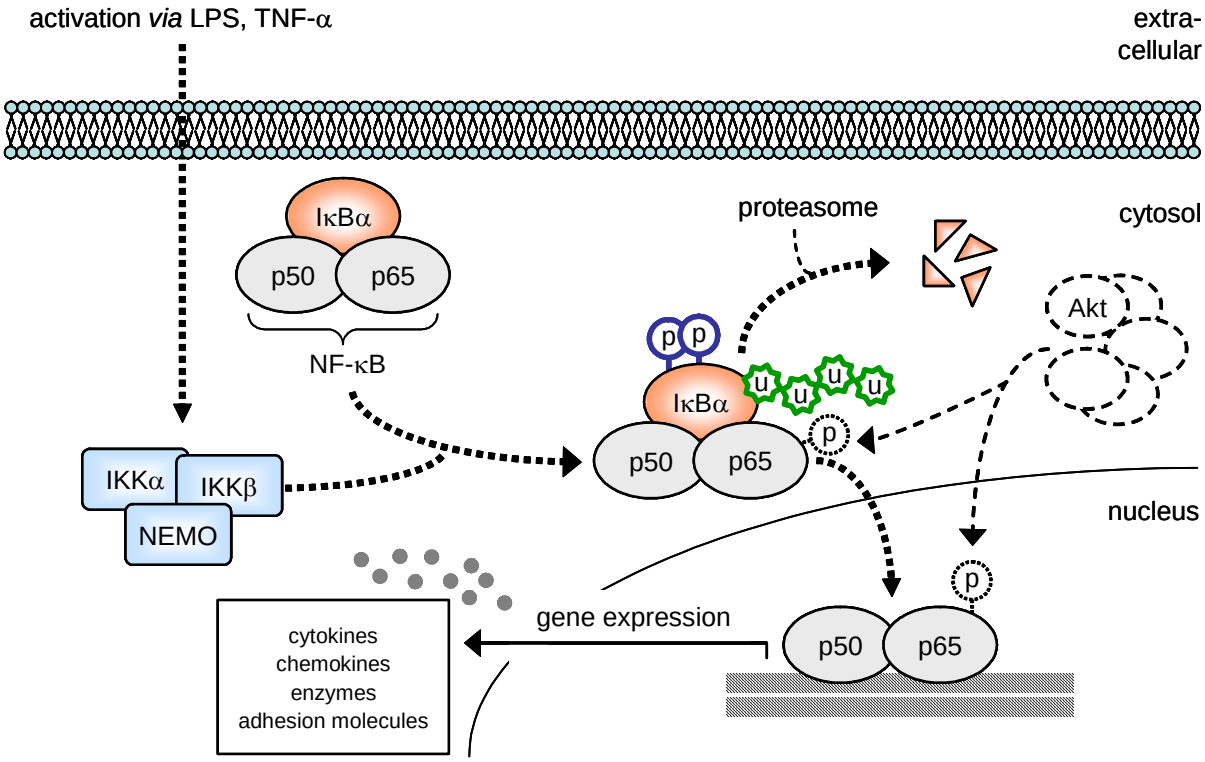


Figure 1:8 *The NF-κB activating system.* After activation of the IKK complex, IκB is subsequently phosphorylated, following immediate ubiquitinylation and degradation by the proteasome. The "free" NF-κB dimer migrates into the nucleus, binds to DNA and activates gene expression.

1.3.2.4 PROTEIN KINASES

After LPS binding, toll-like receptors activate various intracellular signaling molecules in addition to NF-κB discussed previously. These signaling molecules include different protein kinases, such as mitogen activated protein kinases (MAPK), particularly p38 MAPK, phosphatidylinositol 3 kinases (PI3K) and protein kinase Akt.

p38 MAPK

The pro-inflammatory cytokine TNF-α has an important function in cell signaling pathways involved in sepsis. Several of the molecular mechanisms that regulate LPS-induced TNF-α-mediated responses have been discussed in detail (for review see chapter 1.3.2.2). Additionally, mitogen activated protein kinases, are one group of enzymes that respond to various extracellular stimuli, such as LPS. Three subgroups of MAPK have been identified to date:
- extracellular-signal-regulated kinases (Erks)
- c-Jun amino-terminal kinases (JNKs)
- p38 MAPK.

Following stimulation, MAPK are activated by a MAPK activation-cascade. Thereby, the MAPKK-kinase activates the targeted MAPK-kinase by phosphorylation. Proximately, the MAPK-kinase (MAPKK), for instance the MKK3 or MKK6, activates the corresponding MAPK, as p38 MAPK. Interestingly, the p38 MAPK was originally identified in LPS-stimulated murine macrophages, first described as a 38-kDa polypeptide that underwent tyrosine phosphorylation in response to LPS treatment. Since, this peptide has been identified in yeast, these findings primarily linked the LPS-induced mammalian signaling pathway to the pathway in yeast correlated with stress-response ([67] Han, 1994). Nowadays, p38 MAPK has frequently been shown to be critically involved in LPS-induced TNF-α gene expression ([111] Lee, 1994; [171] Sweet, 1996). In human macrophages, inhibition of LPS-induced p38 MAPK activation decreases not only TNF-α protein and TNF-α mRNA expression but also NF-κB activation. Therefore, p38 MAPK is likely to regulate TNF-α gene-transcription *via* a NF-κB dependent pathway ([29] Campbell, 2004; [31] Carter, 1999b; [30] Carter, 1999a; [82] Jiang, 2002). In addition, post-transcriptional regulatory mechanisms leading to reduced TNF-α expression have recently been noticed. Thus, LPS-stimulation of murine macrophages results in TNF-α mRNA stabilization, which was reversed by specific p38 MAPK inhibition ([27] Brook, 2000; [123] Mahtani, 2001). As MAPK signaling is more and more elucidated it is becoming evident that these pathways might be important targets for novel anti-inflammatory drugs in the near future ([64] Guha, 2001; [107] Kyriakis, 2001).

PI3 kinase - Akt pathway

PI3 kinases are activated during toll-like receptor signaling, supposedly, as a result of direct interaction of PI3K with the intracellular domain of the TLR-4 or MyD88 ([103] Koyasu, 2003; [143] Ojaniemi, 2003). Activation of the PI3K occurs *via* phosphorylation, which permits docking of PI3K to the plasma membrane. PI3 kinases are an enzyme family that catalyzes the phosphorylation of cell membrane components, so-called phosphoinositides. These lipid substrates are important second messengers for intracellular signaling pathways. Hence, activated PI3 kinases provoke membrane changes by phosphorylation of phosphoinositides, allowing downstream protein kinase-B, also denoted as Akt to translocate to the cell membrane (Figure 1:9). After membrane recruitment, Akt is activated by dual phosphorylation at serine 473 and threonine 308 residues, further influencing NF-κB dependent gene expression. To date, the PI3K-Akt pathway is supposed to play an important role in regulating different cellular functions involving defense and immune responses ([3] Akira, 2004; [103] Koyasu, 2003).

Studies on the role of the PI3K-Akt pathway in NF-κB dependent cell signaling are controversial. Therefore, this pathway is discussed to act positively, as well as negatively, on NF-κB dependent inflammatory parameters. Recently, it has been demonstrated that inhibition of PI3K strongly enhances LPS-induced inflammation and significantly reduces survival of septic mice ([157] Schabbauer, 2004). Moreover, activation of the PI3K-Akt pathway has been shown to decrease LPS-induced TNF-α expression *via* reduced NF-κB activation in human peripheral blood monocytes ([65] Guha, 2002). Together, these findings indicate that the PI3K-Akt pathway strongly regulates LPS-induced gene expression *in vitro* as well as *in vivo*. However, the downstream mechanisms targeting the expression of pro-inflammatory cytokines, such as TNF-α, are still incompletely understood ([117] Liew, 2005; [179] Viatour, 2005).

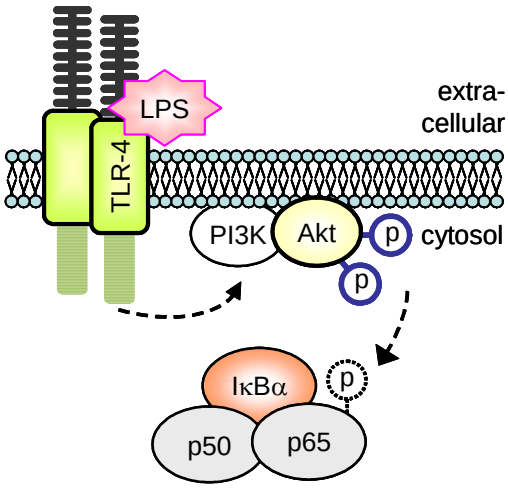


Figure 1:9 *PI3 kinase – Akt pathway.*

Besides classical inflammatory pathways, protein kinases, such as p38 MAPK and Akt, have been demonstrated to influence transcription factor activity and cytokine gene expression, making these proteins very interesting for investigations during inflammation and sepsis.

1.4 SEPSIS & INFLAMMATION - *IN VIVO* AND *EX VIVO*

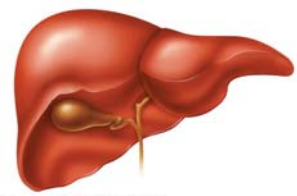
In chapter 1.3, I discussed the major difficulties to clearly stage human sepsis. In order to study the sepsis pathology, various models of sepsis and septic shock have been established to date ([28] Buras, 2005). These experiments are often used as a preliminary testing ground for protective agents and can be divided into three main categories:

- exogenous administration of a toxin (LPS-models)
- exogenous administration of a viable pathogen (bacterial-models)
- alteration of the animals endogenous protective barrier

Human volunteer studies have shown that injection of low doses of endotoxins can induce patho-physiological alterations similar to those reported in septic patient ([50] Fink, 1990). For this reason, amongst all versions, endotoxicosis models (LPS-models) are widely used. Indeed, animal models neither completely copy human sepsis, nor involve identical critical-care delivered to septic patients. However, it will be possible to improve upon animal models by better staging and in conclusion appropriately treating patients with sepsis ([28] Buras, 2005; [49] Esmon, 2004).

In our laboratory, an animal experiment demonstrated that ANP pretreatment highly contributes to survival of LPS-induced sepsis in mice ([91] Keller, 2005a). In order to further analyze ANP interference with different signaling pathways involved in LPS-induced inflammation and sepsis, we decided to focus on two different systems, the liver and blood leukocytes. Both of them are mainly involved in primary immune host responses to microbial infection.

1.4.1 THE LIVER



The liver is the largest solid organ in the human body, constituting of up to 5% of the body weight in adults. *Via* the portal vein, the liver is constantly provided with nutrients from the gastrointestinal tract. The function of the liver is the uptake of these nutrients, their storage, metabolic conversion and their distribution to the circulation. However, the liver does not only participate in metabolic pathways but also comprises various clearance functions. Being the first organ confronted with microbial products derived from the gastrointestinal tract, the liver is highly involved in host-defense mechanisms.

Therefore, this organ is an important source of cytokines, participates in bacterial clearance and is often injured as a result of inflammation and sepsis ([9] Ashare, 2005; [174] van Amersfoort, 2003; [177] van Oosten, 2001).

The liver is made up of several cell populations, whereas the most abundant cell type by mass and by number is the hepatocyte. Besides hepatocytes, the liver is composed of:

- resident immune cells (e.g. Kupffer cells)
- mesenchymal cells with fibrogenic potential (e.g. hepatic stellate cells)
- specialized cells (e.g. endothelial cells).

Since we are mainly interested in immune responses during sepsis, we will now briefly focus on two types of cells within the liver that have frequently been described to strongly interact during inflammatory disorders ([138] Monshouwer, 2003; [151] Ramadori Guiliano, 2005).

Hepatocytes

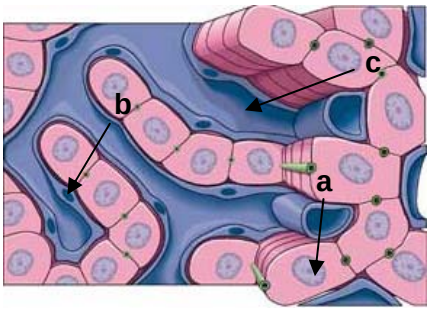


Illustration1:1 *Hepatocytes*

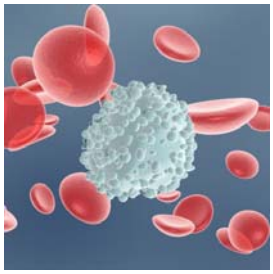
Hepatocytes (a) comprise 60% of the total number and 80% of the whole liver volume. These cells are arranged in plates or cords, that branch and merge in a continuous labyrinth. By forming such a complex cell network, sinusoidal cavities (c) are evolving between these hepatocyte formations. In detail, hepatocytes do not hold direct contact to sinusoidal cavities but rather adjoin to sinusoidal endothelial cells (b). These endothelial cells line the whole sinusoidal space and therefore constitute the primary barrier between the permanent blood-flow in the sinusoids and hepatocytes. Hence, the different sides of hepatocytes are either attached to adjacent hepatocytes, border bile canaliculi or are exposed to the sinusoidal space. Being the “workhorses” of the liver, hepatocytes contain the whole machinery that is necessary to carry out the different vital functions, such as uptake, storage and metabolism of nutritions ([124] Malarkey, 2005; [138] Monshouwer, 2003). Therefore, the degree of hepatic failure, as a result of sepsis, is often related to the extent of hepatocyte damage. LPS has frequently been shown to directly lead to hepatocyte dysfunction. Interestingly, hepatocyte functions are not only influenced by LPS but also by TNF-α from LPS-activated Kupffer cells ([186] Wang, 1995; [187] Wang, 1998).

Kupffer cells

Kupffer cells account for approximately another 15% of total liver cells but constitute the largest amount of resident tissue macrophages in the human body, making up to 90% of these cells. They are attached to the luminal face of liver sinusoids anchored to endothelial cells. Interestingly, Kupffer cells are mainly strategically situated in the periportal area, where the blood enters the liver. Hence, these cells constitute the very first population of macrophages to be faced with bacteria and microbial debris derived from the blood stream ([39] Cotran, 1999). Therefore, clearance of circulating microbial products is one of the most important roles played by Kupffer cells. It is well known that endotoxins are a potent stimulator of Kupffer cells, which then induce the production of various mediators. Once activated, Kupffer cells release cytokines, nitric oxide (NO), proteases and reactive oxygen species (ROS) ([7] Arii, 2000; [45] Decker, 1998; [167] Su, 2002). In respect of cytokines, Kupffer cells are thought to be the principal source of TNF-α and conclusively represent the main cellular mediators of LPS-induced effects in the liver ([16] Bilzer, 2005; [92] Keller, 2005b; [110] Lee, 2004).

1.4.2 BLOOD LEUKOCYTES - MONOCYTES AND NEUTROPHILS

As mentioned above, the survival of humans often depends on their ability to recognize invading pathogenic organisms and to respond rapidly and adequately. These defense mechanisms are innate to the organism. Moreover, the innate immune system includes cellular as well as non-cellular constituents. Neutrophilic granulocytes (neutrophils) and monocytes are the main components in these cellular elements. They both constitute a large quantity amongst white blood cells, also denoted as blood leukocytes. The LPS-stimulated release of proinflammatory cytokines, such as TNF-α, by human leukocytes is an important component of the inflammatory process ([43] Das, 2000; [64] Guha, 2001; [147] Pan, 2000). Through their unique combination of cellular functions, leukocytes represent an important first line defense to invading microorganisms or their products. In our laboratory, it has recently been shown that ANP preconditioning reduces TNF-α expression in LPS-activated whole human blood, *ex vivo* ([95] Kiemer, 2000). Interestingly, these investigations reflect similar effects seen in plasma derived from ANP-preconditioned LPS-treated mice ([91] Keller, 2005a). Thus, we were interested how ANP-preconditioning influences LPS-induced TNF-α expression in whole blood samples of mice as well as of human origin.



2 MATERIAL AND METHODS

2.1 MURINE MODEL OF LPS-INDUCED SEPTIC SHOCK

Animal experiments were kindly performed by Melanie Keller (Ludwig-Maximilians-University of Munich, Department of Pharmacy) and Ulla Gebert (Biochemical Pharmacology, University of Konstanz).

2.1.1 ANIMALS

For the model of LPS-induced septic shock pathogen-free male BALB/c mice were supplied by the house animal breeding facility of the University of Konstanz. The animals had free access to water and chow (Sniff), but were kept of chow with free access to water 12 hours prior to *in vivo* experiments. Animals were housed in a temperature and humidity controlled room under a constant light/dark cycle. All studies were performed with the permission of the government authorities in accordance with the German Legislation on Laboratory Animal Experiments, and followed the directives of the University of Konstanz Ethical Committee.

2.1.2 MATERIAL AND SOLUTIONS

The following materials were used during animal experiments, in the model of LPS-induced septic shock.

Table 2:1 *Material used for animal experiments.*

product		**company**
ANP[1]	- Atrial Natriuretic Peptide (rat)	Calbiochem Novabiochem
LPS[1]	- Lipopolysaccharide Salmonella abortus equi S.	Bioclot
Nembutal®	- Pentobarbital	Sanofi-Ceva
TNF-α ELISA	- enzyme linked immuno sorbent assay	Amersham Pharmacia Biotech

[1] ANP and LPS were diluted in a total volume of 300µl of an endotoxin free sterile 0.9% sodium chloride (NaCl) solution containing 0.1% human serum albumin (HSA).

2.1.3 EXPERIMENTAL SETTING

Septic shock has been induced by injection of a sublethal dose of LPS. Whereas, 15 minutes previous to LPS challenge, mice were treated with either ANP or sterile pyrogen-free 0.9% sodium chloride (NaCl) solution. Following LPS injection, organ and blood samples were obtained after lethal intravenous anesthesia with Nembutal plus administration of Heparin at the indicated time points. Organ samples were snap-frozen in liquid nitrogen and blood was withdrawn by cardiac puncture.

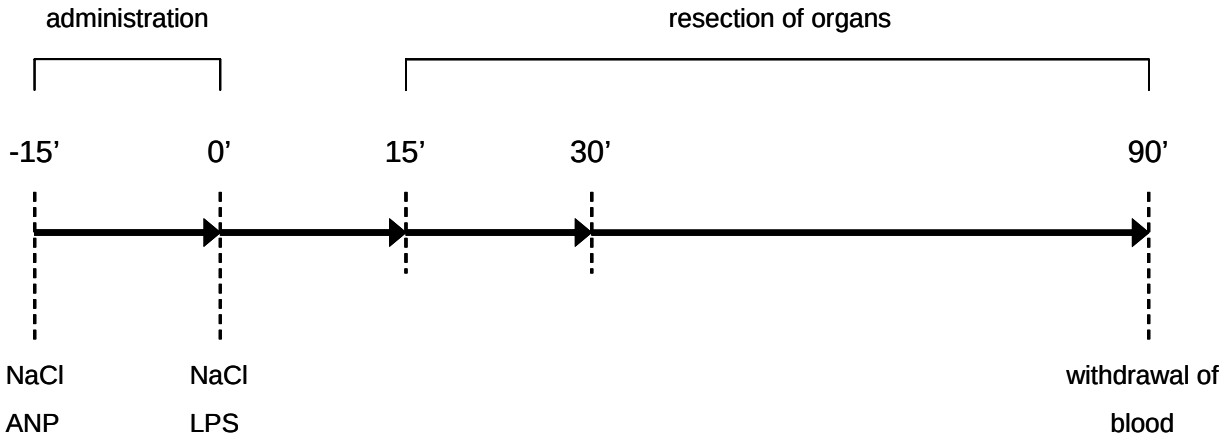


Figure 2:1 *Treatment of animals.*

Table 2:2 *Treatment groups of animals.*

group	**treatment**			**animals**
control	NaCl	0.9 %	(i.v.)	5
ANP	ANP NaCl	5 µg/kg b.wt. 0.9 %	(i.v.) (i.v.)	5
LPS	NaCl LPS	0.9 % 3 mg/kg b.wt.	(i.v.) (i.v.)	5
ANP/LPS	ANP LPS	5 µg/kg b.wt. 3 mg/kg b.wt	(i.v.) (i.v.)	5

2.2 LIVER CELL CULTURE

In order to investigate signaling pathways in specific cell types, we used the model of primary isolated rat hepatocytes.

2.2.1 ANIMALS

For isolation of hepatocytes male Sprague-Dawley rats (220-280g) were purchased from Charles River WIGA GmbH. Animals had free access to water and chow (Sniff) up to the time of experiments. Rats were housed in a temperature and humidity controlled room under a constant light/dark cycle. The study was registered with the local animal welfare committee.

2.2.2 MATERIAL AND SOLUTIONS

The following materials and solutions were used for isolation and culture of primary rodent hepatocytes. Before use, solutions have always been freshly prepared.

Table 2:3 *Material used for isolation of primary hepatocytes.*

product		**company**
ANP	- Atrial Natriuretic Peptide (rat)	Bachem
BD Falcon™	- 100µm Cell Strainer	BD Biosciences
Collagen R		Serva
Collagenase H		Roche Diagnostics GmbH
FCS	- Foetal Calf Serum	Gibco Invitrogen Corporation
Insulin	- Insuman® Rapid 40 I.E./ml	Aventis
LPS,	- Lipopolysaccharide Salmonella abortus equi S.	Bioclot
Medium	- Leibovitz's L-15 special	Pan Biotech GmbH
Percoll™		Amersham Biosciences

Solution A		pH 7.35
115	mM	NaCl
25	mM	$NaHCO_3$
5.9	mM	KCl
1.18	mM	$MgCl_2$
1.18	mM	$MgCl_2$
1.23	mM	NaH_2PO_4
1.2	mM	Na_2SO_4
		H_2O

Solution B		pH 7.35
115	mM	NaCl
25	mM	$NaHCO_3$
5.9	mM	KCl
1.18	mM	$MgCl_2$
1.23	mM	NaH_2PO_4
1.2	mM	Na_2SO_4
2.5	mM	$CaCl_2$
20	mM	Hepes
0.05	%	Collagenase H
		H_2O

Hanks' balanced salt solution		pH 7.35
0.95	mM	$CaCl_2$
5.3	mM	KCl
0.44	mM	KH_2PO_4
0.49	mM	$MgCl_2$
0.41	mM	$MgSO_4$
136.75	mM	NaCl
0.34	mM	Na_2HPO_4
20	mM	Hepes
		H_2O

Leibovitz's L-15 medium		
8.3	mM	Glucose
2	mM	Glutamine
2.5	µg/ml	Dexamethasone
100	U/ml	Penicillin G
100	µg/ml	Streptomycin
50	µg/ml	Gentamycin
50	µg/ml	BSA
5	%	FCS
	in	Leibovitz's medium
add if necessary:		
125	U/l	Insulin

Percoll™ gradient		
136	mM	NaCl
10.2	mM	Na_2HPO_4
2.68	mM	KCl
1.46	mM	KH_2PO_4
0.5	mM	$MgCl_2$
50	%	Percoll™ solution
		H_2O

Trypan blue solution		
0.08	g	Trypan blue
0.03	g	NaCl
20	ml	H_2O

Collagen		
5	ml	Collagen R
45	ml	H_2O

2.2.3 ISOLATION OF PRIMARY HEPATOCYTES

Rat livers for isolation of hepatocytes were kindly provided by Stephanie Kulhanek-Heinze and Florian Hoffmann ((Ludwig-Maximilians-University of Munich, Department of Pharmacy). In brief, hepatocytes were isolated using a modified two step collagenase digestion method. Male Sprague-Dawley rats (200-300g) were anaesthetized by injection of Narcoren. Additionally, 250 I.U. Heparin were administered to avoid blood clotting. The liver was perfused with solution A. Afterwards, the organ was flushed with solution A and subsequently perfused in a circulating manner with solution B containing Collagenase H for several minutes. Both solutions have been oxygenated for 15 minutes with Carbogen (5% CO_2 and 95% O_2) at 37°C. Hereon, the liver was carefully excised and placed into a Petri dish, which contained solution B.

The following procedure was performed in a laminar flow work bench. The hepatic cells were isolated by tearing apart the capsule of the liver and scraping out the cells with a teaspoon. The cell suspension was filtered with a Falcon cell strainer following sedimentation of the cells for 10 minutes. After discarding the supernatant, the hepatocytes were separated from the obtained cell suspension by Percoll density gradient centrifugation (2000rpm, 10 minutes, 4°C). After centrifugation, the three layers of the supernatant were removed and the remaining pellet was suspended in Leibovitz's L-15 Medium. Cell number and viability was determined by staining the hepatocytes with trypan blue (500µl trypan blue solution, 450µl solution B, 50µl cell suspension) in a Neubauer counting chamber. Cells were used for experiments when vitality exceeded 80%. Subsequently 10^6 cells/well were seeded into collagen R coated 6 well plates containing 1ml of Leibovitz's L-15 medium. After waiting for four hours, cells were washed twice with Hanks' balanced salt solution and new medium lacking insulin was added. Hepatocytes were used for experiments after 24h and 48h of culture.

