Inflammation, SIRS and Sepsis after hepatobiliary surgery: Is lipopolysaccharide binding protein the link?

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Haoshu Fang
Aus Anhui (China)
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1. Gutachter: Frau Prof. Dr. Ruth Grümmer
2. Gutachter: Herr Prof. Dr. Matthias Gunzer
3. Gutachter:Frau Prof. Dr. Uta Dahmen

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Dedication

To my dear husband Jiaxiang Zhang,
For his patience and support during my doctoral study period.

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1 Introduction

1.1 Immune system and inflammation

1.1.1 The immune system

The immune system is a complex interwoven system, which is vital for host defense against invading pathogens. When pathogens invade, the immune system distinguishes self from non-self and triggers the immune response to eliminate the invaders. The immune system consists of an innate immune system and an adaptive immune system. The innate immune response is the first line of defense against invading pathogens and is followed by the directly acting cellular response mediated by immune effectors cells such as dendritic cells (DCs), macrophages and neutrophils (Janeway, Jr. and Medzhitov, 2002; Kumar et al., 2011; Medzhitov, 2001; Medzhitov and Janeway, Jr., 2000b). This response is followed by antigen-specific adaptive immune response and contributes further to pathogen elimination (Mogensen, 2009).

Activation of the innate immune system by pathogens is a necessary step in triggering an anti-microbial immune response to pathogens. Recent advances in understanding the mechanism of recognition of pathogens have pointed to conserved molecular pattern called Pathogen Associated Molecular Patterns (PAMPs), such as Lipopolysaccharide (LPS), bacterial DNA and viral RNA (Kumar et al., 2011; Medzhitov and Janeway, Jr., 1997; Mogensen, 2009).

LPS is a component in the outer membrane of gram-negative bacteria, and it is considered to be a prototypical PAMP (Zeytun et al., 2007). Cellular receptors for PAMPs are evolutionarily conserved and called Pattern Recognition Receptors (PRRs), such as the family of Toll-like receptors (TLR) (Medzhitov, 2001; Medzhitov and Janeway, Jr., 2000a; Medzhitov and Janeway, Jr., 1997). PAMPs are recognized by PRRs which are located on the immune cells. Recognition triggers the activation of immune cells and leads to the activation of inflammatory signaling pathways (Medzhitov, 2001; Medzhitov and Janeway, Jr., 2002). Additionally, the innate immune system controls activation of adaptive immune responses via the priming of naïve B and T lymphocytes (Palm and Medzhitov, 2009).

The adaptive immune system is composed of T and B lymphocytes. T lymphocytes recognize processed peptide fragments presented by antigen presenting cells via the expression of T cell receptors (TCR). B lymphocytes receptors (BCR) detect intact molecules. Activated lymphocytes eliminate the invading pathogens through inflammatory cytokines and specific antibodies (Chaplin, 2010).

1.1.2 LPS induced inflammatory response

1.1.2.1 LPS

LPS is an endotoxin. It is the main component of the outer membrane of gram-negative bacteria

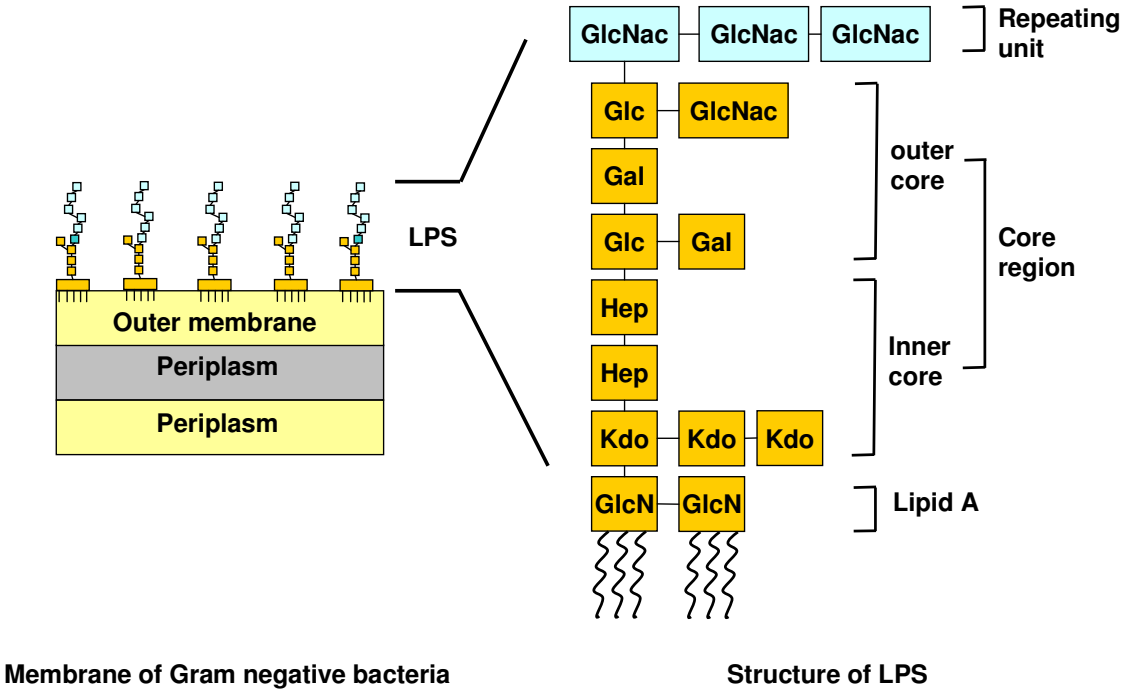

Figure 1.1 Structure of the outer membrane of gram negative bacteria and LPS

LPS is a glycolipid, and consists of an O-specific polysaccharide chain, a core region and a lipophilic phospholipid portion – lipid A.

The lipid A is a hydrophobic lipid protein localized in the outer layer of the bacterial cell membrane. The core region and hydrophilic polysaccharide chains are directed away from the bacterial cell wall. In different strains of gram negative bacteria, the hydrophilic polysaccharide chains are greatly variable (Raetz, 1990).

Biologically, LPS contributes greatly to the structural integrity of the bacteria, and protects the bacteria from chemical damage and helps to stabilize the overall membrane structure (Raetz et al., 2007). When bacteria invading an organism die, LPS is released from the membrane, enters the blood stream and binds to lipopolysaccharide binding protein (LBP), resulting in an inflammatory response.

1.1.2.2 LBP

LBP is named by its ability to bind with LPS. In 1980s, LBP was first described by Ulevitch as an acute phase protein (Tobias et al., 1986).

LBP consists of 452 amino acids and the molecular weight is 60 kDa (Schumann et al., 1990). LBP is mainly produced by hepatocytes, but synthesis of LBP is also detected in epithelial cells of other organs, such as skin, lung, and intestine, as well as in muscle cells of pulmonary arteries and heart (Dentener et al., 2000; Su et al., 1994). LBP is transcriptionally induced by IL-1, IL-6 and glucocorticoids though APRF-STAT-3 signal pathway (Schumann et al., 1996). The gene of human LBP is located in the long arm of chromosome 20 (20q), which is in the same region as the bactericidal/permeability increasing protein (BPI) (Gray et al., 1993).

LBP and BPI belong to the BPI/LBP/PLUNC (palate, lung and nasal epithelium clone) – like domain family (Bingle et al., 2004). In 1997, Beamer has reported that the three-dimensional structure of BPI (Beamer et al., 1997) is homologous with LBP and belongs to the same protein family with LBP. Recently, the existence of similar functional domaisn in other members of this family was demonstrated using computer generated models (Beamer et al., 1998; Mulero et al., 2002).

Current reports indicated that high concentration of LBP inhibited the LPS-induced inflammatory response. Lamping et al. demonstrated that the release of tumor necrosis factor-alpha (TNF-α) by cultured macrophages was decreased when culture medium was supplemented with increasing LBP concentrations (Lamping et al., 1998). This finding was conformed by Hamann and colleagues, who demonstrated that high concentration of LBP led to a decreased activation of nuclear factor-kappa B (NF-κB) after LPS administration in cultured human monocytes (Hamann et al., 2005).

In addition to initiating inflammation (Figure 1.2), LBP also contributes to LPS neutralization via transfer of LPS to high density lipoprotein (HDL) (Murch et al., 2007; Wu et al., 2004). Vesy et al utilized immunoabsorpation techniques to remove of LBP from serum, resulting in a 50% reduction in transfer of LPS to HDL (Vesy et al., 2000). Levels et al demonstrated that most LBP was associated with Apoa-I–containing particles, which may explain the preferential binding of LPS to HDL (Levels et al., 2001). Reports have shown that LBP could extract LPS from gram-negative bacterial cell membranes and transfer LBP to HDL (Wurfel et al., 1994).

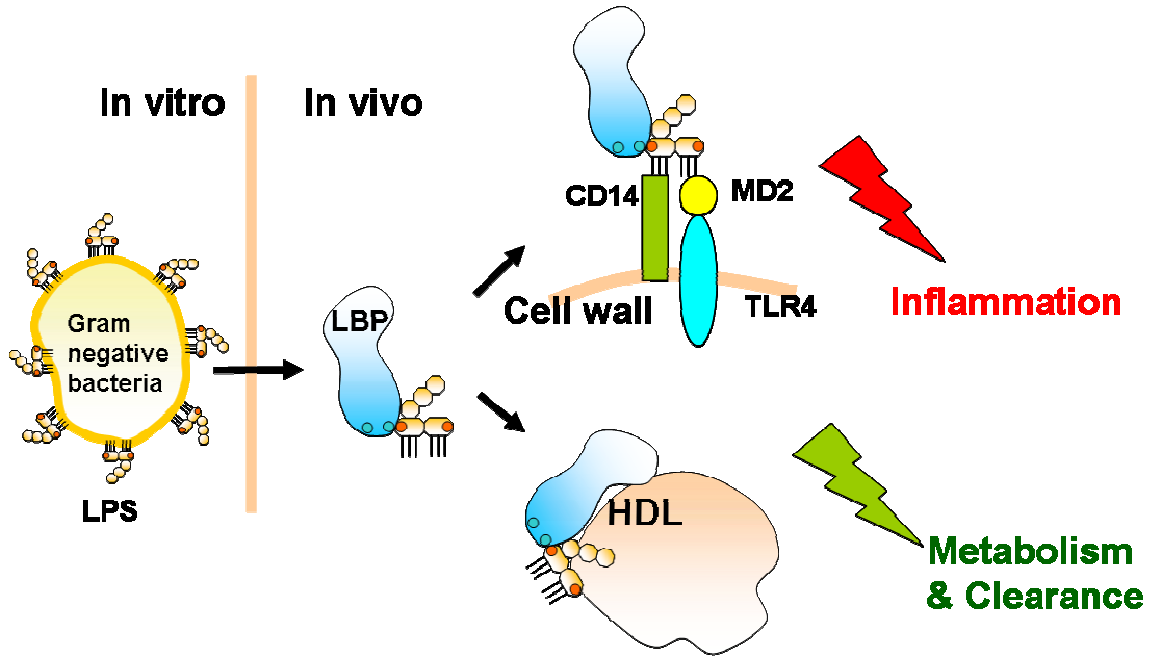

Figure 1.2 Function of LBP

LBP recognizes bacterial LPS and transfers it to TLR4, CD14, and MD2 complex, resulting in inflammatory response. LBP is also involved in detoxification of LPS by transferring LPS into HDL particles.

1.1.2.3 Interaction between LBP and LPS

LBP is a serum glycoprotein and belongs to the lipid transfer/LBP family. The LPS-induced inflammatory response results from binding of LPS to LBP. The high-affinity domain for LPS recognition is contributed to two apolar lipid-binding pockets on the concave surface of a boomerang structure (Beamer et al., 1997). In addition, the tip of the N-terminal domain of LBP contains a cluster of cationic residues, which are essential for the LPS binding and signaling (Jerala, 2007). Hydrophobic interactions between the LBP and acyl chains of lipid A appear to be important for the binding of LBP-LPS. The LPS binding ability to LBP was further identified by the generation of LBP recombinant proteins, where the binding activity was attenuated by inducing a mutation in the interactive domain (Lamping et al., 1996; Schumann et al., 1997).

1.1.3 Inflammation

1.1.3.1 LPS induced downstream signaling pathway

LPS is a strong stimulator of innate or natural immunity. In vitro, LPS can induce macrophages/monocytes to produce a number of cytokines and other inflammatory mediators. Additionally, neutrophils treated with LPS release multiple cytokines, including interleukin (IL)-

6, IL-8, and monocyte chemotactic protein-1. In vivo, within hours after administering small doses of intravenous LPS to humans, changes occur in systemic hemodynamics, ventricular function, pulmonary gas exchange and permeability (Martich et al., 1993). In animal experimental studies, animals administrated with LPS developed signs of endotoxemia, including fever, lethargy, piloerection and diarrhea. The development of this LPS-induced clinical picture can be explained by the help of currently proposed molecular pathways.

The signaling pathway begins with the recognition of LPS by LBP (Figure 1.3). The LPS/LBP complex interacts with cluster of differentiation 14 (CD14) (Frey et al., 1992; Hailman et al., 1994; Tobias et al., 1995; Wright et al., 1990). CD14 is a 55-kDa glycoprotein which is either anchored in the membrane of macrophages/monocytes and neutrophils (mCD14) or presents as soluble form (sCD14) in serum (Haziot et al., 1988). The binding between CD14 and LPS is unspecific because the products from gram positive bacteria and mycobacteria may also react with CD14 (Pugin et al., 1994). The LPS/LBP/CD14 complex is recognized by TLR4/myeloid differentiation 2 (MD2) receptor (Kawai and Akira, 2006; Kawai and Akira, 2009; Leon et al., 2008). MD2 is a 25 kDa glycoprotein that binds to the extracellular domain of TLR4 (Leon et al., 2008) and is indispensable for proper LPS signaling. It is reported that MD2 regulates the surface expression of TLR4 (Nagai et al., 2002). The co-expression of TLR4 and MD2 is necessary for the function of the LPS signaling (Brandl et al., 2005; Kim et al., 2007). This binding could trigger the formation of heterodimer between TLR4 and MD2, and thus activates the signal cascade in the cell (Nagai et al., 2002).

The TLR4 pathway employs signaling through two distinct adaptors, myeloid differentiation primary response gene 88 (MyD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF). In MyD88-dependent signaling pathway, MyD88 binds to the cytoplasmic domain of TLRs via interaction between individual TIR domains. Upon stimulation, MyD88 recruits IL-1 receptor-associated kinase (IRAK) to TLRs. IRAK phosphorylation results in binding with tumour necrosis factor receptor-associated factor 6 in short TRAF6, leading to the activation of transforming growth factor beta (TGF-β) activated kinase-1 (TAK1) and phosphorylation the IκB kinase (IKK) complex. IKK phosphorylation leads to the translocation of NF-κB into the nucleus and promotes gene expression. The MyD88-dependent signaling pathway leads to the activation of pro-inflammatory cytokines, such as TNF-α, IL-1 and IL-6 (Leon et al., 2008). Additionally, the activation of c-Jun N-terminal kinases (JNKs) induces the activation of the transcription factor activator protein 1 (AP-1).

The other signaling pathway is the MyD88-independent pathway (the TRIF pathway). In this pathway, TLR-4 subsequently activates the molecule TIR-domain-containing adapter-inducing interferon-β (TRIF) via the TLR domain. TRIF leads to activation of the transcription factor IRF-3 via TBK1 and IKKε/IKKi. This pathway triggers activation of anti-inflammatory cytokines, such as IFN-b, IL-10, and chemokine ligand (CCL) 5, to protect the host from infection (Biswas and Tergaonkar, 2007).

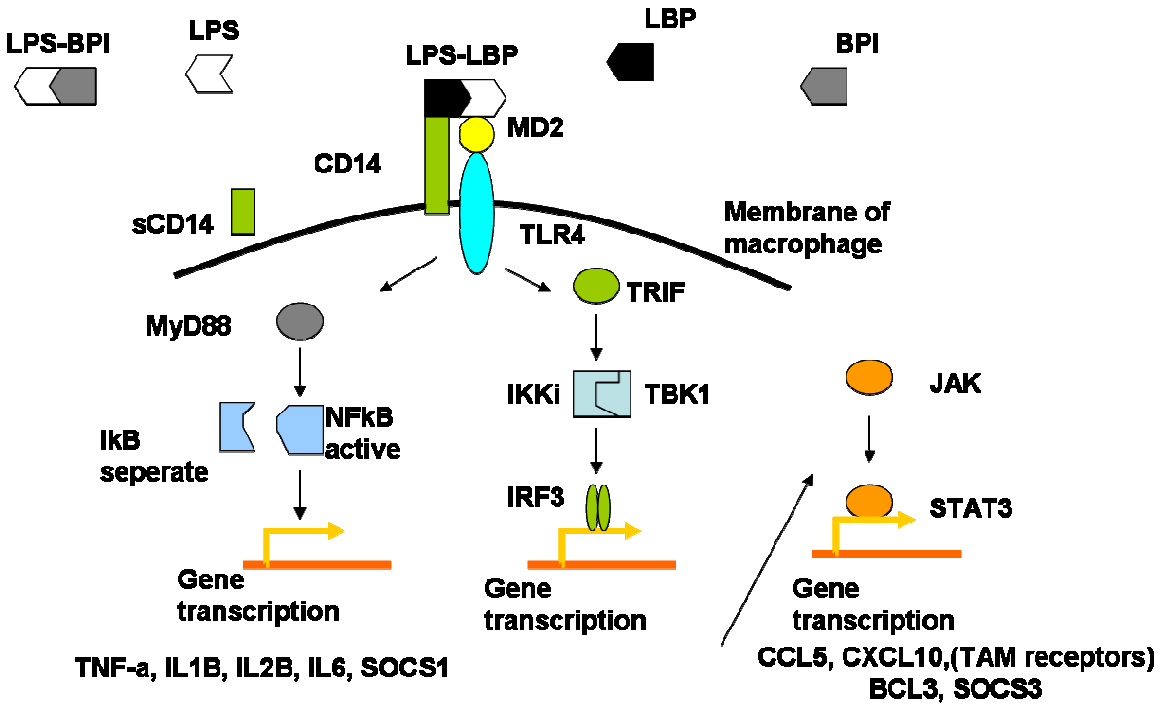

Figure 1.3 LPS induced signaling pathway

LPS binds to LBP, CD14 and TLR4 receptor, which initiates an intracellular signaling pathway leading to the production of pro-inflammatory cytokines.

1.1.3.2 Inflammation

Inflammation is a signal-mediated response to cellular insults by dangerous stimuli like e.g. pathogen or damaged cells. The immune system recognizes these dangerous signals, removes the harmful stimuli, and initiates a recovery procedure. The initiation of the inflammatory response depends on the recognition of PAMPs (Kawai and Akira, 2009; Medzhitov and Janeway, Jr., 2002; Palm and Medzhitov, 2009) and Damage Associated Molecular Patterns (DAMPs) (Matzinger, 2007; Medzhitov, 2008) by the immune system.

1.1.3.3 Pattern recognition theory

Pathogens have long been known to cause local and systemic inflammation. In the pattern recognition theory, immune activation is the result of recognition of PAMPs and DAMPs molecules by PRRs (Matzinger, 2007; Medzhitov, 2008). PRRs are located on the surface of immune cells, such as macrophages, dendritic cells, granulocytes and cytotoxic natural killer cells. TLRs act as PRRs, and play an important role in immune recognition of both pathogen invasion and sterile injury. Upon recognition, PRRs activate the immune cells and trigger a robust inflammatory response by activating signaling pathways to up-regulate gene expression of cytokines, chemokines, cell adhesion molecules and immuno-receptors (Kawai and Akira, 2009; Medzhitov and Janeway, Jr., 2002; Palm and Medzhitov, 2009).

1.1.3.4 Overt inflammation and tissue damage

Invading pathogens or tissue injury causes the stimulation of immune cells within tissues. Activated immune cells release a large amount of pro-inflammatory cytokines, such as high mobility group box -1 (HMGB1), tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1) and IL-6. These pro-inflammatory cytokines subsequently modulate the release and activation of cytokines and chemokines and other inflammatory mediators. HMGB1 has been shown to induce production of pro-inflammatory cytokines by monocytes, macrophages or neutrophils (Andersson et al., 2000; Park et al., 2003). During infection or sterile injury, HMGB1 is released by damaged cells and inflammatory cells. In turn; HMGB1 activates vascular and inflammatory cells to express pro-inflammatory cytokines (Dumitriu et al., 2005; Rovere-Querini et al., 2004).

The release of various inflammatory cytokines and mediators results in vasodilatation, increased blood vessel permeability and decreased blood flow. Increased permeability of the vessels leads to the migration of leukocytes into the tissues along the endothelium. Infiltrating leukocytes can destroy invading pathogens via phagocytosis (Coombes and Robey, 2010).

Although inflammation is important in tissue repair and elimination of harmful pathogens, uncontrolled inflammation leads to extensive tissue damage. Vasodilatation, which is caused during infection, leads to a reduced blood supply. Reduced blood supply results in inadequate oxygen perfusion in tissue, which subsequently leads to tissue destruction and organ failure. Inflammatory cytokines, including TNF-α and IL-1 act as direct cytotoxins to sinusoidal endothelial cells and hepatocytes (Shirasugi et al., 1997; Shito et al., 1997). TNF-α induces Kupffer cells to produce leukocyte chemotaxis and reactive oxygen species (ROS). IL-1 also up-regulates free radical production by neutrophils. ROS formation results in direct cellular and

tissue damage. ROS also play a key role in activation of inflammatory pathways and leads to the migration and accumulation of neutrophils in the liver, promoting the vicious cycle. Activated neutrophils result in additional, prolonged injury via the release of ROS and several proteases (Anaya-Prado et al., 2002).

1.1.3.5 Systemic inflammatory response syndrome (SIRS) and sepsis

Systemic inflammatory response syndrome (SIRS) is characterized by predominant pro-inflammatory response of the host to invading pathogens or sterile injury. Molecular components of this response are cytokines and acute phase proteins whereas cellular components are leukocytes and endothelial cells (Wiersinga, 2011). SIRS represents a serious clinical condition aggravating a systemic inflammatory response. It could result in organ dysfunction and organ failure. The concept of SIRS was defined in 1992 as part of the American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference (Bone et al., 1992). They recommend using uniform diagnostic criteria for the clinical diagnosis of a SIRS. The SIRS is defined by the presence of two or more of clinical criteria listed in Table 1.1(Bone, 1992).

Table 1.1 Criteria for the systemic inflammatory response syndrome (SIRS)

Criterion	Value
Body temperature	> 38 or < 36 °C
Heart beat	> 90 beats/min
Respiratory frequency	> 20 breaths/min
White blood cell count	> 12000 cells/mm3 or < 4000 cells/mm3

SIRS is initiated by the activation of PRRs, such as TLRs and nucleotide-binding oligomerisation domain (NOD)-like receptors (NLRs). The PRRs recognize both PAMPs of invading pathogens and danger-associated molecular patterns (DAMPs) released upon injury, and results in secreting pro-inflammatory cytokines by immune cells. Once released, many of these pro-inflammatory cytokine are able to further amplify the inflammatory response resulting in an exacerbation of SIRS (de Jong et al., 2010).

The SIRS is considered as a hallmark sign of sepsis. Sepsis is defined as SIRS which results from an infection (Kumpf and Schumann, 2010). Sepsis is a major growing health problem. It could be seen as a PRRs-mediated dysregulation of the immune system which results from a harmful or

damaging host response to invading pathogens. Despite an overwhelming increase in our knowledge on the interaction between pathogens and immune system, the mortality rate of sepsis is still high. It is reported that the mortality of sepsis ranges from 18 to 50% (Angus et al., 2001; Levy et al., 2010; Tanaka et al., 2001).

It has become well accepted that sepsis consists of two, often concomitant phases (Figure 1.4): a pro-inflammatory phase called the SIRS and an anti-inflammatory phase called the compensatory anti-inflammatory response syndrome (CARS) (Kumpf and Schumann, 2010). The sepsis begins with SIRS. During the CARS phase, restructuring of the cytokine network, rearrangement of lymphocytes population, and changes in the activation status of these cells were widely observed. Together, changes are associated with immunosuppression (Adib-Conquy and Cavaillon, 2009). CARS is characterized by the activation of several anti-inflammatory mechanisms, including an enhanced production of IL-10 (Marchant et al., 1994), soluble tumor necrosis factor receptor (sTNFR) (Girardin et al., 1992), IL-1 receptor antagonist (IL-1Ra) (Fischer et al., 1992), and TFG-β (Marie et al., 1996). As different phases of the immune response were distinguished along the development of sepsis, it may not possible to treat sepsis as one uniform disease. Therefore, treatment strategies targeting the wrong phase may fail and cause mortality.

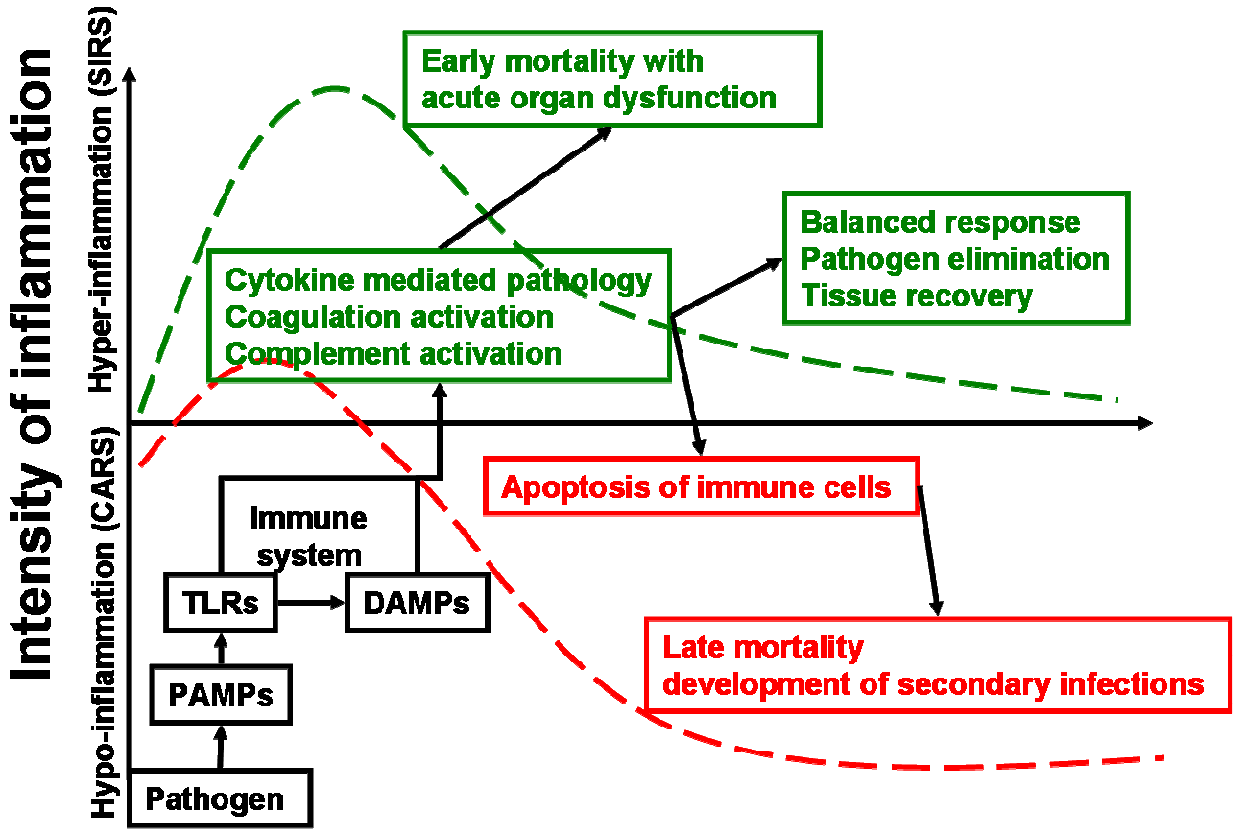

Figure 1.4 Important component of the response during sepsis

PRRs, such as TLRs recognize both PAMPs of invading pathogens and DAMPs released upon injury. The activation of the innate system can lead to a balanced response causing the elimination of invading pathogens and the recovery of tissue, but can also lead to an unbalanced response that can cause hyper-inflammation or immune suppression (Anas et al., 2010).

1.1.4 Use of LBP as a diagnostic marker for infectious diseases

Several diagnostic markers have been investigated to monitor the course of sepsis. However, to date, none of these diagnostic markers have been effectively used in the clinical setting. Therefore, a novel diagnostic marker and therapeutic target is needed for controlling and treating the development of sepsis.