2.2.4 EXPERIMENTAL SETTING

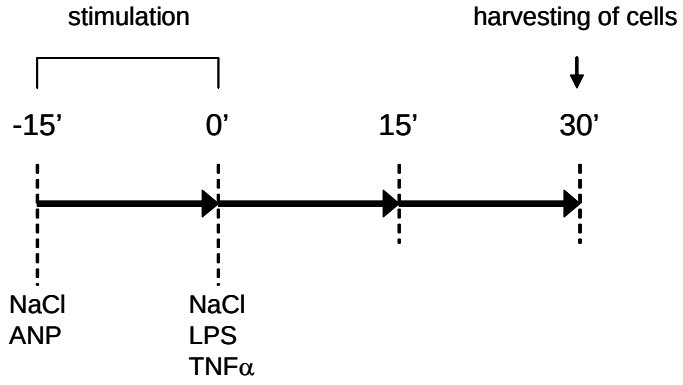


Figure 2:2 *Treatment of primary isolated hepatocytes.*

Table 2:4 *Treatment groups of primary isolated hepatocytes.*

groups	**treatment**	
control	NaCl	0.9 %
ANP	ANP NaCl	25 nM 0.9 %
LPS	NaCl LPS	0.9 % 10 µg/ml
ANP/LPS	ANP LPS	25 nM 10 µg/ml
TNF-α	NaCl TNF-α	0,9 % 10 ng/ml
ANP/TNF-α	ANP TNF-α	25 nM 10 ng/ml
LPS+TNF-α	NaCl LPS TNF-α	0,9 % 10 µg/ml 10 ng/ml
ANP/LPS+TNF-α	ANP LPS TNF-α	25 nM 10 µg/ml 10 ng/ml

2.3 BLOOD

Whole blood experiments were performed to analyze changes on TNF-α protein and mRNA expression in murine and human blood leukocytes.

2.3.1 MATERIAL AND SOLUTIONS

Table 2:5 *Material used for whole blood experiments.*

product		**company**
ANP	- Atrial Natriuretic Peptide (rat)	Bachem
LPS	- Lipopolysaccharide Salmonella abortus equi S.	Bioclot
Medium	- Iscove's MEM	PromoCell
Heparin	- Heparin-Natrium Braun 25000 I.E/5 ml	Braun
BD Cytofix/Cytoperm™ Plus Kit with GolgiPlug™		BD Biosciences

2.3.2 SAMPLE GENERATION

Blood sample generation has always been kindly performed by PD. Dr. Zahler (Ludwig-Maximilians-University of Munich, Department of Pharmacy).

2.3.2.1 SAMPLE GENERATION OF HUMAN BLOOD

Human blood was drawn from healthy male volunteers. Blood was collected by venipuncture into a previously heparinized[1] sterile blood collection tube. To prevent cooling of the samples, subsequently, blood was divided into 200µl portions for FACS analysis alternatively 1ml portions for mRNA extraction and kept at 37°C, 5% CO_2 until used for experiments.

[1] 50IE/ml human blood = 200µl/20ml blood

2.3.2.2 SAMPLE GENERATION OF MOUSE BLOOD

Blood was withdrawn by puncture of the heart. After anesthesia by ether, heparin[1] was injected intraperitoneally, in order to prevent blood clotting. The mouse thorax has been opened very carefully and blood was collected by puncture of the ventricle into a previously heparin coated sterile blood collection tube. For leukocyte experiments, we were able to withdraw approximately 600µl of whole mouse blood. To prevent cooling of the samples, subsequently, blood was diluted 1:1 with medium, divided into 200µl portions and kept at 37°C, 5% CO_2.

[1] 50IE/ml mouse blood = 20µl/mouse

2.3.3 EXPERIMENTAL SETTING

The stimulus was added in the appropriate concentration for the indicated time points. Gained samples were stimulated with LPS and, if indicated, supplementary BD GolgiPlug has been applied. Thereby, 15 minutes previous to LPS challenge, ANP or 0.9% NaCl solution was added to whole blood samples. Following incubation at 37°C, 5% CO_2, vials were gently shaken and whole blood samples were used for FACS staining and PCR analysis.

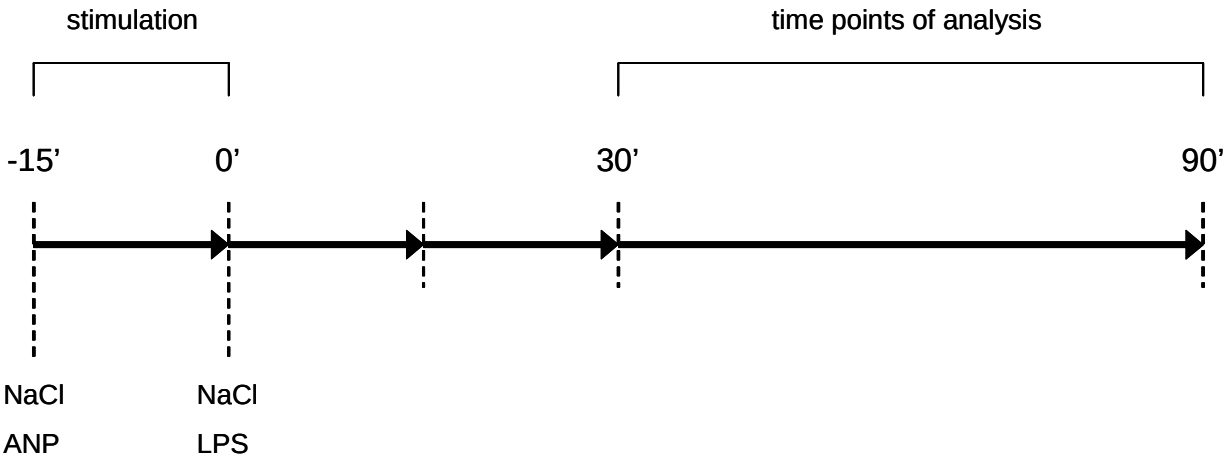


Figure 2:3 *Treatment of whole blood samples.*

Table 2:6 *Treatment groups of whole blood samples.*

group		**treatment**			
control	(+ BD GolgiStop)	NaCl	0.9	%	
		BD GolgiStop	1	µl/ml	= 0.01% Brefeldin A
ANP	(+ BD GolgiStop)	ANP	25	nM	
		ANP	1	µM	
		NaCl	0.9	%	
		BD GolgiStop	1	µl/ml	= 0.01% Brefeldin A
LPS	(+ BD GolgiStop)	NaCl	0.9	%	
		LPS	100	ng/ml	
		LPS	1	µg/ml	
		LPS	10	µg/ml	
		BD GolgiStop	1	µl/ml	= 0.01% Brefeldin A
ANP/LPS	(+ BD GolgiStop)	ANP	25	nM	
		ANP	1	µM	
		LPS	100	ng/ml	
		LPS	1	µg/ml	
		LPS	10	µg/ml	
		BD GolgiStop	1	µl/ml	= 0.01% Brefeldin A

2.4 WESTERN BLOT ANALYSIS

Actually, the term “western blot analysis” only characterizes a process of protein transfer onto a membrane. Usually, “western blot analysis” is termed to describe the whole process of protein separation and protein transfer, following immunological detection of specific proteins.

2.4.1 MATERIAL AND SOLUTIONS

Lysis buffer for liver tissue samples

50	mM	NaCl
50	mM	Hepes
5	mM	EDTA
50	mM	NaF
10	mM	$Na_4P_2O_7$
1	mM	Na_3VO_4
		H_2O

add freshly before use:

1	x	Complete® (25x)
1	mM	PMSF
1	%	Triton® X-100

Laemmli sample buffer 3x

187.5	mM	Tris-HCl
6	%	SDS
30	%	Glycerol
0.015	%	Bromphenolblue
		H_2O

add freshly before use:

5	%	β-Mercaptoethanol

Laemmli sample buffer 1x is prepared by 1:3 dilution of Laemmli sample buffer 3x with H_2O

TBS containing 0.1% Tween pH 8.0

24.6	mM	Tris
188	mM	NaCl
0.2	%	Tween 20
		H_2O

Tris-CAPS 5x pH 9.6

300	mM	Tris
200	mM	CAPS
		H_2O

Anode buffer

12	mM	Tris
20	%	Tris-CAPS 5x
15	%	Methanol
		H_2O

Cathode buffer

12	mM	Tris
20	%	Tris-CAPS 5x
0.01	%	SDS
		H_2O

Electrophoresis buffer

4.9	mM	Tris
38	mM	Glycin
0.1	%	SDS
		H_2O

Stacking gel			
17.15	%	PAA solution	30%
10	%	1,25M Tris	pH 6.8
1	%	SDS solution	10%
0.2	%	TEMED	
1	%	APS solution	10%
		H_2O	

Separation gel 12,5%			
41,6	%	PAA solution	30%
25	%	1.5M Tris	pH 8.8
1	%	SDS solution	10%
0.1	%	TEMED	
0.5	%	APS solution	
		H_2O	

Blotto 5%			
5	%	Blotto	
		TBS-T	pH 8.0

BSA 5%			
5	%	BSA	
		TBS-T	pH 8.0

Coomassie staining solution			
3	%	Coomassie brilliant blue	
10	%	Acetic acid (100%)	
45	%	Ethanol (96%)	
		H_2O	

Coomassie destaining solution			
10	%	Acetic acid (100%)	
33	%	Ethanol (96%)	
		H_2O	

ECL solution A			
25	mM	Luminol	
0.396	mM	p-Coumaric acid	
100	mM	Tris	pH 8.5
		H_2O	

ECL solution B			
0,006	%	H_2O_2	
100	mM	Tris	pH 8.5
		H_2O	

2.4.2 ANTIBODIES

Table 2:7 *Primary antibodies used for western blot analysis.*

primary antibody		**concentration**		**isotype**	**company**
Akt		1 : 1000	in BSA 5%	rabbit IgG	Cell Signaling
Akt phospho	Ser473	1 : 1000	in BSA 5%	rabbit IgG	Cell Signaling
IκBα		1 : 1000	in Blotto 5%	rabbit IgG	Santa Cruz
IκBα phospho	Ser32/Ser36	1 : 1000	in BSA 5%	rabbit IgG	Cell Signaling
p38		1 : 1000	in BSA 5%	rabbit IgG	Cell Signaling
p38 phospho	Thr180/Tyr182	1 : 1000	in BSA 5%	rabbit IgG	Cell Signaling

Table 2:8 *Secondary antibodies used for western blot analysis.*

secondary antibody	**concentration**		**isotype**	**company**
Goat anti mouse	1 : 1000	in Blotto 5%	goat IgG1	Biozol
Goat anti rabbit	1 : 10000	in Blotto 1%	goat IgG	Dianova

2.4.3 SAMPLE PREPARATION

2.4.3.1 PREPARATION OF PROTEIN EXTRACTS FROM MOUSE LIVER TISSUE

40-60mg of tissue were homogenized in 400-600µl of lysis buffer with a homogenizer (Potter S, B. Braun Biotech). After centrifugation of samples (14.000rpm, 4°C, 10 minutes), 10µl of the supernatants were used for protein concentration determination. 3xSDS Laemmli sample buffer was added to the remaining supernatant and probes have been boiled at 95°C for 5 minutes. Samples were kept at -20°C until used for Western Blot analysis.

2.4.3.2 PREPARATION OF PROTEIN EXTRACTS FROM ISOLATED HEPATOCYTES

Cells were cultured in 6-well plates and were stimulated as described previously (chapter 2.2). Cells were washed twice with ice-cold HBSS following addition of 100µl of lysis buffer. Cells have been incubated on ice for 30 minutes, were scraped off and lysates were transferred to Eppendorf tubes. After centrifugation (14.000rpm, 4°C, 10 minutes) of the homogenates 10µl of the supernatants were used for protein concentration determination. 3xSDS Laemmli sample buffer was added to the remaining supernatant and probes have been boiled at 95°C for 5 minutes. Samples were kept at -20°C until used for Western Blot analysis.

2.4.3.3 PROTEIN CONCENTRATION DETERMINATION

In order to employ equal amounts of proteins in all samples, protein concentrations were determined using the Pierce assay (BC assay reagents, Interdim) as described by Smith and colleagues ([166] Smith, 1985). Following 30 minutes of incubation at 37°C, absorbance of the blue complex was measured at 550nm (Tecan Sunrise Absorbance reader, Tecan).

2.4.4 ELECTROPHORESIS

Denaturizing SDS–PAGE allows the separation of proteins according to their molecular weight. All proteins were denaturized by addition of SDS, a highly negative charged detergent, as described under preparation of protein extracts. Equal amounts of proteins were ensured by addition of 1x Laemmli sample buffer. Proteins were loaded and separated by SDS-PAGE (Mini PROTEAN 3, Biorad Laboratories). Proteins have been stacked at 100V for 21 minutes and separated at 200V for 45 minutes.

2.4.5 SEMI-DRY BLOTTING

Following electrophoresis, proteins were transferred from the gels to a PVDF, polyvinylidene fluoride, membrane (Immobilon-P, Millipore) by Semi-dry Blotting using a discontinuous buffer system. Prior to blotting, the membrane has been washed for 5 minutes in methanol, following 30 minutes of incubation in anode buffer. After electrophoresis, gels have been washed in cathode buffer for 5 minutes before they were used for Semi-dry Blotting. Proteins have been transferred to the membrane at 1,5mA/cm^2 for 85 minutes, using the Fastblot B43 (Biometra).

2.4.6 PROTEIN DETECTION

2.4.6.1 SPECIFIC PROTEIN DETERMINATION ON THE MEMBRANE

Prior to the immunological detection of the relevant proteins, unspecific protein binding sites were blocked by incubating the membrane for one hour at room temperature in a 5% Blotto solution. Detection of the proteins of interest was performed by incubating the membrane with the appropriate specific primary antibody overnight at 4°C with continuous shaking. After four washing steps with TBS-T (2 x 30 seconds, 2 x 10 minutes), the membrane has been incubated with the secondary antibody for 1 hour, following 4 additional washing steps (2 x 30 seconds, 2 x 10 minutes). For detection of proteins, the membrane has been incubated in a 1:1 mixture of ECL solution A and solution B for 2 minutes. Luminescence detection was performed by exposure of the membrane to a medical X-ray film (Fuji) and subsequent development with a Curix 60 developing system (Agfa-Gevaert N.V.). Quantification was performed with a Kodak image station (NEN).

2.4.6.2 UNSPECIFIC PROTEIN STAINING WITH COOMASSIE BLUE

In order to ensure equal loading of proteins and uniform blotting of all samples, gels have been stained with Coomassie brilliant blue solution for 20 minutes. The solution nearly stains all kinds of proteins without any specification. Afterwards Gels have been washed for 1 hour with destaining solution. Proteins became visible as blue bands.

2.5 EMSA – ELECTRO MOBILITY SHIFT ASSAY

Electro mobility shift assay (EMSA) was used to determine NF-κB transcription factor activity, by analyzing its ability to form NF-κB-DNA complexes.

2.5.1 MATERIAL AND SOLUTIONS

Table 2:9 *Material used for electro mobility shift assay.*

product	**company**
[γ32P]-ATP 3000Ci/mmol	Amersham
Nuc Trap probe purification columns	Stratagene
T4 polynucleotide kinase	USB

Buffer A

10	mM	Hepes	pH 7.9
10	mM	KCl	
0.1	mM	EDTA	
0.1	mM	EGTA	
		H_2O	

add freshly before use:

1	mM	DTT	
0.5	mM	PMSF	

Buffer B

20	mM	Hepes	pH 7.9
400	mM	NaCl	
1	mM	EDTA	
0.5	mM	EGTA	
25	%	Glycerol	
		H_2O	

add freshly before use:

1	mM	DTT	
1	mM	PMSF	

STE buffer			pH 7.5
10	mM	Tris	
100	mM	NaCl	
1	mM	EDTA	
		H_2O	

Binding buffer 5x		
20	%	Glycerol
5	mM	$MgCl_2$
2.5	mM	EDTA
250	mM	NaCl
50	mM	Tris-HCl
		H_2O

Reaction buffer			
90	%	Binding buffer 5x	
10	%	Gel loading buffer	
2.6	mM	DTT	
		H_2O	

Gel loading buffer		
250	mM	Tris-HCl
0.2	%	Bromphenolblue
40	%	Glycerol
		H_2O

Non-denaturizing PAA gel			
5.3	%	TBE 10x	
15.8	%	PAA solution	30%
2.6	%	Glycerol	
0.05	%	TEMED	
0.08	%	APS	
		H_2O	

TBE 10x			pH 8.3
890	mM	Tris	
890	mM	Boric acid	
20	mM	EDTA	
		H_2O	

2.5.2 RADIOACTIVE LABELING OF CONSENSUS OLIGONUCLEOTIDES

Double-stranded oligonucleotides containing the consensus sequence for NF-κB 5'- AGT TGA GGG GAC TTT CCC AGG C -3' (Promega) was 5' end-labeled with [γ^{32}P]-ATP using the T4 polynucleotide kinase which catalyzes the transfer of the radioactive phosphate to the 5' hydroxyl site of the DNA. After incubation of oligonucleotides with T4 kinase for 10 minutes at 37°C, the reaction was terminated by addition of 0.5M EDTA solution. The radioactive labeled DNA was separated from unlabeled DNA by using Nuc Trap probe purification columns. Radioactive oligonucleotides were eluted from the column with 70µl of STE buffer and frozen at -20°C until used for electro mobility shift assay.

2.5.3 SAMPLE PREPARATION

2.5.3.1 PREPARATION OF NUCLEAR EXTRACTS FROM MOUSE LIVER TISSUE

Tissue samples were prepared with a homogenizer (Potter S, B. Braun Biotech) by homogenizing the probes in Buffer A. After centrifugation (1000rpm, 10 minutes, 4°C) and incubation at 4°C for 10 minutes in 300µl freshly added Buffer A containing 18µl of NP-40 10%, probes were centrifuged (14000rpm, 10 minutes, 4°C). The supernatant has been removed and the pellet was suspended in Buffer B, following incubation at 4°C for 30 minutes with continuous shaking. After centrifugation (14000prm, 10 minutes, 4°C) supernatants were frozen at -80°C and nuclear proteins were kept until used for protein quantification and EMSA.

2.5.3.2 PREPARATION OF NUCLEAR EXTRACTS FROM ISOLATED HEPATOCYTES

Nuclear extracts were prepared from either untreated or stimulated hepatocytes. Following stimulation, cells were scraped on ice after washing twice with ice-cold HBSS buffer. In order to gain a useable amount of nuclear protein, three wells were combined, each containing 200µl of HBSS buffer. After centrifugation (1500rpm, 5 minutes, 4°C), the supernatant was discarded and 400µl of buffer B was added to the remaining pellet. Samples have been incubated at 4°C for 15 minutes, 25µl NP-40 10% were added and samples were centrifuged (12000rpm, 1 minute, 4°C) immediately. The supernatant was removed and the remaining pellet was suspended in 50µl Buffer B. Probes have been incubated at 4°C for 15 minutes with continuous shaking. After centrifugation (12000rpm, 5 minutes, 4°C) supernatants were frozen at -80°C and nuclear proteins were kept until used for protein quantification and electro-mobility shift assay (EMSA).

2.5.3.3 PROTEIN CONCENTRATION DETERMINATION

Protein concentrations in isolated nuclear protein fractions were determined by the method of Bradford ([25] Bradford, 1976) using Coomassie brilliant blue G250. Absorbance of the samples was measured at 590nm (Tecan Sunrise Absorbance reader, Tecan).

2.5.4 BINDING REACTION AND ELECTROPHORETIC SEPARATION

To ensure equal amounts of proteins, 5µg of protein were provided in a total amount of 14µl, containing 2µg poly(dIdC) and 3µl of freshly prepared reaction buffer. Samples have been incubated for 10 minutes at room temperature. To start NF-κB – DNA binding reaction, 1µl of the radioactive labeled oligonucleotide was added and samples were left for 30 minutes at room temperature. The protein-oligonucleotide complexes have been separated by gel electrophoresis (Mini-Protean 3, BioRad) with 0.25 x TBE buffer at 100V for 60 minutes using non-denaturizing polyacrylamide gels.

2.5.4.1 CONTROL EXPERIMENTS

Parallel to each experiment a positive and negative control experiment has been performed in order to ensure specific oligonucleotide-transcription-factor binding. Therefore, different unlabeled oligonucleotides were added to the specific samples;

⇒ positive control NF-κB: - unlabelled AP-1 oligonucleotide was added ⇒ signal

⇒ negative control NF-κB: - unlabelled NF-κB oligonucleotide was added ⇒ no signal.

Now, we are able to determine specific and unspecific protein binding. In the case of unlabeled AP-1 oligonucleotide addition, NF-κB should still quantitatively bind to its corresponding labelled NF-κB oligonucleotide. Therefore, the same signal as previously seen without addition of AP-1 oligonucleotide is supposed to appear. For negative control, the NF-κB signal is mainly supposed to be blocked because of additional binding of NF-κB to the unlabeled NF-κB oligonucleotide. Representative for all performed EMSA experiments, both types of control experiments are once demonstrated in (Figure 3:16).

Additionally “free probe” resulting from the great excess of unbound radioactive oligonucleotide has been detected in each experiment. One representative example is shown in (Figure 3:18).

2.5.5 DETECTION AND EVALUATION

Following electrophoresis, gels have been exposed to Cyclone Storage Phosphor Screens (Canberra-Packard) for 24 hours, followed by analysis with a phosphor imager station (Cyclone Storage Phosphor System, Canberra-Packard).

2.6 HISTOLOGICAL ANALYSIS

Histological analysis was performed to determine changes in localization and presence of different proteins. We have mainly been interested in LPS-induced translocation of the NF-κB subunit p65 into the nucleus. Therefore, histological analysis has been performed by staining murine liver macrophages, the cell nucleus and the NF-κB subunit p65.

2.6.1 MATERIAL AND SOLUTIONS

Table 2:10 *Material used for histological analysis.*

product	**company**
Coverplate™	Thermo Shandon GmbH
Mounting medium	Dako Cytomation GmbH

PBS pH 7.4

10.4	mM	Na_2HPO_4
3.16	mM	KH_2PO_4
132.2	mM	NaCl
		H_2O

1% BSA solution

1	%	BSA
		PBS

2.6.2 ANTIBODIES

The following antibodies were used for immune-histochemical analysis. Antibody dilutions have always been freshly prepared before administration. Secondary antibodies were always carefully kept away from light, and therefore, incubated in the dark.

Table 2:11 *Primary antibodies used for histological analysis.*

primary antibody	**concentration**	**isotype**	**company**
NF-κB p65	1 : 100 in 0.2% BSA in PBS	rabbit IgG	Santa Cruz
F4/80	1 : 100 in 0.2% BSA in PBS	rat IgG	Caltag

Table 2:12 *Secondary antibodies used for histological analysis.*

secondary antibody	concentration	isotype	company
Alexa Fluor®488 anti-rat	1 : 400 in 0.2% BSA in PBS	goat IgG	Molecular Probes
Alexa Fluor®647 anti-rabbit	1 : 400 in 0.2% BSA in PBS	chicken IgG	Molecular Probes
Hoechst 33342 dye	5µg/ml in 0.2% BSA in PBS		Sigma

2.6.3 HISTOLOGICAL ANALYSIS OF LIVER TISSUE

2.6.3.1 IMMUNHISTOCHEMISTRY

Snap frozen organs were cut into 8µm sections, placed on glass slides, and have been fixed immediately in 3% formaldehyde for 3 minutes. Afterwards, slides were washed in PBS. Glass slides were carefully placed in slide holder (Coverplate) and rinsed two times with PBS to ensure that the entire slide is equally moistened. Fixed tissue has been blocked with 1% BSA solution and tissues have subsequently been incubated with primary and secondary antibody for 1 hour at room temperature. Following each step any remaining substances were removed by extensive PBS washing. After antibody incubation, slides were washed twice with PBS and 100µl of Hoechst staining has been applied for 5 minutes, following an additional washing step. Finally, organ sections have been covered with mounting medium, dried for 24 hours and analyzed by confocal laser scanning microscopy.

2.6.3.2 TUNEL STAINING AND INFILTRATION OF LEUKOCYTES

DNA fragmentation is one major sign of apoptotic cell death. To determine strand brakes of genomic DNA, formalin fixed tissue samples were embedded in paraffin, and 5µm sections were cut. These sections were analyzed using the terminal desoxynucleotide transferase-mediated dUTP nick end labelling (TUNEL) assay. TUNEL staining was kindly performed by Dr. Herbert Meissner and Andrea Sendlhofer (Institute of Pathology, Ludwig-Maximilians University, Munich, Germany). Liver sections were stained as described previously ([57] Gerwig, 2003).

Haematoxylin and eosin staining for leukocyte infiltration was kindly performed by Dr. Herbert Meissner and Andrea Sendlhofer (Institute of Pathology, Ludwig-Maximilians University, Munich, Germany). Liver sections were stained as described previously ([57] Gerwig, 2003).

2.7 REAL TIME RT-PCR

Real time RT-PCR is a method used to quantify different mRNA amounts of certain genes. RNA is translated into cDNA, following cDNA replication. Continuant measurement of cDNA amount occurs during cDNA replication.

2.7.1 MATERIAL AND SOLUTIONS

TBE buffer		
89	mM	Tris
89	mM	Boric acid
0.5	mM	Na_2EDTA
		H_2O

Agarose gel		1.2%
1.2	%	Agarose
0.01	%	Ethidiumbromide
		TBE buffer

Table 2:13 *Primer sequences used for real time RT-PCR analysis.*

primer		species	sequence
TNF-α[1]	forward primer	mouse	5'-tgg cct ccc tct cat cag ttc-3'
TNF-α [1]	reverse primer	mouse	5'-ttg gtg gtt tgc tac gac gtg-3'
TNF-α[1]	TaqMan probe	mouse	FAM 5'-tggcccagaccctcacactcagatcatc-3' BHQ
HPRT[1]	forward primer	mouse	5'-gtt aag cag tac agc ccc aaa atg-3'
HPRT[1]	reverse primer	mouse	5'-aaa tcc aac aaa gtc tgg cct gta-3'
HPRT[1]	TaqMan probe	mouse	HEX 5'-agc ttg ctg gtg aaa agg acc tct cga agt-3' BHQ
TNF-α[2]	forward primer	human	5'-cca ggc agt cag atc atc ttc tc-3'
TNF-α[2]	reverse primer	human	5'-agc tgg tta tct ctc agc tcc ac-3'
GAPDH	forward primer	human	5'-ggg aag gtg aag gtc gga gt-3'
GAPDH	reverse primer	human	5'-tcc act tta cca gag tta aaa gca g-3'

[1] ([108] Lambertsen, 2005)

[2] Med. 2, Universitätsklinikum Großhadern, 81377 Munich

The relevant complete mRNA sequences are listed in chapter 5.1.

2.7.2 SAMPLE PREPARATION

2.7.2.1 RNA PREPARATION FROM ORGAN SAMPLES AND HEPATOCYTES

Total RNA was extracted using the RNeasy Mini Kit (Qiagen) according to the instruction manual. In order to guarantee efficient RNA extraction a few steps were added. After disruption of the tissue, homogenization is necessary to reduce the “viscosity” of the lysates. Therefore, the disrupted samples were loaded onto a QIAshredder Spin Column and spun down (14000rpm, 2 minutes, 20°C). Real time RT-PCR is very sensitive to small amounts of DNA. We used the RNase Free DNase Set for digestion of DNA during RNA purification. All additional materials were applied following the users instructions manual. RNA samples were kept at -80°C until used for real time RT-PCR.

2.7.2.2 RNA PREPARATION FROM WHOLE BLOOD SAMPLES

Total RNA was extracted using the RNeasy Mini blood kit from Qiagen according to the instruction manual. Real time RT-PCR is very sensitive to small amounts of DNA. We used the RNase Free DNase Set for digestion of DNA during RNA purification. All additional materials were applied following the users manual instructions. RNA samples were kept at -80°C until used for real time RT-PCR.

2.7.2.3 DETERMINATION OF RNA CONTENT AND RNA INTEGRITY

After RNA elution from the QIAamp Spin Column, 10µl were diluted in 490µl of water and RNA content was determined by measuring the absorption at 260nm and 280nm. The RNA content was calculated from the A_{260} value. The A_{260}/A_{280} ratio was used to determine the RNA purity. Integrity of isolated RNA was checked subjecting 1µg of total RNA to agarose gel electrophoresis (described under 2.7.4.3).

2.7.3 REVERSE TRANSCRIPTION

Reverse transcription was performed with the DyNAmo Probe 2-Step qRT-PCR Kit. A minimum of 400ng and a maximum of 1000ng isolated RNA were transcribed into cDNA according to the users' manual instructions.

Table 2:14 *Cycling protocol for reverse transcription into cDNA.*

purpose	**temperature**	**time**
primer extension	25°C	10 minutes
cDNA synthesis	37°C	45 minutes
reaction termination	85°C	5 minutes

2.7.4 REAL TIME RT-PCR

Real time RT-PCR analysis has always been accomplished according to the DyNAmo Probe 2-Step qRT-PCR Kit users manual. Features that were previously individualized are listed below within the accordant chapter, 2.7.4.1 and 2.7.4.2.

2.7.4.1 PROBE REAL TIME RT-PCR

Real time RT-PCR was performed with the DyNAmo Probe 2-Step qRT-PCR Kit. Real time RT-PCR was accomplished with 100nmol/l probe and 400nmol/l of each forward and reverse primer. 10% of the final mixture was provided by the cDNA sample, which contained 20ng – 100ng cDNA, which has been calculated as RNA. The HPRT1 gene was used as an internal housekeeping gene to normalize the TNF-α data set.

Table 2:15 *Cycling protocol for probe real time RT-PCR analysis.*

purpose	**temperature**	**time**	
initial denaturizing	95°C	15 minutes	
denaturizing	95°C	15 seconds	45 cycles
annealing and extension	60°C	60 seconds	

2.7.4.2 SYBR-GREEN REAL TIME RT-PCR

Real time RT-PCR was performed with the DyNAmo Probe 2-Step qRT-PCR Kit. Real time RT-PCR was accomplished by addition of SYBR-green solution (0.2x final concentration) and 400nmol/l of each forward and reverse primer into the given Master-mix. The GAPDH gene was used as an internal housekeeping gene to normalize the TNF-α data set.

Table 2:16 *Cycling protocol for SYBR-green real time RT-PCR analysis.*

purpose	**temperature**	**time**	
initial denaturizing	95°C	15 minutes	
denaturizing	95°C	30 seconds	45 cycles
annealing	55°C	30 seconds	
extension	72°C	30 seconds	
final extension	72°C	10 minutes	
melting curve	60°C - 95°C		

2.7.4.3 PRODUCT DETERMINATION

In order to check for primer dimers and secondary PCR products, all samples were separated by agarose gel electrophoresis (Owl separation Systems). Ethidiumbromide agarose gels were prepared as described previously (2.7.1). PCR products were supplemented with 6x blue/orange loading dye, loaded onto an agarose gel and electrophoresis has been performed for 2 hours at 100V. Fluorescence of the PCR products was visualized with a Kodak image station (Kodak) at 254nm. Following SYBR-green real time RT-PCR a melting curve has been generated, ranging from 60°C to 95°C. Calculation of the mRNA content was performed with a new mathematical model for relative quantification of real time PCR products developed by Pfaffl and colleagues ([149] Pfaffl, 2001).

2.8 FACS ANALYSIS

Flow cytometry is a useful method for the measurement of cell size and granularity as well as the detection of fluorescent stains. Therefore, we used FACS analysis to determine total TNF-α protein expression in human and mouse whole blood leukocytes as well as on leukocyte cell-surface.

2.8.1 MATERIAL AND SOLUTIONS

Table 2:17 *Material used for FACS analysis.*

product	**company**
FACS™ Lysing solution	Becton Dickinson
BD Cytofix/Cytoperm™ Plus Kit with GolgiPlug™	BD Biosciences

FACS buffer			pH 7.37
140	mM	NaCl	
1.9	mM	KH_2PO_4	
16.5	mM	Na_2HPO_4	
3.8	mM	KCl	
1	mM	EDTA	
10.2	mM	LiCl	
3	mM	NaN_3	
		H_2O	

PBS			pH 7.4
10.4	mM	Na_2HPO_4	
3.16	mM	KH_2PO_4	
132.2	mM	NaCl	
		H_2O	

2.8.2 ANTIBODIES

Table 2:18 *Primary antibodies used for FACS analysis.*

primary antibody		concentration	isotype	supplier
TNF-α	human	1 : 100 in PBS[1]	rabbit IgG	Endogen
TNF-α	mouse	1 : 100 in PBS[1]	rabbit IgG	Endogen

[1] After permeabilization of cells, BD Perm/Wash solution has carefully been used for antibody dilutions to keep cells permeabilized.

Table 2:19 *Secondary antibodies used for FACS analysis.*

secondary antibody	**concentration**	**isotype**	**company**
Alexa Fluor®488 anti rabbit	1 : 400 in PBS[1]	goat IgG	Molecular Probes

[1] After permeabilization of cells, BD Perm/Wash solution has carefully been used for antibody dilutions to keep cells permeabilized.

2.8.3 IMMUNOLOGICAL STAINING OF WHOLE BLOOD LEUKOCYTES

The evaluation of TNF-α expression was carried out using the FACS Lysing solution for lysis of erythrocytes, BD Cytofix/Cytoperm plus Kit for permeabilization of cells, following anti TNF-α staining of whole blood leucocytes. At the end of the stimulation period, cells were pulled down by centrifugation (3500rpm, 10 minutes, 4°C). Supernatants were discarded and the remaining pellets have been suspended in FACS Lysing Solution for 10 minutes in the dark. Cave! Careful fixation had to be provided to prevent permeabilization during cytokine staining when cell surface had to be determined. After centrifugation (2130rpm, 5 minutes, 4°C), supernatants were removed, cells were suspended in the remaining fluid and PBS was used for intensive washing. Washing and centrifugation steps were handled as far as necessary between each treatment. If indicated, cells have been permeabilized using BD Cytofix/Cytoperm plus Kit following the users' instructions manual. Afterwards, cells have been incubated with anti TNF-α antibody for 60 minutes, following Alexa Fluor 488 labeled secondary antibody incubation. Finally, cells were diluted in 500µl PBS and TNF-α expression was determined by FACS analysis.

2.8.4 DETECTION

The FACS instrument settings were adjusted with control cells primarily stained with TNF-α following Alexa Flour 488 staining. The fluorescence of previously-bound labeled antibody can be analyzed in the FL-1 channel (λ_{em}: 530nm), where increased antibody binding results in changes of fluorescence intensity. In order to analyze different cell-types, populations (a: monocytes; b: neutrophils) were gated and analyzed separately. The median (FL1-H) of the curve was used to determine relative TNF-α amounts (Figure 2:4).

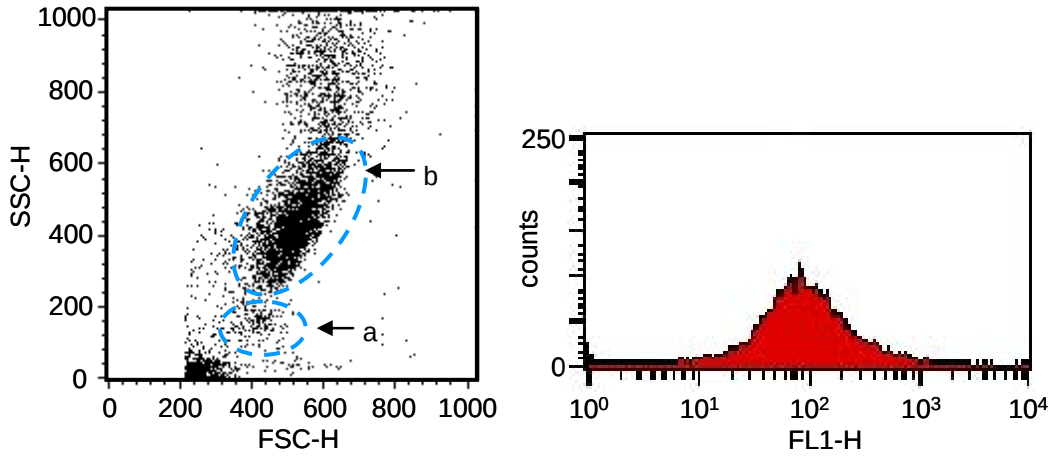


Figure 2:4 *Evaluation of FACS analysis measurements.*

2.9 STATISTICAL ANALYSIS

All cell-experiments were performed at least three times unless indicated otherwise in the respective figure legend. Animal experiments were performed using the accordant number of animals, as shown in the respective table in chapter 2.1. Results are shown as mean ± SEM. Statistical analysis was performed using the GraphPad Prism 3.03 software (GraphPad software Inc). Significance (*) has always been calculated at the 95% confidence interval in comparison to its corresponding control experiment, unless indicated otherwise in the respective figure legend. Because of post-developmental changes, neither western blot analysis nor histological analysis is subjected to any evaluation of significance.

3 RESULTS

3.1 RESEARCH BACKGROUND

Before starting our investigations, a lot of research had already been done in the past. Thereby, the effects of ANP preconditioning on survival of animals suffering from LPS-induced septic shock have quite recently been investigated. Administration of LPS caused an immediate change of animals' state of health. This was observed in LPS as well as in ANP/LPS treated mice. LPS treated animals with administration of ANP initially showed worsening of their state of health but soon recovered from symptoms of LPS-induced septic shock. All animals without ANP preconditioning died within 25 hours. Mice preconditioned with ANP survived LPS-induced septic shock by 75%, as shown in Figure 3:1-A.

TNF-α concentration in blood is an important marker in LPS-induced septic shock ([118] Lin, 2005). Thus, it had been interesting, whether survival after ANP preconditioning is associated with changes in TNF-α expression. Cytokine concentration had been determined in plasma 90 minutes after LPS injection. At this point, TNF-α levels in LPS stimulated animals peaked and were detected by ELISA. As shown in Figure 3:1-B, preconditioning with the cardiovascular hormone ANP caused a significant decrease in TNF-α serum levels by 80% in LPS treated mice. All research has been done by Melanie Keller up to this point ([91] Keller, 2005a).

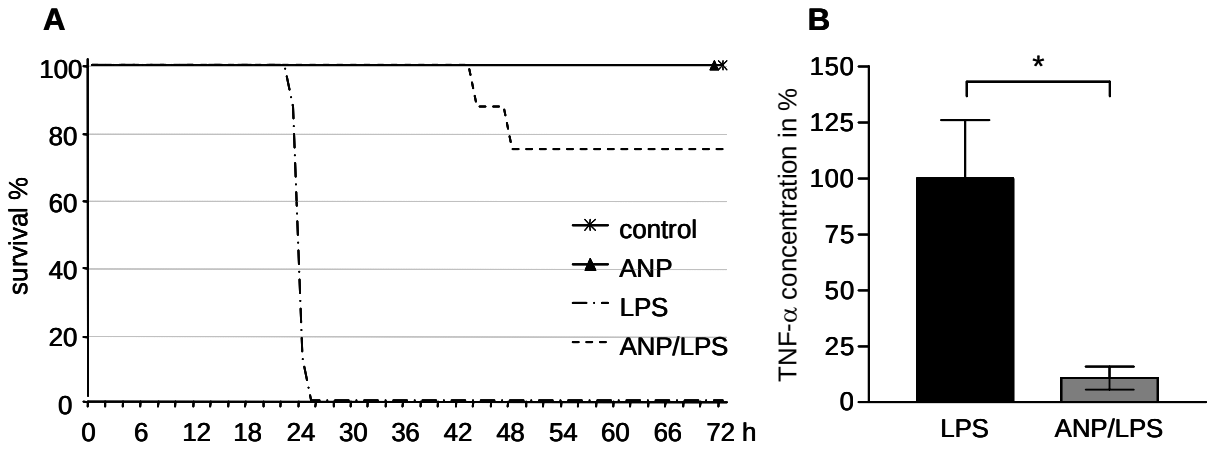


Figure 3:1 *ANP improves survival of mice treated with LPS and reduces TNF-α concentration in serum 90 minutes after LPS injection.* Mice were injected NaCl (control and LPS group) or ANP (ANP and ANP/LPS group). 20 minutes after first injection mice received NaCl (control and ANP group) or LPS (LPS and ANP/LPS group). The animal's state of health had been continuously observed for 72 hours (A). 90 minutes after LPS injection, blood was withdrawn from the heart and used for TNF-α concentration determination by ELISA (B).

3.2 SEPTIC SHOCK MODEL

As described in the previous chapter, pretreatment with ANP significantly reduces mortality in septic shock, which is associated with changes in plasma TNF-α concentrations. Therefore, it has to be investigated, how ANP contributes to minor TNF-α plasma levels in septic mice.

3.2.1 INFLUENCE OF ANP ON LPS-INDUCED TNF-α mRNA EXPRESSION

In order to investigate mechanisms leading to the protective effects of ANP, TNF-α expression in various organs had to be determined. Therefore, we examined mRNA TNF-α expression in selected organs – liver (3.2.1.1), spleen (3.2.1.2) and heart (3.2.1.3). Changes in TNF-α plasma concentrations have primarily been detectable 90 minutes after LPS-induced septic shock. On account of this, we focused on this time point and the previous time point, 30 minutes after LPS injection, to determine TNF-α mRNA expression. To ensure primarily equal cDNA amounts, the hypoxanthine-guanine-posphoribosyl transferase (HPRT) gene was used to normalize the TNF-α data set. As seen in Figure 3:2, TNF-α c_t-values (c_t -cycle of threshold) derived from LPS treated mice are lower than TNF-α c_t-values in organs of untreated animals. HPRT gene expression is completely unaffected by LPS injection, resulting in equal HPRT-c_t values for all animals.

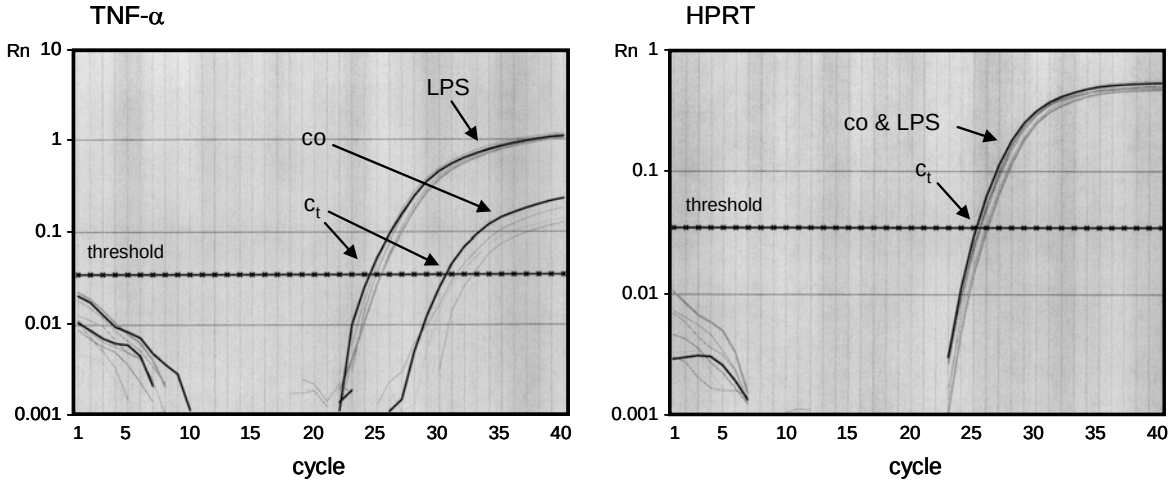
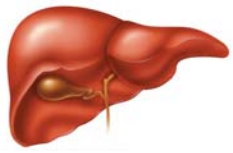


Figure 3:2 *LPS stimulation influences TNF-α gene expression in different organs in mice.* Organ samples of NaCl (co) or LPS treated mice were obtained 90 minutes after LPS injection. Whole liver homogenates were analyzed by real time RT-PCR. Illustrations are demonstrating cDNA replication curves for TNF-α (left) and HPRT (right). Graphs are showing one representative example, with four animals out of five.

3.2.1.1 TNF-α mRNA EXPRESSION IN MOUSE LIVER



The liver is the largest organ in the abdomen. Most bacteria that enter the organism within the blood stream are taken up and eliminated by the liver. Resident liver macrophages, also denoted as Kupffer cells, constitute the largest compartment of tissue macrophages present in the body. They are the first cell population to come in contact with bacteria, bacterial endotoxins and microbial debris ([63] Gregory, 2002b). Therefore, we were interested in changes of TNF-α mRNA expression in livers of ANP pretreated septic mice. Mice have been treated as described previously (2.1.3) and classified into different groups (control, ANP, LPS, ANP/LPS). As shown in Figure 3:3, LPS treatment rapidly accelerates TNF-α mRNA expression, already seen 30 minutes after injection, which steadily consists until the time point of 90 minutes. ANP preconditioning significantly reduces this TNF-α mRNA augmentation by 60% as soon as 30 minutes after LPS injection. Interestingly, this strong reduction has totally been abrogated 90 minutes after LPS-induced septic shock.

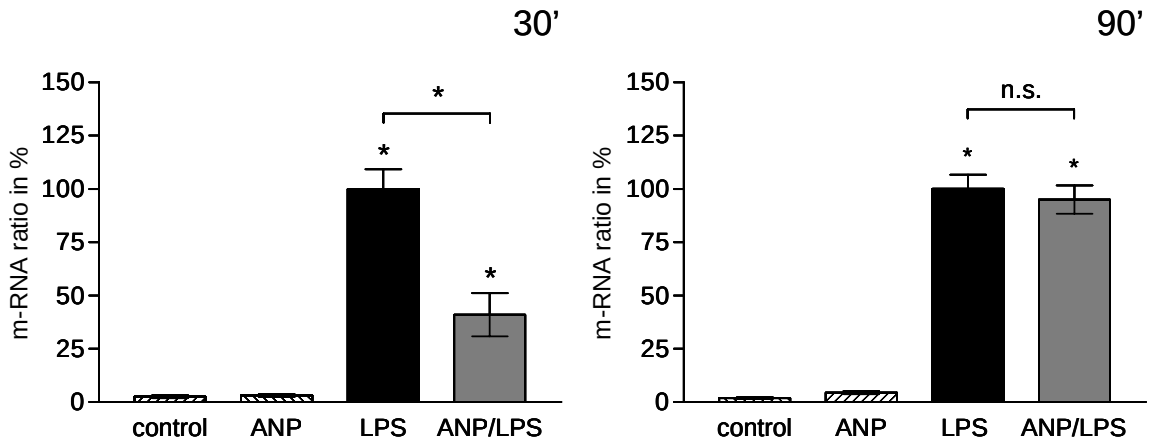


Figure 3:3 *ANP preconditioning contributes to TNF-α mRNA reduction after LPS-induced septic shock in whole liver homogenates.* Animals were preconditioned with ANP (ANP/LPS group) 15 minutes previous to LPS (LPS group) injection. Mice, only receiving NaCl (control group) or ANP (ANP group) were used to identify an impact of these substances on TNF-α mRNA expression. Organ samples were obtained 30 and 90 minutes after LPS injection. Whole liver homogenates were analyzed by real time RT-PCR. Graphs are showing determined TNF-α mRNA expression 30 minutes (left) and 90 minutes (right) after LPS injection. Graphs are representing all animals analyzed for each group including five mice. Real time RT-PCR analysis has been performed in duplicate.

3.2.1.2 TNF-α mRNA EXPRESSION IN MOUSE SPLEEN

The spleen is the bodies' largest filter of the blood. Within this organ innate and adaptive immune system are combined. Therefore, the spleen is the most important organ for antimicrobial reactivity ([130] Mebius, 2005). Thus, we were interested in changes of TNF-α mRNA expression in spleens of ANP pretreated septic mice. Similar to the results determined in whole liver homogenates, LPS injection subsequently leads to elevated TNF-α mRNA expression in the spleen as soon as 30 minutes after LPS administration (Figure 3:4). ANP preconditioning yields 40% - 50% reduction of LPS-induced TNF-α mRNA expression at both investigated time points.

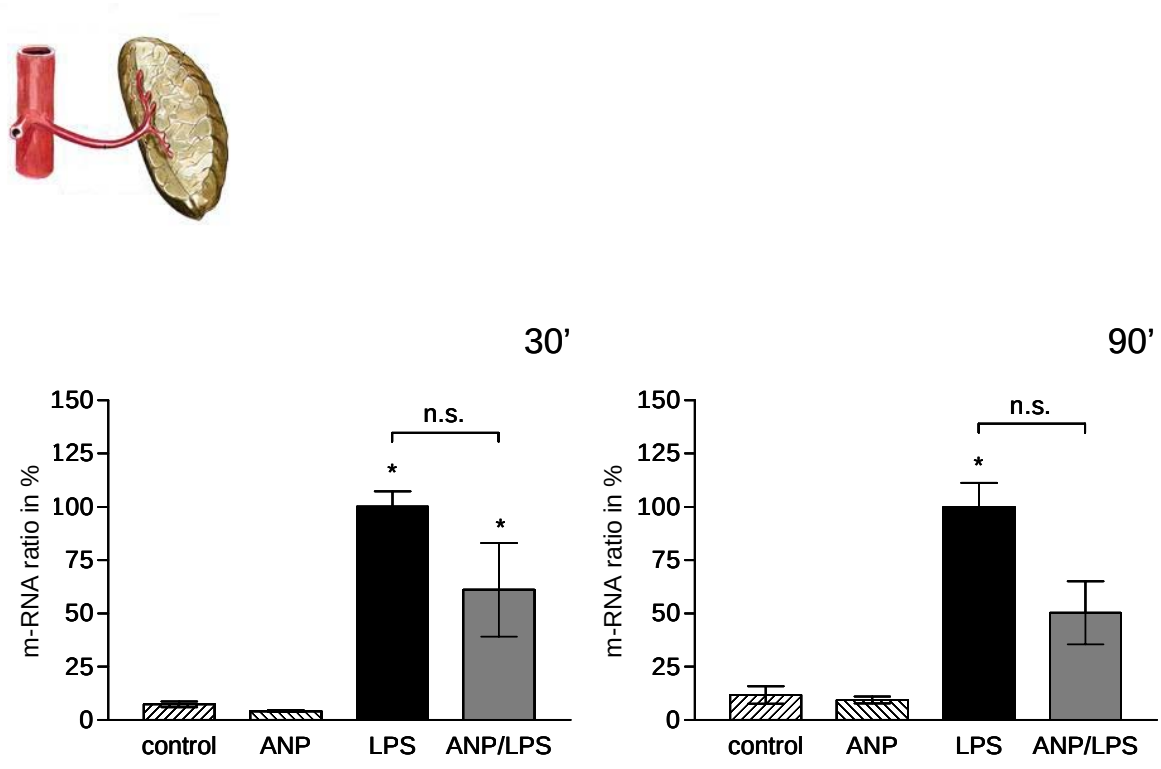


Figure 3:4 *ANP preconditioning contributes to TNF-α mRNA reduction after LPS-induced septic shock in whole spleen homogenates.* Animals were preconditioned with ANP 15 minutes (ANP/LPS group) previous to LPS (LPS group) injection. Mice, only receiving NaCl (control group) or ANP (ANP group) were used to identify an impact of these substances on TNF-α mRNA expression. Organ samples were obtained 30 and 90 minutes after LPS injection. Whole spleen homogenates were analyzed by real time RT-PCR. Graphs are showing determined TNF-α mRNA expression 30 minutes (left) and 90 minutes (right) after LPS injection. Graphs are representing all animals analyzed, each group consisting of five mice. Real time RT-PCR analysis has been performed in duplicate.

3.2.1.3 TNF-α mRNA EXPRESSION IN MOUSE HEART

The polypeptide hormone, atrial natriuretic peptide, is primarily produced in the atrial cardiocytes of the heart ([129] McGrath, 2005). Being the main organ of ANP synthesis, we were interested in changes of TNF-α mRNA expression in the heart of ANP pretreated septic mice. As shown in Figure 3:5, in mice hearts, LPS injection subsequently leads to significant TNF-α mRNA expression as soon as 30 minutes after LPS administration. Interestingly, ANP preconditioning does not have any influence on LPS-induced TNF-α mRNA expression neither 30 minutes nor 90 minutes after LPS injection.