Due to its unique binding abilities in pathogen recognition, LBP plays a key role during infection. Clinically, the upregulation of LBP is observed widely during several infectious diseases (Albillos et al., 2004; Gutsmann et al., 2001; Pavare et al., 2010; Sakr et al., 2008; Vollmer et al., 2009), leading to the perception of LBP as a bio-marker in diagnosis of infectious diseases (Table 6.1). Low or constitutive LBP was detected, and the normal serum concentration in humans range

from 0.5-15 µg/ml. In contrast, the serum LBP levels were strongly elevated up to 40 µg/ml in patients with severe septic shock (Zweigner et al., 2001). Additionally, clinical trials also revealed that the LBP levels in serum of patients with infectious diseases were higher than in serum of healthy humans (Blairon et al., 2003; Meynaar et al., 2011; Oude Nijhuis et al., 2003; Sakr et al., 2008). Albillps et al (Albillos et al., 2004) demonstrated that the elevated LBP levels were correlated with marked immune and hemodynamic derangement in cirrhotic patients with ascites. Jesus Villar et al reported that LBP were elevated and associated with mortality levels in acute respiratory distress syndrome (ARDS) patients (Villar et al., 2009). Su GL et al reported that LBP also contributed to the increased hepatic injury and death following acetaminophen-induced liver injury (Su et al., 2002). Based on the assessment of serum LBP levels in pediatric patients, Pavare, J. suggested that LBP could serve as a useful bio-marker during systemic infectious complications in neonates and children (Pavare et al., 2010; Pavcnik-Arnol et al., 2004). These findings indicated LBP may serve as a biological maker in the diagnostic of infectious diseases.

1.2 LBP in hepatobiliary surgery

The liver acts as an important immune organ against infection. It is an important organ for clearing endotoxin (Bradfield, 1974; Nolan, 1981). Partial hepatectomy (PH) and liver transplantation (LTx) are well established as therapy for both acute and chronic liver diseases. However, there is a high risk of post-operative infection after major hepatobiliary surgery. PH, as well as LTx can cause translocation of bacteria or bacterial compounds (e.g. LPS) into the blood stream. The results both from our group and other groups demonstrated that the production of LBP was elevated after hepatobiliary surgery (Minter et al., 2009; Shi et al., 2008). These findings indicated that LBP may represent the missing link in mediating the inflammatory response after liver surgery.

1.2.1 The liver: a vital organ involved in inflammatory response

The liver is the largest solid organ in the body. It has a dual blood supply. The Liver receives 80% of its blood supply from the intestines through the portal vein. Portal vein blood is rich in food antigens, but potentially also of environmental toxins and bacterial products. The remaining 20% of the blood supply is provided by the hepatic artery.

The liver consists of several cell populations, including hepatocytes, bile duct cells and non-parenchymal cells (NPC), which are organized in a highly structured form. Although the liver

consists of several cell populations, hepatocytes represent 70% of the cell number or 80% of the liver mass. The remaining liver mass consists of non-parenchymal cells, including kupffer cells, sinusoidal endothelial cells, stellate cells and lymphocytes.

The liver is a vital organ controlling the metabolism. The liver is mainly responsible for protein synthesis, detoxification and digestion (Michalopoulos and DeFrances, 1997), but the liver is also involved in the immune response. Growing evidence has been accumulated that the liver acts as an important immune organ, and plays a major role both in hepatic local and systemic inflammatory response (Crispe, 2009; Gao et al., 2008; Racanelli and Rehermann, 2006).

Hepatocytes are involved in the immune response via the production of secreted PRRs, complement and acute phase proteins (Gao et al., 2008). During an acute phase of inflammatory response, pro-inflammatory cytokines, such as TNF-α, IL-6 and IL-1, can induce the production of PRRs and complement by hepatocytes. Both secreted PRRs and complement are part of the innate immune response against local and systemic pathogen infection. Secreted PRRs opsonize pathogens for enhanced phagocytic clearance.

The liver non-parenchymal cells are also involved in the immune response against infection. Kupffer cells, as well as sinusoidal endothelial cells are responsible for elimination of microorganisms through a variety of receptors, cytokines and chemokines. They release both pro-inflammatory mediators (e.g. TNF-α and IL-6) and anti-inflammatory cytokines (e.g. IL-10, TGF-ß) when the liver is under infection and surgical injuries. These inflammatory cytokines then trigger the synthesis and release of acute phase proteins from the hepatocytes (Heinrich et al., 1990; Malik et al., 2002; Moshage, 1997; Schumann and Zweigner, 1999). Liver lymphocytes are enriched in NKT and NK cells that defense against infection and tumor transformation (Cerwenka and Lanier, 2001; Crispe, 2009; Gao et al., 2008; Waldhauer and Steinle, 2008).

1.2.2 Hepatobiliary surgery and postoperative infection

PH, as well as LTx are major hepatobiliary surgery procedures and are performed widely to treat focal and end-stage liver disease (Starzl et al., 1989). Infection is a major cause of morbidity after PH and LTx. The surgical injury may be one of the main causes of infectious complications. Although advances in surgical procedures and perioperative management have contributed to the reduced rate of post-operative complications, infectious complications remain import causes of morbidity and mortality in surgical intensive care unit (ICU).

1.2.3 Partial hepatectomy (PH)

PH is applied in the treatment of liver tumors and during living-related LTx. The postoperative complications after liver resection are stratified into surgical complications (e.g. bleeding, surgical dehiscence and bile leak or biloma) and medical complications (e.g. pleural effusion, renal failure and hepatic failure).

We previously demonstrated that LBP-upregulation after granulocyte colony-stimulating factor (G-CSF) pretreatment improved the lethal outcome after 90% PH using the mass ligation technique (Ji et al., 2009). Rats with and without G-CSF treatment were subjected to 90%PH. The expression of mRNA of LBP was determined by quantitative polymerase chain reaction (qPCR). Survival rate was increased in the G-CSF treatment group. Furthermore, LBP expression was associated with the attenuated of sepsis like syndrome and organ injury. So we speculated that G-CSF-induced LBP expression could also mediate the inflammation triggered by LPS.

1.2.4 Liver transplantation (LTx)

LTx is widely applied to treat both acute liver failure and chronic liver disease. During LTx, the grafts are subjected to cold ischemia (CI) and warm ischemia/reperfusion (WI/R). The cold ischemia is the period between excision and implantation. Warm ischemia occurs during explantation and implantation, when the liver is manipulated. Prolonged ischemia causes hepatic damage, leading to liver failure and mortality (Jin et al., 2011). Liu et al observed an increasing release of inflammatory cytokines and danger signals during prolonged cold storage in a rat model (Liu et al., 2010). The postoperative complications after LTx include infection, rejection, biliary complication and vascular complication (Op et al., 2011).

1.2.5 Post-operative infection

LTx, as well as liver resection has proven to be a successful treatment for patients with end-stage liver diseases respectively hepatic tumors (Starzl et al., 1989). However, both LTx and PH bring along a high risk of post-operative infection (Farid et al., 2010; Saner et al., 2008). Vera et al reported that infectious complications occur in approximately 50% of LTx recipients (Vera et al., 2011). Saner found that the incidence of pulmonary and blood stream infections after LTx were 8% and 24%, respectively (Saner et al., 2008). For patients with liver resection, the incidence of post-operative sepsis may reach close to 15% - with reported ranges from 4.6% to 11.9% (Farid et al., 2010; Simmonds et al., 2006).

The post-operative development of SIRS or sepsis after hepatobiliary surgery is related to translocation of bacteria and bacterial compounds (e.g. LPS). PH, as well as LTx causes portal hypertension, which is associated with an impaired hepatic mononuclear phagocytic system allowing spillover of gut-derived bacteria and endotoxin into the systemic circulation (Boermeester et al., 1995; Wang et al., 1994; Yeh et al., 2003). Bacterial translocation occurs in about 15-20% of patients after liver surgery (Balzan et al., 2007; Capussotti et al., 2009; O'Boyle et al., 1998; Wang et al., 1992; Yeh et al., 2003). Evidence is accumulating that bacteria translocation is associated with post-operative infections.

1.2.6 LBP and Hepatobiliary surgery

Reports have demonstrated that the elevation of serum-LBP is not only observed in sepsis and infectious diseases, but also after surgical procdures. LBP is constitutively expressed at low levels. In contrast, the post-operative levels were up to 20 µg/ml after major abdominal surgery (Hiki et al., 2000). After cardiac surgery serum LBP levels as high as 34 µg/ml were observed (Vollmer et al., 2009). Additionally, LBP mRNA and protein levels were significantly up-regulated after reperfusion in LTx in rats. This finding indicated that the LBP may play an important role in ischemia/reperfusion injury after LTx.

The loss of liver mass via liver disease or hepatobiliary surgery may reduce the functional capacity of the liver. It was reported that the protein synthesis by hepatocytes was reduced (Garcea and Maddern, 2009; Sowa et al., 2008; Wang et al., 1994). Given the liver is a major source of proteins involved in the immune response like LBP, alterations of the balanced immune response subsequent to an extended resection can be expected.

2 Hypothesis and aims of the study

2.1 The hypotheses of the present study

As outlined before, LBP is an acute phase protein, which is currently investigated as marker of sepsis. LBP binds to LPS and activates an inflammatory cascade. LBP is upregulated by LPS and in infectious diseases, but also after major surgery such as extended liver resection. Extended liver resection may be fatal due to the development of liver failure. Liver failure is associated with LPS and bacterial translocation. LPS as well as bacterial translocation represents a major risk for developing SIRS and sepsis. We previously demonstrated that LBP-upregulation after G-CSF pretreatment improved the lethal outcome after 90% PH using the mass ligation technique. Death occurred within 5 days and was attributed to liver and multi organ failure and a sepsis-like clinical picture. Based on these contradictory findings of LBP as an inflammatory mediator and the observed beneficial effect in the lethal PH model, we hypothesized that:

1. LBP mediates/is involved the inflammatory response after hepatobiliary surgery.
2. LBP upregulation prior to the inflammatory stimulus exerted by LPS or partial hepatectomy may aggravate the inflammatory response. Blocking of LBP may reduce this effect.
3. LBP upregulation prior to a septic insult may improve the outcome in poly-microbial sepsis.

2.2 The aims of the present study

2.2.1 Technical aims

To establish a novel LBP-ELISA assay based on the binding between LPS and LBP for fast and economical quantification of LBP-protein levels.

2.2.2 Scientific aims

1. To investigate the relation between LBP expression after hepatobiliary surgery and the postoperative inflammatory response
2. To investigate the role of G-CSF induced LBP upregulation in LPS-SIRS model in naïve and 70% PH rats
3. To investigate the role of G-CSF induced LBP upregulation in poly-microbial sepsis in naïve rats.

3 Material

3.1 Laboratory animals

Male inbred Lewis rats purchased from Central Animal Facility of the University Hospital Essen or Charles River (Sulzfeld, Germany), weighing within 250~350g, were employed in this study. All animals were housed under standard animal care conditions and had free to access to water and rat chow ad libitum. All procedures were carried out according to the German Animal Welfare Legislation. Animal experiments were approved by the Bezirksregierung Düsseldorf.

3.2 Anesthetics

Isofluran	Delta Select, Germany

3.3 Chemicals and reagents

Neupogen 48 (G-CSF)……..	AMGEN, Thousand Oaks, CA, US
Sulfuric acid, 2N……..	AppliChem, Darmstadt, Germany
A. dest , Cutasept F, Glucose 5%, NaCl 0.9%.....	B.Braun, Melsungen, Germany
Leucosept tubes, Tetramethylbenzidine-Set: TMB + hydrogen peroxide……...	BD, Franklin Lakes, NJ, US
Biocoll……..	Biochrom AG, Cambridge, UK
Citric acid, ethanol, HistoFix 4.5 %, Milkpowder, Sodium hydroxide, Tris……..	Carl Roth, Karlsruhe, Germany
Antibody Diluent, Hematoxylin, Protein block solution, TBS (Tris-Buffered Saline) ……..	Dako, Glostrup, Denmark
Phosphate buffered saline…….....	Gibco/Invitrogen, Carlsbad, CA, US
Fetal calf serum……	Invitrogen, Carlsbad, CA, US
Bovine serum albumin, Hydrochloric acid, Mayers Hämalaunlösung……..	Merck, Darmstadt, Germany
peqGOLD Prestained Protein-Marker V……..	PEQLAB Biotechnologie GMBH, Erlangen, Germany
Heparin ……	Ratiopharm, Ulm, Germany
Aprotinin, Leupeptin, Lumilight Western Blotting Substrate, Pepstatin, UniversalProbeLibrary…….	Roche, Basel, Switzerland

1-Butanol, Acrylamide/bisacrylamide 30%, APS, Bromphenol blue, ethanol, ethylenediaminetetraacetic acid, Glycerol, Igepal CA-630, LPS, E.coli O55:B5, Methanol, SDS, Sodium deoxycholate, Sodium orthovanadate, TEMED, Trypsin, Tween 20, β-mercaptoethanol…….……...	Sigma-Aldrich, St. Louis, MO, US
Brilliant QPCR MasterMix…..……..	Stratagene, La Jolla, CA, US
Immu Mount, Restore PLUS Western Blot Stripping Buffer, Xylene substitute……..	Thermo Scientific, Waltham, MA, US

3.4 Antibiotics

Piperacillin fresenius……..……..	Bayer AG, Leverkusen, Germany
Penicillin/Streptomycin……..…….	PAA Laboratories, Austria

3.5 Cell culture media

Rat peritoneal macrophages ……..…...	RPMI 1640 medium
	10% FCS
	2 mM Glutamine
	100 UI/ml penicillin
	100 µg/ml streptomycin

3.6 Buffers and solutions

PBS (pH 7.4) ……..…....….....….....	8 g NaCl
	0.2 g KCl
	1.44 g Na_2HPO4
	1L H_2O
	pH 7.4

Western Blot

RIPA buffer…….…..…....….….....	50 mM Tris-HCl, pH 7.4
	1% Igepal
	0.25% Na-deoxycholate
	150 mM NaCl
	1 mM EDTA
	1 mM PMSF
	Aprotinin, leupeptin, pepstatin: 1 µg/ml each
	1 mM Na_3VO_4
	1 mM NaF
4x SDS loading buffer….....….…....	0.06 M Tris
	5 % Sodiumdodecylsulfat
	100 mM Dithiothreitol
	10 % Glycerol
	0.3 % w/v Bromphenol blue
	in A. dest, pH 6.8
8x Resolving buffer….....….….....	3 M Tris
	0.8 % SDS
	in A. dest, pH 8.8
8x Stacking buffer….....….….....	1 M Tris
	0.8 % SDS
	in A. dest, pH 6.8
10x Running Buffer.....	1.92 M Glycine
	0.5 M Tris
	1% SDS
	in A. dest
10x Transfer Buffer.....	1.92 M Glycine
	0.5 M Tris
	in A. dest
Blocking Buffer.....	PBS
	5% (m/v) milk powder

Enzyme-linked immunosorbent assay (ELISA)

Blocking buffer	PBS
	5% (m/V) BSA
	0.05% (v/v) Tween 20
Washing buffer.....	PBS
	0.05% (v/v) Tween 20
Stop solution	0.5 N H_2SO_4

Silver staining

Fixing solution A.....	40% (v/v) ethanol
	10% (v/v) acetic acid
	H_2O
Fixing solution B.....	5% (v/v) ethanol
	5% (v/v) acetic acid
	H_2O
Ammoniacal silver nitrate solution I.....	2 g silver nitrate
	10 ml deionized water
Ammoniacal silver nitrate solution II.....	53 ml deionized water
	3.3 ml 30% ammonium hydroxide
	0.5 ml 10 N sodium hydroxide
Citric acid developing solution.....	0.05 g citric acid
	0.5 ml formaldehyde
	500 ml H_2O
Stop solution.....	10 ml acetic acid
	H_2O 190 ml

Immunohistochemistry (IHC)

10x TBS.....	5 M Tris
	1.37 M NaCl
	in A. dest, pH 7.6
1x TBST.....	0.5% Tween 20 in 1x TBS

Citrate-EDTA buffer, ph 6.2.....	10mM Citric Acid 2mM EDTA 0.05% Tween 20 in A. dest, pH 6,2

3.7 Commercial kits

Kit	**Company**
Agilent DNA 1000 Kit	Agilent, Santa Clara, CA, US
Agilent RNA 6000 Nano Kit	Agilent, Santa Clara, CA, US
SuperScript™ III First-Strand Synthesis System for RT-PCR	Invitrogen, Carlsbad, CA, US
BCA Protein Assay Kit	Pierce, Rockford, IL, US
CSA II Biotin free Tyramide signal amplification system	Dako, Glostrup, Denmark
DuoSet rat IL-6	R&D Systems, Minneapolis, US
DuoSet rat IL-10	R&D Systems, Minneapolis, US
DuoSet rat TNFα/TNFSF1A	R&D Systems, Minneapolis, US
ECL western blot detection kit	GE Healthcare, UK
Fast Red Kit	Dako, Glostrup, Denmark
HMGB1 ELISA kit	Shino-Test, Kanagawa, Japan
RNeasy® Mini kit (250)	Qiagen, Hilden, Germany

3.8 Standards

Protein-Marker V	peqGOLD, Erlangen, Germany
Agilent RNA 6000 Ladder	Agilent, Santa Clara, CA, US
Agilent DNA 1000 Ladder	Agilent, Santa Clara, CA, US

3.9 Antibodies

Western blot

Monoclonal goat LBP	Santa Curz, CA, US
Polyclonal rabbit HMGB1	Abcam, Cambridge, UK

Polyclonal donkey to goat IgG	Abcam, Cambridge, UK
Polyclonal rabbit to mouse IgG	Abcam, Cambridge, UK
ELISA	
Monoclonal mouse LBP	Cell science, Canton, MA
IHC	
Monoclonal mouse LPS	Abcam, Cambridge, UK
Power Vision poly AP-Anti-mouse IgG,	ImmunoLogic, AD Duiven, Netherlands
Power Vision poly AP-Anti-rabbit IgG,	ImmunoLogic, AD Duiven, Netherlands

3.10 Peptide

Peptide LBPK95A (RVQGRWKVRASFFK) used to block the interaction between LBP and LPS (Arana et al., 2003). The peptide was synthesized in-house using an Fmoc standard procedure on an ABI 433A-peptide-Synthesizer. Lyophilizated peptide was diluted in 0.9% NaCl to the concentration 1 mg/ml, aliquoted and stored at -20 °C.

3.11 Stool batch

Pooled stool from three healthy non-vegetarian donors used for inducing polymicrobial sepsis is kindly provided by PD. Dr. Ralf A. Claus (The center for sepsis control and care, University Hospital of Jena).

3.12 Membranes and films

PVDF membrane	GE Healthcare, UK
High performance chemiluminescence film	GE Healthcare, UK

3.13 Oligonucleotides

All oligonucleotides used in qPCR were synthesized by Microsynth (Balgach, Switzerland). The nucleotide sequence and probe (Roche Diagnostics GmbH, Mannheim, Germany) sequences are presented in Table 3.1.

Table 3.1 Characteristics of Primers and Probes of Selected Genes

Gene	Forward primer	Reverse primer	Probe[a]
LBP	ATCCGGCTGAACACCAAG	TGTCGGGGTACTTTCTGGTT	#82
TNF-α	TGAACTTCGGGGTGATCG	GGGCTTGTCACTCGAGTTTT	#63
IL-6	CCTGGAGTTTGTGAAGAACAACT	GGAAGTTGGGGTAGGAAGGA	#106
IL-10	AGTGGAGCAGGTGAAGAATGA	TCATGGCCTTGTAGACACCTT	#68
IL-1ß	GCTGACAGACCCCAAAAGAT	AGCTGGATGCTCTCATCTGG	#117
TLR-4	GGATGATGCCTCTCTTGCAT	TGATCCATGCATTGGTAGGTAA	#95
MD-2	TGATGATTATTCTTTTTGCAGAGC	ATCCCCAGCAATGGCTTC	#75
CD14	AAAGAAACTGAAGCCTTTCTCG	AGCAACAAGCCGAGCATAA	#26
HPRT	GACCGGTTCTGTCATGTCG	ACCTGGTTCATCATCACTAATCAC	#95

a Universal ProbeLibrary probes

3.14 Consumables

96-well plate, round bottom	eBioscience, San Diego, CA, US
Amersham Hybond™-P	ge-healthcare, Waukesha, WI, US
Cotton buds	Greiner bio-one, Kremsmünster, Austria
Cryo tubes 1.5 ml	Nunc, Langenselbold, Germany
Disposable scalpel	B.Braun, Melsungen, Germany
Eppendorf tubes (0.5 ml, 1.5ml)	Eppendorf, Hamburg, Germany
Finnpipette tips 30-300µl	ThermoScientific, Karlsruhe, Germany
Molinea E 40x60cm	Hartmann, Neuhausen, Switzerland
Mopylen, 7-0	Resorba, Nuremburg, Germany
Needles 27G, 20G	BD, Franklin Lakes, NJ, US
PCR plate 96-well	Sarstedt, Nümbrecht, Germany
PCR plate 96-well, semi-skirted	Eppendorf, Hamburg, Germany
PCR tubes 0.2ml	Greiner bio-one, Kremsmünster, Austria
Polyethylene-splint (12G, 14G, 18G, 22G)	Klinika Medical GmbH, Usingen, Germany
Prolene 6-0	Resorba, Nuremberg, Germany

Pipette tips (1-10µl, 1-100µl, 1-300µl, 100-1000µl)	Sarstedt, Nümbrecht, Germany StarLab, Ahrensburg, Germany
Rotilabo®-Blottingpapiere, Thickness 0,36 mm	Carl Roth, Karlsruhe, Germany
S-Monovette (K3E and Z, 1.2 ml)	Sarstedt, Nümbrecht, Germany
S-Monovette (9NC, 1.4ml)	Sarstedt, Nümbrecht, Germany
S-Monovette Adapter	Sarstedt, Nümbrecht, Germany
Silk 6-0 suture	Resorba, Nuremburg, Germany
Syringe 1ml	BD, Franklin Lakes, NJ, US
Syringe (2ml, 5ml, 10ml, 20ml)	B.Braun, Melsungen, Germany
Tissue-Tek Uni-Cassette Biopsy	Sakura, Torrance, CA, US
Wash basin 25ml	Carl Roth, Karlsruhe, Germany

3.15 Devices

12 channel multipipette 30-300 µl Finnpipette	ThermoScientific, Karlsruhe, Germany
Agilent Bioanalyzer 2100	Agilent, Santa Clara, CA, US
Anesthetic Vaporizer	Penlon, Abingdon, UK
Automated Chemical Analyzer	Bayer; Leverkusen, Germany
Bovie coagulator	Aaron Medical, St. Petersburg, U
CO_2 incubator	Heraeus, Hanau, Germany
Chip Priming Station	Agilent, Santa Clara, CA, US
Chip vortex	Agilent, Santa Clara, CA, US
Electric shaver	Aesculap Favorita 2, BBraun, Melsungen, Germany
ELx 808 ELISA plate reader	Bio-Tek Instruments Inc., Winooski, VT, US
Heating table	Medax GmbH, Kiel, Germany
Hamamatsu slide scanner	L11600, Hamamatsu, Japan
Heraeus Hera Cell CO2-Incubator with Disinfection Stage	Heraeus, Hanau, Germany
IKA Plate Shaker MTS 4	IKA Labortechnik, Staufen, Germany
Leica DM LB	Leica, Wetzlar, Germany
Microsurgical instruments (forceps, scissors)	Aesculap, BBraun, Melsungen,

	Germany
MikroWin 3.0 Software	Mikrotek Laborsysteme GmbH, Overath, Germany
NanoDrop® ND-1000 UV-Vis Spectrophotometer	PEQLAB Biotechnologie GMBH, Erlangen, Germany
Nikon Coolpix 4500	Nikon, Tokyo, Japan
Nikon Coolpix D80	Nikon, Tokyo, Japan
Olympus Color View III	Olympus, Tokyo, Japan
OmniTH Homogenizer	Omni International, Marietta, GA, US
Primus 25 advanced Thermocycler	PEQLAB Biotechnologie GMBH, Erlangen, Germany
Qik Spin Microcentrifuge	United Bioscience, Carindale, Australia
X-ray cassette	Oehmen, Essen, Germany
Rotina EBA 20	Hettich, Tuttlingen, Germany
Scale	Kern 440-33, Bexhill-on-Sea, UK
Sigma Delta Isoflurane Anesthetic Vaporizer	Penlon, Abingdon, UK
Single pipette (2,5µl, 10µl, 20µl, 100µl, 1000µl)	Eppendorf, Hamburg, Germany
single Finnpipette (200 µl, 1000µl)	ThermoScientific, Karlsruhe, Germany
Single 1000µl pipette	Gilson, Middleton, WI, US
Vet ABC	Scil, Gurnee, IL, US
Vortex L46	GLW, Würzburg, Germany
Water bath 1013	GFL, Burgwedel, Germany
Water bath U3/6A	Julabo, Seelbach, Germany
Webomatic E15 basic	WEBOMATIC®, Bochum, Germany
X/16 PowerLab	ADInstruments Inc., Bella Vista NSW, Australia

4 Methods

4.1 Experimental design

From the technical side, we want to establish a LBP ELISA assay based on the binding between LPS and LBP.

From the scientific side, we want to reach the three aims by designing the following experiments.

1. Modulation of LBP levels in hepatobiliary surgery using different models
    1) Different extent of PH model
    2) LTx
        i. Ex-vivo explanted liver cold storage model
        ii. In-vivo warm-ischemia reperfusion model
        iii. In-vivo LTx model
2. G-CSF induced LBP expression in LPS induced inflammation after PH
3. G-CSF induced LBP expression in polymicrobial sepsis

4.1.1 LBP quantification

To establish a LBP ELISA, LPS was used as capture molecule and an anti-LBP antibody as detection antibody (
Figure 4.1). Serum LBP protein was purified from rats after LPS administration to be used as standard in the newly established assay. Twenty-five samples obtained from rats at different time points after injection with LPS or obtained from rats 24h after 30%, 70% or 90% partial hepatectomy were used for western blot and ELISA, to analyze the correlation between both methods.

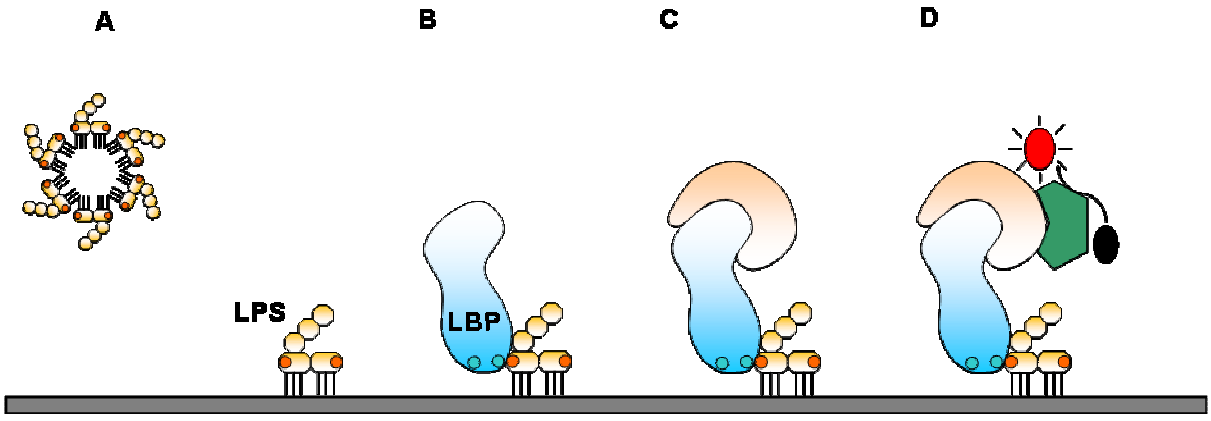

Figure 4.1 Schematic diagram of the LPS-LBP ELISA

LPS aggregates binding to flat-bottom polystyrene microplates using hydrophobic regions (A), LBP-binding to immobilized LPS using barrel domains (B), Primary mouse anti-LBP-antibody binding to LBP-LPS (C), Secondary anti-mouse antibody binding to complex of anti-LBP-LBP-LPS and color reaction (D).

4.1.2 LBP levels in hepatobiliary surgery

4.1.2.1 LBP levels in different extent PH

LBP is mainly synthesized by the liver. We hypothesized that loss of liver mass influenced the LBP levels and caused dysregulation of inflammatory response. To investigate whether the LBP expression and release in livers subjected to different extent of PH. The rats were subjected to 30% PH, 70% PH and 90% PH and sacrificed 24h postoperatively (n=6/group). After 70% PH, additional 12 rats were sacrificed at 1h, 6h, respectively (n=6/group). Serum LBP levels and hepatic LBP mRNA were assessed using the newly established ELISA method and qPCR, respectively. Hepatic injury was identified by histological evaluation and liver enzyme determination. The hepatic expression of inflammatory cytokines was quantified by qPCR, and the correlation between LBP levels with remnant liver mass, liver enzyme and inflammatory response was analyzed.