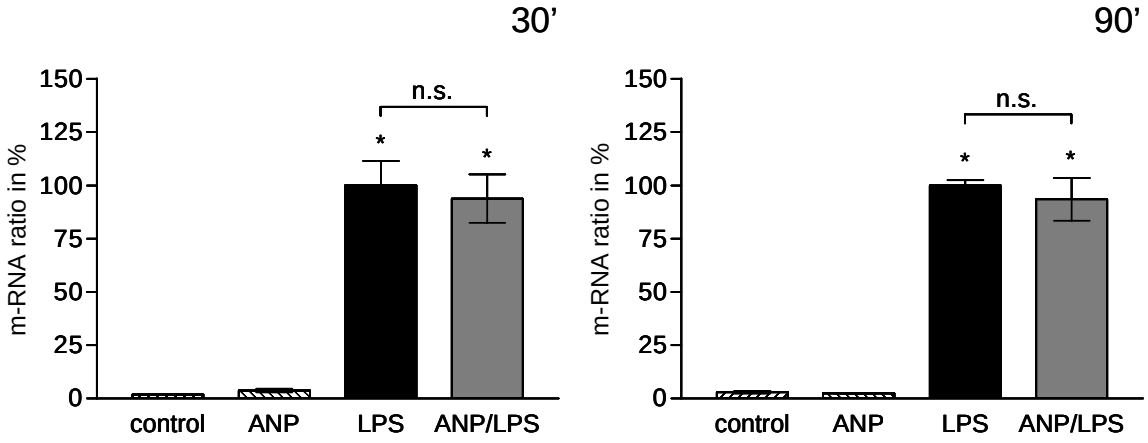


Figure 3:5 *ANP preconditioning does not contribute to TNF-α mRNA reduction after LPS-induced septic shock in whole heart homogenates.* Animals were preconditioned with ANP 15 minutes (ANP/LPS group) previous to LPS (LPS group) injection. Mice, only receiving NaCl (control group) or ANP (ANP group) were used to identify an impact of these substances on TNF-α mRNA expression. Organ samples were obtained 30 and 90 minutes after LPS injection. Whole heart homogenates were analyzed by real time RT-PCR. Graphs are showing determined TNF-α mRNA expression 30 minutes (left) and 90 minutes (right) after LPS injection. Graphs are representing all animals analyzed, each group consisting of five mice. Real time RT-PCR analysis has been performed in duplicate.

3.3 THE LIVER

3.3.1 CHANGES IN LIVER TISSUE

Due to the fact that TNF-α mRNA expression has significantly been reduced in liver tissue, we focused on this organ in order to determine the signaling pathways responsible for TNF-α mRNA and cytokine reduction. In our model of LPS-induced septic shock, the latest time point we are focusing on is 90 minutes of LPS treatment. Therefore, we were interested in morphological changes in liver tissue as far as to this moment.

3.3.1.1 TUNEL STAINING OF APOPTOTIC CELLS

Apoptotic cell death was determined by biochemical and morphological techniques in order to identify early cell damage in the liver during septic shock. DNA fragmentation is one major sign of apoptotic cell death. To determine strand brakes of genomic DNA, formalin fixed tissue samples were embedded in paraffin, and 5µm sections were cut. These sections were analyzed using the terminal desoxynucleotide transferase-mediated dUTP nick end labelling (TUNEL) assay (Figure 3:6). Morphological criteria of apoptosis in TUNEL stained hepatocytes were cell shrinkage and the occurrence of apoptotic bodies. TUNEL staining of 90 minutes LPS treated liver sections did not show any increase in TUNEL positive hepatocytes (a) with characteristic pathologic signs of apoptosis.

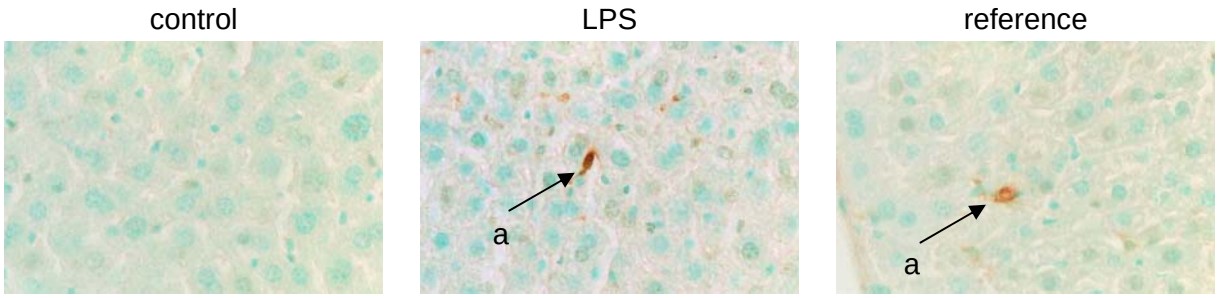


Figure 3:6 *Morphological characteristics of apoptotic cell death.* The figure shows TUNEL staining of liver tissue 90 minutes after LPS injection. LPS treated liver samples were compared to NaCl (control) treated samples. All samples were analyzed in regard to a standard reference, representing healthy mouse liver tissue. Ten high power fields (1,96mm^2) containing approximately 4000 hepatocytes were counted at an original magnification of 400-fold. TUNEL positive hepatocytes (a) are characterized by determination of DNA strand brakes.

3.3.1.2 INFILTRATION OF LEUKOCYTES

After investigating characteristic signs of apoptosis, we have been interested in leukocyte infiltration in liver tissue. The infiltration of leukocytes was determined by Haematoxylin and Eosin (HE) staining (Figure 3:7) of formalin fixed, paraffin embedded liver tissue. Staining of livers derived from 90 minutes LPS treated mice did not display any significant evidence of cell injury and leukocyte infiltration. Interestingly, leukocyte infiltration into the liver could neither been determined in liver tissue around the central vein (left) nor the portal vein (right) as far as to this moment.

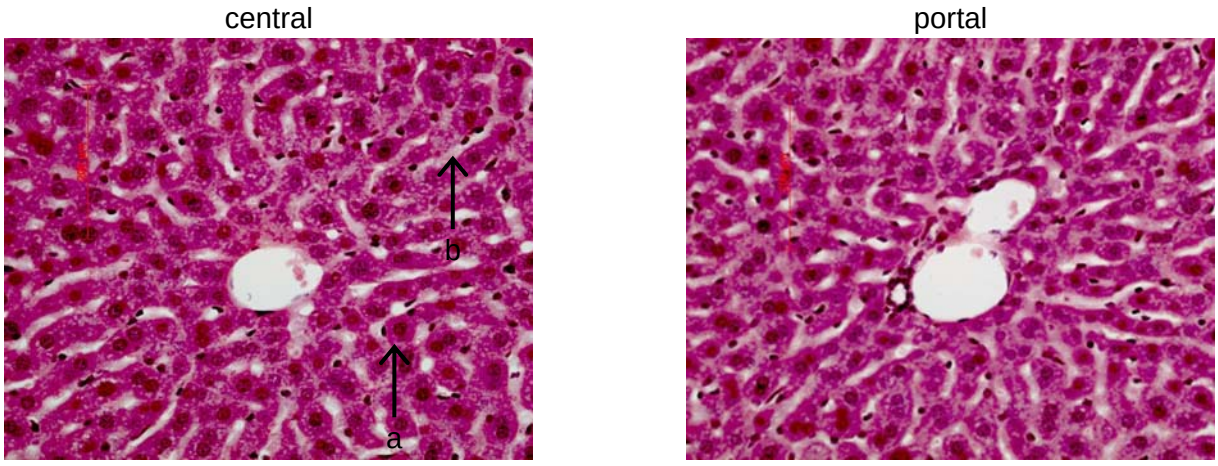


Figure 3:7 *Leukocyte infiltration in liver tissue is not affected 90 minutes after LPS injection.* The figure shows one representative example of Haematoxylin and Eosin stained liver tissue derived from LPS treated animals; hepatocytes (a) sinusoids (b). To demonstrate leukocyte infiltration, different liver sections are shown.

3.3.2 INFLUENCE OF ANP ON THE REGULATION OF PROTEINKINASES

A major effort has been made to investigate the signaling pathways and its key mediators in septic shock. However, the role of LPS activated signaling pathways and their modulation by protective interventions such as ANP preconditioning are still widely unknown ([14] Beutler, 2005). Changes in proteinkinase activation are a characteristic feature during sepsis ([103] Koyasu, 2003; [107] Kyriakis, 2001). Therefore, the changes in phosphorylation of selected proteinkinases were determined in order to investigate the ANP mediated protective effects in LPS-induced septic shock.

3.3.2.1 AKT PROTEINKINASE REGULATION

The PI3 kinase/Akt kinase pathway is known to participate in signaling cascades during inflammation in different cell types. In order to investigate whether ANP pretreatment in septic mice has an influence on proteinkinase Akt activation, we focused on kinase phosphorylation 15, 30, and 90 minutes after LPS injection. Whole liver homogenates were analyzed by western blot and Akt phosphorylation (Ser473) has been determined by specific antibody binding. As presented in Figure 3:8, following LPS injection, mice displayed a strong increase in Akt phosphorylation as soon as 15 minutes after LPS administration. This phosphorylation status clearly increases until 30 minutes of LPS treatment. In regard to the phosphorylated amount detected 30 minutes after LPS administration, phosphorylation decreases to the time point of 90 minutes. In all experiments, the phosphorylation status has been evaluated in according to its corresponding control group at the chosen time point. Phosphorylation slightly changes in control animals most likely due to the influence of stress during the animal experiments. The results displayed in Figure 3:9 obviously demonstrate that ANP pretreatment decreases LPS-induced Akt phosphorylation at all determined time points. These effects lead to the assumption of Akt kinase involvement in ANP mediated protection from LPS-induced septic shock.

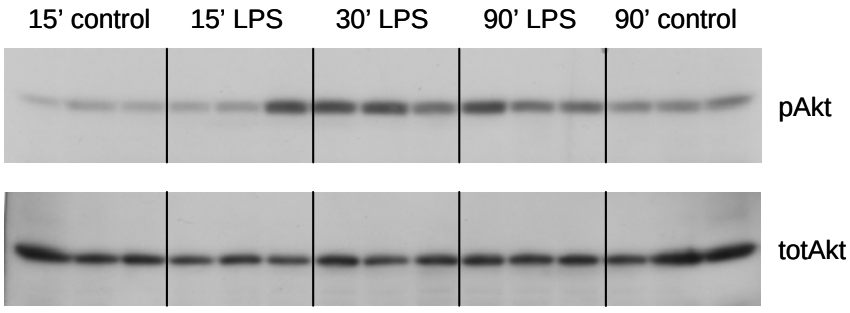


Figure 3:8 *Time dependent Akt kinase phosphorylation in whole liver homogenates 15, 30, and 90 minutes after LPS injection.* Western blot analysis was performed detecting phosphorylated (pAkt) and total (totAkt) amounts of Akt kinase. In further experiments, each phosphorylation status has been evaluated in relation to its corresponding control group (NaCl treatment) at the same time point (15 and 90 minutes shown). The figure shows 3 representative animals of each group out of 5 animals. Western blot analysis has been performed in duplicate. Western blot membranes for total amounts of Akt kinase (totAkt) are shown to ensure equal protein loading.

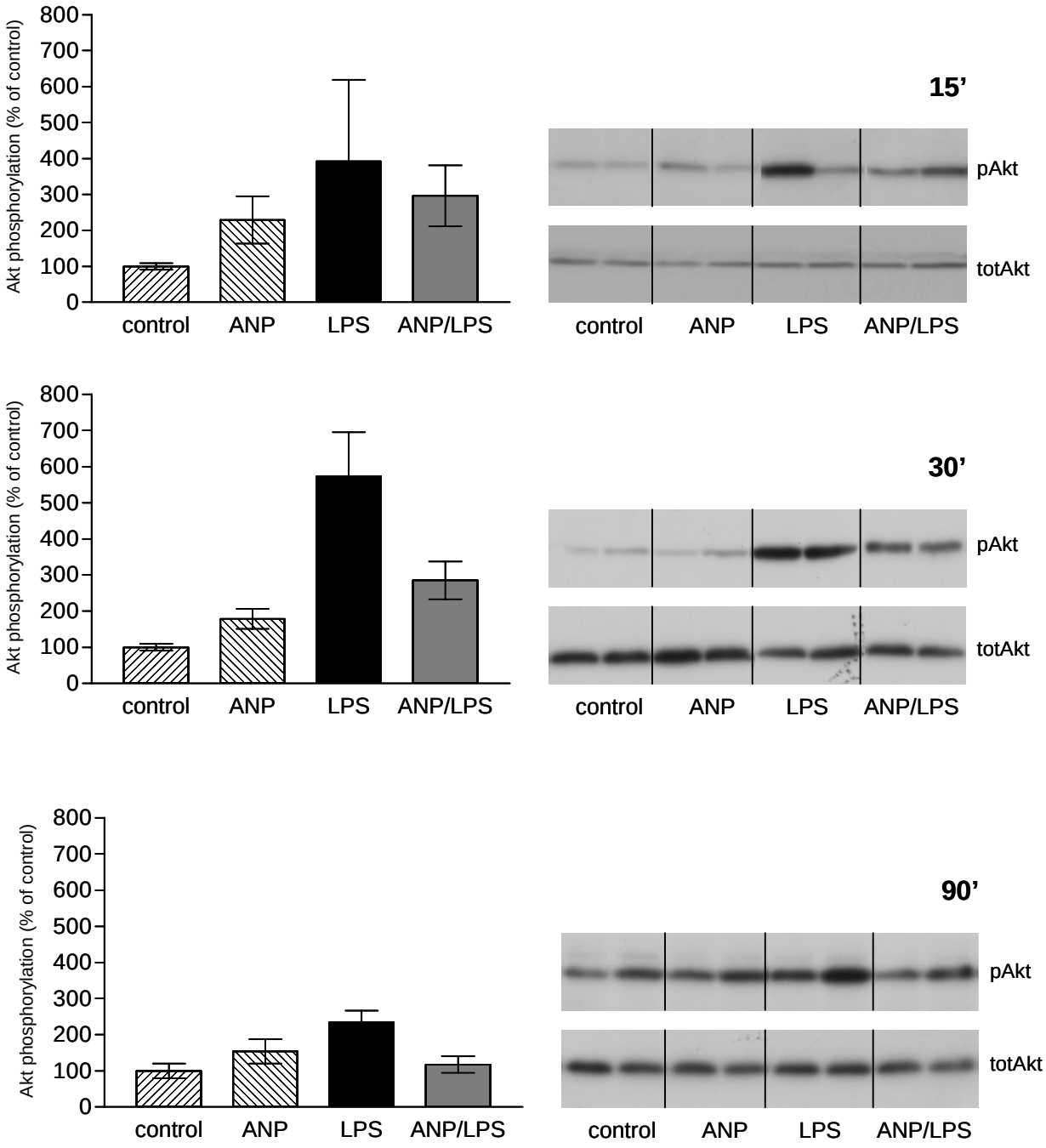


Figure 3:9 *ANP preconditioning decreases LPS-induced Akt phosphorylation in whole liver homogenates*. Animals were preconditioned with ANP (ANP/LPS group) 15 minutes previous to LPS (LPS group) injection. Mice, only receiving NaCl (control group) or ANP (ANP group) were used to identify an impact of these substances on Akt kinase phosphorylation. Organ samples were obtained 15, 30, and 90 minutes after LPS injection. Whole liver homogenates were analyzed by western blot. Graphs are showing determined Akt phosphorylation 15 minutes (top), 30 minutes (middle) and 90 minutes (bottom) after LPS injection. Western blots are representing 2 animals out of each group consisting of five mice. Western blot membranes for total amounts of Akt kinase (totAkt) are shown to ensure equal protein loading. Western blot analysis has been performed in duplicate.

3.3.2.2 p38 PROTEINKINASE REGULATION

Not only the PI3 kinase/Akt pathway, but also the p38 MAPK signaling pathway has frequently been described to be involved in signaling cascades during LPS-induced inflammation and septic shock ([3] Akira, 2004). Therefore, we were interested in the ANP mediated effects on p38 MAPK phosphorylation. Whole liver homogenates were analyzed by western blot techniques and p38 MAPK phosphorylation has been determined by specific antibody binding. The employed antibody determines both required phosphorylation sites (Thr180/Tyr182) for p38 MAPK activation. Following LPS injection, animals displayed a strong decrease in p38 MAPK phosphorylation status already detectable 15 minutes after LPS administration. The reduced p38 MAPK phosphorylation persists until 30 minutes after LPS administration. As displayed in Figure 3:10, the reduced phosphorylation of p38 MAPK still exists but slightly increases 90 minutes after LPS injection in regard to 15 and 30 minutes LPS treated analyzed liver homogenates. During experiments the phosphorylation status has been evaluated in relation to its corresponding control group at the chosen time point. The influence of ANP pretreatment on the phosphorylation of p38 MAPK is shown in Figure 3:11. ANP pretreatment does not seem to have any influence on LPS-induced decrease in p38 MAPK phosphorylation at all determined time points. Conclusively, p38 MAPK might be involved in LPS mediated inflammatory signaling pathways, whereas ANP preconditioning does not interfere with these effects.

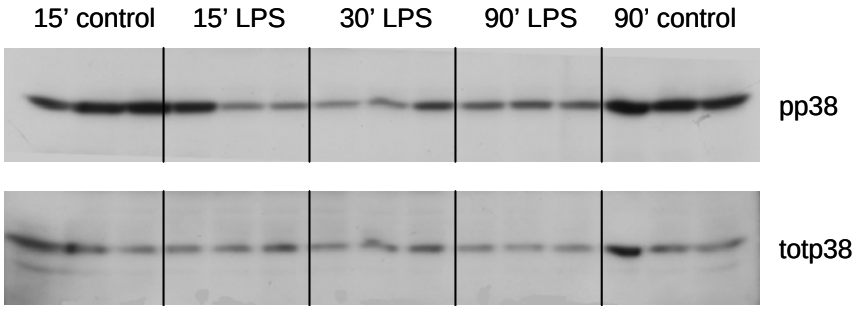


Figure 3:10 *Time dependent p38 proteinkinase phosphorylation in whole liver homogenates 15, 30, and 90 minutes after LPS injection.* Western blot analysis was performed detecting phosphorylated and total amounts of the proteinkinase p38 MAPK. In further experiments, each phosphorylation status has been evaluated in response to its corresponding control group (NaCl treatment) at the same time point (15 and 90 minutes shown). The figure shows three representative animals out of five animals in each group. Western blot analysis has been performed in duplicate.

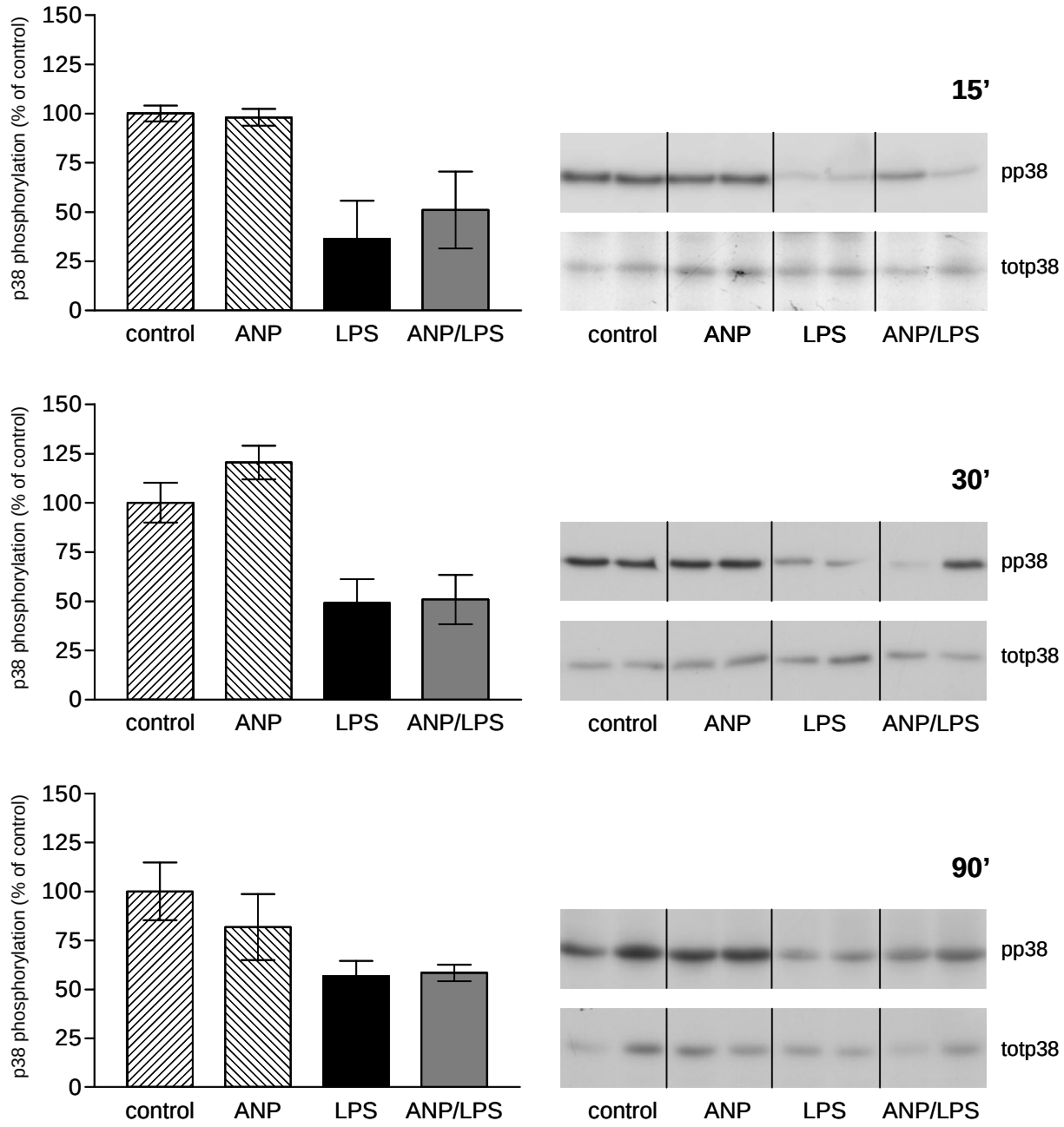


Figure 3:11 *ANP preconditioning does not influence LPS-induced decreased p38 MAPK phosphorylation in whole liver homogenates.* Animals were preconditioned with ANP (ANP/LPS group) 15 minutes previous to LPS injection (LPS group). Mice only receiving NaCl (control group) and ANP (ANP group) were used to identify an impact of these substances on p38 MAPK phosphorylation. Organ samples were obtained 15, 30, and 90 minutes after LPS injection. Whole liver homogenates were analyzed by western blot. Graphs are showing determined p38 MAPK phosphorylation (pp38) 15 minutes (top), 30 minutes (middle), and 90 minutes (bottom) after LPS administration. Western blots are representing two animals out of each group consisting of five animals. Western blot membranes for total amounts of p38 MAPK (totp38) are shown to ensure equal protein loading. Western blot analysis has been performed in duplicate.

3.3.3 INFLUENCE OF ANP ON THE REGULATION OF NF-κB AND IκBα

The transcription factor NF-κB is one of the most important "players" within the LPS signaling pathway ([3] Akira, 2004). Following activation, NF-κB translocates into the nucleus, binds to its corresponding DNA-sequence, and finally promotes expression of several cytokines (Figure 3:12). Therefore, ANP mediated influences on NF-κB binding activity might be responsible for final differences in TNF-α expression.

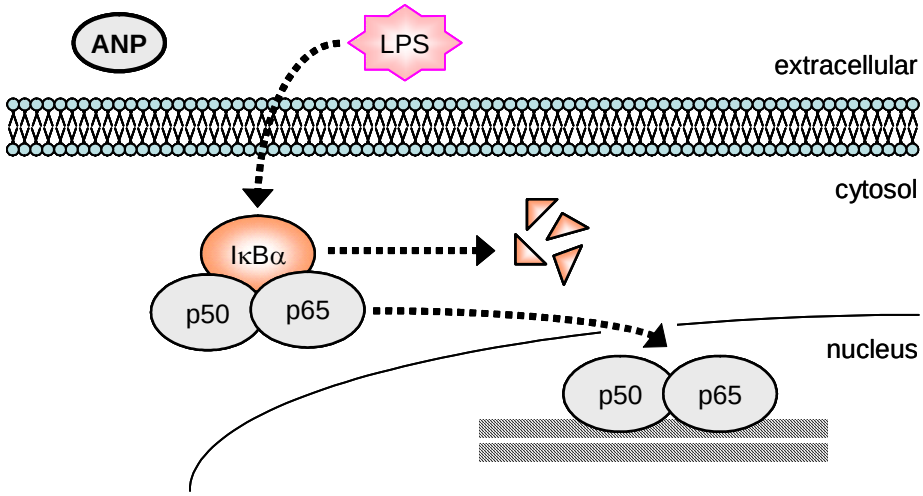


Figure 3:12 *The NF-κB signaling cascade*. After IκBα phosphorylation subsequent degradation occurs of this protein. The transcription factor NF-κB, consisting of two subunits, translocates into the nucleus, and finally promotes expression of several cytokines, such as TNF-α.

3.3.3.1 REGULATION OF THE TRANSCRIPTION FACTOR NF-κB

Changes in TNF-α plasma levels already occur 90 minutes after LPS injection. Thus, we focused on the early effects on NF-κB binding activity in the nucleus caused by ANP preconditioning in the model of LPS-induced sepsis. As demonstrated in Figure 3:13, LPS treatment significantly increases NF-κB binding activity in whole liver homogenates. This characteristic effect has previously been described, in the model of LPS-induced sepsis ([3] Akira, 2004). Neither NF-κB binding activity in tissue of NaCl (control) treated animals has been detectable, nor does ANP injection seem to have any influence on binding activity of the transcription factor. Preconditioning with ANP in LPS-induced septic shock provoked a decrease in NF-κB binding activity as soon as 15 minutes after LPS injection. This effect is still detectable 30 minutes after LPS-induced endotoxemia.

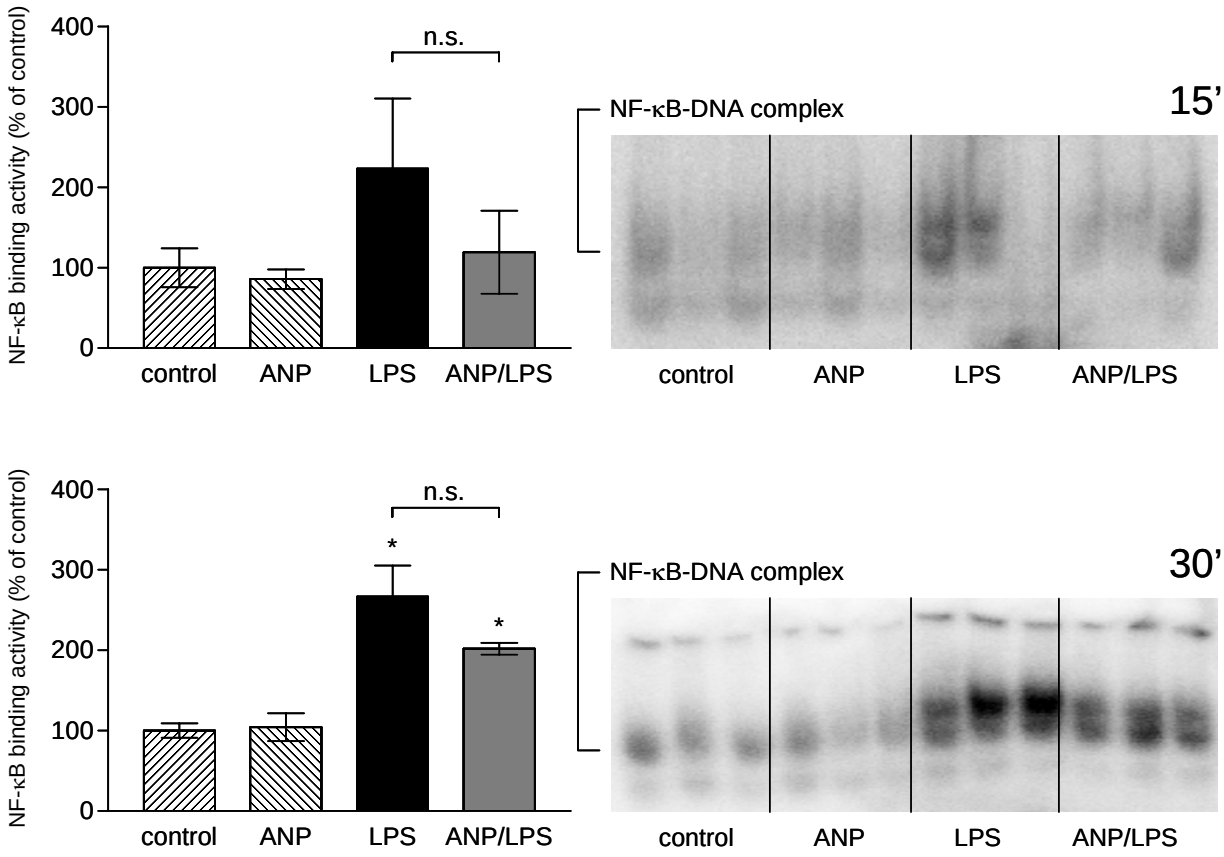


Figure 3:13 *ANP pretreatment reduces NF-κB binding activity in whole liver homogenates after LPS-induced septic shock.* Animals were preconditioned with ANP (ANP/LPS group) 15 minutes previous to LPS (LPS group) injection. Mice, only receiving NaCl (control group) or ANP (ANP group) were used to identify an impact of these substances on NF-κB binding activity. Organ samples were obtained 15 (top) and 30 (bottom) minutes after LPS injection. Whole liver homogenates were analyzed by electro-mobility-shift assay. Pictures are representing three animals out of each group consisting of five animals. EMSA has been performed in duplicate.

3.3.3.2 REGULATION OF THE NF-κB INHIBITORY FACTOR IκBα

Following determination of NF-κB activation, we were interested in upstream mechanisms leading to ANP mediated inhibition of NF-κB binding activity. IκBα, the inhibitory factor of NF-κB prevents its translocation from the cytosol into the nucleus, and therefore inhibits transcription of respective genes. Following phosphorylation by its corresponding kinase IKK, IκBα is subsequently ubiquitinylated and degraded within the proteasome. As demonstrated in Figure 3:14, corresponding to NF-κB binding activity, we observed increased IκBα phosphorylation 15 and 30 minutes after LPS injection. Decreased levels of total amounts of IκBα have been detected 30 minutes after LPS injection.

This effect points to an increased degradation of the NF-κB inhibitory factor. ANP preconditioning causes strong decreases in LPS-induced IκBα phosphorylation. This finally results in a higher content of the inhibitory factor, most likely due to minor degradation of IκBα 30 minutes after LPS injection.

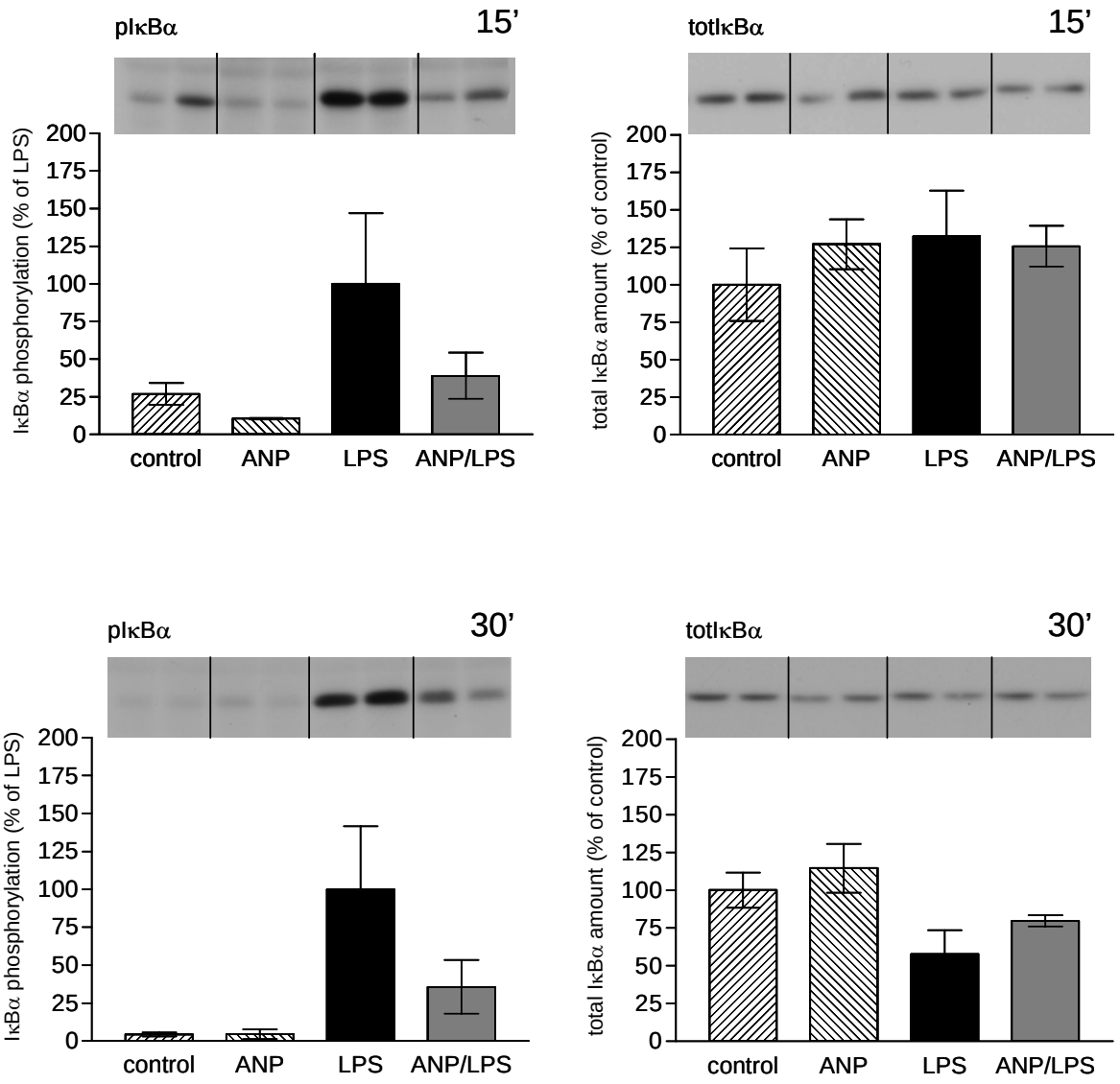


Figure 3:14 *ANP pretreatment reduces IκBα phosphorylation, resulting in minor degradation of IκBα protein in whole liver homogenates after LPS-induced septic shock.* Animals were preconditioned with ANP (ANP/LPS group) 15 minutes previous to LPS (LPS group) injection. Mice, only receiving NaCl (control group) or ANP (ANP group) were used to identify an impact of these substances on IκBα phosphorylation and degradation. Organ samples were obtained 15 (top) and 30 (bottom) minutes after LPS injection. Whole liver homogenates were analyzed by western blot and IκBα has been determined using specific antibodies, detecting phosphorylated (pIκBα) and total amounts of IκBα (totIκBα). Blots are representing two animals out of each group consisting of five animals. Western blot has been performed in duplicate.

3.3.4 CELL DEPENDENT REGULATION OF TRANSCRIPTIONAL ACTIVITY

Effects on the nuclear factor-κB and its inhibitory factor IκBα have always been seen in whole organ homogenates. In order to further investigate signaling cascades involved in ANP mediated TNF-α reduction in septic-shock, cell dependent investigations had to be done. Thus, we were interested in the cell type responsible for changes in NF-κB activity, which might finally contribute to decreased TNF-α mRNA expression in ANP preconditioned endotoxemic liver samples.

3.3.4.1 p65 LOCATION IN LIVER TISSUE

NF-κB, a known heterodimer, mainly consists of the p65 and the p50 subunits. In order to determine the cell type involved in changes within NF-κB binding activity, we focused on the distribution of the NF-κB subunit p65. Therefore, “NF-κB” translocation into the nucleus has been investigated by staining of p65 in frozen slices of liver tissue. Parallel, resident liver macrophages, also denoted as Kupffer cells, were stained to differentiate between Kupffer cells and hepatocytes. Images were obtained by confocal-microscopy. As frequently described ([16] Bilzer, 2005), we expected Kupffer cells to be responsible for changes in NF-κB binding activity. Surprisingly, both Kupffer cells (Figure 3:15-a) as well as hepatocytes (Figure 3:15-b) show a significant increase in p65 translocation into the nucleus, seen 30 minutes after LPS injection. Interestingly, ANP preconditioning reduces translocation of p65 in Kupffer cells as well as in hepatocytes, shown by decreased p65 appearance in the nucleus of both cell types (Figure 3:15).

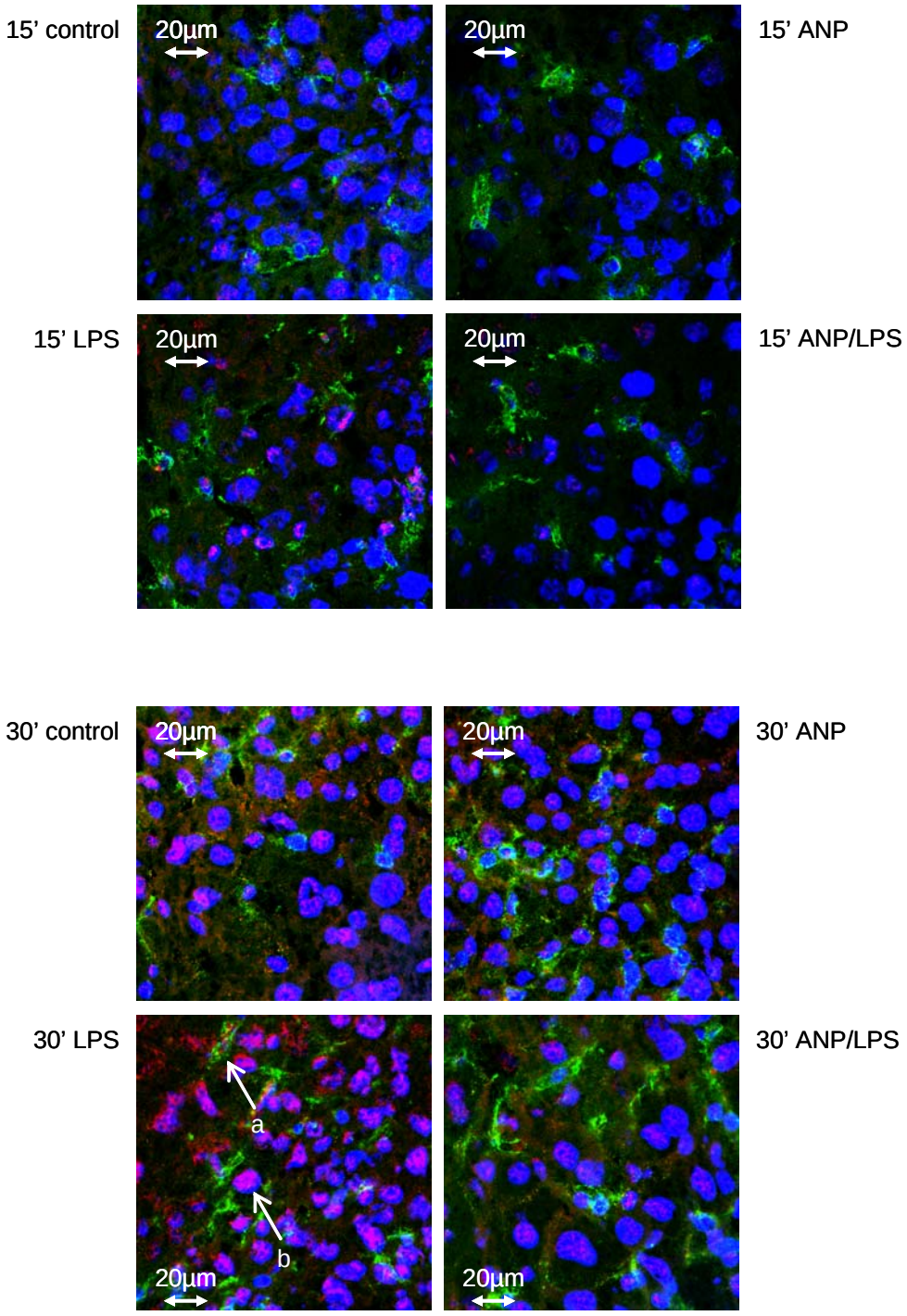
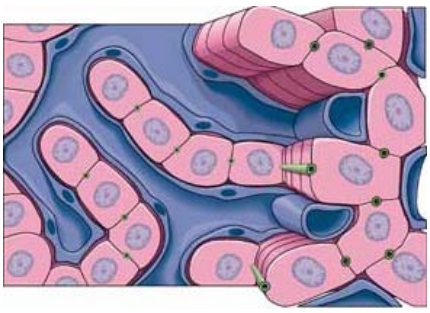


Figure 3:15 *ANP prevents translocation of the NF-κB subunit p65 into the nucleus.* Animals were preconditioned with ANP (ANP/LPS group) 15 minutes previous to LPS (LPS group) injection. Mice, only receiving NaCl (control group) or ANP (ANP group) were used to identify an impact of these substances on p65 translocation. Organ samples were obtained 15 (top) and 30 (bottom) minutes after LPS injection. Liver slices were stained with specific conjugated antibodies to determine p65 location by microscopy; blue: nucleus of Kupffer cells (a) and hepatocytes (b); green: macrophages/Kupffer cells (a); red: NF-κB subunit p65.

3.4 HEPATOCYTES



The liver is made up of different cell populations. The most abundant cell type by mass and by number are the hepatocytes. Surprisingly, not only Kupffer cells but also hepatocytes showed elevated p65 translocation into the nucleus after LPS injection. This effect is supposed to be associated with NF-κB activity. ANP preconditioning prevents p65 translocation into the nucleus in both cell types, pointing to an involvement of hepatocytes in ANP mediated protection from LPS-induced septic shock. Much research has been done to investigate the response of Kupffer cells to bacterial products, such as LPS. But only little is known about the LPS response of the livers major cell type, the hepatocytes.

3.4.1 INFLUENCE OF ANP ON THE REGULATION OF NF-κB AND IκBα

The regulation of NF-κB is tightly controlled. Activation of the transcription factor NF-κB is mediated through phosphorylation and degradation of its inhibitory factor IκBα. We investigated the nuclear factor-κB binding activity, and phosphorylation as well as degradation of its inhibitory factor IκBα in whole liver homogenates (3.3). Now, we were interested in the microbial response of primary isolated hepatocytes. Effects on TNF-α serum levels have been seen to peak 90 minutes after LPS-induced septic shock in mice (3.1). As previously determined, changes in NF-κB binding activity have already been detectable 15 minutes after LPS injection, in whole liver homogenates (3.3.3.1). Therefore, we focused on NF-κB binding activity and corresponding changes in IκBα phosphorylation and degradation, 30 minutes after LPS stimulation of isolated hepatocytes. Cells were used for experiments 24 hours after isolation. In primary isolated hepatocytes, we investigated NF-κB binding activity and IκBα phosphorylation and degradation. Hence, we were conscious about receptor and surface molecule reduction during the process of hepatocyte isolation. Complete ANP receptor recovery has previously been described in hepatocytes 24 hours after isolation ([104] Kulhanek-Heinze, 2004). But, to exclude an influence by cells lacking receptor and surface molecules, hepatocytes have additionally been cultivated for 48 hours and NF-κB binding activity has comparatively been determined.

3.4.1.1 REGULATION OF NF-κB IN 24 HOURS CULTIVATED HEPATOCYTES

Transcription factor binding activity has hardly been detectable in NaCl and ANP treated cells 30 minutes after stimulation. Interestingly, as seen in Figure 3:16, 30 minutes of LPS stimulation does not cause any changes in NF-κB binding activity. Stimulation with TNF-α alone as well the combination of LPS and TNF-α significantly increases binding activity of the nuclear factor to the same extends. Interestingly, neither an impact on NF-κB binding activity by ANP preconditioning in TNF-α nor in LPS plus TNF-α stimulated isolated hepatocytes has been detectable.

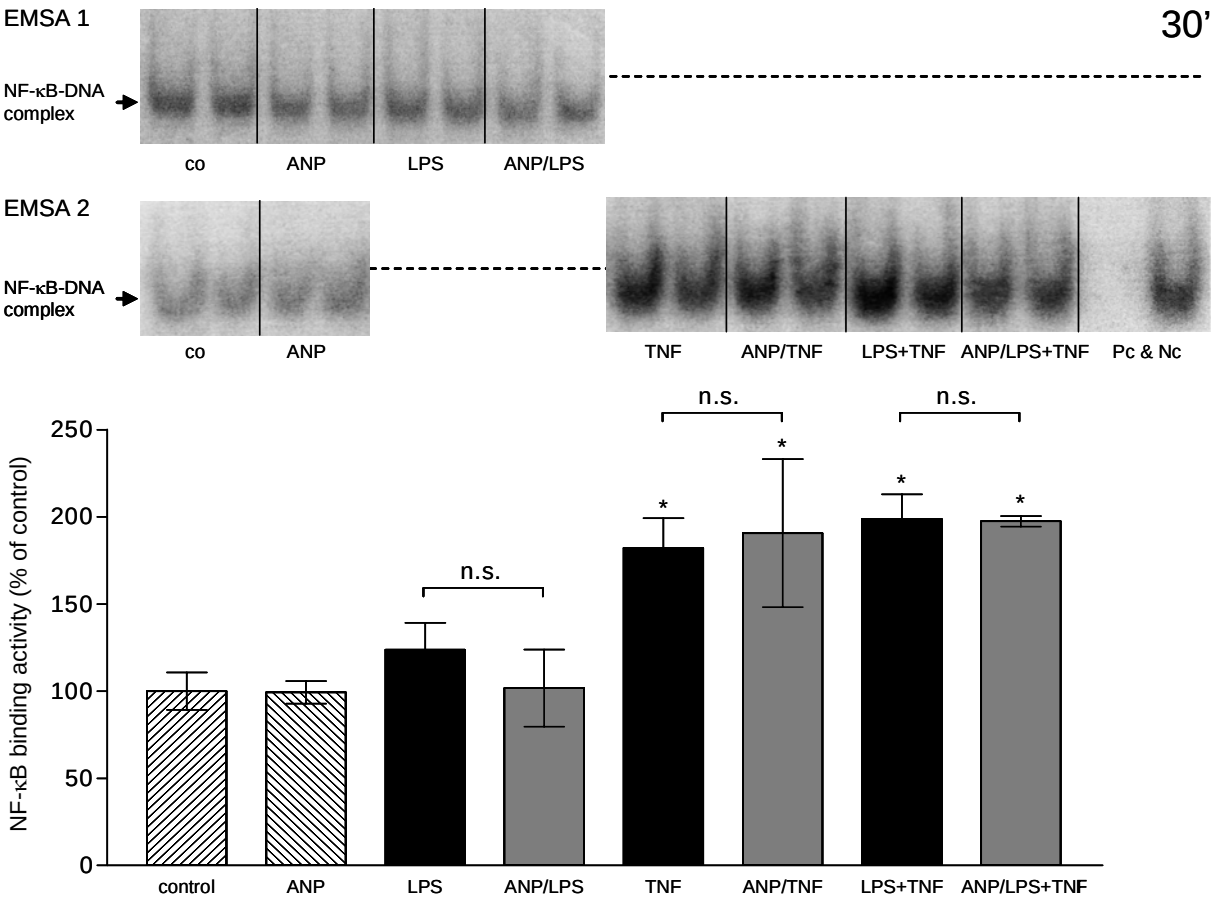


Figure 3:16 *ANP pretreatment does not show any changes on TNF-α and LPS+TNF-α induced NF-κB binding activity in 24 hours cultivated hepatocytes.* Cells were preconditioned with ANP (ANP/LPS, ANP/TNF-α and ANP/LPS+TNF-α group) 15 minutes previous to LPS and/or TNF-α stimulation (LPS, TNF-α and LPS+TNF-α group). Cells receiving NaCl (control group) or ANP (ANP group) were used to identify an impact of these substances on NF-κB binding activity. Cells were harvested 30 minutes after LPS and/or TNF-α administration. NF-κB binding activity was determined by EMSA. Illustrations show one representative experiment out of three performed experiments. Positive control (Pc) and negative control (Nc) were performed as described under 2.5.4.1.

3.4.1.2 REGULATION OF IκBα IN 24 HOURS CULTIVATED HEPATOCYTES

Following phosphorylation by its corresponding kinase, IκBα is subsequently ubiquitinylated and degraded within the proteasome. After determining NF-κB binding activity, we were interested in changes in IκBα degradation. Corresponding to NF-κB binding activity observed 30 minutes after LPS administration, we did not detected any decrease in total amounts of total IκBα protein 30 minutes after LPS stimulation. This effect does not point to an increased degradation of the NF-κB inhibitory factor in LPS stimulated hepatocytes. Conclusively, solely LPS treatment did not only display unaffected NF-κB binding activity but also unaffected amounts of total IκBα protein in the cytosol.

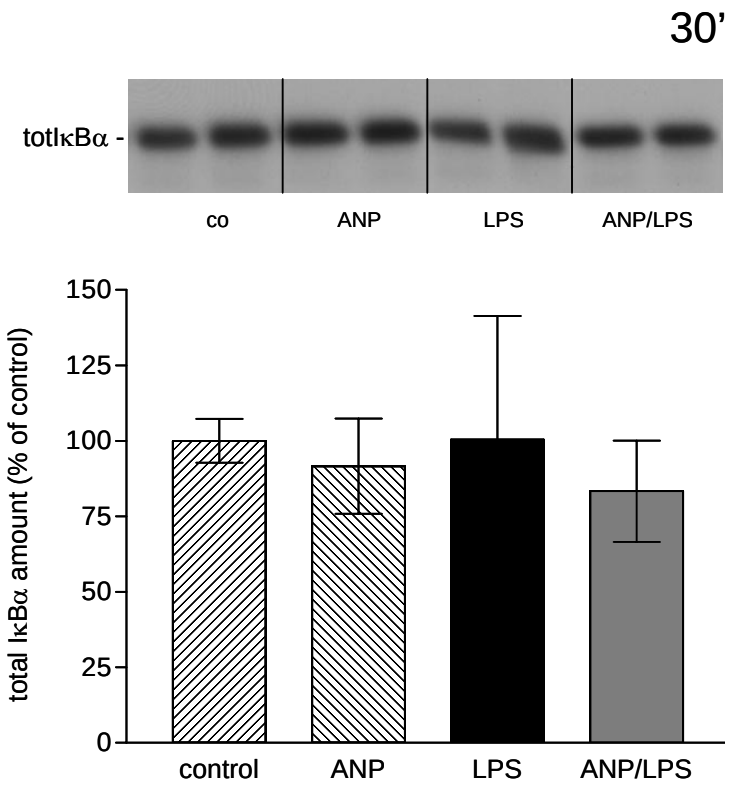


Figure 3:17 *LPS stimulation does not show any decrease in total amounts of IκBα in 24 hours cultivated primary hepatocytes.* Cells were preconditioned with ANP (ANP/LPS group) 15 minutes previous to LPS and/or TNF-α (LPS, TNF-α and LPS+TNF-α group) stimulation. Cells, only receiving NaCl (control group) or ANP (ANP group) were used to identify an impact of these substances on total amounts of IκBα. Cells were harvested 30 minutes after LPS and/or TNF-α administration. Cell extracts were analyzed by western blot. Blots are showing one representative experiment out of three performed experiments.