4.1.2.2 LBP levels in I/R injury and LTx

In this study, three animal models were used to cover different situations - CI, WI/R, and LTx - modeling the different pathophysiological aspects of the surgical procedure during LTx.

As a CI model, livers were explanted and stored in saline at 4 °C. Effluents were collected every hour during cold ischemia and liver tissue was obtained at 0h, 4h, 8h, and 12h. As shown in

Figure 4.2, selective warm I/R injury was induced by clamping the vascular blood supply to the median and left lateral lobe of the liver for 90min followed by 0.5h, 6h and 24h reperfusion (n=6/group). Six rats were subjected to LTx after cold-preservation of the graft for 6h and sacrificed 24h postoperatively. Another set of 6 rats was included as normal control group. Rats subjected to 2 mg/kg LPS injection and an observation time of 1h, 6h, 24h were used as control for LBP elevation (n=6/group).Serum and effluent protein levels as well as hepatic-mRNA and protein levels of LBP were examined after warm I/R, CI and LTx. LPS translocation and hepatic mRNA expression of inflammatory cytokines was observed after LTx.

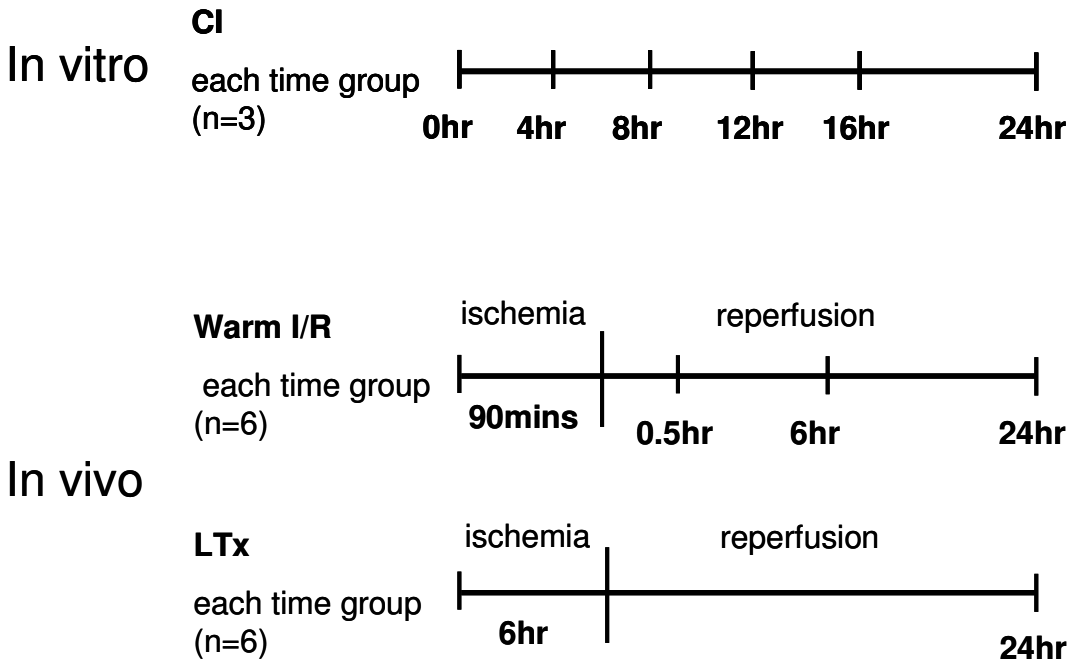

Figure 4.2 Experimental design to investigate LBP expression after LTx

In in-vitro experiment, the effluent was used to co-stimulate rat peritoneal macrophages with LPS (Figure 4.3). Effluent was collected at defined time-points of cold liver storage. The LBP in effluent was measured by western blot. The time point with highest LBP concentration was chosen for macrophage stimulation. The macrophages were co-cultured with effluent (50 µl) and 0.33 ng/ml LPS. The same volume 0.9% NaCl and 0.33 ng/ml LPS was added in control group. In the LBP blockade experiments, macrophages were stimulated with effluent and LPS in combination with LBP inhibitory peptide (80 µg/ml). The culture suspensions were taken after 4 h stimulation and the TNF- α levels was detected by ELISA.

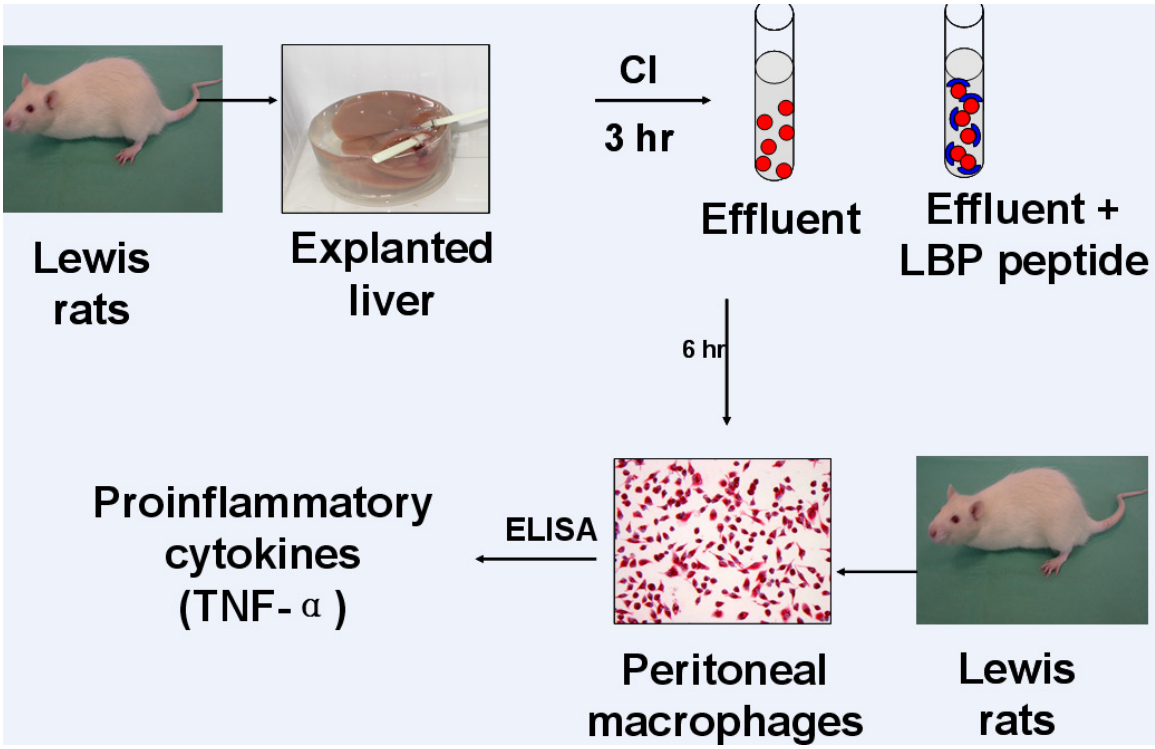

Figure 4.3 Experimental Design of LPS-macrophage-stimulation in vitro

4.1.3 G-CSF induced LBP expression in LPS-SIRS model and 70% PH

4.1.3.1 G-CSF induced LBP expression in vivo

To investigate the G-CSF induced LBP levels, rats were pretreated with G-CSF for 5 days. Several organs were harvested including heart, lung, muscle, brain, liver, spleen, kidney, skin, small bowel, and testis at the sixth day. The peritoneal macrophages, bone marrow cells, peripheral blood mononuclear cell (PBMC) were isolated respectively. The gene expression of LBP was detected in different organs and cells. IHC staining was performed on the bone marrow cells, PBMC, and peritoneal macrophages.

4.1.3.2 G-CSF induced LBP expression in LPS induced inflammatory response and 70% PH

To investigate the role of G-CSF induced LBP in inflammatory response after liver resection, elevation of LBP was induced via G-CSF pretreatment (100 μg/kg/day) for 5 days. The inflammatory response was induced by intravenous injection of 2 mg/kg LPS. 70% PH was performed by removing the left lobe and median lobe. The LBP blockade was performed by intraperitoneal administration of 1.75 mg of the inhibitory peptide-LBPK95A-2h before LPS

administration. The treatment was performed alone or in combination as described in Table 4.1. Observation time was 1h, 6h, and 24h. Each group consisted of 6 rats. If animals did not reach one observation time point, the planned longer observation period was omitted. Serum and hepatic LBP levels, mortality and hepatic injury (liver enzyme, histological evaluation), and inflammatory cytokines were observed in each experimental group to monitor the inflammatory response.

To investigate LPS-binding in the liver, a stepwise 97% PH was performed in G-CSF treatment rats and non-treated rats. Liver lobes were removed sequentially one after the other in 5 min intervals until 97% PH was reached. LPS-binding to the liver was visualized by IHC in each removed liver lobe.

Table 4.1 Group distribution of investigation the role of G-CSF induced LBP expression in LPS-SIRS model and 70% PH

Group (n=6/group)	Treatment				Observation time
	G-CSF	LPS	70% PH	Peptide	
Normal	-	-	-	-	1h, 6h, 24h
LPS	-	+	-	-	1h, 6h, 24h
G-CSF	+	-	-	-	0h
G-CSF + LPS	+	+	-	-	1h, 6h
G-CSF + LPS + peptide	+	+	-	+	1h, 6h, 24h
NaCl + 70% PH	-	-	+	-	1h, 6h, 24h
LPS + 70% PH	-	+	+	-	1h, 6h, 24h
G-CSF + 70% PH	+	-	+	-	1h, 6h, 24h
G-CSF + LPS + 70% PH	+	+	+	-	1h, 6h

4.1.4 G-CSF induced LBP expression in polymicrobia sepsis

For survival analysis, control animals (s.c. injection of 100 µl 5% glucose for 5 days, i.p. injection of human stool suspension, n=25) and G-CSF pretreated animals (s.c. injection of 100 mg/kg G-CSF for 5 days, i.p. injection of human stool suspension, n=25) were monitored clinically followed up every 3h until death. To investigate the biological effects in different phases after infection, rats were sacrificed at 2h (n=4), 12h (n=4), 3d (n=6), and 7d (n=3), for G-CSF +bacteria group and control + bacteria group.

4.2 Animals

4.2.1 Anesthesia

Surgical procedures were performed under inhalation anesthesia using vaporized isoflurane. The anesthesia was induced in a chamber and maintained using a face mask with a 0.5 L/min oxygen flow mixed with 3% Isoflurane. The operation started when the rat had no more pain reflexes, e.g. no response to clamping the skin using surgical forceps.

4.2.2 Surgical models

4.2.2.1 Partial hepatectomy

Different liver lobes were removed according to the description of Madrahimov N et al (Madrahimov et al., 2006). The left lateral lobe (LLL), superior caudate lobe (SCL) and inferior caudate lobe (ICL) was removed after clamping and ligating the narrow pedicle with a 6-0 prolene suture. To resect the right superior lobe (RSL), right inferior lobe (RIL), left median lobe (ML) and right ML, the mosquito clamp was placed around the base of the respective lobe, and the liver lobe was dissected along the instrument. Piercing sutures were performed below the clamp. The distribution of different extent liver resection was described in Table 4.2.

Table 4.2 Different extent of PH

Type of PH	Resected liver lobe
30% PH	LLL
70% PH	LLL, ML
90% PH	LLL, ML, RSL, RIL
97% PH	LLL, ML, RSL, RIL, SCL, ICL

4.2.2.2 Ex-vivo liver ischemia model

The ex-vivo liver ischemia model was performed as described by Liu et al (Liu et al., 2010). After opening the abdomen with a transversal incision, the liver was freed from its ligaments and flushed with cold saline solution. Infrahepatic vena cava and portal vein were cannulated with 12G and 14G catheters, respectively. The cannulated livers were placed in refrigerator (4 °C). At every hour, the livers were flushed with cold saline at a constant pressure of 10 cm H_2O through the portal vein. For each time point, 1.5 ml effluent was collected from the infrahepatic vena cava during 24h. Protease inhibitors were added to the effluents immediately after collection. Effluents were centrifuged (300g ×5 min) to remove the red blood cells. Fresh effluent from 3 liver of cold ischemia was pooled together, aliquoted and frozen at -20 °C for further studies.

4.2.2.3 In-vivo selective warm liver I/R injury model

The selective warm ischemia was performed as described by Liu et al (Liu et al., 2011). Heparin (150 U in 1.5 ml saline) was given through the penile vein at least 10 min before clamping. After opening the abdomen, the portal tract containing hepatic artery, portal vein and bile duct of left lateral and median liver lobes was clamped in the liver using a micro vascular clamp.

4.2.2.4 In-vivo LTx

The non-arterialized rat orthotopic LTx procedure was performed following the method described by Kamada (Kamada and Calne, 1979). Briefly, after dissecting all hepatic ligaments, the portal vein, infrahepatic and suprahepatic vena cava (VC) were mobilized and the left phrenic vein, hepatic artery and adrenal artery and vein were ligated. The common bile duct was transacted and cannulated by a 5mm 22 gauge biliary cannula and secured with 6-0 silk suture. After heparinization by using 2ml saline mixed with 100 IU heparin, the donor liver was immediately flushed by 4 °C saline under a pressure of 10 cm H_2O until the entire liver became evenly yellowish.

The liver graft was preserved in ice-cold 0.9% NaCl solution bath. Cuffs made of 14-gauge and 10-gauge catheter were installed in the portal vein and the infrahepatic inferior vena cava respectively.

Immediately after the explantation of the liver from recipient's own liver, the graft was orthotopically placed in abdominal cavity covered by cool saline soaked gauze to avoid additional warm ischemia. Subsequently, the suprahepatic VC was reconstructed by a simple running suture using 7-0 polyproplene suture material. The portal vein and infrahepatic VC were anastomosed employing a cuff technique. The bile duct was reconstructed by a 5mm cannula and secured both in the donor side and recipient side. Part of the great omentum was used to wrap the bile duct anastomosis using a 6-0 silk suture.

The abdomen was checked carefully for bleeding and stenosis of anastomosis and washed with room-temperature-saline. The abdomen was closed with 3-0 absorbable suture in two layers.

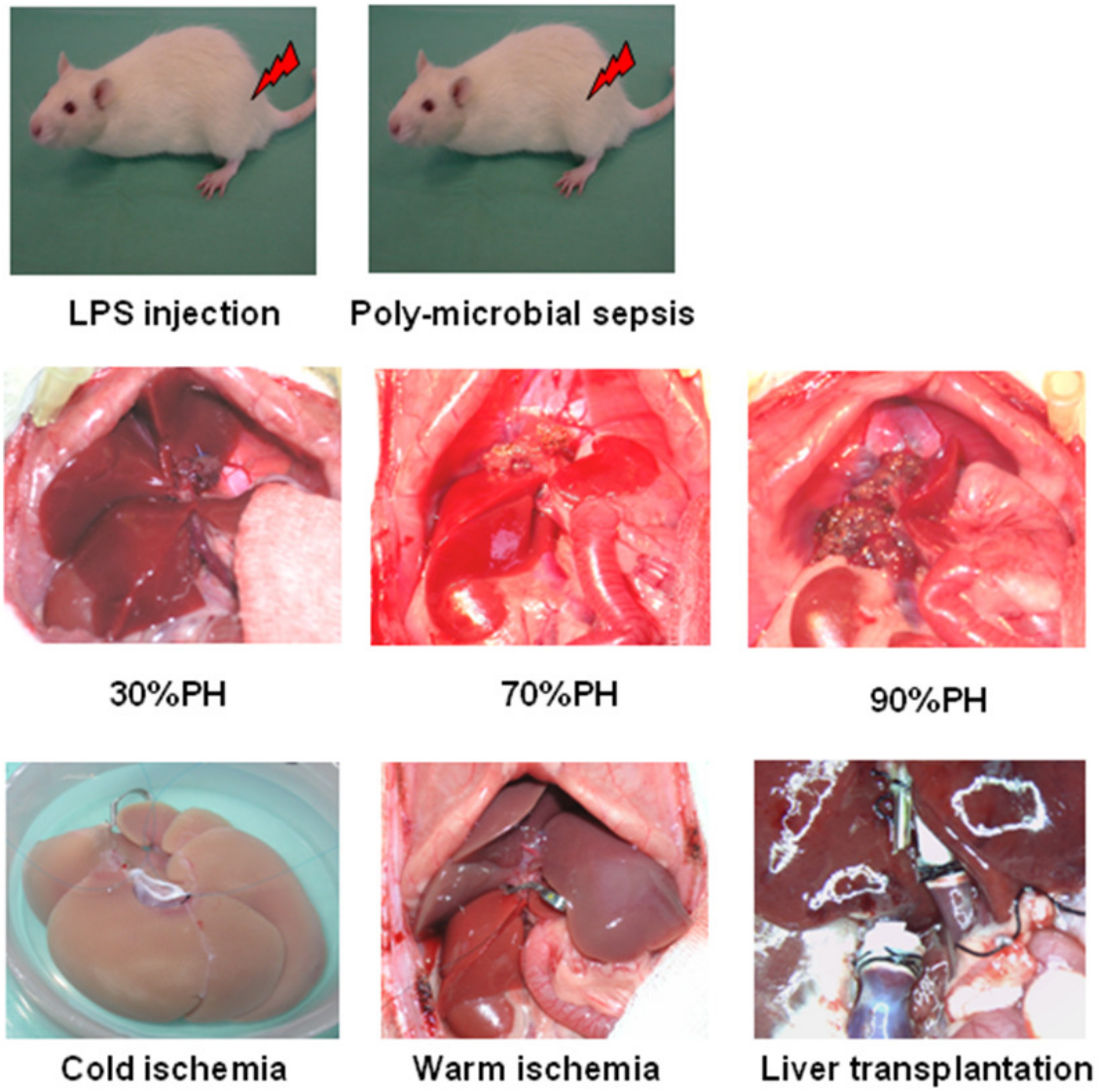

Figure 4.4 Surgical models used in this study

4.2.3 Post-operative treatment

After operation, buprenorphine (0.01 mg/kg) was injected subcutaneously to achieve the postoperative analgesia (Avsaroglu et al., 2008). To prevent the post-operative infection, 0.01 mg/kg piperacillin was given by intramuscular injection (Madrahimov et al., 2006) after LTx and PH. To keep the body temperature, the rats were maintained on a heated table for 12 h.

4.2.4 Monitoring and sampling

At the defined time point, rats were sacrificed under anesthesia. Blood was taken from the inferior vena cava for blood count, clinical chemistry and protein biochemical detection. All

abdominal organs were sampled for histological evaluation. The liver tissues were stored in -80 °C for further investigation.

4.3 Primary cell isolation and culture

4.3.1 Isolation and culture of peritoneal macrophages

Isolation and culture of peritoneal macrophages were performed as described by Liu et al (Liu et al., 2012).Peritoneal macrophages were harvested by two times of peritoneal washings with 20ml of PBS (3 U/ml heparin). The cells were washed three times (300g ×2min) with PBS and then Cultured in RPMI1640 medium supplemented with 10% fetal bovine serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were plated in 24 well plates at a density of $3x10^5$ cells and cultured at 37 °C under a gas phase of air/CO_2 (95:5). Three hours later, the non-adherent cells were discarded, and the adherent cells were used for further experiments. The non-adherent cells were removed after 3h attachment. The resulting adherent population consisted of >96% macrophages as judged by CD68 staining Figure 4.5. The cell viability typically exceeded >96% as determined by trypan blue exclusion assay.

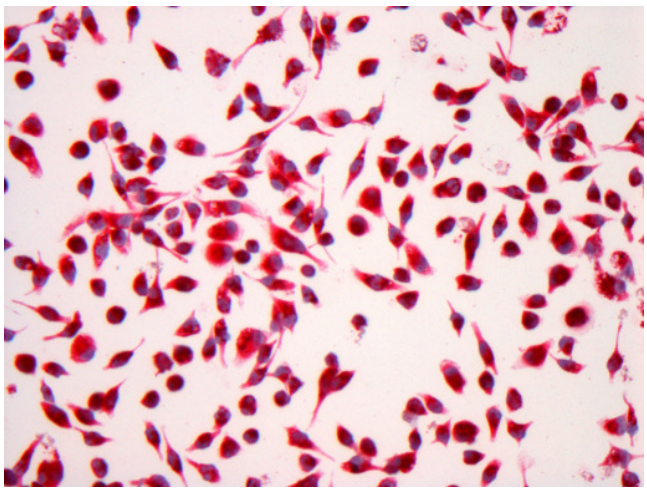

Figure 4.5 Expression of CD68 in peritoneal macrophages

CD68 localization in macrophages was visualized by immunostaining with anti-CD68 (red).

4.3.2 Isolation of bone morrow cells

Bone marrow cells were harvested from rat femurs by two times washings with 10ml PBS (3 U/ml heparin). The red blood cells were removed by suspending cells in deionized water for exactly 10 sec, and restored isotonicity by 2×PBS. The bone marrow cells were re-suspended in PBS for further investigation.

4.3.3 Isolation of PBMC cells

PBMCs were separated by Biocoll density gradient centrifugation. Add 15 ml Biocoll into a new 50 ml leucosept separation tubes, centrifugation 1000g for 30 second. PBS diluted blood was transferred into the tube, centrifugation at break (1200g × 10 min). The PBMC-containing middle layer was transferred to a fresh tube. Cells were washed twice with PBS.

4.3.4 Collection of cells from peritoneal cavity

The infected rats were sacrificed at 2h, and 12h respectively. All ascites was collected from peritoneal cavity, and the volume of ascites was recorded. Cells in ascites were pelleted by centrifugation at 300g × 10 min and re-suspended in 500µl PBS. The number of cells was determined using a cell counting chamber.

4.4 Protein-biochemical methods

4.4.1 Western blot

4.4.1.1 Sample preparation

Serum samples were diluted for 5 times using deionized water. Liver tissue was lysed in lysis buffer (Tris 50 mM pH7.4, NaCl 150 mM, 1% Igepal CA-630, 1 mg/ml leupetin, 1 mg/ml pepstatin) at 4 °C. Protein was quantified with the BCA protein Assay Kit.

4.4.1.2 SDS-PAGE

An equal amount of protein (15 µg) of the total liver samples or equal volume (10 µl) of serum samples were loaded on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. Samples were separated for 90 min at 120 volts and transferred to Amersham Hybond^{TM}-P membrane.

4.4.1.3 Immunoblotting

The membranes were blocked in 5% milk solution (5% non-fat milk powder, 0.1%Tween 20 in PBS) 1h at room temperature. The membranes were then probed with a goat polyclonal antibody to LBP (1:100, Santa Cruz) for LBP expression. Signals were detected with Lumilight western blot substrate and exposed to X-ray film for autoradiography. The same membranes were stripped with stripping buffer for 15 min at room temperature and immunoblotted with rabbit anti-GAPDH (1:20000). Digitalization of films was performed using a film scanner. The signal intensity was quantified with Image J program, and compared with a calibration curve constructed with serially diluted (1:5 - 1:160) from rat serum obtained 6 hours after LPS injection.

4.4.2 Silver staining

Following electrophoresis, gels were subjected to silver staining as previously described (Liu et al., 2010). Quantification of bands was performed using Image J program (NIH, Bethesda, USA).The ratio of LBP protein in the eluate was calculated using the following formula: (Area under curve of target band) / (Area under total curve) ×100%. The concentrations of LBP in eluate then were calculated based on the total protein concentrations and the ratio of LBP protein to total protein in the eluate.

4.4.3 Immunoprecipitation

Immunoprecipitation experiments were performed according to the instructions of the manufacturer. Briefly, serum samples (1mg) were firstly pre-cleared using the control agarose resin to reduce nonspecific protein binding. The serum samples then were incubated with protein A/G plus agarose resin (20µl) coupled with 10 µg polyclonal goat anti-LBP antibody at 4 °C overnight. After three times washing with Lysis/Wash buffer (0.025 M Tris, 0.15 M NaCl, 0.001 M EDTA, 1% NP-40, 5% glycerol; pH 7.4), serum proteins were then eluted from beads by using antigen elution buffer. The protein concentration of the eluate was measured by BCA method using bovine serum albumin as calibrators, and the purity was estimated by SDS-PAGE and silver staining.

4.4.4 ELISA

4.4.4.1 LBP ELISA

Standard 96 well ELISA plates were coated with 10 µg/ml of LPS in PBS (pH 9.8) (Plested et al., 2000). The plates were subsequently washed three times with PBST (0.01% Tween20, pH 7.4) and blocked with 1% BSA in PBST (0.01% Tween20, pH 7.4) at room temperature for 2 hour. After the plates were washed, 100µl of each dilution of the calibrator and samples were added to the wells and incubated for 2 hour at room temperature. The plates were washed three times with PBST (0.01% Tween20, pH 7.4) and then incubated with monoclonal mouse anti-LBP antibody (1:10000, cell science) for 2 hours. After the plates were washed again for three times, rabbit anti-mouse IgG-H&L antibody (1:5000) was added to each well, and the plates were incubated at room temperature for 1 h. After the plates were washed, 100 µl 1:1 mixtured of H_2O_2 and Tetramethylbenzidine was added to each well and incubated for 12-15 min in the dark at room temperature without shaking. The color reaction was stopped by adding 50 µl of sulfuric acid and the plates were measured with the ELx 808 ELISA plate reader at 450 nm.

The non-linear regression standard curve was created for quantification using sigma plot. We determined the detection limit, the working range as well as intra- and inter-assay variability as parameter of assay quality. The intra-assay variability was tested by repeating single sample in 6 separate plates. The inter-assay variability was tested by repeating single sample up to 9 times in one plate.

4.4.4.2 TNF-α, IL-6, IL-10 and HMGB1 ELISA

For analysis of serum or ascites TNF-α, IL-6, IL-10 and HMGB1 levels, a commercial available Enzyme-linked Immunosorbent Assay kits were used. The procedure was performed according to the manufactures suggestions. Measurements of the ELISA were performed in 96-well polysterene plates using an ELx 808 ELISA plate reader at 450 nm.

4.5 Histopathology

4.5.1 Histological processing and Hematoxylin-Eosin (HE) staining

Liver tissue was fixed in 4.5% buffered formalin for at least 24h. Paraffin embedding was performed using standard techniques. Sections (4µm) were cut and stained with Hematoxylin-Eosin. Whole slides scans were obtained at 200x magnification using the Hamamatsu slide scanner. Three pictures at a magnification of 200 x - one from each lobular zone - were selected randomly to analyze tissue damage. Histological evaluation was performed by an experienced pathologist (PD Dr. Olaf Dirsch, Institute for Pathology, University Hospital of Jena). The liver tissues were analyzed regarding sinusoidal infiltration, sinusoidal dilation, vacuolization of hepatocytes, and pericentral bleeding.

4.5.2 LPS immunohistochemical staining

After deparaffinization and rehydration, antigen retrieval was performed in a water bath using citrate buffer (10mM Citric Acid, pH 6.0) for 20mins at 100 °C. Slides were washed 3 times with TBST (0.5% Tween-20). Nonspecific protein binding was blocked using 100ul serum free blocking buffer. Sections were incubated with diluted (1/100) polyclonal mouse anti-LPS antibody for 15 min at room temperature. Slides were washed 3 times with TBST (0.5% Tween-20) and detection was performed using Anti-Mouse Immunoglobulins-HRP. Slides were washed 3 times with TBST (0.5% Tween-20) and incubated in amplification reagent for 15 min and followed by 15min anti-fluorescein-HRP incubation. After washing slide 3 times and employing liquid DAB substrate-chromogen as substrate, sections were counterstained with Hematoxylin for 5mins. Whole slides scans were obtained at 200×magnification using the Hamamatsu slide

scanner. Three pictures at a magnification of 200× - one from each lobular zone - were selected randomly to analyze LPS staining.

4.5.3 LBP immunohistochemical staining

Antigen retrieval was performed as described above. Nonspecific protein binding was blocked using 100ul serum free blocking buffer. Sections were incubated with monoclonal mouse anti-LBP antibody (1:100, cell science) for 1 h. Detection was performed using Bright Vision rabbit-anti-mouse-AP and employing Fast-red as substrate. Slides were visualized as described above.

4.5.4 ASDCL staining

The ASDCL (naphthol-AS-D-chloroacetate esterase) staining is used to highlight granulocytes and granulocyte precursor cells. The ASDCL staining is based on the ability of cytoplasmic esterases produced by granulocytes to hydrolyze the substrate naphthol AS-D chloracetate (Leder, 1970; MOLONEY et al., 1960; Schlayer et al., 1988). This hydrolysis separates a naphthol compound which reacts with a diazonium compound in the reaction solution. This complex develops a red/violet color and accumulates at the site of esterase activity.