3.4.1.3 REGULATION OF NF-κB IN 48 HOURS CULTIVATED HEPATOCYTES

Above mentioned (3.4.1.1), NF-κB binding activity has hardly been detectable in NaCl and ANP treated hepatocytes. As displayed in Figure 3:18, 48 hours of cultivation after isolation, had no impact on NF-κB activation 30 minutes after LPS stimulation. TNF-α stimulation significantly increases transcription factor NF-κB binding activity in nuclear extracts of hepatocytes. However, as seen before, there have not been any detectable changes in NF-κB binding activity, by ANP preconditioning 30 minutes after TNF-α stimulation. Though, 48 hours cultivated hepatocytes seem to be more constant, but don't show any changes, within their recognition pattern in 30 minutes of LPS or TNF-α treatment.

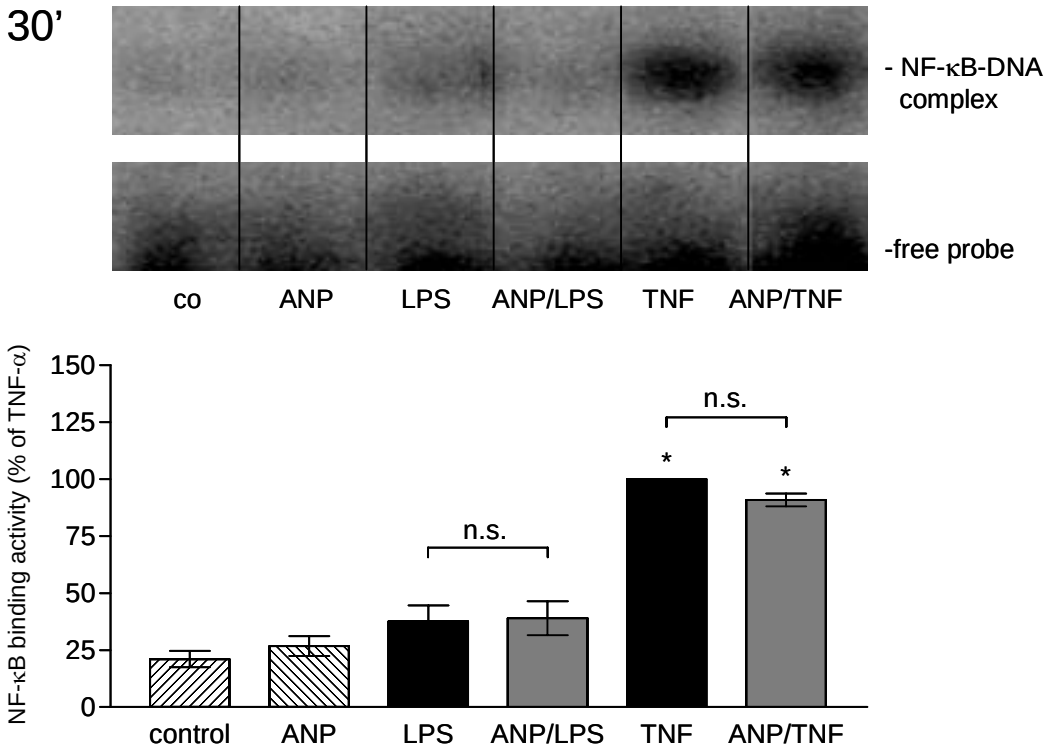
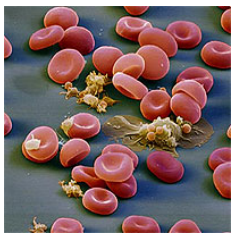


Figure 3:18 *ANP pretreatment does not show any changes in TNF-α induced NF-κB binding activity in 48 hours cultivated hepatocytes.* Cells were preconditioned with ANP (ANP/LPS, ANP/TNF-α group) 15 minutes previous to LPS or TNF-α (LPS and TNF-α group) stimulation. Cells, only receiving NaCl (control group) or ANP (ANP group) were used for control experiments. Cells were harvested 30 minutes after LPS or TNF-α administration. NF-κB binding activity was determined by EMSA. Graphs are representing the evaluation of three performed experiments. Illustration shows one representative experiment. EMSA for each experiment has been performed in duplicate.

3.5 BLOOD



In our LPS-induced septic shock model we observed reduced TNF-α plasma levels in ANP preconditioned mice, peaking 90 minutes after LPS administration (3.1). For this reason, we investigated contributions of heart, spleen and liver to decreased TNF-α expression. Hereon, we analyzed signaling pathways, consequently leading to previous determined TNF-α mRNA expression in murine liver samples. Interestingly, there is still one major “organ system” connecting all just mentioned organs. Thus, we were mainly interested how ANP preconditioning influences LPS-induced TNF-α expression in murine leukocytes *ex vivo*. In order to verify effects primarily determined in murine blood samples, we further investigated TNF-α expression in human blood leukocytes, in particular monocytes and granulocytes.

3.5.1 MURINE BLOOD LEUKOCYTES

The determination of whole TNF-α protein contents including the permeabilization of samples receiving BD golgi-plug has been accomplished according to 2.3.3 descriptions. Analysis of TNF-α expression exclusively on the cell surface was performed by allowing leukocytes to release TNF-α upon stimulation. As displayed in Figure 3:19, following FACS measurement, cells have been gated and TNF-α expression has been analyzed in murine leukocytes. The results shown in Figure 3:20 demonstrate that ANP administration does not seem to have any impact neither on cell surface nor on total amounts of TNF-α expression compared to control experiments.

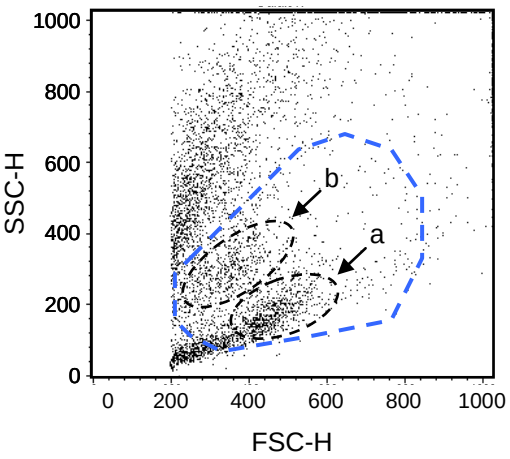


Figure 3:19 *Dot plot of murine blood leukocytes gained by FACS analysis*. Gated cell population represents murine leukocytes, respectively monocytes (a) and granulocytes (b) (neutrophils).

Interestingly, 90 minutes of LPS stimulation increases total amount of TNF-α, which are not reflected in TNF-α contents expressed on cell surface. ANP preconditioning slightly decreases total amounts of TNF-α in murine leukocytes. Additionally, preconditioning with ANP in 90 minutes LPS stimulated blood samples almost completely inhibits expression of TNF-α on leukocyte cell surface. These findings point to an involvement of ANP in LPS-induced TNF-α expression in murine blood cells.

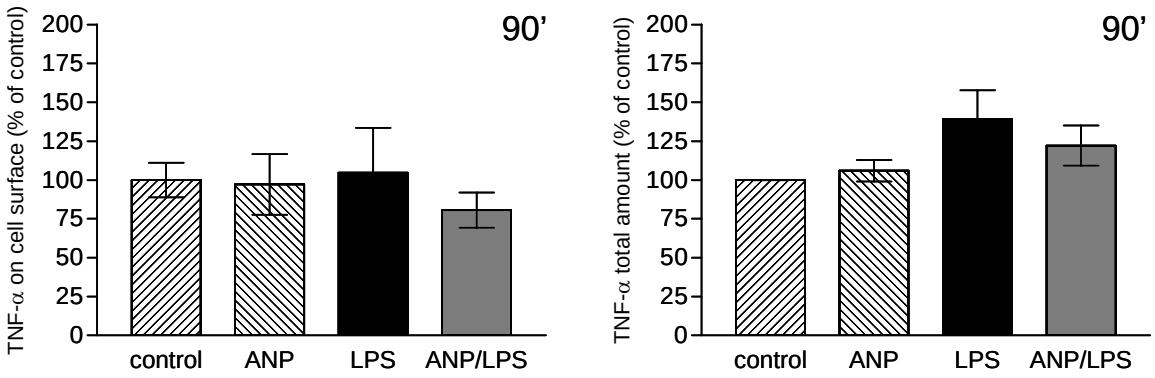


Figure 3:20 *ANP pretreatment of 90 minutes LPS stimulated whole blood samples shows slight changes on TNF-α expression in murine leukocytes.* Whole blood samples were preconditioned with ANP (ANP and ANP/LPS group) 15 minutes previous to LPS administration (LPS group). Samples, only receiving NaCl (control group) were used for control experiments. Following 90 minutes of stimulation cells were immediately fixed and TNF-α amounts were analyzed using antibody staining and FACS analysis. Graphs are representing TNF-α expression determined on cell surface (left) and total TNF-α contents (right) in murine blood leukocytes. Analyses are representing the evaluation of ten performed experiments.

3.5.2 HUMAN BLOOD LEUKOCYTES

Investigating influences of ANP in LPS treated murine blood samples displayed one major drawback, the amount gained from each animal. In order to investigate influences of ANP on leukocytes on the one hand and to determine parallels between murine and human blood samples on the other hand, we analyzed different cell populations of human blood leukocytes. Human blood samples were used for TNF-α mRNA determination and FACS analysis. These samples were taken from healthy male volunteers and subsequently stimulated with different ANP and LPS concentrations. Leukocytes were treated with 25nM and 1µM ANP, and 100ng/ml, 1µg/ml and 10µg/ml LPS, respectively. As demonstrated in, for FACS analysis, two leukocyte populations, in detail monocytes (a) and neutrophils (b) were gated and analyzed separately.

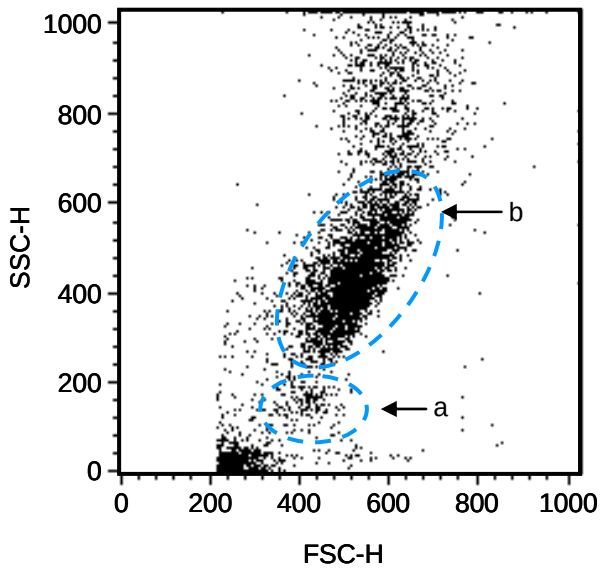


Figure 3:21 *Dot plot of human blood leukocytes gained by FACS analysis.* Different cell populations are representing monocytes (a) and neutrophils (granulocytes) (b).

3.5.2.1 INFLUENCE OF ANP ON LPS-INDUCED TNF-α mRNA EXPRESSION

Since the previous results determined in murine blood samples indicate changes in TNF-α expression on the cell surface, the question arises, which mechanism contributes to this effect? In order to investigate, if the determined changes result from divergences of transcriptional activity, TNF-α mRNA content has been evaluated using real time RT-PCR analysis. After 30 minutes of LPS administration, significant increases in TNF-α mRNA expression have been detected compared to control and ANP treated blood samples. However, analyzed mRNA contents in ANP preconditioned human blood leukocytes did not display any differences compared to non-pretreated LPS stimulated blood samples.

This indicates that previously determined changes in TNF-α cell surface expression in mouse derived blood samples are most likely not a result of transcriptional regulation, but rather underlie posttranscriptional processes.

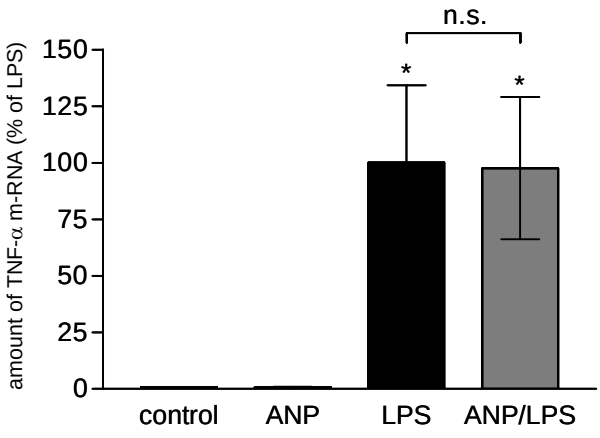


Figure 3:22 *ANP preconditioning of whole blood samples does not show any changes in LPS-induced TNF-α mRNA expression*. Whole blood samples were preconditioned with ANP 15 minutes (ANP and ANP/LPS group) previous to LPS injection (LPS and ANP/LPS group). Samples, only receiving NaCl (control group) or (ANP group) were used to identify an impact of these substances on TNF-α mRNA expression. Leukocyte mRNA was isolated 30 minutes after LPS stimulation. Samples were analyzed by real time RT-PCR. The graph represents real time RT-PCR analysis from four experiments.

3.5.2.2 CHANGES IN TNF-α EXPRESSION ON CELL SURFACE IN LEUKOCYTES

As demonstrated in Figure 3:23, LPS stimulation of whole human blood samples influences the TNF-α expression on cell surface in human blood neutrophils. Graphs (control and LPS) are demonstrating one representative example of two different stimulations, respectively NaCl (control) and LPS (100ng/ml). Thereby, following 30 minutes of LPS stimulation, TNF-α protein accumulates on the cell-surface of human blood derived neutrophils compared to NaCl treated blood samples. This effect is clearly detectable by analyzing the FL1-H histogram plot overlay of both stimulations. This overlay displays a slight sideward shift of the histogram plot representing LPS treated neutrophils.

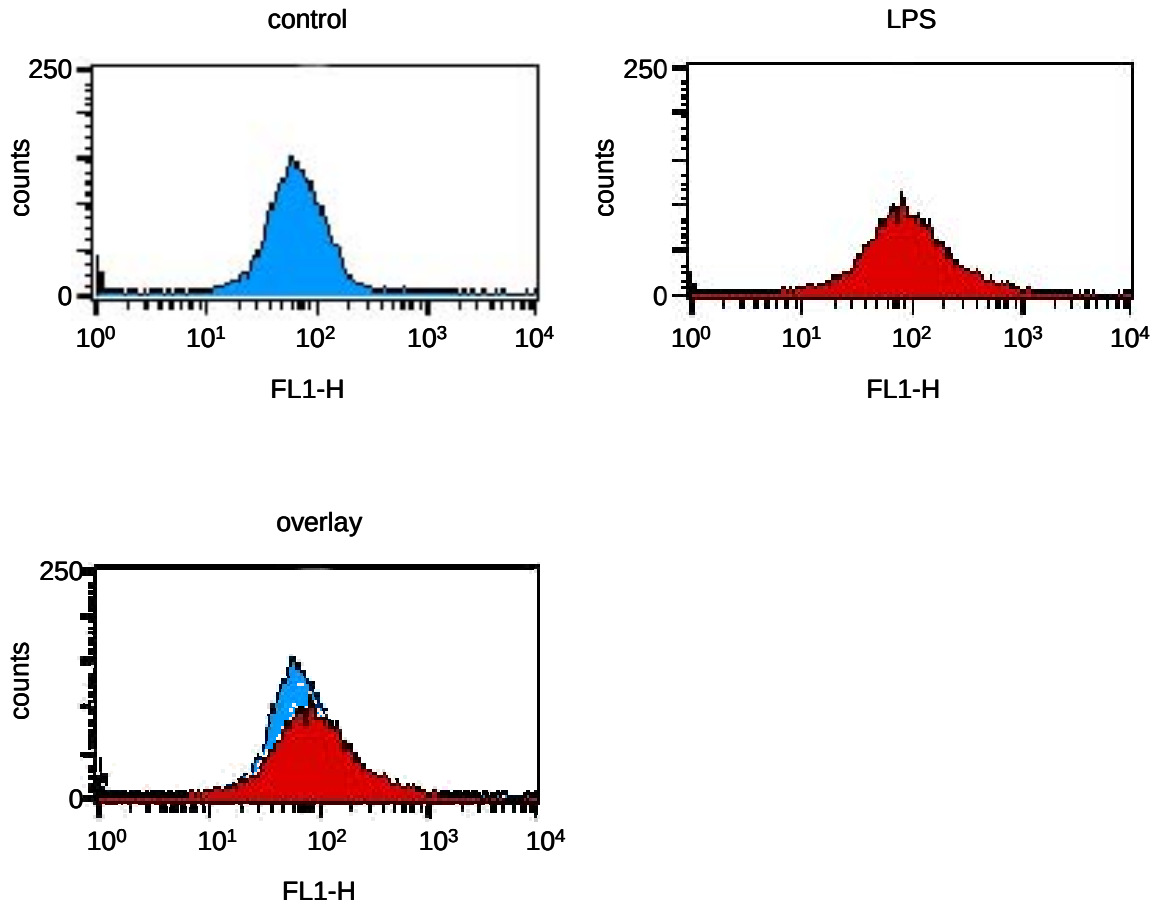


Figure 3:23 *LPS stimulation influences TNF-α expression on cell surface in human blood neutrophils.* Granulocytes of NaCl (control) or LPS treated blood samples were immediately fixed 30 minutes after LPS (100ng/ml) administration. TNF-α amounts on cell surface were determined using antibody staining and FACS analysis. Histogram plots demonstrate one representative example out of all performed experiments.

Figure 3:24 and Figure 3:25 show that LPS stimulation of whole blood samples significantly influences TNF-α appearance on leukocyte cell surface. Interestingly ascending LPS concentrations from 100ng/ml to 10µg/ml finally lead to complete removal of cell surface TNF-α protein in neutrophils as well as in monocytes. ANP preconditioning with different concentrations, diversely influences TNF-α contents *via* further reduction of detectable TNF-α amount on cell surface. This additive reduction caused by ANP pretreatment has been detectable in 100ng/ml and 1µg/ml LPS stimulated blood neutrophils and monocytes.

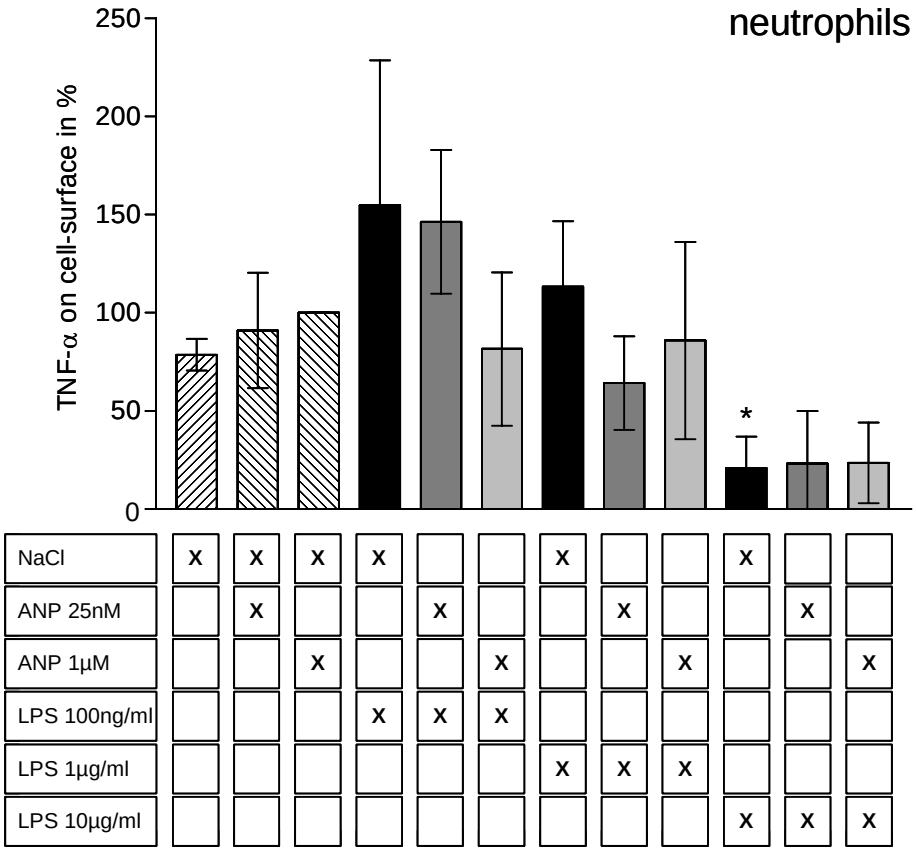


Figure 3:24 *ANP pretreatment of LPS stimulated whole human blood shows slight changes on TNF-α expression on cell surface of human blood granulocytes.* Whole human blood samples were preconditioned with ANP (ANP and ANP/LPS group) 15 minutes previous to LPS (LPS group) administration. Cells, only receiving NaCl (control group) were used for control experiments. Following 30 minutes of LPS stimulation, cells were immediately fixed and TNF-α amounts on cell surface were determined using antibody staining and FACS analysis. TNF-α cell-surface expression is calculated in percent, assuming 100% for the solely ANP (1µM) stimulated group. Graphs are representing the evaluation of four performed experiments. Each experiment has been performed in duplicate.

Surprisingly, 10µg/ml LPS treated blood samples preconditioned with ANP do not display any additional effects in TNF-α reduction on cell surface. Moreover, high dose LPS treated blood samples, irrespective of ANP preconditioning, completely remove TNF-α from the cell surface. Leukocytes treated with 100ng/ml LPS, which represents the lowest applied LPS concentration, benefit most of all from ANP preconditioning in a dose dependent manner. Following ANP preconditioning, reduced TNF-α cell surface expression is not only detectable in neutrophils but also in monocytes. Finally, we can reason that human derived blood samples mainly differ from murine blood samples regarding its more sensitive inflammatory response.

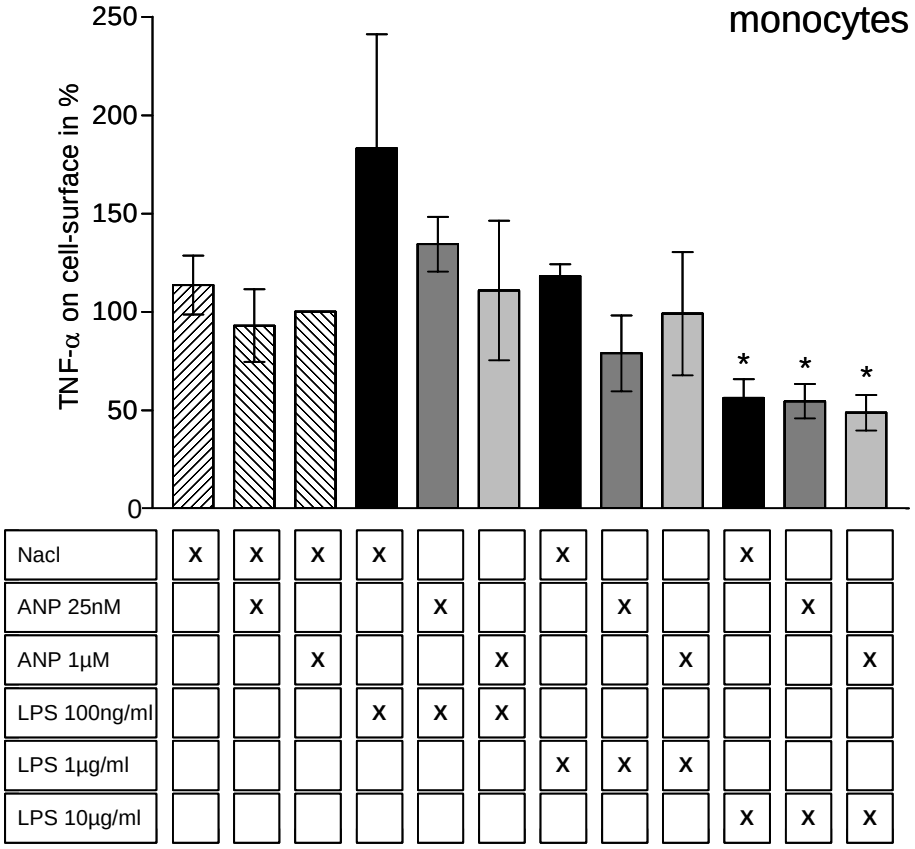


Figure 3:25 *ANP pretreatment of LPS stimulated whole human blood shows slight changes on TNF-α protein expression on cell surface of human blood monocytes.* Whole human blood samples were preconditioned with ANP (ANP and ANP/LPS group) 15 minutes previous to LPS (LPS group) administration. Cells, only receiving NaCl (control group) were used for control experiments. Cells were immediately fixed 30 minutes after LPS administration. TNF-α amounts on cell surface were determined using antibody staining and FACS analysis. TNF-α cell-surface expression is calculate in percent, assuming 100% for the solely ANP (1µM) stimulated group. Graphs are representing the evaluation of four performed experiments. Each experiment has been performed in duplicate.