For evaluation of neutrophil infiltration, Slides were visualized as described above, and 5 pictures were taken with magnification of 200×. Hepatocytes and ASDCL staining positive cells were counted manually. The result was expressed as ratio of ASDCL positive cells to hepatocytes. .

4.6 Biological chemistry methods

4.6.1 Clinical chemistry

Blood was sampled using 1.2 mL serum collection tube. The blood was static settlement for 30 min and centrifuged (400g × 10 min). The serum was precipitated and transferred to a fresh 1.5 ml EP tube. To collect the effluent after cold ischemia, samples were centrifuged (300g × 5 min) to remove the red blood cells. To assess hepatocellular injury, aspartate aminotransferase (AST) and alanine transaminase (ALT) were measured in serum or fresh effluent using an Automated Chemical Analyzer.

4.7 Molecular biological methods

4.7.1 Quantitative Polymerase Chain Reaction (qPCR)

Total RNA was isolated from tissues or cells using the RNeasy kit. Complementary DNA (cDNA) was synthesized by using the First-Strand cDNA synthesis kit and quantified using Agilent bio-analyzer with RNA nano6000 kit. cDNA-stock solution was diluted to 1ng, and equal amounts of cDNA were used for each PCR reaction as described previously (Xing et al., 2009). Primers and probes were mixed with Brilliant probe-based qPCR Master Mix kit and then diluted with deionized water up to 20 µl. The sense and antisense primers and the probes from the universal probe library are indicated in Table 3.1. Samples were run on an Mx3000P qPCR System. Thermal cycling conditions consisted of a 10 min template denaturizing step at 95 °C, followed by 50 cycles of 95 °C for 30 s, 50 °C for 30 s and 72 °C for 30 s. Standard curve was generated using a serial dilution of a normal sample. Gene expression was normalized using hypoxanthine guanine phosphoribosyltransferase (HPRT) to compensate for errors when diluting the cDNA stock solution. The fold change was calculated using a normal liver tissue sample as reference sample.

4.8 Microbiological analysis

4.8.1 Bacterial load in ascites and tissues

Rats were infected with the stool suspension, and sacrificed at 2h and 12h postintervention, respectively. Ascites was collected and diluted with Brain Heart Infusion (BHI) broth (Institute of Microbiology, University Hospital Jena). The right superior liver lobe, right lung lobe, and half of the spleen was harvested. The tissues were weighed and homogenized in 3 ml NaCl. The homogenate was diluted to 5 ml with BHI bouillon. Each sample had 3 dilutions from 1:10 to 1:1000. Ten µl supernatant was taken for bacterial culture in a blood agar filled culture plate. Plates were incubated for 24 h at 37 °C and colonies were counted by an experienced microbiologist (PD. Dr. Jürgen. Rödel, Institute for Microbiology, University Hospital of Jena). The results of bacterial culture were expressed as number of colonies/gram tissue.

4.9 Statistical analysis

Data were expressed as mean ± standard deviation (SD). Differences between paired groups were analyzed using the two tailed paired samples Student's t-test and differences between independent groups were analyzed using the two tailed independent-samples Student's t- test.

Multiple groups were compared using the one way independent ANOVA test. Bivariate correlations were tested with Spearman's rank correlation. All statistical analyses were performed using Sigma Stat v.3.5 (Systat Software, Inc., Chicago, USA). Graphs were generated using Sigma Plot v.10 (Systat Software, Inc., Chicago, USA). A p-value below 0.05 was considered statistically significant.

5 Results

5.1 Establishment of LPS-LBP ELISA

5.1.1 Purification and quantification of serum LBP protein

For serum LBP quantification (Figure 5.1), LBP protein was purified from rat serum obtained 6h after LPS administration using immuno-precipitation. 13.2 ± 2.6 µg of protein complex was obtained from 80 µl serum. Immunoblotting showed that the bands with 55kDa corresponded to LBP. Based on the area under the curve, the ratio of LBP protein to total protein in the eluate was 37 ± 7.3%, as determined in 3 separate experiments. The LBP concentration in the serum was 60.0 ± 11.8 µg/ml.

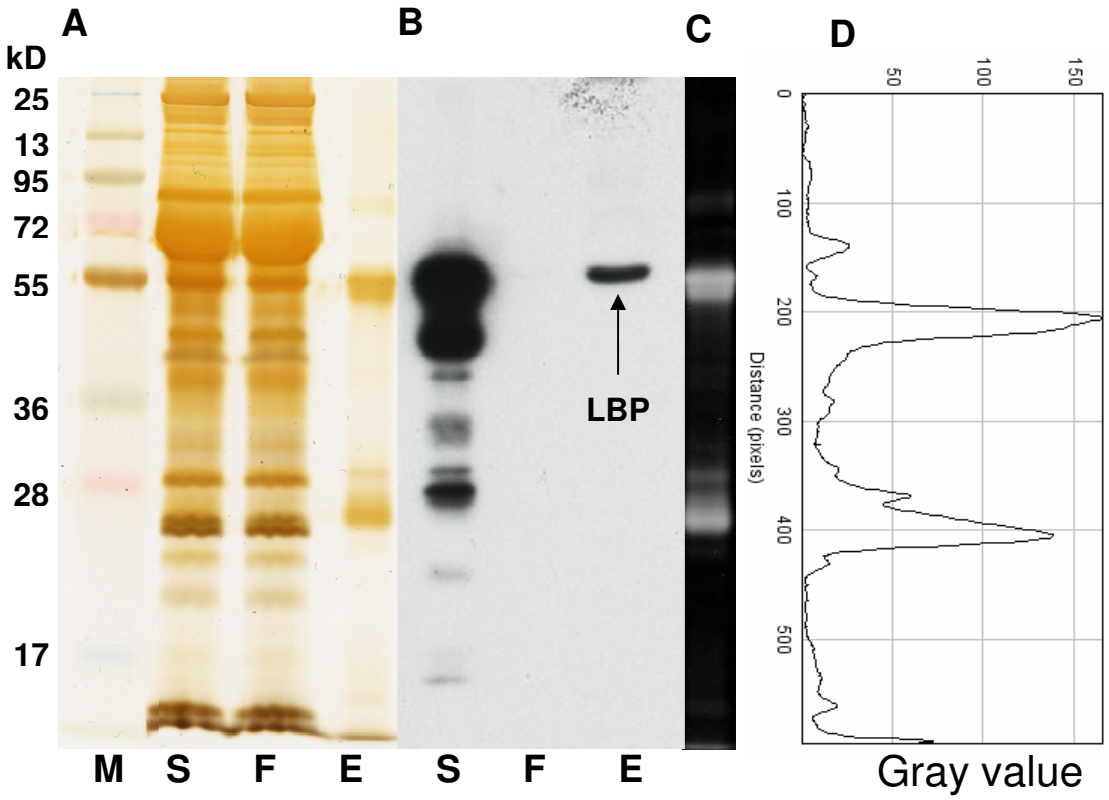

Figure 5.1 Purification of LBP

LBP was immunoprecipitated and eluted from Protein A/G beads. (A) The eluate containing LBP-protein was separated using SDS-PAGE and the gel was subjected to silver staining. (B) LBP was detected by western blot. (C) and (D) protein profile was analyzed by image J after silver staining. The experiment was performed in triplicates with similar results. Representative images from 2 independent experiments were selected. M=marker, S=serum, F=flow through, E=eluate

5.1.2 Establishment of LBP ELISA

To optimize the LBP assay system, different amounts of LPS were used for coating. As shown in Figure 5.2, there was no significant difference when coating the plate with 1 – 10 µg (in 100 µl) LPS. Different BSA concentrations were tested to reduce non-specific binding. Further optimization of binding condition was evaluated by adding of the non-ionic detergent tween-20 to the blocking buffer respectively to the diluted sample (Figure 5.2). The assay showed optimal blocking efficiency and stable results when using a blocking buffer consisting of 5% BSA, 0.05% Tween-20 in PBS and a sample dilution buffer containing 1% BSA in PBS. The working range of the ELISA was 0.1- 60ug/ml. The inter- and intra-assay coefficient of variation was 4.71% and 10.70%, respectively, which is within the range of commercially available ELISA-assays.

We then tested the correlation between the LPB ELISA method and western blot. Twenty-five serum samples obtained from rats 1h, 6h, and 24h after LPS injection and after PH were used. As shown in Figure 5.3, upregulation of serum LBP levels were observed when using both methods. The correlation between the values obtained by both methods was strong ($r=0.885$, $p<0.001$).

5.2 LBP and hepatobiliary surgery

5.2.1 LBP-levels after PH are related to the remnant liver mass

5.2.1.1 Hepatic injury after PH is related to the extent of resection

As shown in Figure 5.4, liver enzymes were increased in all experimental groups. Serum AST after 30% PH was 505.33 ± 102.58 IU/L. Release of liver enzymes after 70% PH and 90% PH was even higher, indicating severe hepatocellular injury in both models.

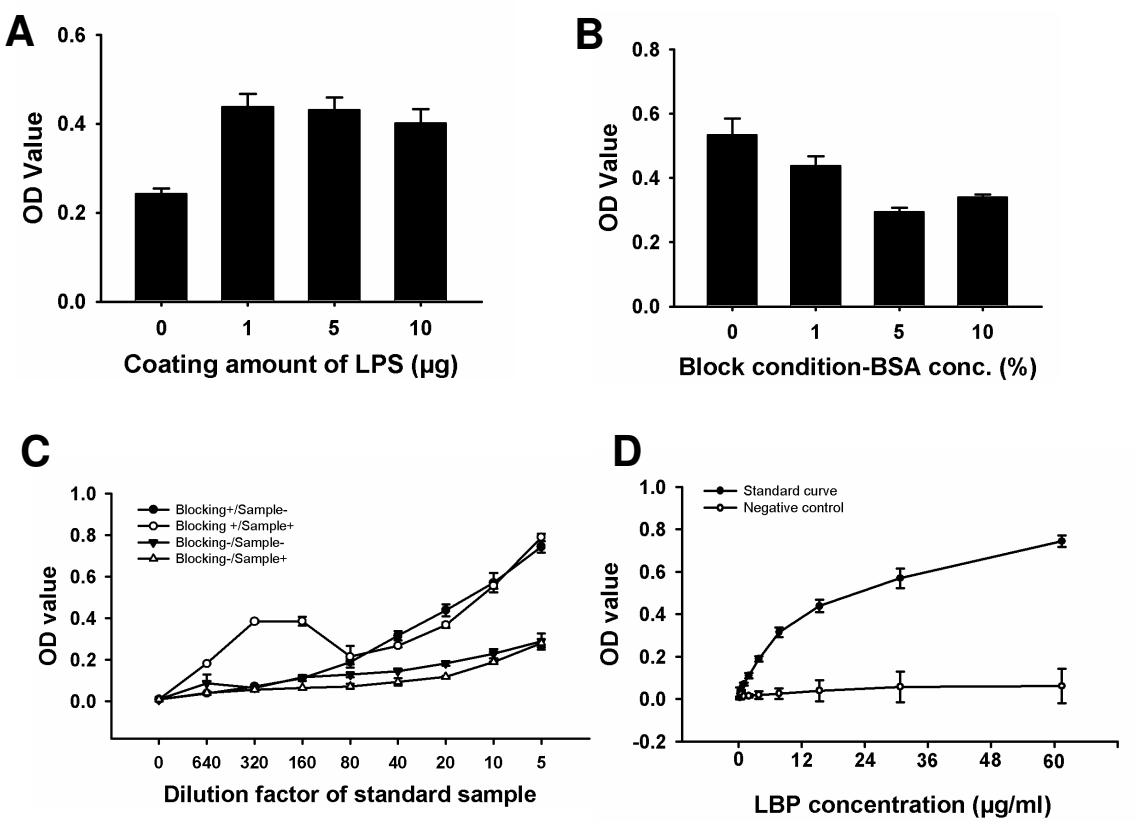

Figure 5.2 Optimization of the ELISA-assay

Titration of LPS (1-10 µg) for coating (A) showed similar results irrespectively of concentration. Titration of BSA in blocking buffer revealed lowest background signal when using 5%BSA (B). Assay establishment: Sensitivity of assay (C) was optimized when adding low amount of Tween-20 to the blocking buffer, but not to the sample (-●-). The sensitivity was decreased when blocking without tween-20 (-△-, -▲-). Tween-20 influenced the binding between LBP and LPS (-○-), indicated by an extra "spike" in the curve. Standard curve (D) obtained following the optimized protocol and applying non-linear regression analysis (SigmaPlot 10.0). Data are representative of 3 independent experiments. Data are shown as mean ± SD.

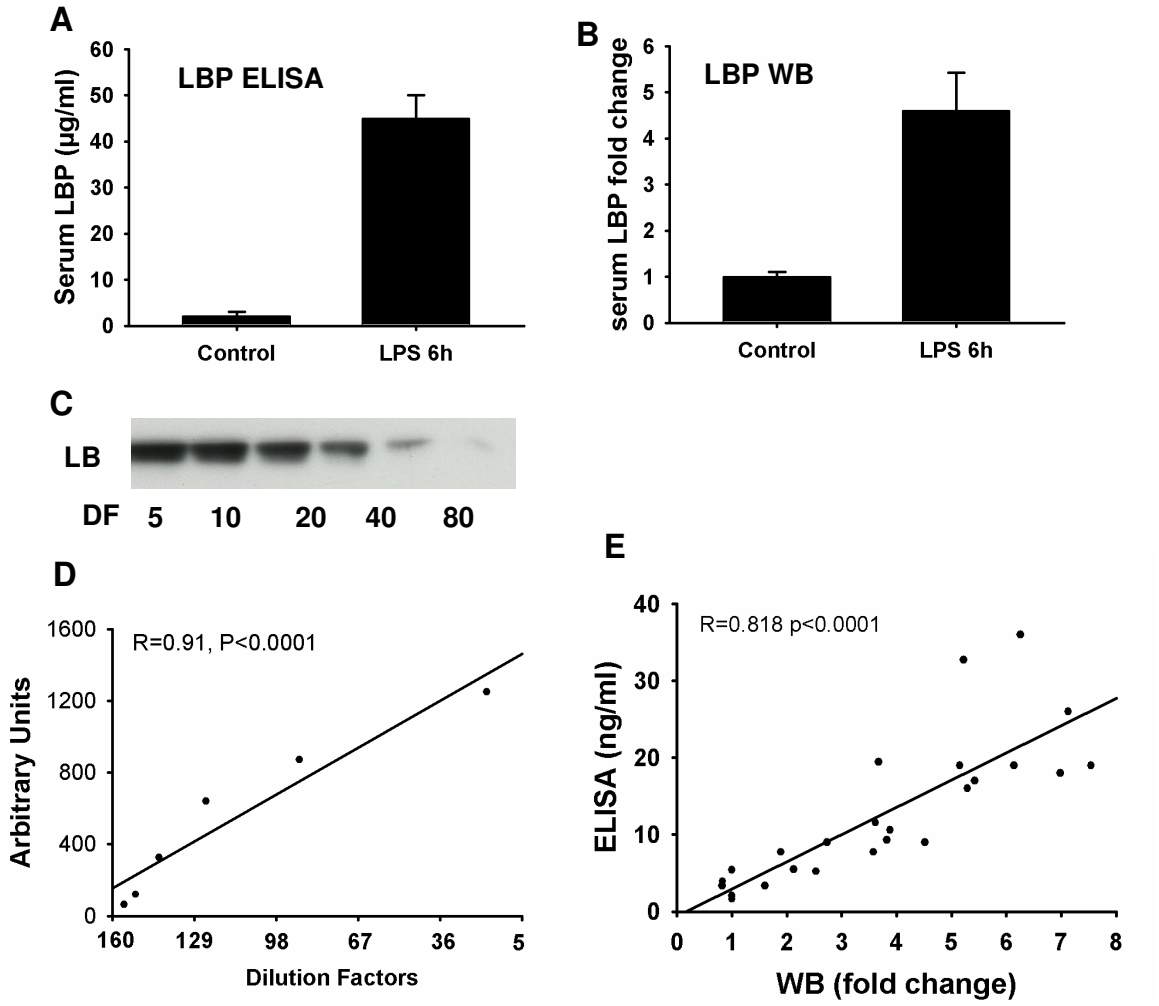

Figure 5.3 Correlation of LPS-LBP ELISA with western blot

Serum LBP levels using serum obtained 6 hours after LPS injection assessed with LPS-LBP ELISA (A) and western blot (WB) (B). LBP-levels were determined by WB using a calibration curve constructed with serially diluted rat serum obtained 6 hour after LPS administration (C), and linear regression was analyzed (D). Strong correlation of serum LBP levels measured with western blot and LPS-LBP ELISA (E). Results are shown as mean obtained in 3 independent experiments. Data are shown as mean ± SD, n= 6 per group. DF=dilution factor

5.2.1.2 Elevation of pro-inflammatory cytokines mRNA-expression is correlated to extent of resection

The expression of mRNA of TNF-α and IL-6 were quantified by qPCR. As shown in Figure 5.4, compared with normal control rats, PH significantly up-regulated the expression of TNF-α and IL-6 in livers. TNF-α and IL-6 expression was significantly increased at 24h after resection. TNF-α and IL-6 mRNA levels were correlated to the extent of resection.

5.2.1.3 Hepatic LBP mRNA expression is upregulated in livers after PH, irrespectively of extent of resection

The hepatic LBP mRNA expression levels were measured by qPCR. As shown in Figure 5.5, LBP mRNA expression increased to 15-fold after surgery when compared with normal controls. There was no significant difference in mRNA expression among the 3 groups of animals subjected to various degree of liver resection.

5.2.1.4 Elevation of serum LBP protein levels is correlated to the remnant liver mass

In order to investigate the serum LBP levels after hepatobiliary surgery, LBP concentration was measured in serum by ELISA. As shown in Figure 5.5, in the normal control group, very low LBP levels were detected (0.8 µg/ml ± 1.0). After PH, serum LBP levels were significantly upregulated when compared with normal value (30% PH: 23.6 µg/ml ± 6.7, 70% PH: 17.5 µg/ml ±1.4, 90% PH: 4.5 µg/ml ± 2.2, vs. normal control, $p<0.05$). However, elevations of serum protein levels were positively correlated to the remnant liver mass ($R=0.821$, $p<0.0001$), suggesting that serum levels may reflect the impaired synthetic capacity of the small remnant liver.

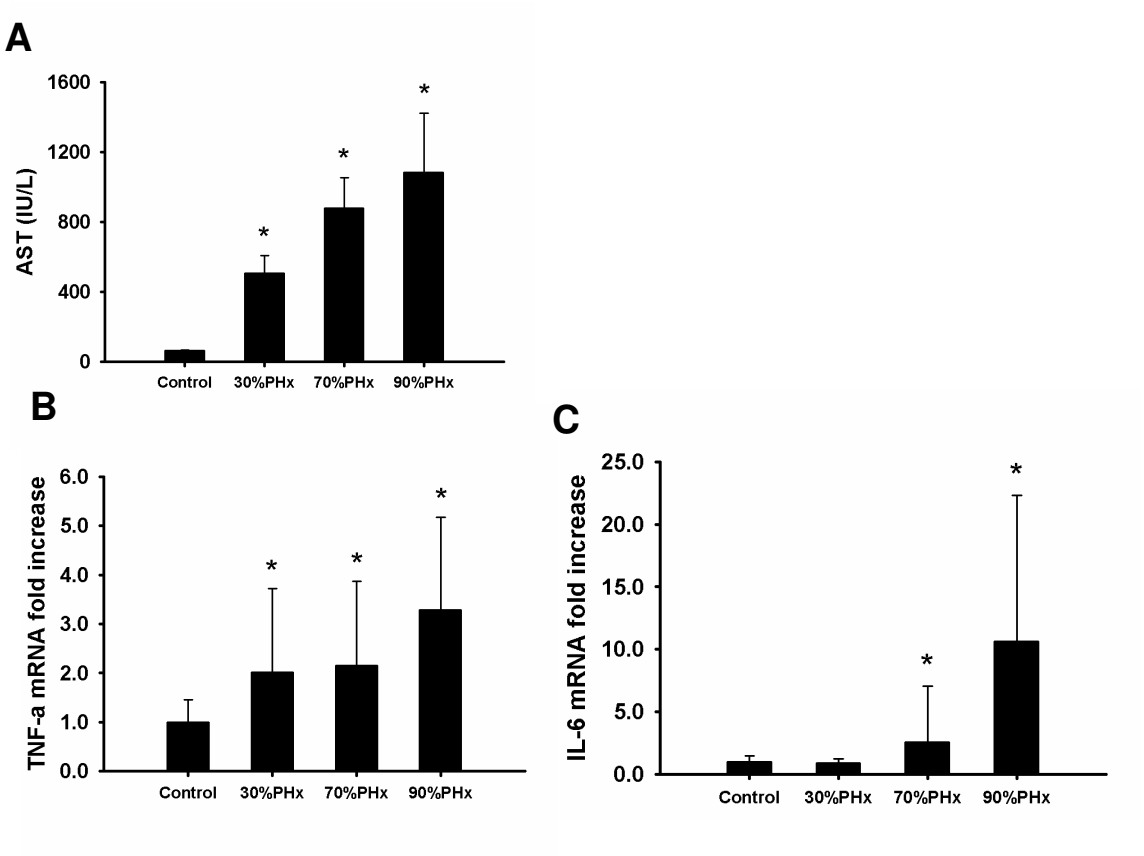

Figure 5.4 Liver damage and the expression of pro-inflammatory cytokines after different extent of liver resection

Serum AST levels were analyzed to indicate hepatocellular injury. In contrast to normal controls, serum AST levels were significantly increased in rats subjected to different extent of liver resection. Release of liver enzymes after 90% PH was significantly higher than in other groups (A). Expression of hepatic pro-inflammatory cytokines was measured by qPCR in normal liver tissue, PH liver tissue. mRNA levels for TNF-α (B) and IL-6 (C) were significantly increased in livers subjected different extent liver resection. *$p<0.05$ vs normal controls. Data are shown as mean ± SD, n= 6 per group.

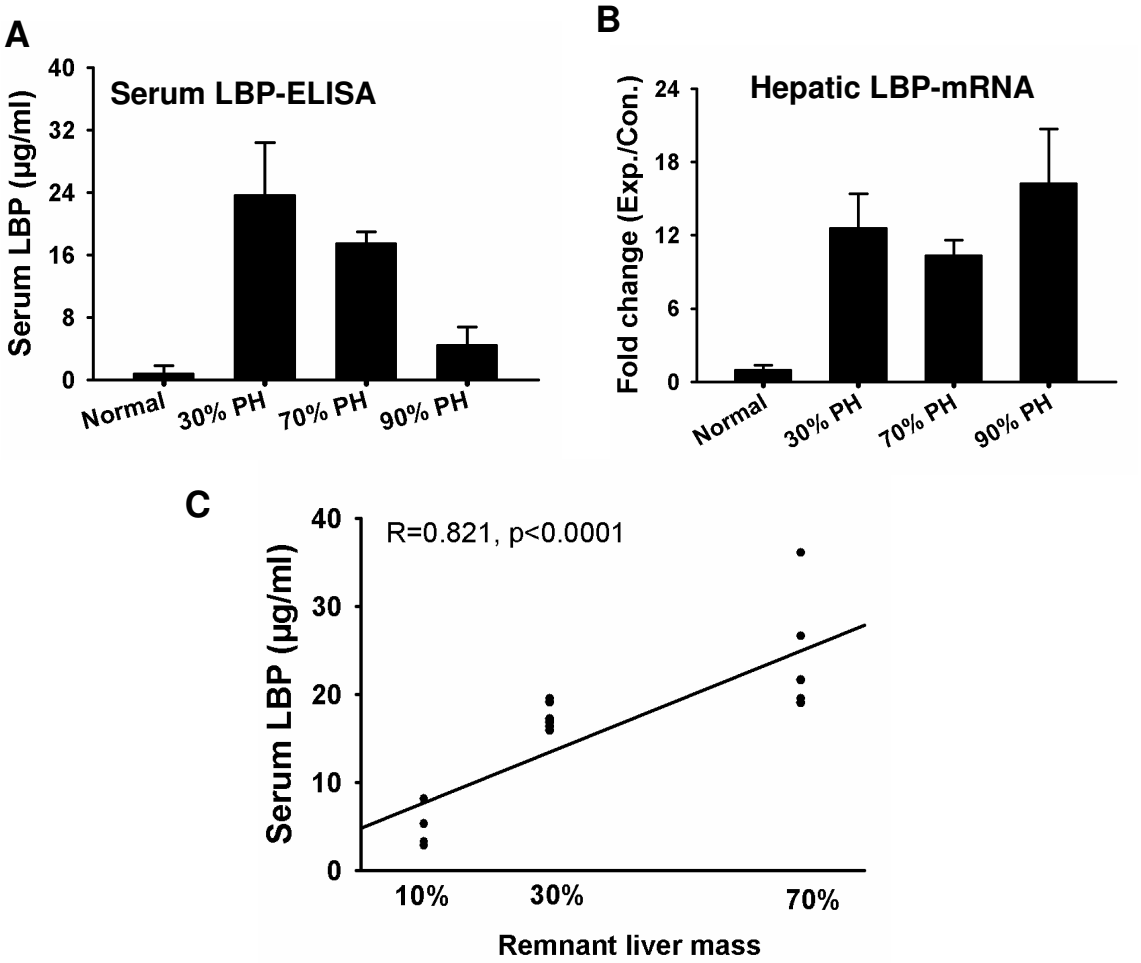

Figure 5.5 Expression of LBP after PH in rats

Serum LBP levels measured with LPS-LBP ELISA (A) after 30% PH, 70% PH and 90% PH in rats in comparison to hepatic mRNA (B). Elevations of serum protein levels were positively correlated to the remnant liver mass (C). Data are shown as mean ± SD, n= 6 per group.

5.2.2 LBP expression is associated with inflammatory response after I/R and LTx

5.2.2.1 Cold preservation of liver grafts causes release of LBP into the effluent

Cold ischemia of the explanted liver caused hepatic injury, which became apparent within 8 h as indicated by a release of AST and ALT into the effluent. To determine whether LBP was also released during cold ischemic storage of liver, the effluent was subjected to the LBP-ELISA. As shown in Figure 5.6, the release of LBP was detected as early as 1 h, and then peaked at 3 h. After 7 h, LBP was undetectable in the effluent during cold storage.

We also investigated whether cold ischemia upregulated LBP-expression in the cold preserved organ. As shown in Figure 5.6, hepatic LBP mRNA expression was not induced during cold ischemic storage.

5.2.2.2 LBP in effluent enhances LPS induced pro-inflammatory cytokine synthesis

LBP has been implicated in LPS induced inflammation. We wanted to determine, whether LPS released from ischemic liver could modulate LPS induced inflammation. Effluent with the highest concentration of LBP, obtained after 3 h of cold storage, was selected for the macrophage stimulation assay. Effluent with or without addition of LBP inhibitory peptide was added to cultured peritoneal macrophage prior to LPS stimulation. After 6h of stimulation with LPS, the cell culture supernatant was removed and the release of TNF-α was measured by ELISA. As shown in Figure 5.7, TNF-α levels were significantly increased after stimulation with 3h-effluent and LPS, when compared with LPS only (3h-effluent group: 11.09 ± 0.58 ng/ml, non-effluent group: 9.07 ± 0.22 ng/ml, $p<0.001$). However, the synthesis of TNF-α was significantly attenuated when pretreating the effluent with LBP inhibitory peptides (5.14 ± 0.91 ng/ml, $p<0.001$). This finding indicated that LBP released during CI could indeed modulate the LPS induced inflammatory response. The use of inhibitory peptides confirmed that the modulation of the LPS induced inflammatory response was partially due to LBP release in the effluent of livers subjected to cold ischemia.