4 DISCUSSION

4.1 TNF-α mRNA EXPRESSION

The kinetics of cytokine release into the circulation has frequently been studied in experimental models of infection and inflammation. In these models tumor necrosis factor-α (TNF-α) has always been the very first cytokine appearing in the blood stream ([42] Damas, 1997c; [172] Tom van der Poll, 1999). Interestingly, infusion of low doses of LPS into healthy human volunteers resulted in sepsis like symptoms and rapid release of TNF-α into the circulation, peaking 90 minutes after endotoxin administration ([126] Martich, 1993; [133] Michie, 1988). Therefore, TNF-α can be considered to be one of the most prominent cytokines released during bacterial infection and sepsis ([41] Damas, 1997b). Besides its intensively studied proinflammatory functions, TNF-α still represents one of the exceedingly discussed proteins associated with inflammation and septic-shock. On the one hand, increased TNF-α levels have frequently been associated with enhanced mortality in septic shock in humans ([36] Collighan, 2004a). The reduction of TNF-α plasma levels contributes to improved survival in a murine LPS-induced shock model ([60] Gorgen, 1992). On the other hand, with respect to efficiency of anti-TNF-α treatment, several studies revealed only little benefit on the outcome of sepsis and septic shock. Within these randomized trials, neither anti-TNF-α antibodies nor soluble TNF-α receptors have been demonstrated to significantly improve survival in septic patients ([152] Reinhart, 2001).

Besides all controversy, it has been shown that the peak concentrations of TNF-α, determined in plasma, were initially significantly different between patients suffering from severe sepsis and patients being affected by septic shock. Exclusively, patients with rapid onset of septic shock demonstrated high but transient TNF-α concentrations ([37] Collighan, 2004b; [40] Damas, 1997a). Therefore, the rapid and early intervention might be very beneficial for the outcome of sepsis. In our model of LPS-induced sepsis, we demonstrated that ANP preconditioning rescued mice from septic shock by 80%. Further on, the survival was accompanied by significantly reduced TNF-α levels determined in plasma 90 minutes after LPS injection ([91] Keller, 2005a). Hereon, in a separate group of animals, we analyzed various selected organs, heart, spleen and liver, for determination of TNF-α gene expression following ANP preconditioning in a murine septic shock model.

- Atrial cardiocytes in the heart of mammals produce the polypeptide hormone ANP ([129] McGrath, 2005). Interestingly, recent findings indicate that patients suffering from septic shock are characterized by significantly elevated ANP plasma levels ([191] Witthaut, 2003).

Notably, early studies in 1988 already recognized enhanced ANP levels in endotoxin infected sheep. Moreover, at that time it has primarily been concluded that ANP should be considered as a potential mediator during the pathogenesis of sepsis ([121] Lubbesmeyer, 1988).

- The spleen is one of the most important organs in the innate immune system for antimicrobial reactivity. Its unique structure enables it to an efficient remove of blood-borne microorganisms and their products, as LPS. Patients without spleens have an increased risk of infection ([130] Mebius, 2005). Additionally, various studies have shown that spleenectomy significantly influences TNF-α expression of different populations of macrophages, such as peripheral blood mononuclear cells and resident liver macrophages ([164] Shih-Ching, 2004).
- Besides the spleen, being the first organ, confronted with microbial products absorbed by the gastrointestinal tract, the liver is mainly involved in host-defense mechanisms. In particular, Kupffer cells are activated by bacterial stimuli, as LPS, and subsequently produce various cytokines, foremost TNF-α ([8] Ashare, 2006). Interestingly, Kupffer cells, derived from mice that survive LPS-induced septic shock have been demonstrated to significantly produce less TNF-α upon LPS stimulation compared to solely LPS-treated mice ([60] Gorgen, 1992).

Conclusively, these three organs most likely seem to be involved in ANP mediated protective effects during LPS-induced sepsis. Examination of TNF-α mRNA expression exhibits different effects in heart, liver and spleen. Following ANP preconditioning spleen and liver showed significantly decreased LPS-induced TNF-α mRNA expression. This result does not appear in the heart, where gene expression has not been affected by ANP pretreatment. This shown effect indicates that protective mechanisms, leading to TNF-α reduction seem to be linked to immunoreactive organs, comprising a great value of resident tissue macrophages. According to our investigations, we can ascertain that following ANP preconditioning, spleen and liver might contribute to decreased LPS-induced TNF-α protein levels determined in plasma. As mentioned above, plasma levels of ANP and its precursor protein pro-ANP have been demonstrated to be highly increased in septic patients. Therefore, endogenous ANP is supposed to be an important regulatory mediator in inflammation and sepsis ([191] Witthaut, 2003). Interestingly, we can specify that the heart, which represents the main compartment liberating ANP into the circulation, significantly contributes to TNF-α mRNA expression in LPS-induced sepsis. However, this organ does not display any changes in TNF-α mRNA expression in ANP preconditioned organs in LPS-induced septic shock.

4.2 THE LIVER

Approximately 50% of septic patients develop evidence of organ injury, including liver dysfunction, which is an important determinant of survival ([35] Cohen, 2002). As previously described, the liver clears the blood of bacterial endotoxins and subsequently produces cytokines in response to these stimuli during the course of infection (see chapter 1.4.1). In addition, it has been shown, that impaired clearance of injected particulates from the blood stream by the liver is associated with subsequent increased severity of infection ([8] Ashare, 2006; [154] Rimola, 1984). Consequently, the liver seems to play a major role in mediating inflammatory diseases and hepatic dysfunction that may greatly influence the outcome of sepsis. In the murine model of LPS-induced sepsis, we were able to detect a great impact of ANP preconditioning on TNF-α mRNA expression in whole liver homogenates. Therefore, we decided to further investigate signaling mechanisms, leading to or arising from reduced TNF-α mRNA expression in murine liver tissue.

4.2.1 HISTOLOGICAL CHANGES IN LIVER TISSUE

For the purpose of identification of early histological changes, we focused on apoptosis as well as on leukocyte infiltration in liver tissue derived from endotoxemic mice.

Different studies indicted a major impact of immigrated leukocytes in nonspecific host defenses to microbial infections within the liver. They postulate that the majority of pathogens is subsequently killed by immigrating leukocytes mobilized rapidly to the liver in response to infection. Additionally, immunohistochemical analysis of liver sections revealed the massive immigration of leukocytes two hours after infection ([62] Gregory, 1996; [61] Gregory, 2002a; [63] Gregory, 2002b). Interestingly, following 90 minutes of LPS treatment, we did not detect any immunohistochemical changes, respectively leukocyte infiltration as far as to this moment. Conclusively, we firstly presume that ANP mediated protective effects determined in the liver do not arise from infiltrated leukocytes but rather originate from liver cells. Secondary, there can not be ascertained any pathologic apoptotic cell death of hepatocytes focusing on 90 minutes after LPS administration. This time point is supposedly too early to detect any apoptotic cell death. Indeed, in regard to apoptosis, our investigations are in great consistence with recent findings, which propose that hepatocyte apoptosis only seems to be a minor phenomenon in endotoxic shock in mice. Furthermore, liver failure due to high doses of endotoxins was mainly characterized by single cell necrosis at later time points ([22] Bohlinger, 1996; [186] Wang, 1995).

4.2.2 THE NF-κB AND IκBα SIGNALING PATHWAY IN THE WHOLE LIVER

Many of the LPS caused effects are mediated through the activation of the transcription factor NF-κB. To date, over 200 genes are known to be regulated by NF-κB ([2] Aggarwal, 2003). Amongst these genes, NF-κB is mainly involved in modulating the expression of TNF-α ([68] Hanada, 2002). It is known, that LPS administration subsequently leads to NF-κB activation resulting in the production of TNF-α by resident liver macrophages, also referred to as Kupffer cells ([45] Decker, 1998). Conclusively, the inhibition of NF-κB transcriptional-activity in the liver was regarded as a potential therapeutic target to prevent NF-κB mediated liver injury. Unfortunately, the NF-κB signaling pathway appears to be central to innate immunity and mediates not only cellular responses to LPS but also to TNF-α. Production of TNF-α is rapidly induced by LPS, and consequently contributes to NF-κB activation by binding to its TNFR-1 in the liver. Therefore, TNF-α acts to amplify inflammatory signaling ([102] Koay, 2002). Additionally, the possibility that NF-κB activation in hepatocytes is protective following liver injury points to even more complexity of events following global activation of NF-κB in all hepatic cell types.

Besides all complexity, recent studies indicate that NF-κB binding activity is significantly diminished in liver, following LPS administration in TNFR-1 knock out mice ([102] Koay, 2002; [142] Nowak, 2000). Interestingly, cell-associated TNF-α was shown to contribute small effects to hepatic injury. Whereas, secreted TNF-α, also denoted as soluble TNF-α (solTNF-α) was proven to mainly mediate mechanisms leading to hepatic dysfunction ([142] Nowak, 2000). Moreover, various studies demonstrate that blocking the TNF-α processing with matrix metalloproteinase inhibitors protects against endotoxin induced liver injury and lethality in mice ([56] Gearing, 1995; [137] Mohler, 1994). Together, all these findings suggest that endotoxin induced NF-κB activation at least partially requires soluble TNF-α and its corresponding receptor TNFR-1 in liver tissue.

We analyzed the endotoxin-induced binding activity and translocation of the transcription factor NF-κB. In the present study our investigations of NF-κB binding activity in murine liver homogenates clearly prove a significant increase in NF-κB-DNA binding as soon as 15 minutes after LPS administration. Interestingly, ANP preconditioning compensates this inflammatory effect by causing a reduction of LPS provoked NF-κB-DNA binding. Additionally, determination of NF-κB activation in regard to cell-type dependency exhibits most interesting effects on p65 translocation. Dimerization of the p50 and p65 NF-κB subunits is essential for transcription factor activation.

Interestingly, the p50 subunit lacks this transactivating domain, which enables NF-κB to activate gene transcription ([20] Blackwell, 1997). However, the p65 subunit seems to be crucial for NF-κB transcriptional activity ([179] Viatour, 2005). Following activation, NF-κB translocates from the cytosol into the nucleus. Hence, to determine cell type specific NF-κB activation, we focused on the NF-κB subunit p65 in antibody stained liver slices. Surprisingly, p65 translocation not only occurred as primarily suggested in Kupffer cells but has also been greatly detectable in hepatocytes. Furthermore, in both cell types, ANP preconditioning diminished LPS-induced p65 translocation into the nucleus. These findings reveal the close interaction of Kupffer cells and hepatocytes in LPS-induced hepatic inflammatory disorders.

Regulation of NF-κB transcriptional activity is facilitated by reversible binding of IκBα to the NF-κB complex. Nevertheless, concepts about NF-κB separation, its translocation into the nucleus and mechanism finally leading to activation have recently been discussed intensively. On the one hand, various studies indicate the dependency of NF-κB activation by phosphorylation and degradation of its inhibitory protein IκBα ([86] Karin, 2000). To date, this theory seems to be the most privileged NF-κB signaling pathway. On the other hand, it has been postulated that NF-κB activation primarily depends on IκBα degradation without requirement of former phosphorylation ([72] Henkel, 1993; [86] Karin, 2000). Independent of all confusions, it has been shown that LPS-induced increase in NF-κB mediated luciferase activity was dependent on the intact IκBα protein by selective expression of an IκBα dominant negative form (IκBα-DN) in the liver. Blackwell and colleagues demonstrated that they were able to completely suppress LPS-induced luciferase expression in IκBα-DN organs ([21] Blackwell, 2000). For this reason, we were interested in signaling pathways leading to decreased LPS-induced NF-κB binding activity in ANP preconditioned livers. Therefore, we focused on changes in IκBα phosphorylation and degradation.

As seen in our experiments a strong phosphorylation of the NF-κB inhibitory protein IκBα occurs as soon as 15 minutes after LPS injection. Besides, LPS-induced increased phosphorylation, decreased levels of total IκBα protein can be determined 30 minutes after LPS administration. This clearly points to an ensuing degradation of IκBα. To agree with determined influences on NF-κB binding activity, LPS-induced phosphorylation of IκBα has been reduced in ANP pretreated liver homogenates. Furthermore, reduced phosphorylation in ANP preconditioned organs is accompanied by exceeding IκBα protein levels compared to organs derived from septic mice, leading to assumption of decreased IκBα degradation.

Altogether, we suppose that the ANP-mediated diminished NF-κB binding activity in endotoxemic livers is evoked *via* the cardinally described NF-κB and IκBα signaling pathway.

In brief, ANP inhibits the phosphorylation and degradation of IκBα resulting in the prevention of NF-κB transcriptional activity in whole liver homogenates. This effect might be a result of direct ANP interference within the LPS-induced NF-κB activating pathways (Figure 4:1). Furthermore, our findings indicate various parallels to a body of pharmacological data, describing the termination of primarily endotoxin-induced TNF-α signaling protecting against further NF-κB activation, and consecutively reducing liver inflammation and organ failure.

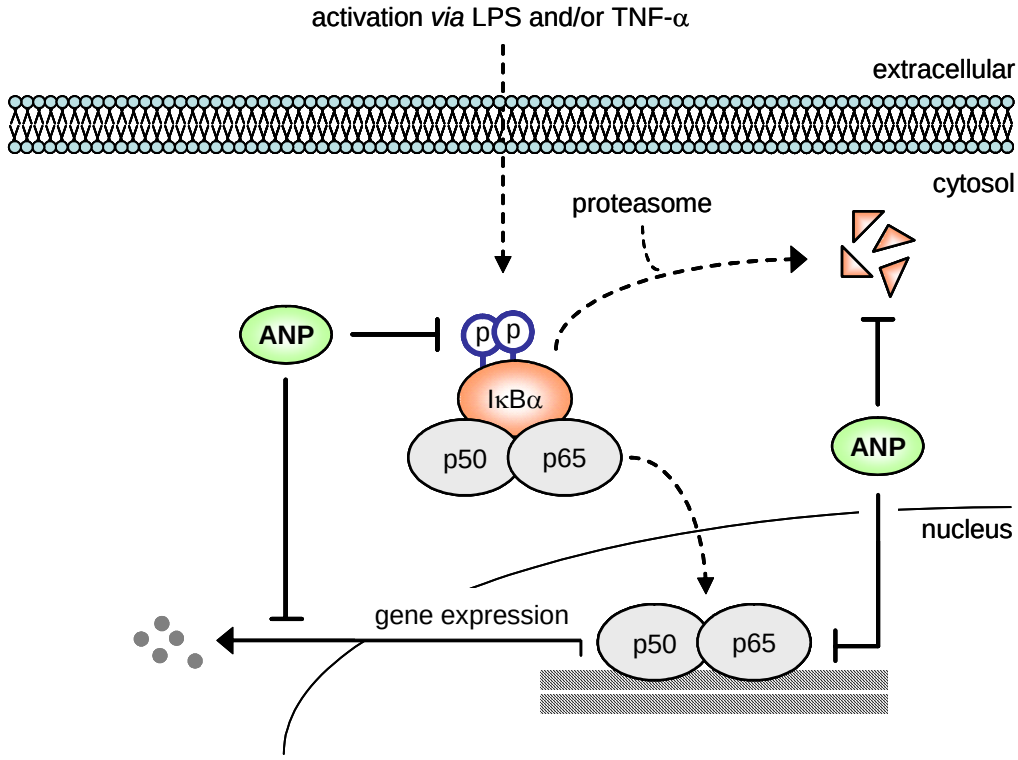


Figure 4:1 *Influences of ANP on the NF-κB & IκBα signaling pathway in the liver.*

4.2.2.1 INFLUENCES OF ANP ON LPS STIMULATED HEPATOCYTES

After investigating NF-κB subunit p65 translocation into the nucleus, we have been astonished, detecting p65 not only in Kupffer cells but also in the nucleus of hepatocytes as soon as 30 minutes after LPS injection. Superiorly, in both cell types, ANP preconditioning markedly reduced LPS-induced p65 translocation. In the intact liver, hepatocytes are always in direct contact to Kupffer cells. Therefore, the present experiments were firstly designed to assess whether direct contact between hepatocytes and Kupffer cells is of influence for LPS-induced augmented NF-κB binding activity.

Secondly, we were interested, if previously determined ANP mediated reduction of LPS-induced NF-κB binding activity, will still be detectable in isolated hepatocytes.

Besides LPS-induced Kupffer cell activation, the direct responsiveness of hepatocytes to LPS stimulation has been widely discussed. Interestingly, hepatocytes express the whole molecular necessities to respond to LPS, such as TLR-4, CD14 and MyD88 ([115] Li, 2002b; [181] Vodovotz, 2001). Based on this knowledge, hepatocytes have been demonstrated to directly respond to LPS by activation of NF-κB ([119] Liu, 2002). Conclusively, it has been suggested that hepatocytes respond to LPS in a manner at least qualitatively similar to Kupffer cells ([119] Liu, 2002). However, in contrast to Kupffer cells, where NF-κB activation is responsible for cytokine production, NF-κB activation in hepatocytes is basically supposed to result in induction of several acute phase proteins and iNOS ([109] Laskin, 1995; [138] Monshouwer, 2003; [156] Saad, 1995). However, besides the direct activation of hepatocytes by LPS, hepatocytes are also activated by proinflammatory cytokines released by LPS activated Kupffer cells. For this reason, hepatocytes exhibit several cytokine receptors, for instance TNFR-1. The activation of this receptor does not only activate NF-κB but also plays a central role in the pathogenesis of LPS-induced liver injury.

For this reason, several studies have focused on the importance of direct contact between hepatocytes and Kupffer cells during the hepatic inflammatory response. In these experiments the LPS-induced inflammatory response of hepatocytes has been compared to co-cultures, comprising hepatocytes and Kupffer cell. Interestingly, these studies revealed that only co-cultures exhibit a great production of TNF-α, whereas TNF-α release was mainly accomplished by Kupffer cells ([74] Hoebe, 2001). Today, it is commonly accepted that Kupffer cells are among the first cells that respond to LPS ([52] Freudenberg, 1982). Cellular communication between Kupffer cells and hepatocytes is thought to occur mainly by production of cytokines and inflammatory mediators ([45] Decker, 1998).

Therefore, high levels of LPS can induce increased NF-κB activation and pronounced secretion of TNF-α by Kupffer cells, ultimately leading to endotoxin induced liver injury ([69] Hartung, 1997; [167] Su, 2002). In detail, TNF-α release from LPS-treated Kupffer cells contributes to hepatocyte necrosis as well as to apoptosis during sepsis ([66] Hamada, 1999; [106] Kurose, 1996). Indeed, not only Kupffer cells are supposed to influence hepatocytes but also hepatocytes are discussed to mainly enhance LPS-induced effects on Kupffer cells by close cell-to-cell interactions ([163] Scott, 2005). Although, we did not use the model of co-cultivation, we believe that TNF-α mRNA expression determined in whole liver tissue derives mainly from activated Kupffer cells. This assumption is based on two facts. The first aspect concerns the NF-κB binding activity.

We observed, on the one hand an elevated NF-κB binding activity in LPS treated whole liver homogenates and TNF-α stimulated isolated hepatocytes. On the other hand, primary isolated hepatocytes have not been responsive to high doses of LPS, implying no effects on NF-κB activation and IκBα degradation. The second aspect regarding our investigations on isolated hepatocytes do not indicate any changes in TNF-α mRNA expression following 30 minutes of LPS stimulation (data not shown). Hence, we conclude that only close cell-to-cell interactions, between hepatocyte and Kupffer cells, modulate ANP mediated protective effects in hepatic inflammation during sepsis.

Recently published studies indicate, that in hepatocytes the blockage of TNF-α induced NF-κB activity causes apoptosis, indicating the existence of cell-dependent NF-κB regulated anti-apoptotic genes ([193] Xu, 1998). Moreover, several reports demonstrate that in inflammatory conditions NF-κB activation is essential for hepatocyte survival. This activation results in the transcription of anti-apoptotic genes, in detail cIAP2 and Bcl-2 ([34] Chu, 1997; [198] Zong, 1999). In particular, cIAP2 has been demonstrated to inhibit effector caspase-3 activity and apoptosis ([158] Schoemaker, 2002).

As a result of the protective anti-apoptotic function of NF-κB in the liver, only targeting the proinflammatory TNF-α induced NF-κB pathway has been excluded. However, tools that allow the inhibition of both, the death as well as the NF-κB signaling pathway might still be relevant (Figure 4:2). In this context, the usage of the zinc finger protein A20 prevents TNF-α induced hepatocyte apoptosis despite its strong inhibitory effect on NF-κB activation ([15] Beyaert, 2000; [73] Heyninck, 2003). Therefore, primary reduction of TNF-α expression by macrophages accomplished by ANP pretreatment might be a protective tool in the inflammatory signaling cascade.

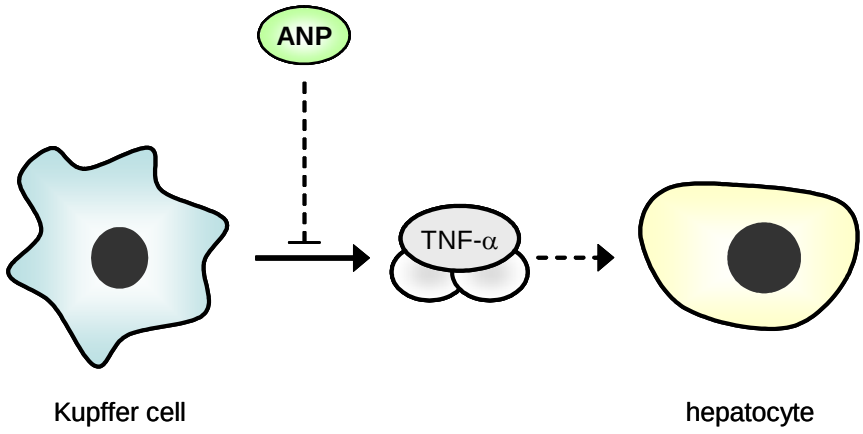


Figure 4:2 Hypothesis of the ANP mediated beneficial effects by Kupffer cell and hepatocyte interaction.

4.2.2.2 INVOLVEMENT OF PROTEINKINASES

Akt proteinkinase

Regarding endotoxemia, various studies have analyzed the role of the PI3K/Akt pathway in LPS-induced inflammation. It has been shown that LPS-induced TLR-4 signaling activates the PI3K/Akt pathway in human monocytic cells ([54] Fukao, 2003). Additionally, these studies indicated that the inhibition of the PI3K/Akt pathway enhanced LPS-induced NF-κB activity and consequently increased NF-κB dependent gene expression. Furthermore, inhibition of the PI3K pathway strongly increased LPS-induced inflammation and significantly reduced the survival time of endotoxemic mice ([157] Schabbauer, 2004; [190] Williams, 2004). Therefore, the PI3K pathway is supposed to negatively regulate the LPS-induced gene expression and plays an important role as a negative feedback regulator that limits proinflammatory responses ([65] Guha, 2002). Conclusively, the PI3K/Akt pathway is supposed to play a pivotal role in the maintenance of homeostasis and the integrity of the immune response during sepsis.

In our study, we clearly demonstrate that LPS treatment is accompanied by increased Akt phosphorylation in whole liver homogenates as soon as 15 minutes after LPS injection. Hence, we presume that our data are in consistence with the hypothesis assuming that the PI3K/Akt pathway might be a process that serves as a beneficial mechanism which compensates proinflammatory responses. Regarding these protective mechanisms, surprisingly, we analyzed reduced proteinkinase Akt phosphorylation in murine liver samples derived from ANP pretreated septic mice. This effect might be related to recent findings in reduction of LPS-induced NF-κB activation in ANP pretreated liver samples. For this reason, besides LPS signaling pathways, we might also have to focus on TNF-α mediated inflammatory responses in the liver. As previously discussed, hepatocytes are mainly resistant to apoptosis following TNF-α stimulation ([186] Wang, 1995). This effect is explained by the fact that the TNF-α-TNFR-1 pathway not only triggers downstream signals leading to apoptosis but also induces an NF-κB dependent anti-apoptotic signaling cascade ([158] Schoemaker, 2002). Additionally, several studies focused on the role of the PI3K/Akt pathway within this protective anti-apoptotic mechanism. These studies indicate that not only the inducible activation of NF-κB but also the constitutively activation of Akt signaling regulated hepatocyte survival against TNF-α stimulation ([79] Imose, 2003). Moreover, it has been cited, that Akt activation protects murine hepatocytes from TNF-α mediated apoptosis through NF-κB activation. These findings primarily link the TNF-α-induced NF-κB mediated anti-apoptotic signaling cascade to Akt activation in hepatocytes ([70] Hatano, 2001).

Whether Akt mediated NF-κB activation is caused by enhanced IκBα phosphorylation is still discussed ([122] Madrid, 2000; [145] Ozes, 1999). Indeed, most studies have not found any evidence for the involvement of Akt activation in IκBα degradation. These studies rather propose a mechanism independent of IκBα. In this pathway activation of Akt affects the transcriptional activity of NF-κB by phosphorylating the NF-κB subunit p65 ([122] Madrid, 2000).

However, besides this controversy, we can conclude that on the one hand determined Akt activation is initiated by direct responding to LPS administration. On the other hand, increased phosphorylation might also be a result of preceding TNF-α expression. Constitutively, regarding the latter, the reduction in Akt phosphorylation determined in our studies could consequentially be connected to impaired TNF-α expression in ANP pretreated endotoxemic liver samples.

p38 MAPK

As mentioned in chapter 1.3.2.4 the p38 MAPK has firstly been described in LPS stimulated murine macrophages ([67] Han, 1994). Hereon, the p38 MAPK was shown to be critically involved in LPS-induced TNF-α gene and consecutive protein expression ([31] Carter, 1999b; [111] Lee, 1994). Interestingly, in human macrophages, the inhibition of LPS-induced p38 MAPK activation decreases NF-κB activation as well as TNF-α gene expression. Consequently, p38 MAPK mediated gene expression is supposed to require NF-κB activation ([30] Carter, 1999a). Furthermore, LPS-induced p38 MAPK activation in murine macrophages is widely discussed to influence TNF-α mRNA stability. Thus, stimulation of a murine macrophage cell line with endotoxin resulted in stabilization of TNF-α mRNA, which has been reversed by specific inhibition of p38 MAPK.