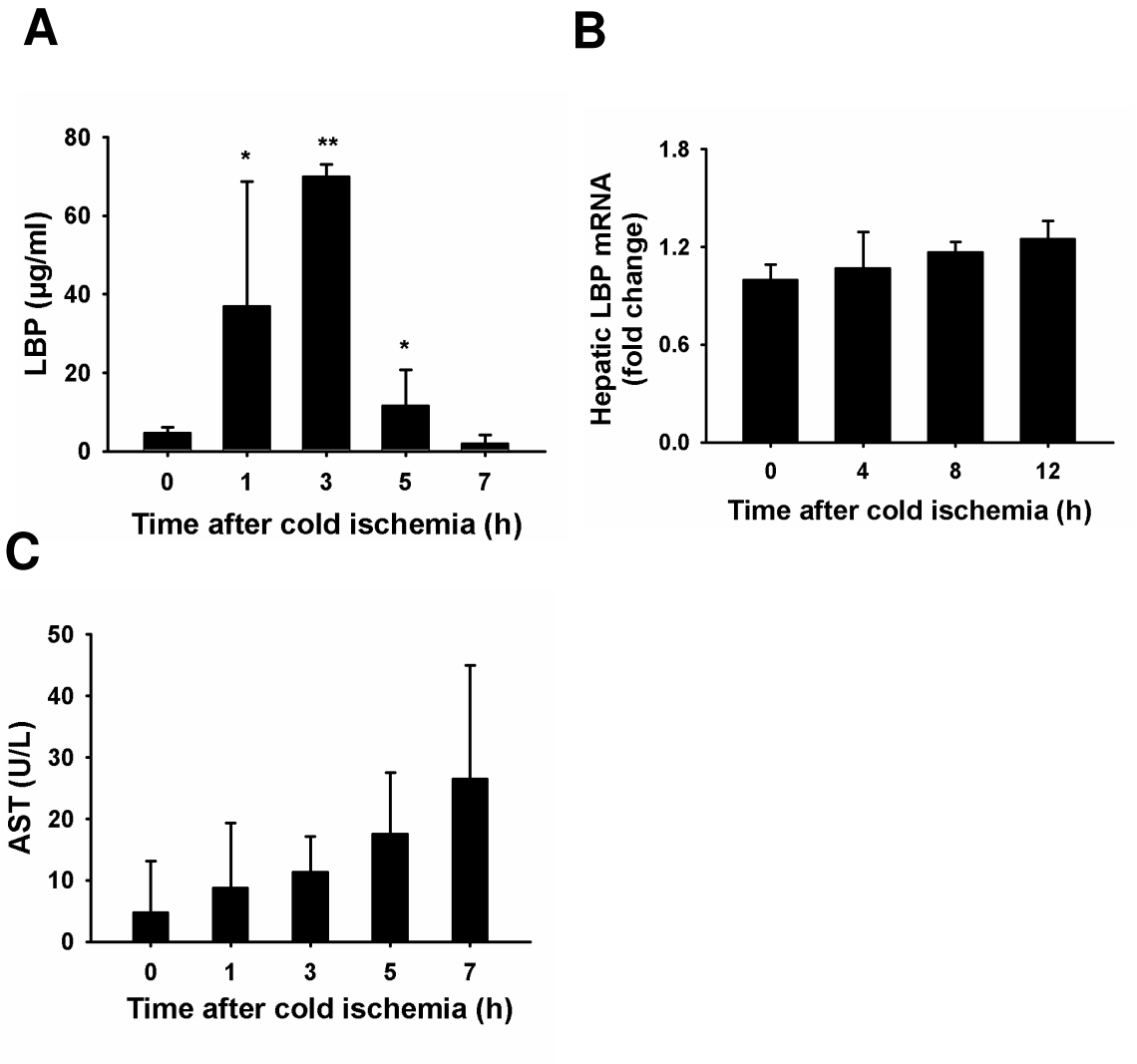

Figure 5.6 Release of LBP during CI

During cold ischemia storage, hepatic effluents and liver tissues were collected hourly for 24h cold storage (graph only shows LBP-levels at selected observation time points: 1, 3, 5 and 7 hours. After 7 hour cold preservation, no LBP was detected in effluent). (A) LBP levels in effluents as quantified by ELISA were highest in samples obtained after 3h (B) LBP mRNA expression was not significantly altered during cold storage of liver. (C) Release of AST increased slightly, but not significantly during the 8h cold ischemic storage when compared with 0h effluent. *$p<0.05$;**$p<0.01$, compared with 0h effluent. Data are shown as mean ± SD, n= 3 per group.

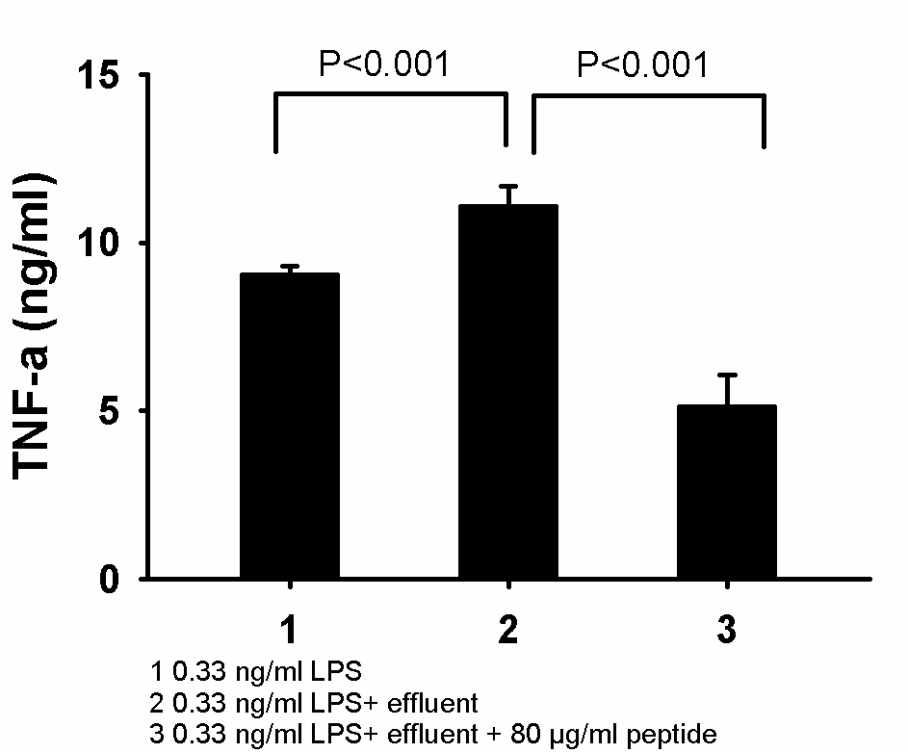

Figure 5.7 Induction of TNF-α expression in peritoneal macrophage assay after stimulation with LPS

The effluent with the highest LBP concentration, obtained after 3h of cold storage, was used. Macrophages were stimulated with LPS (0.33 ng/ml), effluent and LBP inhibitory peptide. The concentration of TNF-α was significantly increased after stimulation, when effluent was used in addition to LPS. The elevation of TNF-α was inhibited markedly when adding the LBP inhibitory peptide..Data are representative of 3 independent experiments. Data are shown as mean ± SD.

5.2.2.3 WI/R causes release of LBP into serum

To assess the extracellular release of LBP after WI/R injury, serum LBP concentrations were quantified using the newly developed ELISA-Assay. LBP was released into the serum as early as 0.5 h, and reached a peak 6h after reperfusion (0.5 h: 13.83 ±1.39 µg/ml, 6 h: 45.00 ± 9.20 µg/ml, 24h: 20.30 ± 7.16 µg/ml, vs. normal controls: 2.05 ± 1.00 µg/ml, $p<0.05$). We also detected the hepatic LBP-mRNA and protein expression after WI/R by qPCR and western blot, respectively. A significant elevation of hepatic mRNA (fold increase to normal: 7.16 ± 1.93, $p<0.01$) and protein (fold increase to normal: 3.27 ± 2.00, $p<0.05$) was observed after 6h, and increased with reperfusion time up to 24 h after reperfusion (fold increase to normal: mRNA 12.77 ± 3.04; protein: 10.30 ± 7.69, $p<0.01$) (Figure 5.8).

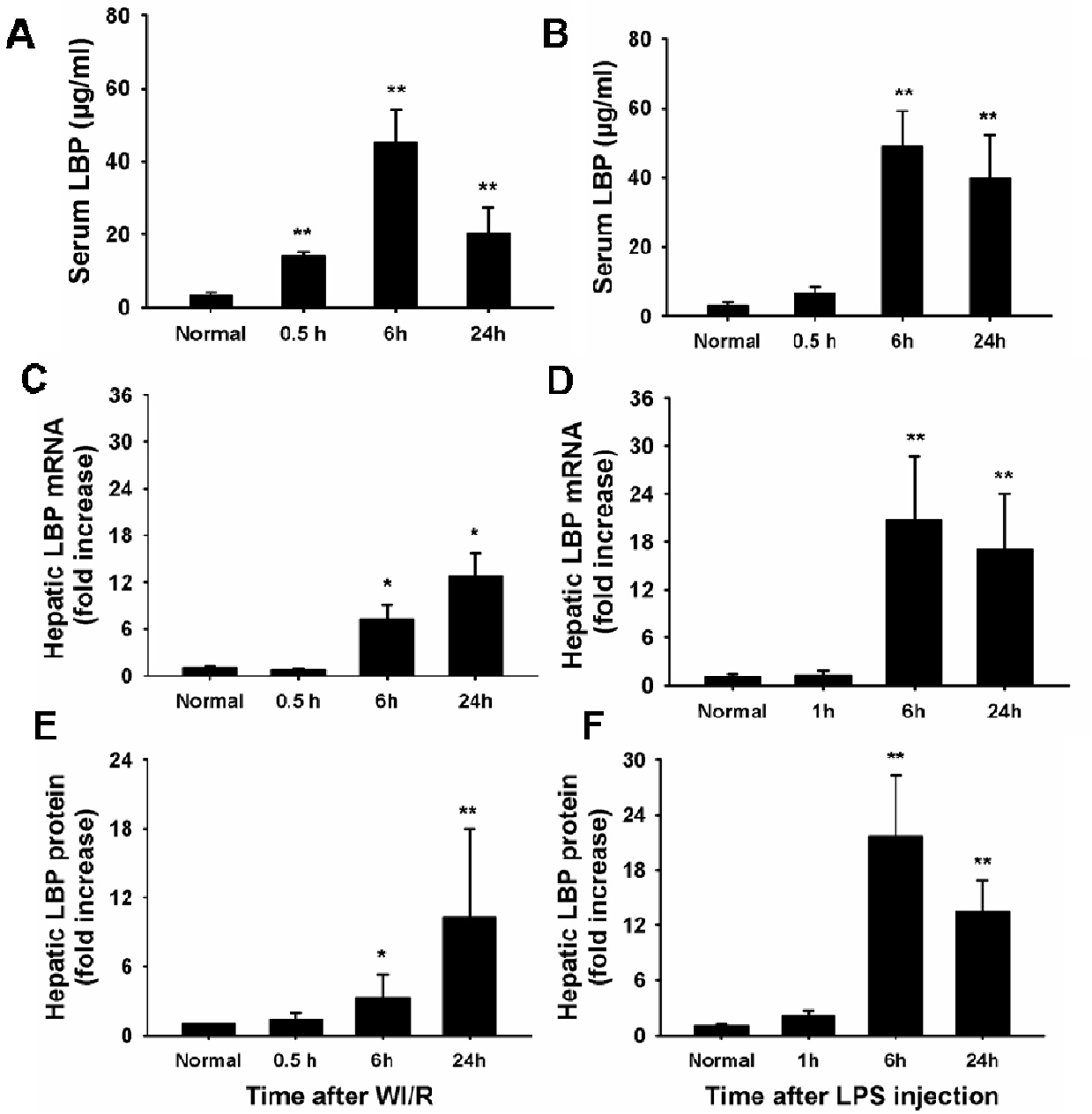

Figure 5.8 Elevation of serum LBP levels after WI/R or LPS injection

Serum LBP levels were measured by ELISA. Upregulation of serum LBP levels was observed after WI/R (A) and after LPS injection (B) and followed a similar kinetic. After WI/R, Serum LBP levels were significantly increased as early as 1h, reached a peak at 6h and remained high 24h after reperfusion. The expression of hepatic LBP mRNA (C, D) and protein was investigated by qPCR and western blot (E, F), respectively. Hepatic LBP mRNA and protein expression was significantly increased after 6h of reperfusion. *$p<0.05$;**$p<0.01$, compared with normal controls. Data are shown as mean ± SD, n= 6 per group.

5.2.2.4 LTx causes release of LBP into serum

To determine whether LBP expression was upregulated after LTx, rats were subjected to LTx, and serum and hepatic LBP were detected. Serum LBP levels were elevated approximately ten fold at 24 h after LTx using ELISA-assay (24 h: 24.90 ± 3.47 µg/ml, vs. normal controls: 2.05 ± 1.00 µg/ml, $p<0.01$). In parallel, the hepatic expression levels of LBP, both mRNA and protein, were also significantly upregulated, almost reaching 10-fold, at 24 h after LTx (fold increase to normal: mRNA 9.08 ± 3.01, $p<0.01$; protein: 6.59 ± 5.18, $p<0.05$) (Figure 5.9).

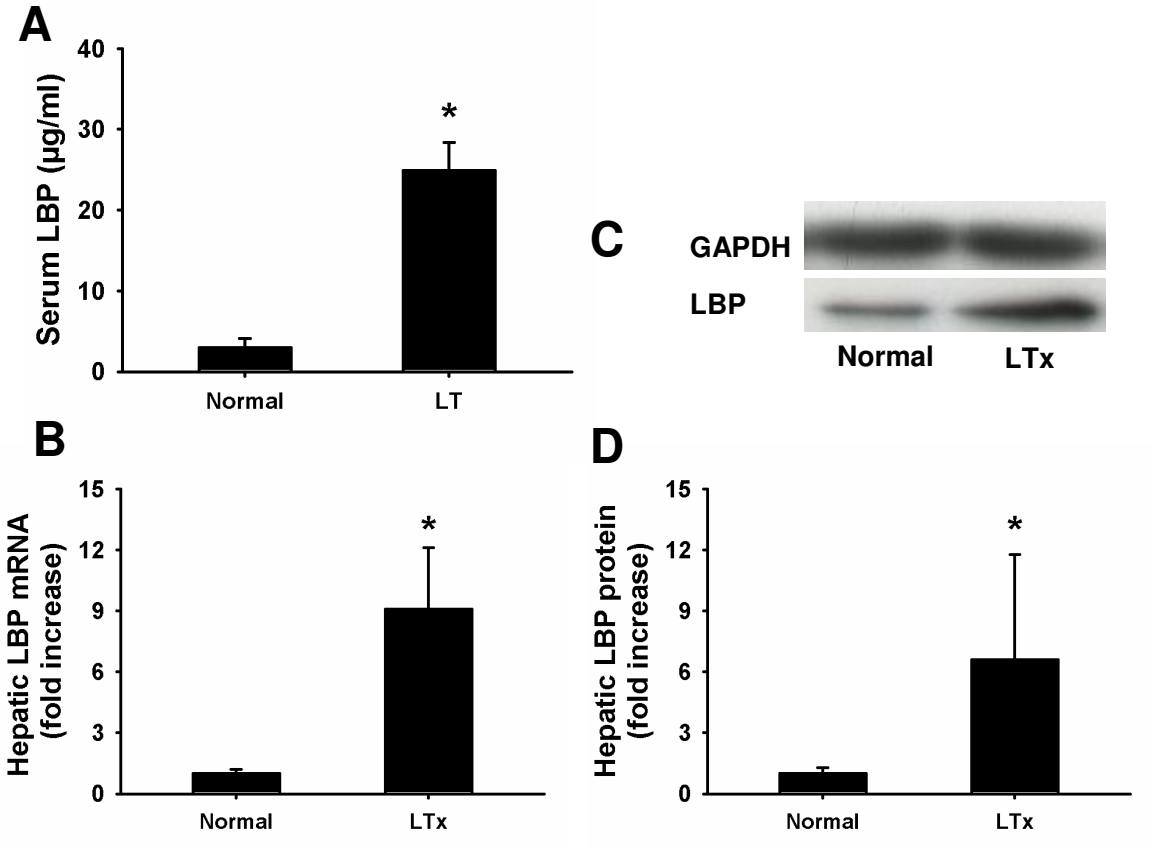

Figure 5.9 Elevation of serum LBP levels after LTx

LBP levels were observed 24h after rat liver trasplantation. Serum LBP (A), hepatic LBP mRNA (B) and protein (C, D) were measured using ELISA, qPCR and western blot, respectively. After LTx, significant upregulation of both serum and hepatic LBP levels were observed. *$p<0.001$, compared with normal controls. Data are shown as mean ± SD, n= 6 per group.

5.2.2.5 LTx causes translocation of LPS into hepatocytes

To determine whether LPS was translocated after LTx, LPS immunohistochemical staining was employed in normal liver tissue and in livers subjected to 6 h CI, LTx and 24 h observation time. As shown in Figure 5.10, no positive staining signals were detected in normal liver tissue. However, in liver sections obtained after LTx, cytoplasmic staining in almost all hepatocytes, but not in other cell types became visible. This finding suggested that the LPS did translocate to the liver and was internalized into hepatocytes.

5.2.2.6 LBP upregulation is associated with post-operative inflammation

The expression of mRNA of TNF-α, IL-6 and IL-1ß was determined by qPCR. As expected, an increased expression of hepatic TNF-α, IL-6 and IL-1ß was observed after LTx in comparison to the control group (Figure 5.10).

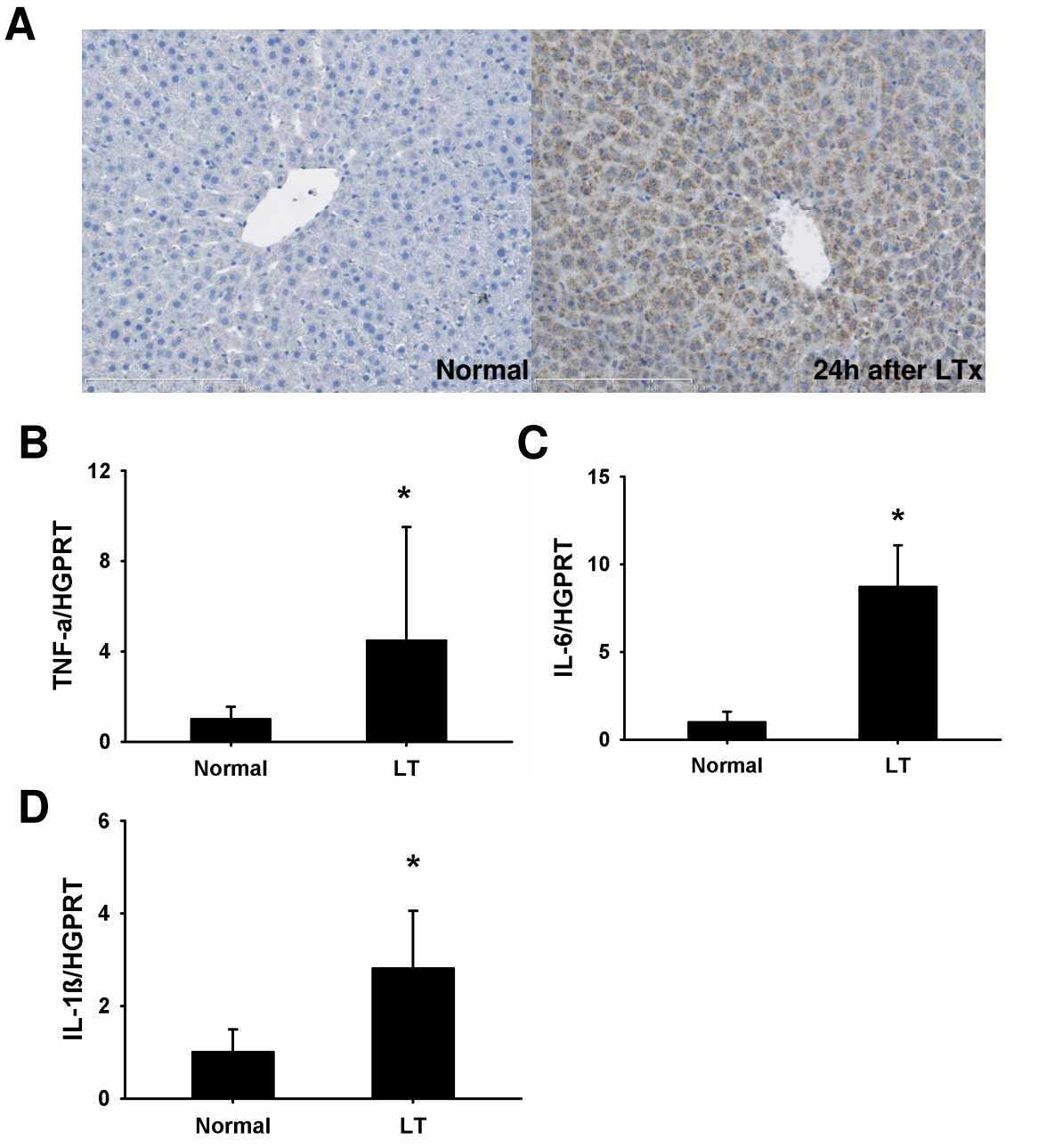

Figure 5.10 Translocation of LPS and expression of hepatic inflammatory cytokines after LTx

Strong cytoplasmic signals were detected in almost all hepatocytes after Ltx but not in livers from naïve animals suggesting translocation of LPS after Ltx (n=6 animals) (A). TNF-α, IL-6 and IL-1ß mRNA expression levels were measured by qPCR in normal liver tissue, and tissue obtained after LTx. mRNA levels for TNF-α (B) IL-6 (C) and IL-1ß (D) were significantly elevated after LTx. *p<0.05 vs normal controls. Data are shown as mean ± SD, n= 6 per group. Representative images from 6 rats per group were selected.

5.3 G-CSF induced LBP expression is deleterious in LPS-SIRS model

5.3.1 G-CSF pretreatment induces LBP expression

To investigate whether G-CSF pretreatment could enhance LBP expression, we firstly investigated the LBP gene expression after G-CSF pretreatment in different organs of rats. LBP was ubiquitously expressed in observed organs of rats. Interestingly, a significant elevation of LBP expression was observed after G-CSF pretreatment ($p<0.001$). The result was confirmed by the LBP staining in the smear of bone morrow cells (Figure 5.11). Moreover, the G-CSF pretreatment also led to an about 3-fold increase of both hepatic and serum LBP-protein levels (Figure 5.12).

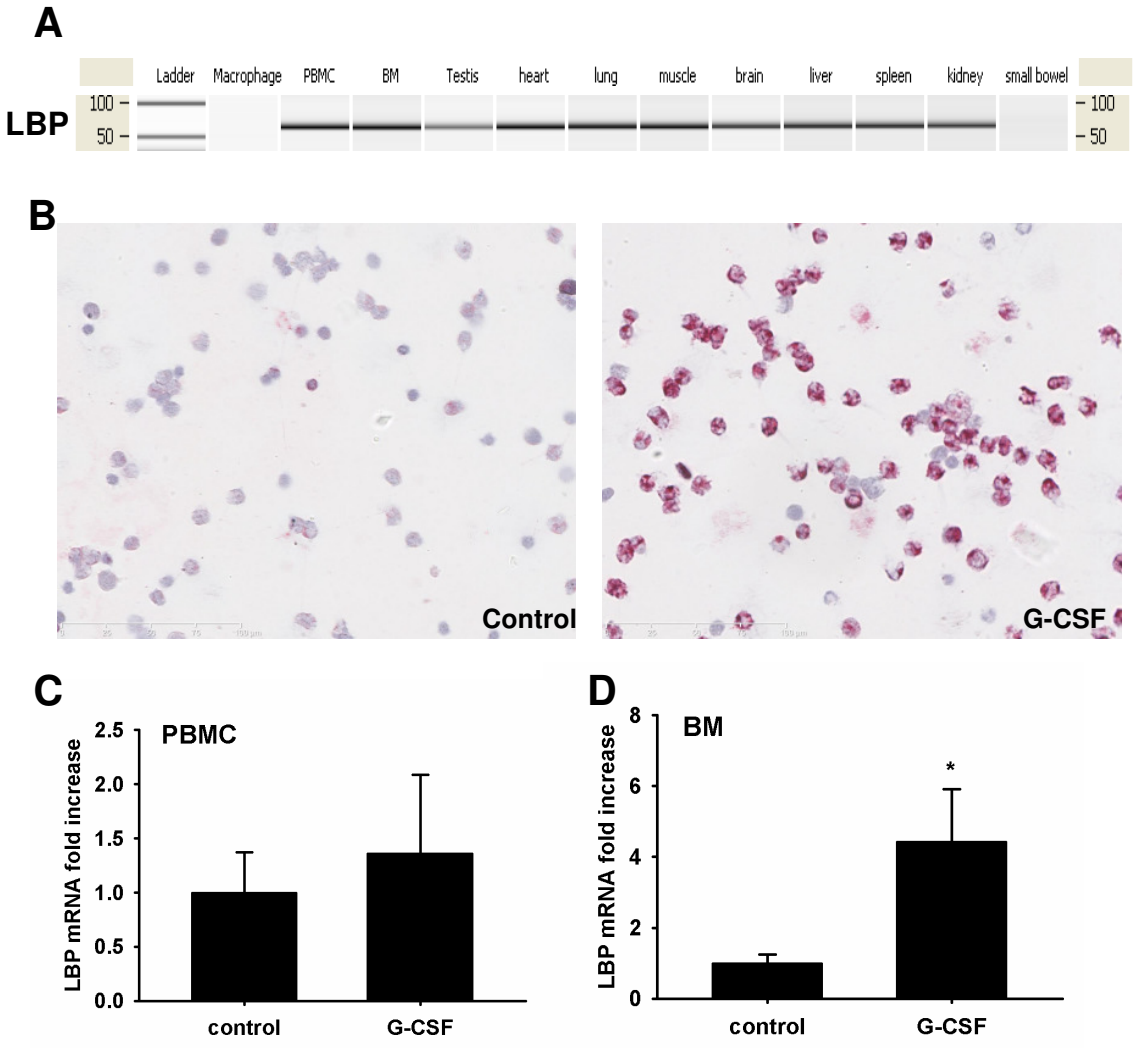

Figure 5.11 G-CSF pretreatment induces the expression of LBP in granulocytes in rats

(A) Expression of LBP mRNA was detected by qPCR in various organs of rats. The gene expression of LBP was upregulated ubiquitously except in macrophages. (B) After G-CSF pretreatment, the expression of LBP was upregulated in BM cells. (C) Expression of LBP was increased slightly in blood mononuclear cells but significantly in bone marrow cells ($p<0.001$). Data are shown as mean ± SD, n= 6 per group.

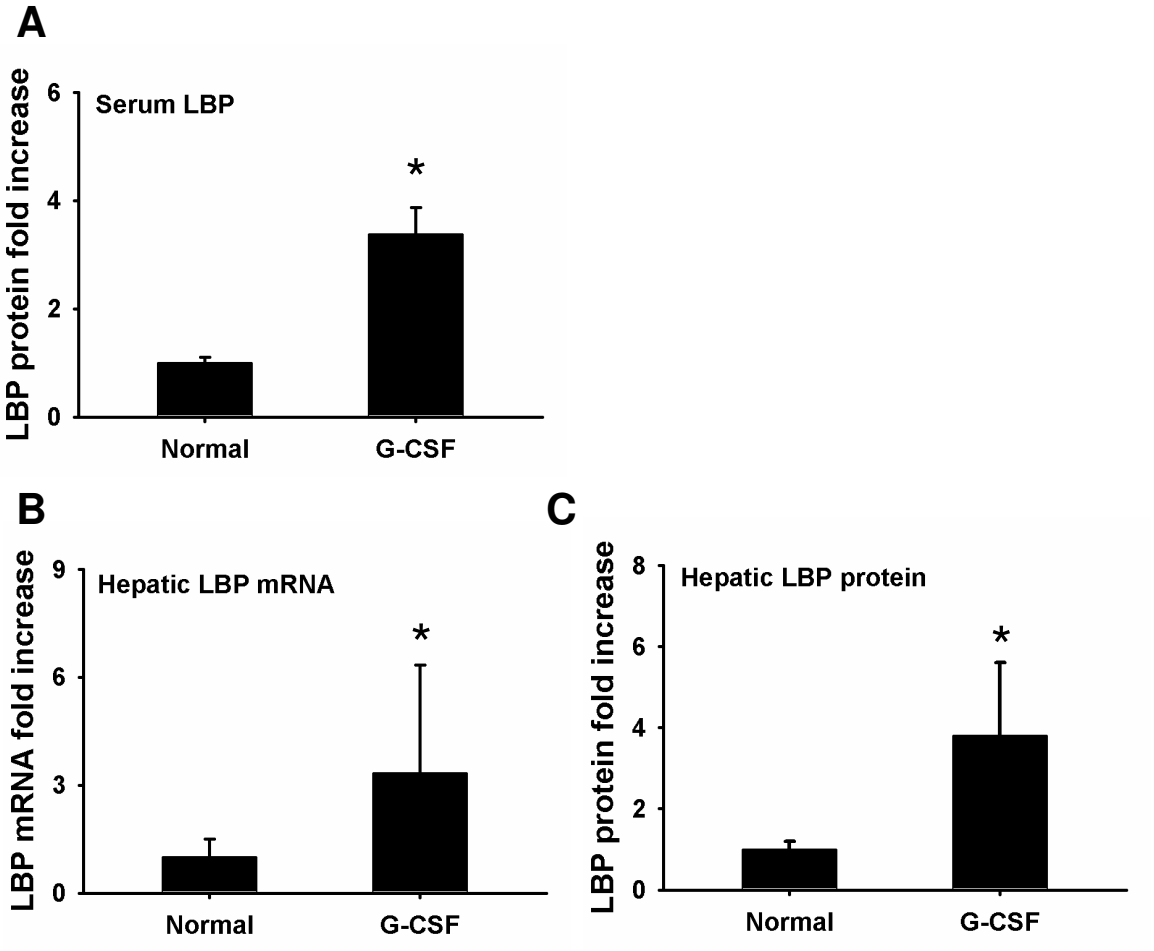

Figure 5.12 G-CSF pretreatment upregulates hepatic and serum LBP levels

Hepatic LBP mRNA, protein and serum LBP levels were measured using qPCR and western blot in normal rats and rats pretreated with G-CSF, respectively., Low or constitutive LBP levels were detected in normal rats. In G-CSF pretreated rats, 3-fold increase of both hepatic and serum LBP levels were observed. *$p<0.05$ vs normal controls. Data are shown as mean ± SD, n= 6 per group.

5.3.2 G-CSF induced LBP expression sensitizes to a subsequent LPS challenge

We demonstrated that G-CSF pretreatment induced the LBP expression. Next, we tested the effect of elevated LBP prior to LPS challenge. Of note, the G-CSF induced LBP expression caused mortality to a subsequent LPS challenge. As shown in Figure 5.13, all rats survived after LPS administration. However, in G-CSF pretreatment group, all rats died within 6h after the same LPS challenge. To further confirm whether the impaired survival rate resulted from LBP elevation, we treated rats with LBP inhibitory peptide (LBPK95A) 2h before LPS injection to block the interaction between LBP and LPS. As shown in Figure 5.13, rats treated with LBP inhibitory peptide did not experience the deleterious effect as did those in the G-CSF pretreated group. A survival advantage was observed when rats underwent LBP blockade prior to LPS challenge. Administration of LBPK95A increased survival rate and prolonged survival time to 18 hours ($p<0.001$).

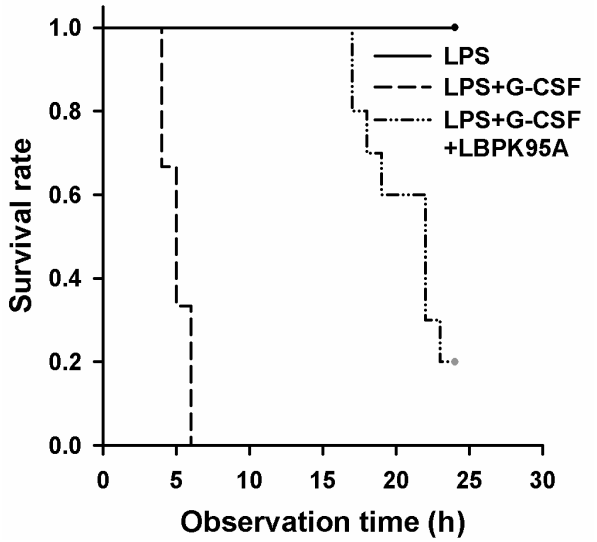

Figure 5.13 G-CSF induced LBP expression is associated with death of rats

Up-regulation of LBP via G-CSF was associated death of all pretreated rats upon the LPS-challenge. All rats died within 6h after LPS-injection in LPS+G-CSF. Survival rate was significantly increased in rats treated with the LBP inhibitory LBPK95A peptide ($p<0.001$). Data are shown as mean ± SD, n= 6-10 per group.

5.3.3 G-CSF induced LBP expression is associated/causes severe liver damage

The results of the assessment of liver damage paralleled the survival data. Severe liver injury was demonstrated by significantly increased AST levels in G-CSF+LPS group. In contrast, serum AST levels were significantly reduced in rats treated with LBP inhibitory peptide LBPK95A (G-

CSF+LPS+ LBPK95A: 524.50 ± 301.66 U/L vs. G-CSF+LPS 5413.50 ± 3951.13 U/L, $p < 0.005$). The results were confirmed by the histological evaluation. As shown in Figure 5.14, in G-CSF+LPS group, severe bleeding, sinusoidal congestion and infiltration were present in liver tissues from rats that were treated with G-CSF+LPS, whereas minimal damage was noted in liver tissues from G-CSF+LBPK95A+LPS-trated rats. Blockade of LBP using the inhibitory peptide resulted in less liver damage, indicating that pretreatment with LBP inhibitory peptide to LBP protected against LPS induced liver injury.

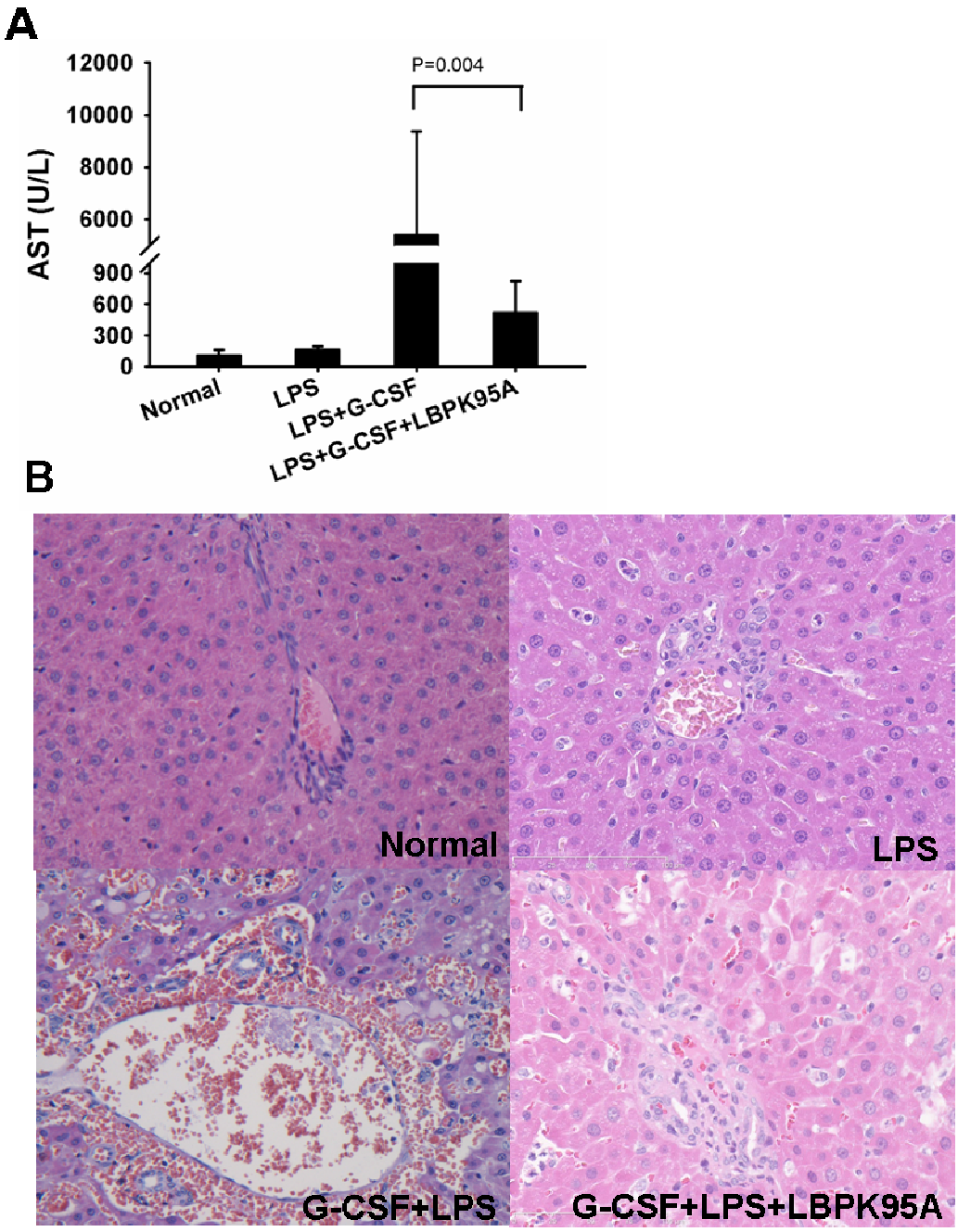

Figure 5.14 G-CSF induced LBP expression increases the liver injury

Blocking the elevated LBP reduced hepatic damage as indicated by substantially lower liver enzymes (A) and moderate histological damage (B). Untreated animal died within 8h and showed severe histological damage such as confluent necrosis and bleeding. Animals treated with both, GSCF and peptide showed less damage. Data are shown as mean ± SD, n= 6 per group. Representative images from 6 rats per group were selected.

5.3.4 G-CSF induced LBP expression enhances expression of inflammatory cytokines

Inflammatory cytokines, such as TNF-α, IL-6 and IL-1ß, play an important role in the pathophysiology of hepatic injury in SIRS and sepsis. The expression of mRNA for these cytokines in liver was measured by qPCR. These cytokines were significantly increased 1h and 6h after LPS administration in G-CSF+LPS groups. However, animals that were treated with LBP-inhibitory peptide LBPK95A exhibited lower increases in hepatic TNF-α, IL-6 and IL-1ß mRNA levels (Figure 5.15).

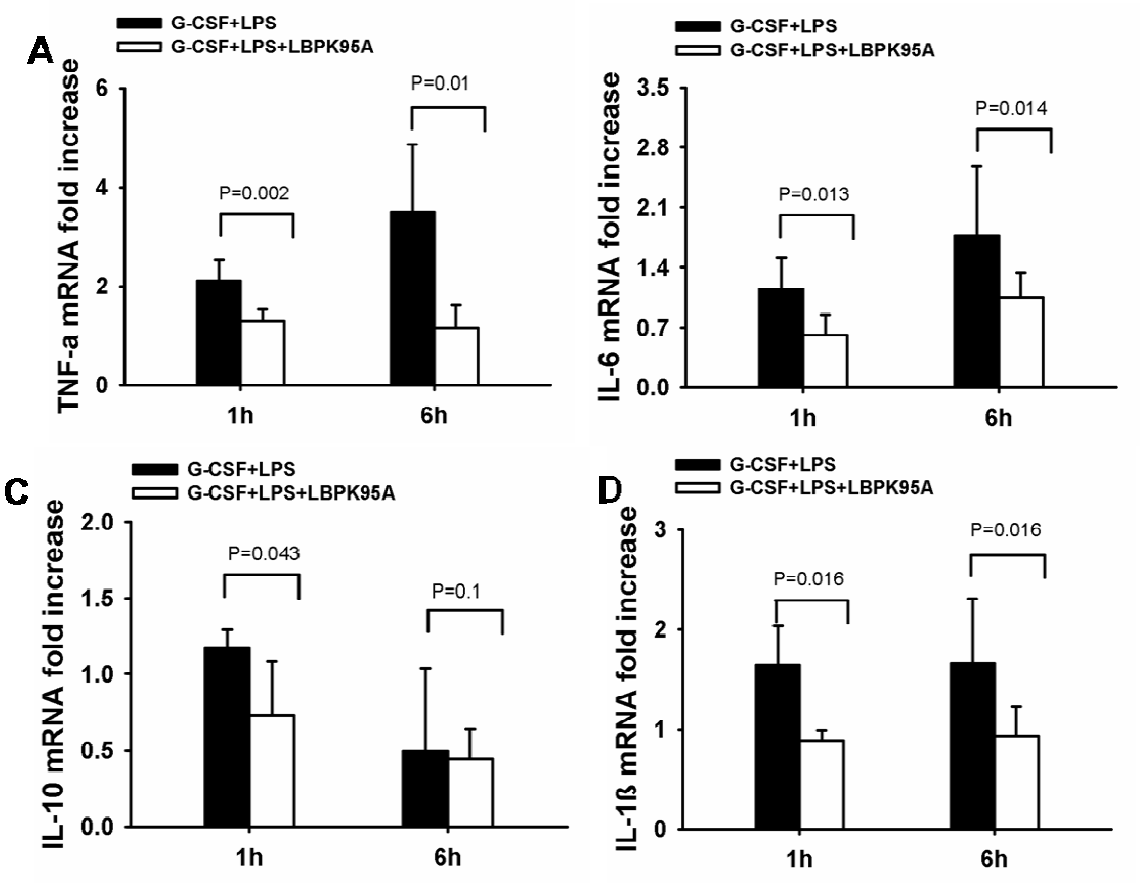

Figure 5.15 G-CSF induced LBP expression amplifies the LPS induced inflammatory response

Up-regulation of LBP-levels prior to the LPS-challenge via G-CSF caused an overt inflammatory response, as indicated by an increased of hepatic mRNA expression of TNF-α (A), IL-6 (B), IL-10 (C) and IL-1ß (D). Pretreatment with the LBP inhibitory peptide LBPK95A prior to the LPS-challenge significantly reduced the mRNA expression of these pro-inflammatory cytokines. Data are shown as mean ± SD, n= 6 per group.

5.3.5 G-CSF induced LBP expression enhances the LPS-binding in the liver

G-CSF induced LBP expression caused systemic inflammation and death of animals subsequent to a LPS-challenge. To determine whether the upregulated LBP could enhance LPS trapping in the liver, LPS immunohistochemical staining was performed in livers tissues that were obtained from GSF +LPS treated rats or LPS only treated rats, respectively. As shown in Figure 5.16, LPS-binding to the liver was visible within 5 min after LPS injection. The staining intensity of LPS increased with time in both groups. Furthermore, the staining was accentuated in zone 2. The intensity of LPS staining in G-CSF+LPS group was higher than in non-treated group throughout the observation period. The maximal staining intensity was observed 35min after injection. Both the staining intensity and the number of positive cells in G-CSF+LPS group were obviously higher than in LPS only group.

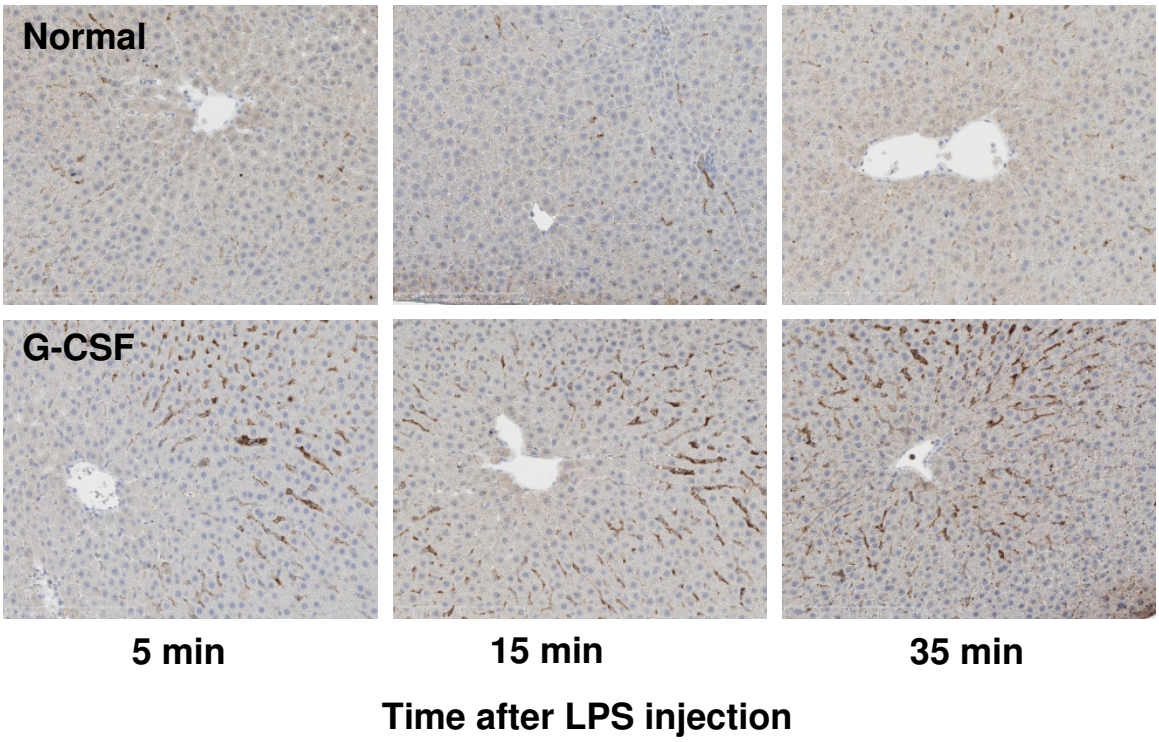

Figure 5.16 G-CSF induced LBP expression increases LPS-binding to the liver

Immunohistochemical staining for LPS in liver tissues from rats treated with LPS with or without G-CSF pretreatment (original magnification ×200). Compared to G-CSF non-pretreatment group, the LPS positive staining was significantly increased in G-CSF pretreatment group as early as 5 min after LPS injection.

5.3.6 G-CSF induced LBP expression aggravates the LPS associated liver injury after liver resection

Studies have demonstrated that bacterial translocation and an increased responsiveness to LPS were observed after liver resection (Xu et al., 2007). In our previous study, we demonstrated that increased serum LBP levels were observed after 90% PH (Ji et al., 2009). To determine whether the increased responsiveness to LPS mainly was mediated by LBP, we pretreated rats with G-CSF and then performed 70% PH, which caused an elevation of LBP both in systemic circulation and liver. As shown in Figure 5.17, the G-CSF induced LBP expression markedly shortened the survival time (all rats died within 2-4 hour) and aggravated the liver damage, indicated by significant increase of AST levels (G-CSF+LPS+70% PH: 3503.75 ± 948.95 U/l vs. LPS+70% PH: 2044.40 ± 663.47 U/l, $p<0.05$). This result was confirmed by histological findings. As shown in Figure 5.17, in LPS+PH group, moderate sinusoidal congestion and infiltration were observed. In G-CSF+LPS+PH group, the maximum liver damage was indicated by severe sinusoidal congestion and pronounced signs of bleeding. This finding suggested that the peri-operative LBP elevation sensitized strongly to a subsequent LPS challenge in 70% PH model.

5.3.7 G-CSF induced LBP expression aggravates the LPS associated inflammatory response after liver resection

To determine whether the G-CSF induced LBP expression could enhance the LPS-induced inflammatory cytokine expression, the production of TNF-α, IL-6 and IL-1ß were examined. As shown in Figure 5.18, the G-CSF induced LBP expression augmented the expression of hepatic inflammatory cytokines. The expression of hepatic TNF-α, IL-6, and IL-1ß mRNA was significantly increased in G-CSF+LPS+70% PH group when compared with LPS+70% PH group at 1h and 6h, respectively. These data indicated that the G-CSF induced LBP expression enhanced the LPS induced inflammatory response in 70% PH model in rats.

Because the intensity of the inflammatory response also can be mediated by other LPS receptors, such as TLR4, MD2 and CD14, we then further analyzed the gene expression of these receptors. The result showed that the hepatic expressions of other LPS receptors-TLR4, CD14, and MD2-were not significantly influenced by G-CSF pretreatment (Figure 5.19).

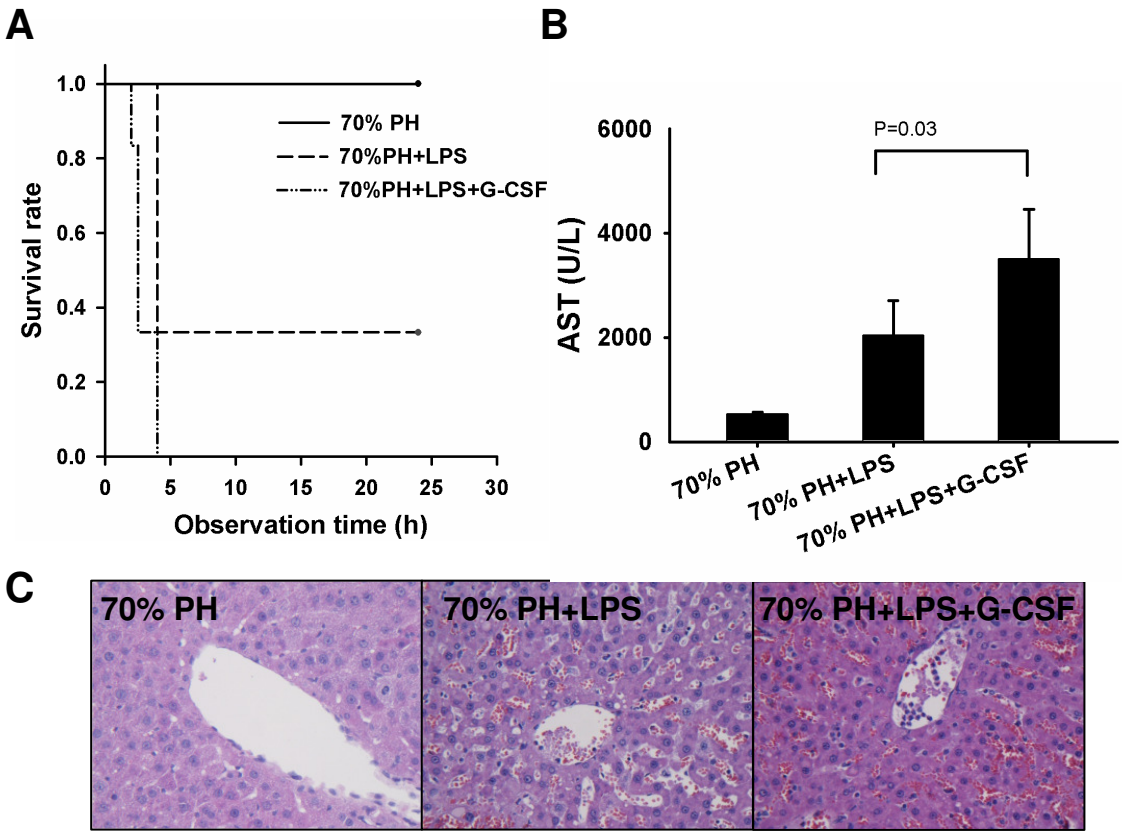

Figure 5.17 G-CSF induced LBP expression causes mortality and severe liver damage after 70% PH and subsequent to LPS-challenge in rats

The survival rate was about 35% in 70% PH +LPS group. However, the G-CSF induced LBP expression prior to 70% PH and subsequent to LPS-challenge caused death of all rats within 4h (A). G-CSF pretreatment prior to 70% PH and subsequent to LPS-challenge caused higher elevation of liver enzyme (B) and more severe histological alternation (C) when compared with 70% PH +LPS group. Data are shown as mean ± SD, n= 6 per group. Representative images from 6 rats per group were selected.

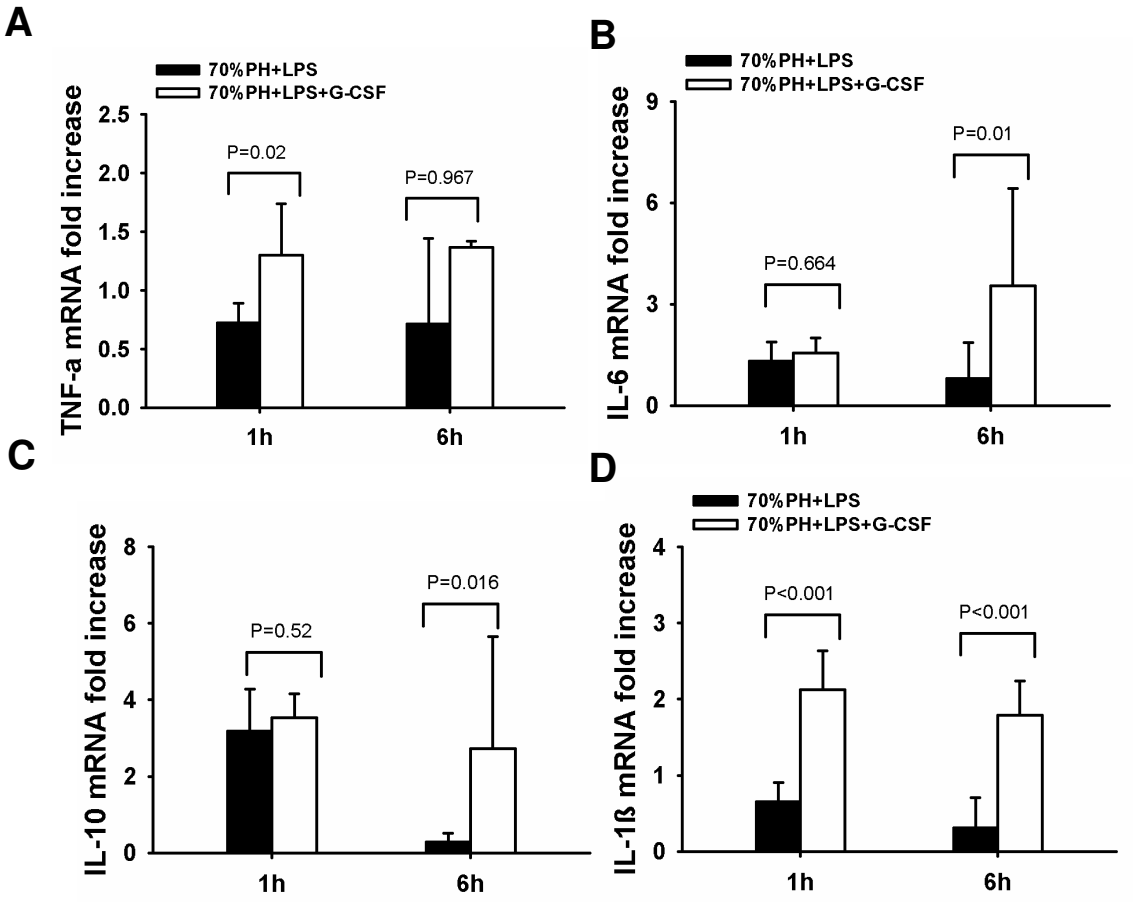

Figure 5.18 G-CSF induced LBP expression increases the expression of pro-inflammatory cytokines after 70% PH and subsequent to LPS-challenge

The expression of hepatic pro-inflammatory cytokines (TNF-α (A), IL-6 (B), IL-10 (C) and IL-1ß (D) mRNA was examined by qPCR. G-CSF induced LBP expression prior to PH and LPS-challenge enhanced the hepatic inflammatory response, indicated by the significantly higher levels of hepatic mRNA expression. Data are shown as mean ± SD, n= 6 per group.

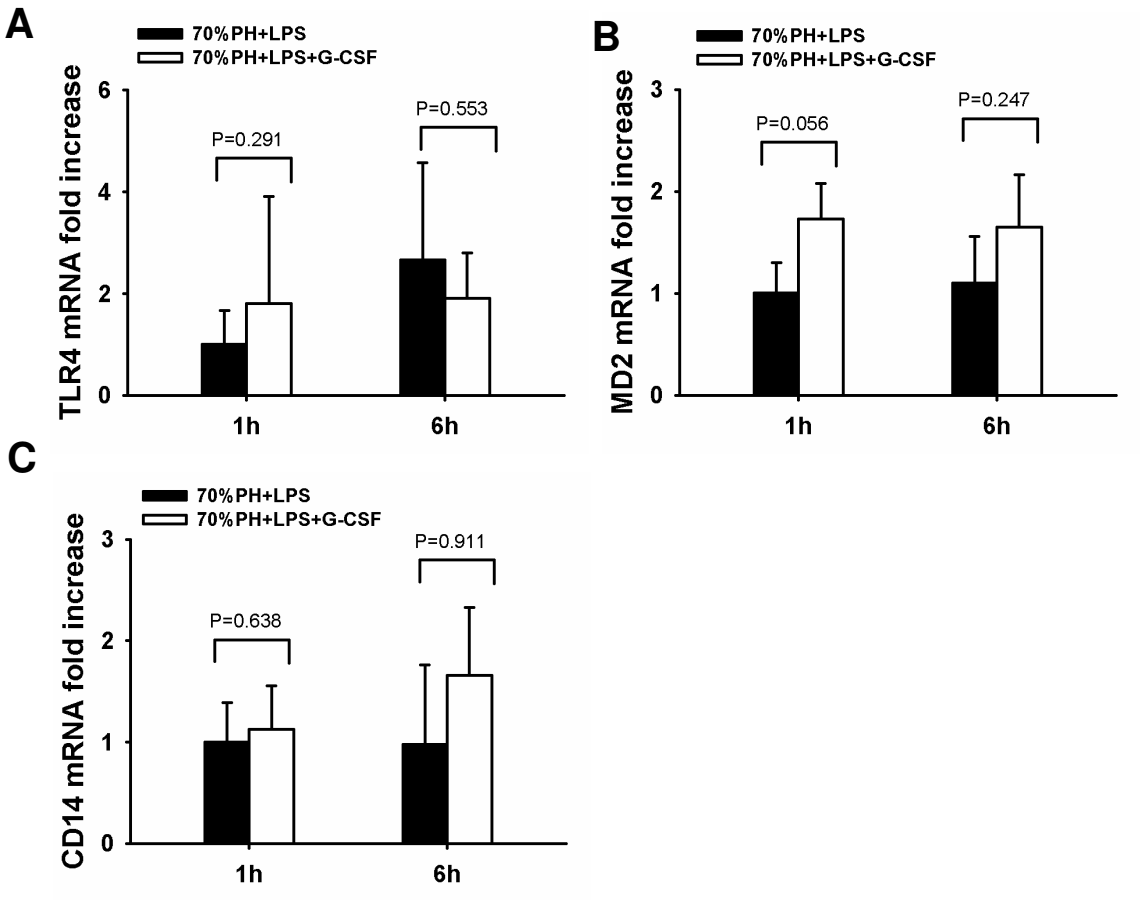

Figure 5.19 G-CSF induced LBP expression does not affect the expression of other LPS-receptors after 70% PH and subsequent LPS-challenge

Hepatic mRNA expression of LPS receptors TLR4, CD14, and MD2 was measured by qPCR, respectively. Hepatic TLR4, CD14 and MD2 mRNA levels were slightly higher in G-CSF+LPS+70% PH than in LPS+70% PH, albeit the difference between two groups did not reach statistical significance. Data are shown as mean ± SD, n= 6 per group.