As our studies clearly indicate, p38 MAPK phosphorylation is highly decreased in whole liver homogenates derived from LPS treated mice, pointing to reduced p38 MAPK activation. Most investigations exclusively focus on activated macrophages. Therefore, regarding the entire organ, our effect might be beneficial by limiting p38 MAPK activation, following TNF-α expression in inflammatory and septic disorders. Unexpectedly, ANP preconditioning does not have any influence on p38 MAPK activation compared to solely LPS treated liver samples. Conclusively, we can say that reduction of p38 MAPK activation is supposedly an important parameter in inflammatory processes, whereas ANP preconditioning does not mediate its protective effects by influencing the signaling cascades leading to changes in p38 MAPK activation.

4.3 BLOOD DERIVED LEUKOCYTES

As mentioned above, the induction of TNF-α expression in macrophages represents an important pathomechanism in various inflammatory processes. Therefore, based on our effects determined in murine liver tissue, we are especially interested in the ANP mediated regulatory mechanism of LPS-induced TNF-α production. In order to corroborate the hypothesis of the special relevance of macrophages in ANP mediated beneficial effects, we focused on blood derived leukocytes. In our laboratory, recent findings indicate the major impact of macrophages on reduction of LPS-induced TNF-α expression. ANP was shown to attenuate TNF-α production in LPS-activated murine bone marrow derived macrophages *via* a cGMP dependent mechanism at the transcriptional level ([95] Kiemer, 2000). This synthesis is mainly regulated *via* the proinflammatory transcription factor NF-κB ([182] Vollmar, 2005). Additionally, in resident liver macrophages, also denoted as Kupffer cells, ANP has also been demonstrated to specifically interact with TNF-α cytokine expression. Interestingly, regulatory mechanisms determined in Kupffer cells clearly differ from those preliminary determined in murine bone marrow macrophages. LPS-induced TNF-α mRNA expression was not significantly affected by ANP treatment. Therefore, an influence of ANP on posttranscriptional processing of LPS-induced TNF-α production has primarily been suggested. Moreover, ANP was shown to markedly increase cell associated TNF-α in LPS stimulated Kupffer cells ([94] Kiemer, 2002a). TNF-α is initially synthesized as a membrane bound, cell associated protein (*mem*TNF-α) that is further cleaved to yield the soluble form (see chapter 1.3.2.2). Above mentioned, these two forms of TNF-α have apparently different activities. In regard to LPS-mediated liver injury, secreted TNF-α, also referred to as soluble TNF-α (*sol*TNF-α), was shown to mainly mediate mechanisms finally leading to hepatic dysfunction ([142] Nowak, 2000). Therefore, a therapeutic agent which primarily inhibits the expression or the release of TNF-α from the cell surface might preserve the benefits of these cytokines, by preventing TNF-α induced damage. In former experiments, including blood derived leukocytes from human donors, it has been demonstrated that ANP significantly inhibited the production of TNF-α in LPS stimulated blood samples ([95] Kiemer, 2000). On the base of these findings, we primarily aimed to investigate changes in TNF-α protein expression in murine leukocytes. Our studies demonstrate, that total LPS-induced TNF-α protein concentrations determined in murine leukocytes seem to be slightly decreased in ANP pretreated murine leukocytes. Additionally, determination of cell membrane associated TNF-α protein contents revealed this slight reduction.

Conclusively, we can ascertain that reduced total TNF-α protein levels determined in murine leukocytes are likely to be reflected in cell surface TNF-α protein contents.

As commonly accepted, cytokines are not accumulated intracellularly and their secretion mainly depends on new protein synthesis. However, it should be noted, that neutrophils that comprise approximately 80% of all leukocytes, contain very low levels of total messenger RNA compared to mononuclear cells ([93] Kermarrec, 2005). Therefore, mRNA isolation for accurate analysis of changes in TNF-α gene expression by real time RT-PCR would have required to large murine blood volumes. Consequently, we aimed to investigate the influence of ANP on LPS-induced TNF-α mRNA expression in human blood leukocytes. As previously determined in whole liver-tissue, we supposed reduced TNF-α mRNA expression in ANP pretreated LPS stimulated white blood cells. Unexpectedly, as our studies clearly demonstrate, we have not been able to determine any changes in TNF-α mRNA expression in ANP pretreated human leukocytes compared to solely LPS stimulated cells. Interestingly, recent studies demonstrated that linking the murine and human LPS-models into one definitive comparison, such as TNF-α expression proves to be very difficult. However, although differences exist, there are several similarities in the inflammatory response to endotoxin in mice and humans. In detail, humans clearly demonstrated a physiological response which has not been evident in mice. However, the cytokine induction patterns in mice and humans were similar, although greater concentrations of endotoxin were needed to induce the same response in mice ([38] Copeland, 2005; [132] Mestas, 2004). Consequently, we analyzed the same parameter as preliminary determined in murine blood samples, in particular TNF-α cell surface expression by utilizing varying LPS and ANP concentrations. In our studies, we examined the expression of *mem*TNF-α in stimulated neutrophils and monocytes. Cell associated TNF-α has clearly been detectable on the cell surface of resting neutrophils as well as on monocytes. Interestingly, upon stimulation with low dose of endotoxin, TNF-α accumulated on the cell surface, indicating primarily elevated transport of TNF-α protein to the cell surface. In contrast, high LPS concentrations subsequently resulted in decreased *mem*TNF-α levels in both investigated cell types, pointing to immediately increased TNF-α cleavage from the cell surface. Supporting these effects, the very identical phenomenon has recently been described for LPS treated human blood monocytes by Robertshaw and colleagues ([155] Robertshaw, 2005). As mentioned above, in Kupffer cells, ANP has primarily been suggested to interfere in posttranscriptional TNF-α protein processing, resulting in enhanced cell-associated TNF-α contents ([94] Kiemer, 2002a). Regarding the latter, there are two assumed ways of interference, either inhibiting the transport to, or preventing the cleavage of TNF-α from the cell membrane.

Both effects finally cause accumulated cell associated TNF-α amounts. Interestingly, the theoretical consequence of blocking the TNF-α cleavage would be the accumulation of membrane bound TNF-α on the cell surface ([56] Gearing, 1995). As previously determined in murine blood samples, ANP preconditioning in LPS treated human blood leukocytes did not yield enhanced TNF-α cell surface expression, but rather minimized detectable *mem*TNF-α protein amounts compared to low dose LPS stimulated cells in a dose dependent manner. Interestingly, following inhibition of TNF-α processing, previous studies focusing on TNF-α in peripheral blood mononuclear cells showed an accumulation of *pro*TNF-α in the golgi-apparatus rather than on the cell surface ([128] McGeehan, 1994). These findings might be in consistence with determined cell associated TNF-α levels in murine Kupffer cells, as membrane bound TNF-α has not been itemized ([94] Kiemer, 2002a). This might also be a possible mechanism responsible for the outcome of our experiments. These complex details will certainly be very interesting to be analyzed in the future. Moreover, high dose LPS concentrations employed for experiments with murine blood samples entirely removed *mem*TNF-α from the cell surface, whereas any ANP mediated beneficial effect has completely been abolished. Finally, these findings might elucidate preliminary unexplainable determined TNF-α mRNA expression in human blood leukocytes. Therefore, we are not able to clearly exclude ANP interference in TNF-α gene expression up to this point. Based on these primary investigations of ANP interference in blood leukocytes, further investigations have to be accomplished. These studies will focus on whether ANP mediated effects on LPS-induced TNF-α expression are rather caused on the transcriptional or the posttranscriptional level.

4.4 SUMMARY

Altogether, as shown in Figure 4:3, we have proven that ANP mediated effects are diverse yet similar in different organs derived from endotoxemic mice and in isolated blood leukocytes. These ANP mediated interactions are proceeding at the onset of LPS-induced inflammation and sepsis.

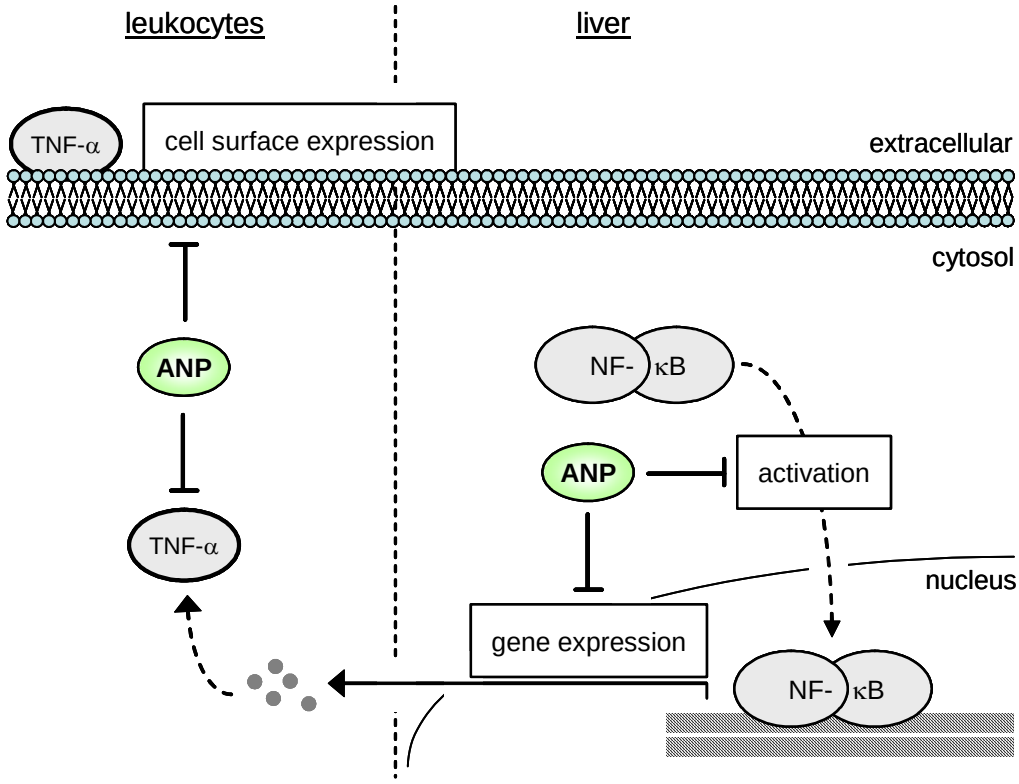


Figure 4:3 *Determined ANP mediated protective effects in early inflammatory pathways.*

Primarily, we can clearly demonstrate that ANP preconditioning in endotoxemic mice yields TNF-α m-RNA reduction, determined in the spleen as well as in the liver.

As investigated in whole liver tissue, ANP preconditioning mediates its beneficial effects by reducing LPS-induced transcription factor NF-κB activation. This reduction is caused by decreased phosphorylation of the NF-κB inhibitory factor IκBα, proximately leading to impaired degradation of IκBα protein. Thus, enhanced IκBα protein level in the cytosol prevent NF-κB translocation into the nucleus, and subsequently transcription factor activity and gene expression. These effects might be caused by or lead to the reduction in TNF-α gene expression, finally preventing liver failure.

Secondly, besides the transcriptional regulation of TNF-α gene expression determined in spleen and liver tissue, we focused on ANP mediated effects in LPS stimulated murine and human blood derived leukocytes. Following LPS stimulation, we observed reduced total TNF-α protein levels as well as decreased TNF-α amounts on the cell-surface in ANP preconditioned blood leukocytes, respectively monocytes and neutrophils. These initial investigations indicate that the reduced TNF-α protein levels in leukocytes might either be evoked by interference of ANP in transcriptional or posttranscriptional processes.

Eventually, due to its effects on key events of cell activation, such as the reduction of LPS-induced TNF-α expression, ANP may represent a promising beneficial autocrine substance in modulating early inflammatory signaling pathways.

5 APPENDIX

5.1 mRNA SEQUENCES FOR REAL TIME RT-PCR ANALYSIS

```
DEFINITION:     tumour necrosis factor-α (TNF-α) mRNA
ACCESSION:      M10988
SOURCE:         Homo sapiens (human)
ORIGIN   1 cacaccctga caagctgcca ggcaggttct cttcctctca catactgacc cacggctcca
        61 ccctctctcc cctggaaagg acaccatgag cactgaaagc atgatccggg acgtggagct
       121 ggccgaggag gcgctcccca agaagacagg ggggccccag ggctccaggc ggtgcttgtt
       181 cctcagcctc ttctccttcc tgatcgtggc aggcgccacc acgctcttct gcctgctgca
       241 ctttggagtg atcggccccc agagggaaga gtcccccagg gacctctctc taatcagccc
       301 tctggccccag gcagtcagat catcttctcg aaccccgagt gacaagcctg tagcccatgt
       361 tgtagcaaac cctcaagctg aggggcagct ccagtggctg aaccgccggg ccaatgccct
       421 cctggccaat ggcgtggagc tgagagataa ccagctggtg gtgccatcag agggcctgta
       481 cctcatctac tcccaggtcc tcttcaaggg ccaaggctgc ccctccaccc atgtgctcct
       541 cacccacacc atcagccgca tcgccgtctc ctaccagacc aaggtcaacc tcctctctgc
       601 catcaagagc ccctgccaga gggagacccc agaggggct gaggccaagc cctggtatga
       661 gcccatctat ctgggagggg tcttccagct ggagaagggt gaccgactca gcgctgagat
       721 caatcggccc gactatctcg actttgccga gtctgggcag gtctactttg ggatcattgc
       781 cctgtgagga ggacgaacat ccaaccttcc caaacgcctc ccctgcccca atccctttat
       841 taccccctcc ttcagacacc ctcaacctct tctggctcaa aaagagaatt gggggcttag
       901 ggtcggaacc caagcttaga actttaagca acaagaccac cacttcgaaa cctgggattc
       961 aggaatgtgt ggcctgcaca gtgaagtgct ggcaaccact aagaattcaa actggggcct
      1021 ccagaactca ctggggccta cagctttgat ccctgacatc tggaatctgg agaccaggga
      1081 gcctttggtt ctggccagaa tgctgcagga cttgagaaga cctcacctag aaattgacac
      1141 aagtggacct taggccttcc tctctccaga tgtttccaga cttccttgag acacggagcc
      1201 cagccctccc catggagcca gctccctcta tttatgtttg cacttgtgat tatttattat
      1261 ttatttatta tttatttatt tacagatgaa tgtatttatt tgggagaccg gggtatcctg
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      1381 acaataggct gttcccatgt agccccctgg cctctgtgcc ttcttttgat tatgtttttt
      1441 aaaatattat ctgattaagt tgtctaaaca atgctgattt ggtgaccaac tgtcactcat
      1501 tgctgaggcc tctgctcccc agggagttgt gtctgtaatc ggcctactat tcagtggcga
      1561 gaaataaagg ttgcttagga aagaa  //


DEFINITION:     glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA
ACCESSION:      NM_002046
SOURCE:         Homo sapiens (human)
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       121 ggagtcaacg gatttggtcg tattgggcgc ctggtcacca gggctgcttt taactctggt
       181 aaagtggata ttgttgccat caatgacccc ttcattgacc tcaactacat ggtttacatg
       241 ttccaatatg attccaccca tggcaaattc catggcaccg tcaaggctga gaacgggaag
       301 cttgtcatca atggaaatcc catcaccatc ttccaggagc gagatccctc caaaatcaag
       361 tggggcgatg ctggcgctga gtacgtcgtg gagtccactg gcgtcttcac caccatggag
       421 aaggctgggg ctcatttgca ggggggagcc aaaagggtca tcatctctgc cccctctgct
       481 gatgccccca tgttcgtcat gggtgtgaac catgagaagt atgacaacag cctcaagatc
       541 atcagcaatg cctcctgcac caccaactgc ttagcacccc tggccaaggt catccatgac
       601 aactttggta tcgtggaagg actcatgacc acagtccatg ccatcactgc cacccagaag
       661 actgtggatg gcccctccgg gaaactgtgg cgtgatggcc gcggggctct ccagaacatc
       721 atccctgcct ctactggcgc tgccaaggct gtgggcaagg tcatccctga gctgaacggg
       781 aagctcactg gcatggcctt ccgtgtcccc actgccaacg tgtcagtggt ggacctgacc
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       901 gagggccccc tcaagggcat cctgggctac actgagcacc aggtggtctc ctctgacttc
       961 aacagcgaca cccactcctc caccttgac gctggggctg gcattgccct caacgaccac
      1021 tttgtcaagc tcatttcctg gtatgacaac gaatttggct acagcaacag ggtggtggac
      1081 ctcatggccc acatggcctc caaggagtaa gacccctgga ccaccagccc cagcaagagc
      1141 acaagaggaa gagagagacc ctcactgctg gggagtccct gccacactca gtccccccacc
      1201 acactgaatc tcccctcctc acagttgcca tgtagacccc ttgaagaggg gaggggccta
      1261 gggagccgca ccttgtcatg taccatcaat aaagtaccct gtgctcaacc  //
```

```
DEFINITION:     tumour necrosis factor-α (TNF-α) mRNA
ACCESSION:      NM_013693
SOURCE:         Mus musculus (house mouse)
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       61 gaaatagctc ccagaaaagc aagcagccaa ccaggcaggt tctgtccctt tcactcactg
      121 gcccaaggcg ccacatctcc ctccagaaaa gacaccatga gcacagaaag catgatccgc
      181 gacgtggaac tggcagaaga ggcactcccc caaaagatgg ggggcttcca gaactccagg
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      301 tgtctactga acttcggggt gatcggtccc caaagggatg agaagttccc aaatggcctc
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      661 gtcaacctcc tctctgccgt caagagcccc tgccccaagg acacccctga gggggctgag
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     1081 ccaccatcaa ggactcaaat gggctttccg aattcactgg agcctcgaat gtccattcct
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     1201 ctcaggcctt cctaccttca gacctttcca gactcttccc tgaggtgcaa tgcacagcct
     1261 tcctcacaga gccagccccc ctctatttat atttgcactt attatttatt atttatttat
     1321 tatttattta tttgcttatg aatgtattta tttggaaggc cggggtgtcc tggaggaccc
     1381 agtgtgggaa gctgtcttca gacagacatg ttttctgtga aaacggagct gagctgtccc
     1441 cacctggcct ctctaccttg ttgcctcctc ttttgcttat gtttaaaaca aaatatttat
     1501 ctaacccaat tgtcttaata acgctgattt ggtgaccagg ctgtcgctac atcactgaac
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//


DEFINITION:     hypoxanthine guanine phosphoribosyl transferase(HPRT-1) mRNA
ACCESSION:      NM_013556
SOURCE:         Mus musculus (house mouse)
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     1141 aagctctcga tttcctatca gtaacagcat ctaagaggtt ttgctcagtg gaataaacat
     1201 gtttcagcag tgttggctgt attttcccac tttcagtaaa tcgttgtcaa cagttccttt
     1261 taaatgcaaa taaataaatt ctaaaaatt  //
```

5.2 ABREVIATIONS

Akt	Proteinkinase B
ANP	Atrial natriuretic peptide
AP1	Activator protein-1
APC	Activated protein C
APS	Ammonium persulfate
AT	Anti-thrombin
BNP	Brain natriuretic peptide
BSA	Bovine serum albumin
CAPS	Cyclohexylamino-1-propane sulfonic acid
cDNA	Complementary DNA
cGMP	Cyclic-guanosise-monophosphate
CHAPS	3-[(3-Cholamidopropyldimethylammanio]-1-propanesulfonate
CNP	C-type natriuretic peptide
COX_2	Cyclo-oxygenase 2
DNA	Desoxyribonucleic acid
DNP	*Dendroaspis* natriuretic peptide
DTT	Dithiothreitol
ECL	Enhanced Chemoluminescence
EDTA	Ethylendiamintetraacetic acid
EGTA	Ethylene-glycol-bis(2-aminoethylether)tetraacetic acid
ELISA	Enzyme linked immuno sorbent assay
EMSA	Electro mobility shift assay
Erk	Extracellular-signal-regulated kinase
FACS	Fluorescence activated cell sorter
FADD	FAS-associated death domain protein
FCS	Foetal calf serum
FL-1	Fluorescence channel 1
FSC	Forward scatter
GCase	Guanylate-cyclase
h	hour
HBSS	Hank's balanced salt solution
HEPES	N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
IκBα/β/ε	Inhibitory factor-κBα/β/ε
IKK	IκB-kinase complex
IL-1	Interleukin-1
iNOS	Inducible NO-synthase
IRAK	IL-1 receptor associated kinase
JNK	c-Jun amino-terminal kinases
KC	Kupffer cells
LBP	LPS-binding protein
LPS	Lipopolysaccharide
LRR	Leucine rich repeats
LTA	Lipoteichoic acid
MAPK	Mitogen-activated protein kinase
MAPKK	Mitogen-activated protein kinase kinase

MAPKKK	Mitogen-activated protein kinase kinase kinase
*mem*TNF-α	Membrane bound tumor necrosis factor-α
MKK3/6	Mitogen-activated protein kinase kinase 3/6
mRNA	Messenger ribonucleic acid
MyD88	Myeloid differentiation factor-88
NEMO	Nuclear factor-κB essential modulator
NF-κB	Nuclear factor-κB
NO	Nitric oxide
NP	Natriuretic peptide
NPR-A	Natriuretic peptide receptor-A / A-type natriuretic receptor
NPR-B	Natriuretic peptide receptor-B / B-type natriuretic receptor
NPR-C	Natriuretic peptide receptor-C / C-type natriuretic receptor
PAA	Polyacrylamide
PAI	Plasminogen-activator inhibitor
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGE_2	Prostaglandine-E_2
PI3K	Phosphatidylinositol 3 kinase
PMSF	Phenylmethanesulfonylfluoride
PVDF	Polyvinylidene fluoride
RAAS	Renin-angiotensin-aldosteron system
RIP	Receptor interacting protein
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean value
*sol*TNF-α	Soluble tumor necrosis factor-α
STE	Sodium chloride, Tris, EDTA buffer
TACE	TNF-α cleaving enzyme
TBE	Tris, borate, EDTA buffer
TBS-T	Tris buffered saline with Tween-20
TE	Tris-EDTA buffer
TEMED	N, N, N', N' tetramethylethylene diamine
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TIR	Toll/IL-1 receptor homology domain
TLR	Toll-like receptors
TNF-α	Tumor necrosis factor-α
TNFR-1/2	Tumor necrosis factor receptor-1/2
TNP	Taipan venom natriuretic peptide
TRADD	Tumor necrosis factor receptor-1 associated death domain
TRAF2	Tumor necrosis factor receptor associated factor 2
TRAF6	Tumor necrosis factor receptor associated factor 6

5.3 ALPHABETICAL ORDER OF COMPANIES

Agfa-Gevaert AG	Munich	Germany
Alexis Biochemicals	Grünberg	Germany
Amersham Pharmacia Biotech	Uppsala	Sweden
Aventis	Frankfurt	Germany
B. Braun Melsungen	Melsungen	Germany
Bachem	Heidelberg	Germany
BD Biosciences	Heidelberg	Germany
BD Labware	Bedford	USA
Beckmann Instruments	Munich	Germany
Bioclot	Aidenbach	Germany
Biomers	Ulm	Germany
Biometra	Göttingen	Germany
Biorad Laboratories	Munich	Germany
Biozol GmbH	Eching	Germany
Calbiochem Novabiochem	Schwalbach	Germany
Canberra-Packard GmbH	Dreieich	Germany
Cell Signaling	Frankfurt/Main	Germany
Charles River WIGA GmbH	Sulzfeld	Germany
Dako Cytomation GmbH	Glostrup	Denmark
Dianova	Hamburg	Germany
Endogen	Rockford	USA
Finnzymes/New England Biolabs	Espoo	Finnland
Fuji Photo Film GmbH	Düsseldorf	Germany
Gibco Invitrogen Corporation	Karlsruhe	Germany
GraphPad Software Inc.	San Diego	USA
Kodak	Rochester	USA
Millipore	Bedford	USA
Molecular Probes/Invitrogen/Caltag	Karlsruhe	Germany
NEN Life Science Products	Cologne	Germany
Owl separation Systems	Porthsmouth	USA
Pan Biotech GmbH	Aidenbach	Germany
PromoCell	Heidelberg	Germany
Qiagen GmbH	Hilden	Germany
Roche Diagnostics GmbH	Mannheim	Germany
Sanofi-Ceva	Düsseldorf	Germany
Santa Cruz Biotechnology	Heidelberg	Germany
Serva	Heidelberg	Germany
Sigma-Aldrich	Taufkirchen	Germany
Sniff GmbH	Soest	Germany
Ssniff	Soest	Germany
Stratagene	Heidelberg	Germany
Thermo Shandon GmbH	Frankfurt	Germany

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6.1 PUBLICATIONS

6.1.1 ORIGINAL PUBLICATION

Omalizumab: Vom IgE zum Anti-IgE
Kathrin Ladetzki-Baehs, Dr. Verena M. Dirsch, Prof. Dr. Angelika Vollmar
Pharmazie in unserer Zeit, 2004;33(2):116-22. Review

6.1.2 ORAL PUBLICATION

Influence of ANP on different pathomechanisms involved in LPS-induced sepsis
Deutsche Pharmazeutische Gesellschaft (DPhG), März 2004

6.1.3 POSTER PRESENTATION

Preconditioning with ANP modulates LPS-induced NF-κB activation *in vivo*
Elke Koch, Kathrin Ladetzki-Baehs, Melanie Keller, Alexandra K. Kiemer, Albrecht Wendel Angelika M. Vollmar
Deutsche Gesellschaft für experimentelle und klinische Pharmakologie und Toxikologie (DGPT), April 2004

Preconditioning with the cardiovascular hormone ANP rescues mice from LPS-induced sepsis
Kathrin Ladetzki-Baehs, Melanie Keller, Elke Koch, Stefan Zahler, Alexandra K. Kiemer, Albrecht Wendel, Angelika M.Vollmar
Deutsche Gesellschaft für experimentelle und klinische Pharmakologie und Toxikologie (DGPT), April 2006

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8 CURRICULUM VITAE

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Schulbildung

Sep. ’91 – Juli ’97	Abitur am Geschwister-Scholl-Gymnasium Magdeburg
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