5.4 G-CSF induced LBP expression modulates the inflammatory response in polymicrobial sepsis

5.4.1 G-CSF induced LBP expression improves the survival rate after septic insult

Pooled stool from three healthy non-vegetarian donors was used for inducing polymicrobial sepsis. The microbiological analysis of the stool batch was showen in Figure 5.20. The survival rate in G-CSF group was significantly higher than in control group after the septic insult ($p<0.05$, n=25 rats/group). In control group, rats began to die at as early as 14 hours after the induction of sepsis, and the mortality rate increased to 50% at 20 hour after injection. In contrast, 90% of the rats with G-CSF pretreatment survived at least 20 hours. A delayed 'death storm' occurred at 30-40 hours, but the mortality in G-CSF group was still lower than in control group. Moreover, the 7-day survival rate in G-CSF group was 56%, whereas the survival rate in the control group only reached 36% (Figure 5.21).

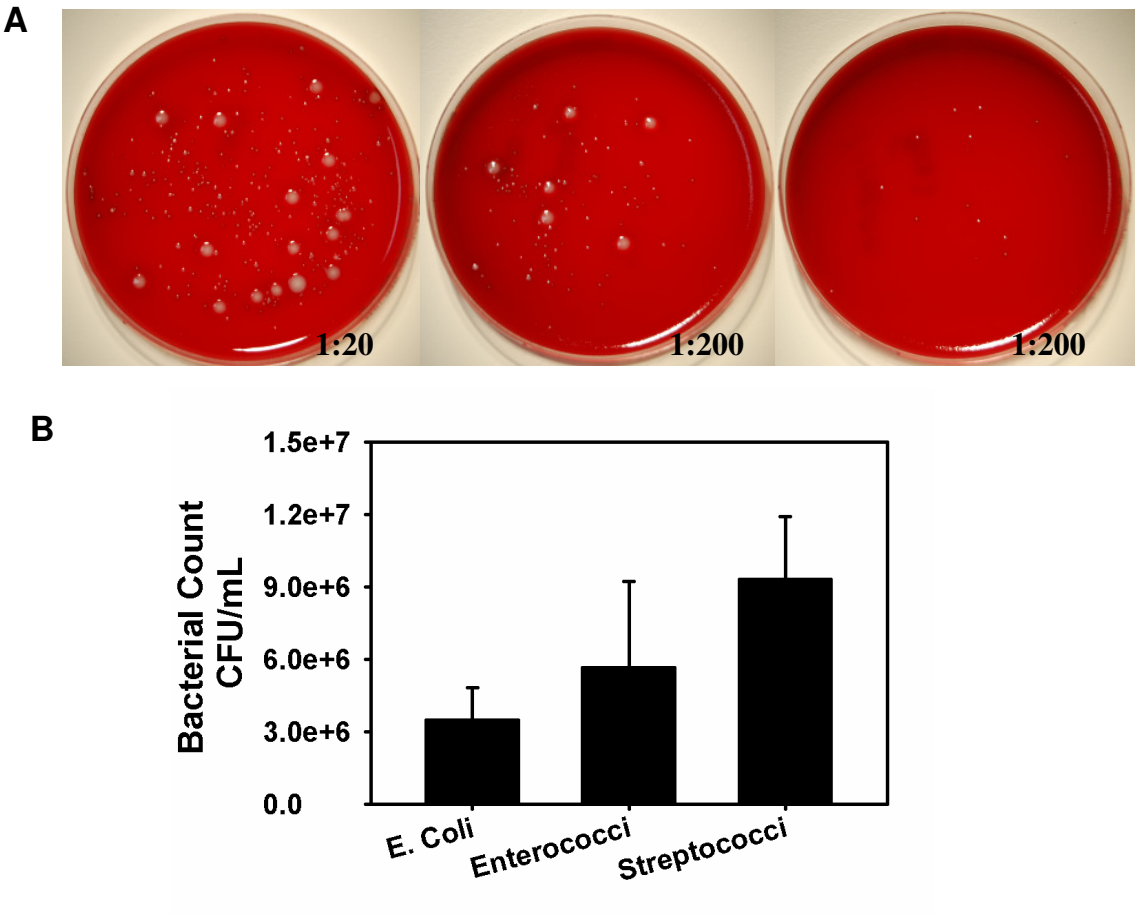

Figure 5.20 Microbiological analysis of the stool batch

The stool batch was diluted to 1:200, 1:2000, and 1:20000, and then cultured in petri dishes of blood agar (A). The microbiological composition of the stool suspension was analyzed. The major microbiological composition was streptococci. Representative images from 2 independent experiments were selected. Data are shown as mean ± SD.

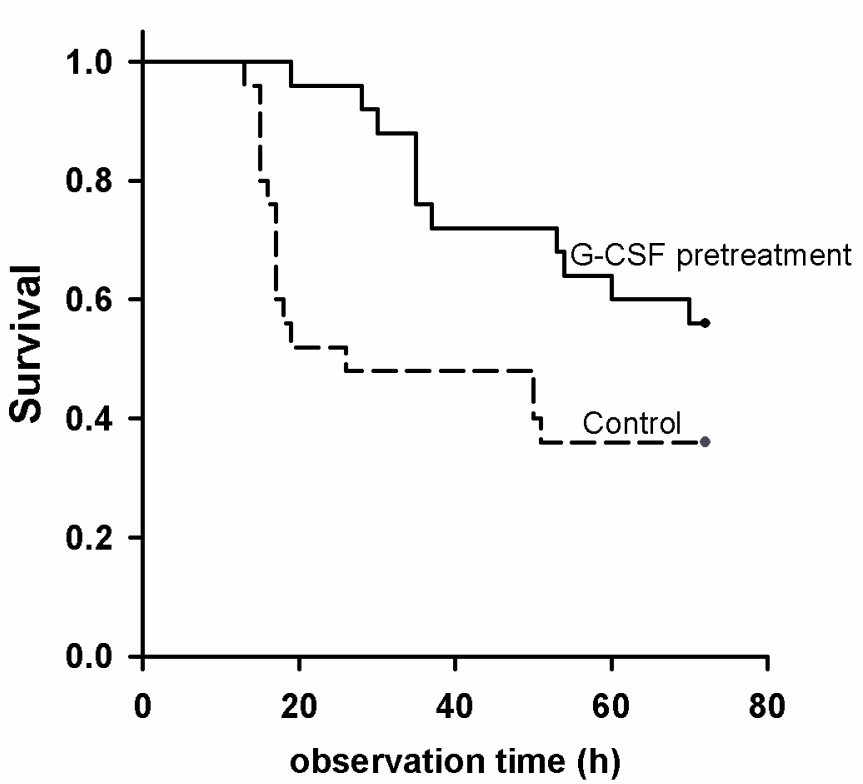

Figure 5.21 G-CSF induced LBP expression protects against the lethality in polymicrobial sepsis

In control group, 36% survival rate was observed at 72 h after the induction of sepsis, however, the survival rate was significantly increased after G-CSF pretreatment ($p<0.05$). n=25 per group.

5.4.2 G-CSF induced LBP expression enhances the hepatic neutrophil infiltration after septic insult

Injection of stool suspension resulted in a time-dependent induced ascites and increased of the total number of cells in the fluid filled peritoneal cavity (Figure 5.22). The maximal effect was observed 12h after injection. By 2 and 12 h after injection, the total number of cells in G-CSF group increased to about 2-fold and 4-fold over control, respectively. Differential cell counting revealed that this increase could be accounted for by a substantial rise in the number of polymorphonuclear neutrophils (PMN). In G-CSF group, the influx of PMN was significantly increased when compared with control. The ascites volume in two groups had no difference.

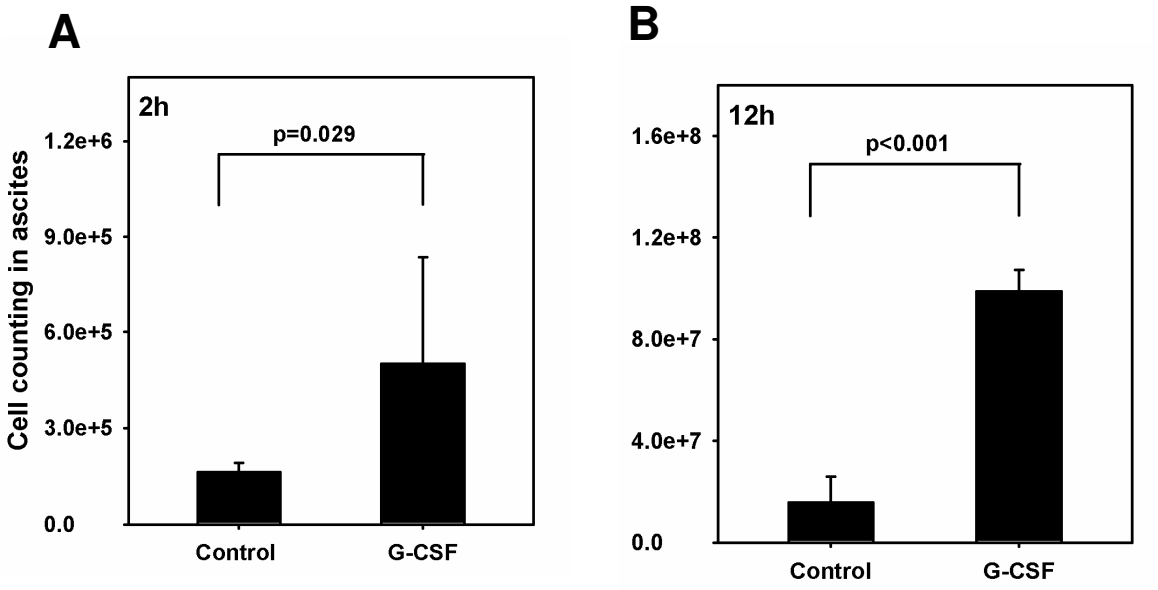

Figure 5.22 G-CSF induced LBP expression increases the influx of neutrophil in the peritoneal cavity

The total number of cells in the ascites was calculated 2h and 12 h after the septic insult, respectively. The influx of neutrophil in the peritoneal cavity was increased in both groups after injection. However, G-CSF pretreatment significantly increased neutrophil infiltration when compared with control group, while the ascites volume in two groups had no difference ($p<0.05$). Data are shown as mean ± SD, n= 4 per group.

5.4.3 G-CSF induced LBP expression decreases the production of inflammatory cytokines

Inflammatory cytokines, such as IL-6 and TNF-α, act as major player in sepsis. The levels of these cytokines in ascites were measured using ELISA-assay. As shown in Figure 5.23, TNF-α levels, as well as IL-6 levels were increased at 2 and 12h, and the highest levels were observed at 12h. Both TNF-α and IL-6 levels in G-CSF group were significantly lower than in control group 12 h after the induction of sepsis. HMGB1 was recently identified as an inflammatory cytokine that is involved as a late mediator in sepsis. Ascites-HMGB1 levels were also measured using ELISA-assay, as shown in Figure 5.23C, HMGB1 increased along with the observation time after injection time and the maximal levels were observed at 12 h. Of note, the concentration of HMGB1 in G-CSF group was significantly lower than in control group at 2h.

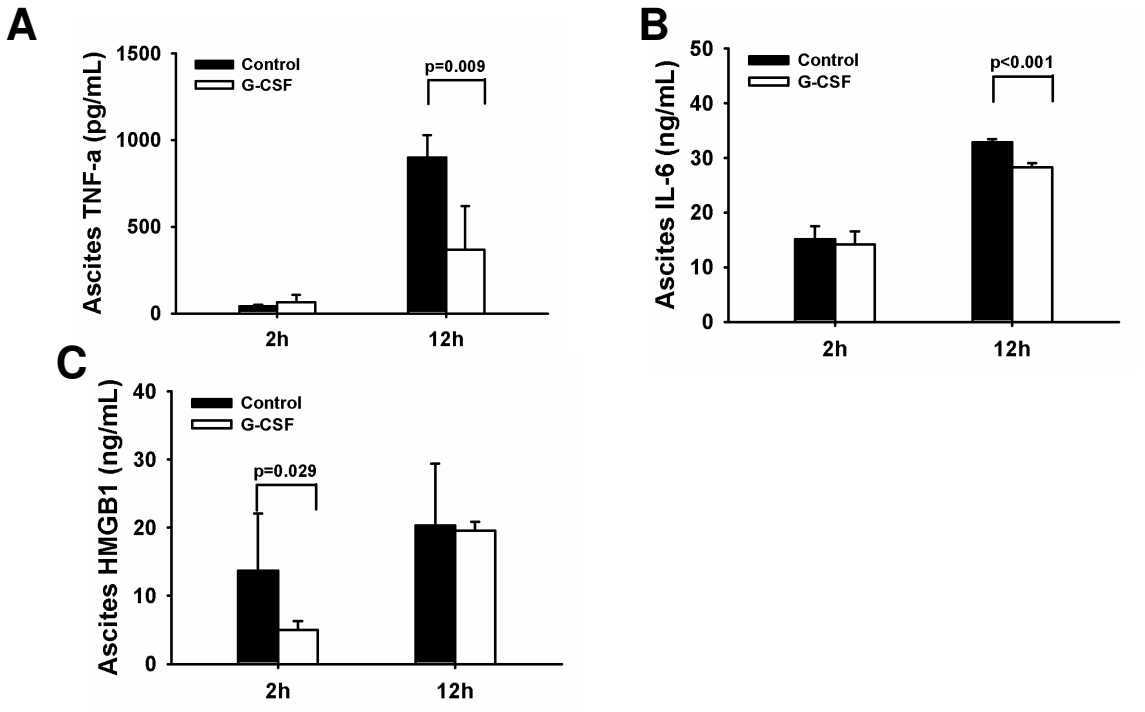

Figure 5.23 G-CSF induced LBP expression attenuates the production of pro-inflammatory cytokines in ascites

The concentration of pro-inflammatory cytokines (TNF-α, IL-6, and HMGB1) in ascites was measured using ELISA-assay. These cytokines were increased after septic insult both in G-CSF group and control group. However, the concentration of TNF-α, IL-6, and HMGB1 was lower in G-CSF pretreatment group than in without G-CSF pretreatment group ($p<0.05$). Data are shown as mean ± SD, n= 4 per group.

5.4.4 G-CSF induced LBP expression decreases the bacterial infiltration

The protective effect of G-CSF pretreatment was also reflected in the bacterial load in different organs. As shown in Figure 5.24, the lungs harvested at 2 h were negative for bacterial growth in both groups. Bacterial infiltration in lung was observed at 12 h after septic insult, however, rats pretreated with G-CSF showed a significant lower bacteria count (con: 14.5 ± 0.8×10^6 clones/gram tissue vs. G-CSF: 2.24 ± 0.97×10^6 clones/gram tissue, $p<0.05$). We also observed bacterial infiltration in liver. G-CSF pretreatment led to a decreased bacterial infiltration in liver both at 2 h and 12 h after the septic insult (2h: con: 0.18 ± 0.06×10^6 clones/gram tissue vs. G-CSF: 0.09 ± 0.0310 $\times10^6$ clones/gram tissue, $p=0.08$; 12h: con: 14.5 ± 0.8×10^6 clones/gram tissue vs. G-CSF: 2.24 ± 0.97×10^6 clones/gram tissue, $p<0.05$). G-CSF pretreatment decreased bacterial counting in ascites at 2h and no difference was observed at 12h when compared with control group. Predominant bacteria strains in liver and lung were E. coli, enterococci, and streptococci. The data indicated that G-CSF pretreatment decreased the bacterial infiltration in lung and liver.

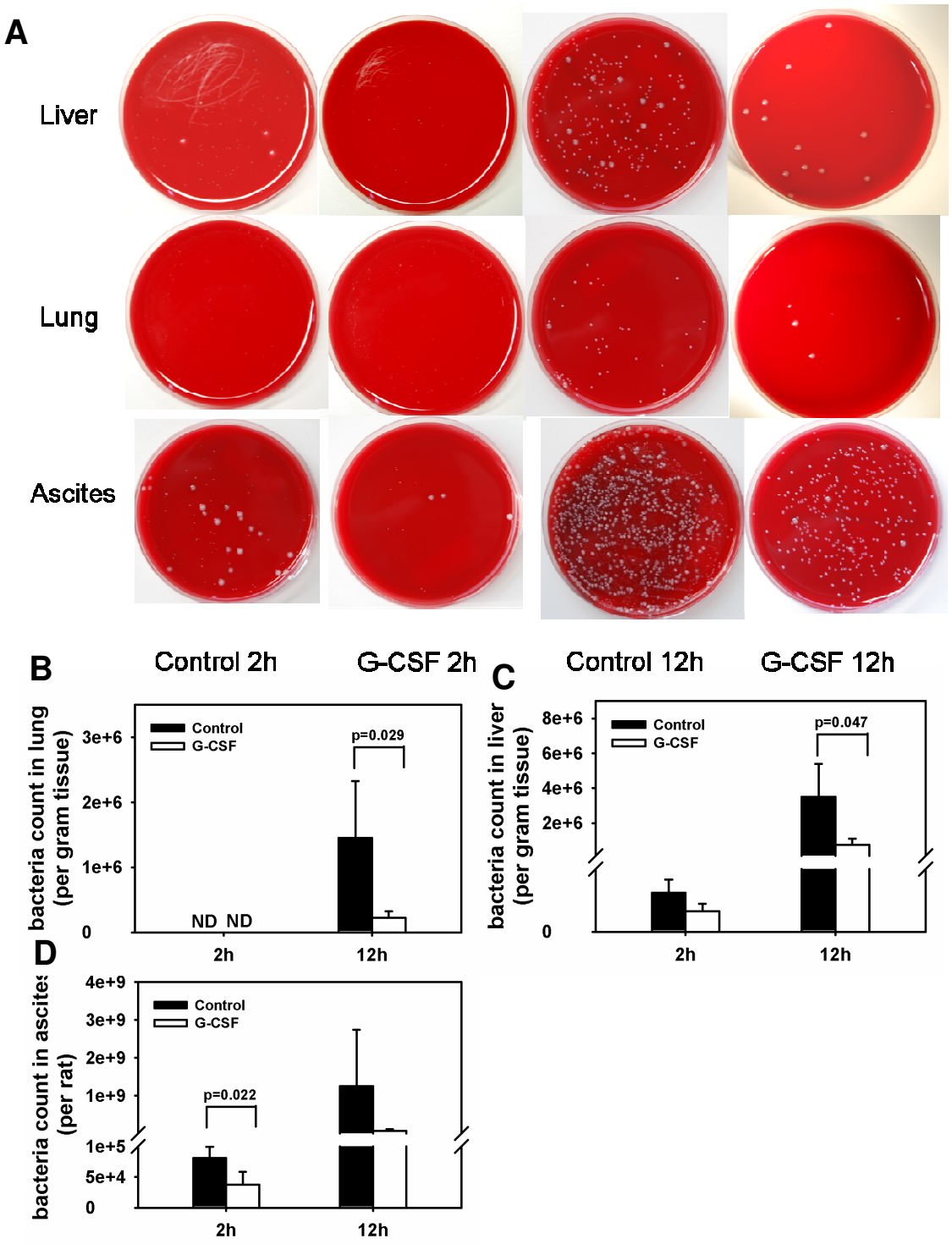

Figure 5.24 -CSF induced LBP expression decreases the bacterial infiltration in liver and lung

Bacterial infiltration was observed in liver tissue, but not in lung 2h after sepsis. At 12 h, the bacterial infiltration both in liver and lung was increased in both groups. G-CSF pretreatment decreased the bacterial infiltration in liver, ascites and lung when compared with control group ($p<0.05$). Data are shown as mean ± SD, n= 4 per group. Representative images from 4 rats per group were selected.

5.4.5 G-CSF induced LBP expression slightly decreases the production of inflammatory cytokines after recovery

Rats recovering from sepsis between 3 d and 7 d after the septic insult presented with a "clean" peritoneal cavity without ascites and normal liver enzyme levels. At d 7, animals showed no signs of illness, although moderate expression of inflammatory cytokines was present both in liver and lung. The cytokines levels at 7 d were lower in G-CSF group that in control group, albeit the difference between the two groups did not reach significance (Figure 5.25).

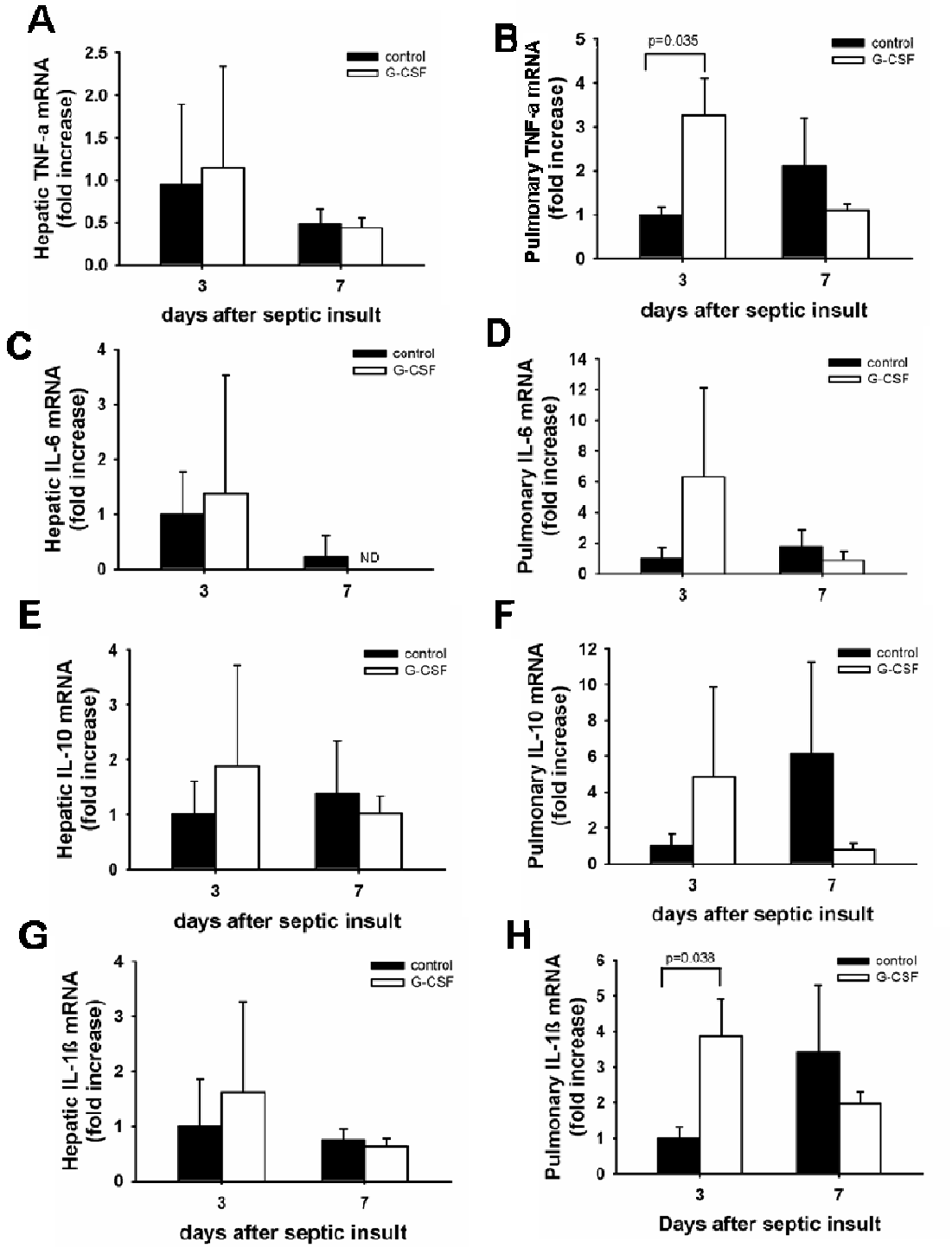

Figure 5.25 G-CSF induced LBP expression protects rats from long time recovery

The expression of hepatic and pulmonary pro-inflammatory cytokines (TNF-α, IL-6, IL-10 and IL-1ß) mRNA was examined by qPCR. G-CSF pretreatment showed slightly higher levels of these cytokines than in control group at 3 d after infection. However, the expression of these cytokines was slightly higher in control group than in G-CSF pretreatment group at 7 d after insult. Data are shown as mean ± SD, n= 4 per group.

6 Discussion

We conducted this study to better understand the role of LBP in the inflammatory response and for the development of infectious complications, SIRS and sepsis after hepatobiliary surgery.
We achieved our technical aim and established successfully a novel and economic ELISA assay to quantify LBP-protein in body fluids.
The key findings of our study can be summarized as follows:
1. We observed that LBP was an important trigger for the inflammatory response in W/I and LTx injury, but apparently not the key trigger for the inflammatory response after PH. Serum LBP-levels after PH were not related to the severity of hepatocellular injury, but to the size of the remnant liver. In contrast, the severity of the inflammatory reaction was not associated with the LBP-levels but with the size of the remnant liver. Taken together, our findings suggested that the inflammatory response after PH was not LBP dependent.
In contrast, we found that LBP was one of the triggers of the inflammatory response after I/R injury and liver transplantation. LBP released from the cold-stored organ triggered the cytokine production of macrophages in-vitro. LBP blocking peptide reduced this effect.
2. We observed that G-CSF induced LBP expression prior to a challenge with LPS aggravated the systemic inflammatory response and induced lethal SIRS, in other words, sensitized to LPS. Sensitization was even more pronounced in liver resected rats.
3. We observed in contrast, that G-CSF induced LBP expression improved the outcome of sepsis and delayed the course of disease. Bacterial load both in liver and lung was lower, as well as the local inflammatory response in terms of hepatic and pulmonary cytokine expression. However, neutrophil infiltration to the peritoneal cavity as primary site of infection was more pronounced.

6.1 Establishment of LPS-LBP ELISA assay

LBP contributes to the recognition of LPS by cells, which triggers the inflammatory response. Bacterial infections are frequently observed as complication in patients who undergo liver resection, which calls for a scientific work-up in animal experiments. However, the choice of commercially available kits for quantitative determination of rat LBP concentration in serum is limited and kits are rather costly. In the first part of this experiment, we aimed to establish a novel and economical LBP-ELISA assay method. We successfully established a LBP-ELISA assay

based on the biological binding between LPS and LBP. We detected LBP in serum after PH using the LBP-ELISA assay and western blot. The results from two methods had a high correlation.

6.1.1 Interaction between LBP and LPS

LBP is a serum glycoprotein and belongs to the lipid transfer/LBP family. Lipid transfer/LBP proteins have high-affinity of binding to lipopolysaccharide (LPS), which contributes to two apolar lipid-binding pockets on the concave surface of a boomerang structure (Beamer et al., 1997). In addition, the tip of the N-terminal domain of LBP contains a cluster of cationic residues, which are essential for the LPS binding and signaling (Jerala, 2007). Hydrophobic interactions between the LBP and acyl chains of lipid A appear to be important for the binding of LBP-LPS. As reported by Jun Kohara (Kohara et al., 2006), the binding between LPS and LBP was abolished by low concentrations of detergent. In the establishment of our assay system, the influence of detergent tween-20 was tested in different steps. We found that tween-20 affected the binding between LPS and LBP at low LBP concentrations when sample dilution buffer with tween-20, which displayed as an extra "spike" in the standard curve. We also optimized our system using different concentration LPS as capture molecules, and found that the system showed good results when the coating amount was 1 μg per well.

We defined the working range of the LBP-ELISA using purified serum LBP from rats after LPS administration 6h. We determined the working range of the LBP-assay was 0.1-60ug/ml. Accurate measurement of serum LBP can be achieved at concentrations >0.1ug/ml. We measured LBP concentrations in serum samples from rats with LPS injection and rates subjected to PH by western blot and ELISA. The results from LPS-LBP ELISA and western blot were strongly correlated.

6.2 LBP levels and liver surgery

Liver resection and liver transplantation are performed for liver diseases. Loss of liver mass renders patients increasingly susceptible to subsequent liver failure and inflammatory response (Farid et al., 2010; Jarnagin et al., 2002). Increased portal vein pressure is associated with a reduced function of the hepatic mononuclear phagocytic system, which allows spillover of gut-derived bacteria and endotoxin into the systemic circulation (Boermeester et al., 1995; Wang et al., 1994; Yeh et al., 2003).

Liver resection as well as liver transplantation is an established therapy for liver tumors. Bacterial translocation increases after liver resection. Bacterial translocation after liver resection is

explained by the impairment of intestinal barrier and bacterial overgrowth (Wang et al., 1992; Wang et al., 1993). Post-operative infection is a serious complication after liver resection and LTx (Table 6.2). Reports have demonstrated that bacterial translocation occurs in about 15-20% of patients and infections are frequently observed as complications in patients who undergo major liver resection (Balzan et al., 2007; Capussotti et al., 2009; O'Boyle et al., 1998; Wang et al., 1992; Yeh et al., 2003). Vera et al reported that infectious complications occur in approximately 50% of LTx recipients (Vera et al., 2011). Saner found that the incidence of pulmonary and blood stream infections after LTx were 8% and 24%, respectively (Saner et al., 2008). Infectious complications remain an import cause of mortality in these patients.

Increased serum levels of LBP are not limited to infections, but do also occur after surgery itself as reported by Kudlova et al. They observed that LBP levels increased subsequent to cardiac surgery (Kudlova et al., 2007). We confirmed that upregulation of LBP also occurred after partial hepatectomy, and observed that serum LBP levels were associated with the remnant liver mass. These and similar findings in the past led to the perception that LBP was an acute phase protein and prompted investigation of LBP as biomarker.

As LBP is an acute phase protein, it was discussed whether serum LBP levels might reflect the severity of the postoperative acute phase response after liver surgery. Serum LBP was elevated up to 20 µg/ml after major abdominal surgery such as gastrectomy, pancreatectomy, and colectomy, upregulation of LBP to 30 µg/ml was also observed after cardiac surgery as reported by Vollmer et al (Vollmer et al., 2009). Recently, Sakr et al reported that serum LBP concentrations were increased in patients in surgical intensive care unit (Sakr et al., 2008). Our results do not support, that postoperative serum levels are indicative of the severity of the inflammatory response.

6.2.1 LBP and liver resection

As LBP is synthesized in the liver, postoperative circulating LBP levels could also be related to the synthetic capacity of the remnant liver after surgery. Our results suggested that hepatic LBP synthesis was induced upon surgical stress such as liver surgery and eventual concomitant bacterial/endotoxin translocation, but its systemic level were related to size of the remnant liver and thereby to the synthetic capacity of the small remnant liver. In this case, postoperative LBP-levels would not reflect the severity of the acute phase response after hepatobiliary surgery, but the levels would be related to the combination of both the severity of the acute phase response and the impairment of liver function.

Our findings confirm this line of arguments. We observed that serum LBP concentrations were elevated in response to hepatobiliary surgery, but were negatively correlated to the extent of PH. This finding suggested that the systemic LBP levels were influenced by the remnant liver size. This finding leads to the conclusion that determination of serum LBP levels may not be helpful to assess the severity of the postoperative acute phase response or the risk of sepsis after liver resection or in case of an impaired liver function.

6.2.2 LBP and hepatic ischemic injury and LTx

In this experiment, we provided additional evidence for the importance of LBP as inflammatory mediator by investigating the role of LBP after LTx and I/R injury. We demonstrated that LBP was upregulated during CI storage in vitro and was also increased after WI/R and LTx in vivo.

The capacity of LBP, released by the ischemic liver, to augment an inflammatory response in vitro was determined in the macrophage stimulation assay and further clarified by the attenuation of the LPS induced inflammatory response via LBP inhibitory peptide. Our finding is inline with the observation of Lamping et al., who reported that LBP augmented the LPS-induced inflammatory response in-vitro, indicated by an increased in TNF-α level of cultured macrophages (Lamping et al., 1998). These observations were further supported by Arana et al (Arana et al., 2003). They reported that the release of TNF-α was inhibited when using the LBP inhibitory peptide. These results were confirmed by others in-vivo. Su et al. found that blocking LBP-LPS interactions using LBP inhibitory peptide protected from liver injury induced by acetaminophen induced hepatotoxicity (Su et al., 2010). Minter et al. reported that high LBP-levels were deleterious in a mouse bile duct ligation (BDL) model, where LPS levels were presumingly high (Minter et al., 2009). In a separate study, we observed that the inflammatory response to LPS was massively enhanced in animals with elevated LBP-levels induced by G-CSF pretreatment. In the same study, we found that the use of the inhibitory peptide attenuated inflammation and improved the outcome.

LPS and bacterial translocation are observed after liver transplantation, as reported by Yokohama (Yokoyama et al., 1989) in patients and by Moritaka (Goto et al., 1992) in an experimental study. In the present study, we detected intracytoplasmic signals for LPS in hepatocytes by immuno-histochemical staining indicating that LPS was translocated to the cytoplasm of hepatocytes after liver transplantation. We could demonstrate that LPS translocation was associated with an increased hepatic expression of LBP. In our experiment, we observed that increased LBP levels were associated with an enhanced inflammatory response induced by LPS. Hiki et al

demonstrated that a significant increase of serum LBP levels was observed in patients after major abdominal surgery, and correlated with the systemic levels of inflammatory cytokines (Hiki et al., 2000). Both observations imply that LBP may play a role in regulating the biologic activity of circulating LPS.

In conclusion, as LTx caused LPS-translocation as well as upregulation of LBP, this pro-inflammatory pathway could contribute to the ischemia-reperfusion associated inflammatory response. Interfering with this pathway by blocking LBP could represent a novel anti-inflammatory strategy in this LTx setting.

6.3 G-CSF induced LBP expression is deleterious in LPS induced SIRS both in naïve rats and in liver resected rats.

To reduce the sepsis and SIRS in patients after liver resection, investigating of the mechanism in LPS induced liver injury is important. Our present findings strongly suggested that G-CSF induced LBP expression was associated with an enhanced liver injury and mortality to a subsequent LPS challenge in naive and even more in liver resected animals. Of note, the expression of inflammatory cytokines, liver damage and mortality was attenuated by blocking LBP using inhibitory peptide in LPS-induced SIRS model. These data confirm that the G-CSF induced LBP expression plays an important role in initiation and aggravation of the LPS-induced inflammatory response. Given that the enhanced mortality of rats was associated with the peri-operative upregulation of LBP in in naive and even more in the 70% PH-model, the preoperative LBP levels before hepatobiliary surgery may be used as a risk indicator to predict the severity of post-operative inflammation.

6.3.1 Role of G-CSF induced LBP expression

G-CSF is a hematopoietic cytokine that acts on neutrophil proliferation and differentiation (Moore, 1991). It is reported that G-CSF may be involved in the host inflammatory response (Pollmacher et al., 1996). G-CSF is increased in blood of patients with infection (Cebon et al., 1994). It is reported that circulating G-CSF levels were increased in patients with infection, and tumor (Cebon et al., 1994; Joshita et al., 2010; Matsuda et al., 2009). Furthermore, circulating levels of G-CSF were increased after endotoxin administration (Taveira da Silva et al., 1993).

G-CSF modulates the neutrophil production in the bone marrow and the delivery of neutrophils into the blood (Moore, 1991). Berner R et al demonstrated in septic neonates that G-CSF expression was highly elevated and strongly correlated to LBP levels (Berner et al., 2002). In a previous study of our group, which was used as basis for this study; we demonstrated that G-CSF

pretreatement induced hepatic LBP expression. Here we confirmed and extended these observations. We hypothesized that the G-CSF pretreatment may not only facilitate neutrophil differentiation, but also augment the expression and eventually release of LBP from neutrophils and liver tissue after sterile and/or infectious insult.

Kang K et al found that LBP deficient mice showed delayed neutrophils influx in case of a peritoneal infection (Yang et al., 2002). These data indicated that G-CSF may modulate the inflammatory response through LBP during infection.

6.3.2 G-CSF induced LBP expression - new model to investigate the function of LBP in LPS-SIRS model

We previously demonstrated that G-CSF induced LBP expression improved the outcome in the lethal model of 90% PH using the mass ligation technique (Ji et al., 2009). This finding suggested that upregulation of LBP could be associated with an increased host defense. Based on this finding and the theory that LBP is a mediator in LPS-induced inflammatory response, we speculated that G-CSF induced LBP expression could also modulate the inflammation triggered by LPS. Using recombinant LBP to investigate LPS inflammatory response as done by others (Lamping et al., 1998) has some limitations: 1) recombinant LBP is isolated from Escherichia coli, it is hard to rule out remaining bacterial/LPS contamination, 2) recombinant LBP is easily degraded in-vivo after administration, 3) recombinant LBP is very costly, especially in doses needed for rats. Therefore, in this project, we used G-CSF induced LBP expression instead of recombinant LBP. To confirm that our observations are not related to other functions of this cytokine, we used LBP inhibitory peptides to block the interaction LBP between LPS.

6.3.3 G-CSF induced LBP expression aggravates LPS induced SIRS

Our present findings strongly suggested that G-CSF induced LBP expression was associated with an enhanced liver injury, inflammation and mortality in response to a subsequent LPS challenge with or without 70% PH. Of note, the expression of inflammatory cytokines, liver damage and mortality was attenuated by blocking LBP using inhibitory peptide in LPS-induced SIRS model. These data confirmed that the elevation of LBP plays an important role in initiation and aggravation of the LPS-induced inflammatory response. Given that the mortality of rats was associated with the pre-operative upregulation of LBP in 70% PH, the LBP levels may be used as a risk indicator to predict the severity of post-operative inflammation.

LBP plays an important role in the pathogenesis of SIRS after operation. LBP transfers LPS to CD14, and then triggers the inflammatory cascade though TLR4 signaling pathway (Blomkalns et al., 2011), causes the release of cytokines and liver damage. In in-vitro experiments, LBP augmented the LPS induced inflammatory response, indicated by higher increased TNF-levels after LPS stimulation by cultured macrophage (Lamping et al., 1998). What's more, a survival benefit was observed in mice receiving the LBP inhibitory peptide, LBPK95A (Arana et al., 2003). Wurfel et al reported that LBP protein deficiency in mice was beneficial for survival with LPS shock (Wurfel et al., 1997). Similar results were observed in a bile duct ligation model, inhibition of LBP via LBP inhibitory peptide enhanced resistance to a subsequent LPS challenge, indicated by an increased survival rate (Minter et al., 2009). These findings suggest that LBP acts as a soluble 'pattern-recognition molecule' and transports and pesents the LPS to the appropriate TLR, and then triggers the inflammatory response.

Based on the observations in present study, we argued that the LBP levels before LPS administration or infection were important for the severity of the inflammatory response. Upregulation of peri-inflammatory LBP levels seemed to increase the susceptibility to LPS.

According to the pattern recognition theory, binding of LPS to its receptor-TLR4 and forming the complex under the association with CD14 and MD2 is necessary to activate its inflammatory signal cascade (Jerala, 2007). LBP is responsible for LPS transfer and presentation LPS to TLR4 (Hamann et al., 2005). After G-CSF pretreatment, the expression of LPS receptors-TLR4, CD14, and MD2 did not increase significantly. However, G-CSF induced LBP expression was observed both in bone marrow cells and liver. The hepatic elevation of LBP was associated with the rapid binding of LPS in liver tissue, suggesting that LBP could be a major mediator for LPS recognition and initiation of the inflammatory response in liver.

Activation of the TLR4 signal pathway causes a cascade of events, including translocation of NF-kB to the nucleus and leads to production/release of inflammatory cytokines. Agustin et al showed that high TNF-α and IL-6 levels were observed in cirrhotic patients with high LBP levels and severe infectious complications (Albillos et al., 2003). In our experiment, the G-CSF induced LBP expression was crucial for the enhanced LPS induced inflammatory response, indicated by upregulation the production of pro-inflammatory cytokines.

Preoperative upregulation of LBP as observed in several liver diseases treated by PH, puts the patients at risk for SIRS after the operation. The upregulation of LBP is not limited to infectious disease, but occurs also in patients with liver disease. Albillps et al demonstrated that serum LBP levels were significantly increased in cirrhotic patients with ascites when compared with healthy

controls (Albillos et al., 2004). Montes-de-Oca et al reported that the serum LBP levels were significantly higher in hepatitis C virus (HCV) infected patients with cirrhosis than those with compensated liver disease (Montes-de-Oca et al., 2011). Elevation of LBP in non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) patients was reported by Ruiz et al (Ruiz et al., 2007). Su GL et al reported that LBP contributed to an increased hepatobiliary injury and death following acetaminophen-induced liver injury (Su et al., 2002).

Studies from animals have demonstrated that an increased responsiveness to LPS was observed after liver resection (Mochida et al., 1990; Takayashiki et al., 2004). In the present study, post-operative SIRS like syndrome were observed subsequent to the peri-operative LBP elevation, which correlated with the mortality and the overt inflammatory response.

We also observed that G-CSF induced LBP expression sensitized the rat to a subsequent LPS-challenge, which was attenuated by LBP blocking peptide. The elevated LBP did aggravate the post-operative inflammatory response.

We concluded that LBP could act as a major mediator in LPS induced inflammatory response. Our data also suggested that the elevation of LBP during liver resection could enhance the subsequent LPS induced inflammation. Monitoring the dynamic changes of LBP pre and peri-operatively may reflect the pending risk for postoperative systemic inflammatory response syndrome.

6.4 G-CSF induced LBP expression is protective in poly-microbial sepsis in naïve rats

Growing evidence indicates that LPS induced SIRS model can not fully reflect the situations during sepsis. To investigate the role of G-CSF induced LBP expression during sepsis, we used the poly-microbial sepsis model where sepsis is induced by inoculation of a defined stool suspension. In contrast to the results in the LPS induced SIRS model, G-CSF induced LBP expression had a protective effect after the septic insult, as indicated by the increased survival rate, decreased liver and kidney damage, decreased bacterial infiltration and lower systemic inflammatory response. Interestingly, a higher neutrophil influx into the peritoneal cavity and lower expression of pro-inflammatory cytokine in liver and lung was observed in G-CSF pretreatment group when compared with control group.

It has been shown that circulating LBP levels are up-regulated in several infectious diseases, such as septic shock, acute lung inflammation and infection (Sakr et al., 2008; Villar et al., 2009;

Vollmer et al., 2009). LBP is not only upregulated in Gram negative infection, but also in infections induced by Gram positive bacteria (Sakr et al., 2008) and fungi (Blairon et al., 2003) Recently, LBP was suggested as a biological marker for diagnosis and prognosis of patients with infectious disease. However, the serum levels of LBP did not correlate strongly with the severity of disease. Sakr et al demonstrated that LBP moderately discriminated patients without infection from patients with severe sepsis but not from patients with sepsis without organ dysfunction (Sakr et al., 2008). The contradictory observations reveal that the role of LBP for the LPS and the bacterial induced inflammatory response is not fully investigated and understood.

Studies demonstrated that low or constitutive LBP levels initiate the LPS induced inflammatory response, whereas high levels LBP neutralize LPS to prevent overstimulation of the immune system (Heumann and Roger, 2002). Jack and his colleagues reported that LBP protein deficiency in mice was deleterious in prolonged Salmonella infection (Jack et al., 1997), but was beneficial in an acute model of LPS shock (Wurfel et al., 1997), both observations are complementary to our findings.

G-CSF is a hematopoietic cytokine that acts on neutrophil proliferation and differentiation (Moore, 1991). Yang K. et al reported that LBP is necessary for the fast neutrophil infiltration (Yang et al., 2002). In our study, the increased neutrophil infiltration was observed as early as 2 h after infection in G-CSF group, which correlated to a decreased inflammatory response. Clinically, G-CSF is used widely to treat leucopenia, which is encountered following liver and renal transplantation. Survival benefits were observed after G-CSF in animal sepsis models (Dunne et al., 1996). However, treatment with G-CSF did not have a therapeutic benefit in the clinical treatment of infectious diseases (Bo et al., 2011; Mohammad, 2010).

6.5 Conclusion

Taken together, LBP was increased and modulated the inflammation after liver surgery. Blocking LBP using LBP-inhibitory peptide might represent a novel strategy to reduce the I/R-induced inflammatory response. G-CSF induced LBP expression sensitized to a subsequent LPS-challenge and caused lethal SIRS. In contrast, G-CSF induced of LBP expression had a protective effect in poly-microbial sepsis. These seemingly contradictory results suggest, that the effect of LBP is dependent on the type of inflammatory/infectious insult and the phase of disease. This findings help to explain the limited value of LBP-levels as diagnostic and predictive marker of SIRS and sepsis when determined after the insult. However, LBP levels may be potentially useful to assess the risk when measured prior to the defined insult.

Table 6.1 Upregulation of LBP levels after infection and operation

Reference	Disease	Pati. Nr.	LBP level (µg/ml)	Conclusion
(Pavare et al., 2010)	SIRS in Children	36	14.7 (8,7-26)	LBP may serve as good biomarkers for identifying children with
	Sepsis in children	91	26.4 (17,5-42,2)	severe sepsis and bacteraemia
	Severe sepsis in children	13	79.7 (57,8-90,6)	
(Sakr et al., 2008)	No sepsis	208	14.2 (7.7–22.2)	The correlation of LBP concentrations with disease severity and
	Sepsis	64	15.2 (10.4–25.7)	outcome is weak when compared with other markers
	Severe sepsis	55	20.5 (8.1–38.8)	
(Meynaar et al., 2011)	SIRS	-	16.3 (10.8-22.2)	LBP is not as good as serum procalcitonin when setting up as a
	Sepsis	-	30.9 (14.7-41.5)	bio-marker
(Pavcnik-Arnol et al.,	SIRS/sepsis	33	12.4–82.8	LBP on the first day of suspected infection is a better marker of
2007)	SIRS/no sepsis	27	2.1–30.6	sepsis in critically ill neonates
(Villar et al., 2009)	Severe sepsis- ARDS	99	112.56±71.8	LBP serum levels are similar in survivors and non-survivors
	Severe sepsis- ALI	56	76.66±55.9	Significant differences are observed at 48 h and at d 7 between
	Severe sepsis-Non	25	51.9± 630.5	ALI and ARDS patients
	ALI/ARDS			ARDS: acute respiration distress syndrome
	Survival	106	117.4 ± 75.7	ALI: acute lung injury
	Non-survival	74	129.8 ± 71.3	
(Blairon et al., 2003)	Gram-negative infection	13	40.80 (20.30–132.00)	LBP is a specific marker of sepsis (upregulated during sepsis, but
	Gram-positive	17	35.55 (7.30–108.50)	have no difference among G-, G+, fungi)
	Yeast infection	5	39.9 (18.15–82.70)	
	Control	18	2.30–10.00	
(Vollmer et al., 2009)	Infectious endocarditis	57	33.41 ± 32.10	Serial LBP provides an effective and useful tool for evaluating the
	(IE)			response to therapy in IE patients
	HVDs	40	6.67 ± 1.82	HVDs: noninfectious heart valve diseases
(Hiki et al., 2000)	Major abdominal surgery	15	21 (18.5-25)	LBP plays a role in regulating the circulating LPS

Table 6.2 Infectious risk after hepatobiliary surgery

Reference	Op.	Liver Disease	Complications	Absolute Nr.	%
(Trotter et al., 2006)	PH	Healthy liver donors	Sepsis	4/4500	0.2%
			Sepsis/liver failure	8/4500	0.4%
(Jarnagin et al., 2002)	PH	Tumor patients	Sepsis	39/1083	3.6%
(Malek et al., 2010)	PH	Malignant tumor in children	Sepsis	6/54	11.0%
(Farid et al., 2010)	PH	Colorectal metastasis	Sepsis	84/705	11.9%
			Sepsis + MOF	96/705	13.6%
(Saner et al., 2008)	LDLT	Blend	Sepsis	18/55	32.3%
	CDLT	-	Sepsis	36/173	20.8%
(Vera et al., 2011)	LDLT	-	Sepsis	52/94	55.3%

LDLT: living donor liver transplantation

CDLT: cadaveric donor liver transplantation

MOF: multiple organ failure

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8 Summary

Background:
LBP binds to LPS and activates an inflammatory cascade. LBP is an acute phase protein, which is currently investigated as marker of sepsis. LBP is upregulated by LPS and in infectious diseases, but also after major surgery.
Extended liver resection may be fatal due to the development of liver failure. Liver failure is associated with LPS and bacterial translocation. LPS as well as bacterial translocation represents a major risk for developing systemic inflammatory response syndrome (SIRS) and sepsis after extended liver resection. We previously demonstrated that LBP-upregulation after G-CSF pretreatment improved the outcome in the lethal model of 90% PH using the mass ligation technique.
We want to understand the role of LBP in postoperative inflammation, SIRS and sepsis. We established a novel LBP-ELISA assay to first determine the expression level after hepatobiliary procedures. Second, we tested the effect of LBP upregulation in a SIRS and third in a Sepsis model.

Method:
An LBP-ELISA assay was established based on the binding between LPS and LBP.
1. Liver samples and serums were obtained after different degrees of PH, WI/R and LTx to investigate LBP expression, liver injury and the expression of pro-inflammatory cytokines. LBP released into the effluent during CI was used in a macrophage-LPS-stimulation assay to investigate the role of LBP in vitro. Blocking experiments using an LBP-inhibitory peptide were performed to confirm the relevance of LPS/LBP for the induction of the inflammatory response.
2. After pretreatment with G-CSF to upregulate LBP-expression, rats were subjected to subsequent LPS challenge with or without 70% PH. LBP inhibitory peptide was used to block the interaction between LBP and LPS. Serum and hepatic LBP levels, mortality, hepatic injury (AST, histological evaluation), and inflammatory cytokines were used to assess the severity of inflammatory response. LPS IHC staining was performed to observe the time-dependent process of LPS-binding in the liver.
3. The role of GCSF-induced LBP-upregulation was also investigated in poly-microbial sepsis in naïve rats. Mortality, hepatic injury (AST, histological evaluation), and inflammatory cytokines were used to assess the severity of inflammatory response.

Results:
(1) LBP elevation was related to the size and thereby the synthetic capacity of the small remnant liver after liver resection. Translocation of LPS and upregulation of LBP occured after LTx, but also after PH and WI/R and was associated with the postoperative inflammatory response after LTx.
(2) Pre-operative upregulation of LBP via G-CSF sensitized to a subsequent LPS challenge and enhanced the LPS-induced inflammatory response. In contrast, blocking the interaction between LBP and LPS attenuated the overt inflammatory response.
(3) Pretreatment with G-CSF attenuated the inflammation in the poly-microbial sepsis model. We speculated that LBP-blocking peptide would aggravate the outcome in sepsis.

Conclusion:
Taken together, LBP was increased and modulated the inflammation after liver surgery. Blocking LBP using LBP-inhibitory peptide might represent a novel strategy to reduce the I/R-induced inflammatory response.
G-CSF induced LBP expression aggravated the clinical course in LPS-induced SIRS and but had a protective effect in poly-microbial sepsis. These seemingly contradictory results suggest, that the effect of LBP is dependent on the type of inflammatory/infectious insult and the phase of disease. Our findings may help to explain the limited value of LBP-levels as diagnostic and predictive marker of SIRS and sepsis when determined after the insult. However, LBP levels may be potentially useful to assess the risk when measured prior to the defined insult.

9 Abbreviations

ALI……	Acute lung injury
ALT……	Alanine transaminase
ARDS…….	Acute respiratory distress syndrome
ASDCL……..	Naphthol-AS-D-chloroacetate esterase
AST……	Aspartate aminotransferas
BCR……	B cell receptors
BDL……	Bile duct ligation
BHI…….	Brain heart infusion
BPI…….	Bactericidal/permeability increasing protein
CARS…….	Compensatory anti-inflammatory response syndrome
CCL……	Chemokine ligand
CD……..	Cluster of differentiation
CDLT…….	Cadaveric donor liver transplantation
CI……	Cold ischemia
DAMPs	Danger-associated molecular patterns
DCs……	Dendritic cells
E……	Eluate
EDTA……	Ethylenediaminetetraacetic acid
ELISA……	Enzyme-linked immunosorbent assay
F…….	Flow through
FCS……	Fetal bovine serum
GAPDH……..	Glyceraldehyde-3-phosphate dehydrogenas
G-CSF…….	Granulocyte colony-stimulating factor

HCV……	Hepatitis C virus
HDL……	High density lipoprotein
HE	Hematoxylin-eosin
HMGB1	High mobility group box -1
HPRT……..	Hypoxanthine-guanine phosphoribosyltransferase
HVDs……..	Noninfectious heart valve diseases
ICL…….	Inferior caudate lobe
ICU…….	Intensive care unit
IE……	Infectious endocarditis
IFN…….	Interferon
IHC…….	Immunohistochemistry
IKK…….	IκB kinase
IL…….	Interleukin
IL-1Ra…….	IL-1 receptor antagonist
iNOS……	Inducible NO synthase
I/R……..	Ischemia-referfusion
IRAK……...	IL-1 receptor-associated kinase
I/R……	Ischemia reperfusion
JNKs……	c-Jun N-terminal kinases
LBP……...	Lipopolysaccharide binding protein
LBPK95A…….	LBP inhibitory peptide
LDH…….	Lactate dehydrogenase
LDLT……..	living donor liver transplantation
LPS……..	Lipopolysaccharide
LLL…….	Left lateral lobe
LTx…….	Liver transplantation

M…….	Marker
MD2……	Myeloid differentiation 2
ML……..	Median lobe
MOF…….	Multiple organ failure
MyD88……	Myeloid differentiation primary response gene 88
NAFLD……	Non-alcoholic fatty liver disease
NASH……..	Non-alcoholic steatohepatitis
NF-κB	Nuclear factor-κB
NOD…….	Nucleotide-binding oligomerisation domain
NPC……..	Non-parenchymal cells
NLRs	NOD-like receptors
PAMPs…….	Pathogen-associated molecular pattern
PBMC……..	Peripheral blood mononuclear cell
PLUNC……	Palate, lung and nasal epithelium clone
PMN……	Polymorphonuclear neutrophils
PRRs	Pattern recognition receptors
PH……	Partial heptectomy
qPCR……	Quantitative Polymerase chain reaction
RAGE……..	Receptor for advanced glycation end products
RIL……..	Right inferior lobe
ROS…….	Reactive oxygen specie
RSL…….	Right superior lobe
S……..	Serum
SCL…….	Superior caudate lobe
SD……	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel

	electrophoresis
SIRS……	Systemic inflammatory response syndrome
sTNFR……..	Soluble tumor necrosis factor receptor
TAK1	TGF-β activated kinase-1
TCR……..	T cell receptor
TGF-ß……..	Transforming growth factor beta
TLR……..	Toll-like receptor
TNF-α…….	Tumor necrosis factor-alpha
TIR…….	Toll/interleukin-1 receptor/resistance
TRIF……	TIR-domain-containing adapter-inducing interferon-β
VC……	Vena cava
WB……	Western blot
WI/R…….	Warm ischemia-reperfusion

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12 Acknowledgement

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13 Curriculum vitae

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14 Declaration (Erklärungen)

Erklärung:

Hiermit erkläre ich, gem. § 6 Abs. 2, f der Promotionsordnung der Math.-Nat. Fakultäten zur Erlangung der Dr. rer. nat., dass ich das Arbeitsgebiet, dem das Thema „*Inflammation, SIRS and Sepsis after hepatobiliary surgery: Is Lipopolysaccharide binding protein the Link?“* zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von *Haoshu Fang* befürworte.

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