Modulation of Gene Expression of Iron Regulatory Proteins, Hemeoxygenase-1 and Lactoferrin, in Mice' Liver and Muscle by Different Cytokines, In Two Models of Acute Phase Reaction.

Dissertation
zur Erlangung des Doktorgrades
der Mathematisch-Naturwissenschaftlichen Fakultäten
der Georg-August-Universität zu Göttingen

vorgelegt von
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Göttingen 2010

D7

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Tag der mündlichen Prüfung: 28.04.2010

1 Abbreviations

AP-1	Activating protein-1
APP	Acute-phase proteins
APR	Acute-phase response
BCA	Bicinchoninic acid
Bp	Base pair
cDNA	Complementary deoxyribonucleic acid
Ci	Curie
Ct.	Threshold cycle
dd H_2O	Double distilled water
DEPC	Diethylpyrocarbonate
dNTP	Deoxyribonucleoside triphosphate
DTT	Dithiothreitol
EDTA	Ethylendiaminetetraacetic acid
EGTA	*E*thylene glycol tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FCS	Fetal calf serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HO-1	Heme oxygenase-1
i.p	Intraperitoneal
IFN-γ	Interferon gamma
IL-1ß	Interleukin 1 beta
IL-6	Interleukin 6
Kb	Kilobase
kDa	Kilodalton
KO	Knockout
LB-medium	Luria Bertani medium

LPS	Lipopolysaccharide
Ltf	Lactoferrin
MAPK	Mitogen Activated Protein Kinase
MOPS	3-(N-morpholino) propanesulfonic acid
mRNA	Messenger deoxyribonucleic acid
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
OD	Optical density
P/S	Penicillin / streptomycin
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMSF	Phenylmethyl sulfonylfluoride
RNase	Ribonuclease
ROS	Reactive Oxygen Species
rpm	Revolutions per minute
RT	Room temperature
RT-PCR	Reverse transcriptase-PCR
SAA	Serum amyloid A
SEM	Standard error of the mean
STAT-3	Signal transducer and activator of transcription 3
TAE	Tris acetate EDTA buffer
TBS-T	Tris-buffered saline containing tween 20
Tf	Transferrin
TNF-α	Tumor necrosis factor alpha
TO	Turpentine oil
Tris	Tris-(hydroxymethyl)-aminomethane
UV	Ultraviolet
WT	Wild type

2 Introduction

The immune system is an association of cells and molecules with specialized roles to defend the organism against infections and traumas. Fundamentally, the immune system responds in two ways:

1. Innate (natural) responses are the instant outcome of any infection or injury and occur to the same extent however many times the infectious agent is encountered. The molecular components of innate responses include complement, acute phase proteins and cytokines such as IL-6, IL-1ß, TNF-α and the interferons. The interferons are released by phagocytic cells (natural killer cells, neutrophils, monocytes and macrophages).

2. Acquired (adaptive) responses follow the innate immune response and improve on repeated exposure to a given infection. Acquired responses involve the proliferation of antigen-specific B and T cells.

Innate and acquired responses usually work together to eliminate pathogens (Delves and Roitt, 2000).

2.1 Acute phase reaction (APR)

The acute phase reaction (APR) is an innate immune response and is a physiological reaction of the body to injury, trauma or infection. It has been defined as 'the entire array of metabolic and physiologic changes which occur in response to tissue injury or infection (Bayne and Gerwick, 2001;Fulop, 2007). Its pattern varies depending on the type and intensity of the causative inflammatory agents (Siewert et al., 2004). From a clinical perspective, endotoxic shock and tissue trauma represent two severe homeostasis disturbances, leading towards the development of an acute phase response(Klein et al., 2003). The inflammatory process resulting from infection or tissue injury is required for innate immune surveillance, optimal tissue recovery and subsequent restoration of normal physiological homeostasis (Vodovotz et al., 2006). This process is responsible for the general symptoms of acute phase reaction such as fever, somnolence, anorexia, muscular and joint pain, and adinamia (Ramadori and Christ, 1999).

The usual outcome of the acute phase reaction is to successfully resolve the inflammatory process along with repair of damaged tissue, rather than persistence of inflammation which can lead to scarring and loss of organ function (Serhan and Savill, 2005;Sheikh et al., 2007). It is an important pathophysiologic phenomenon that replaces the normal homeostatic mechanisms with new set points that contribute to defensive or adaptive capabilities of the immune system. The functions of these changes are highly variable and diverse: some participate in initiating or sustaining the inflammatory process, others modulate it and still others have adaptive roles (Gabay and Kushner, 1999).

2.2 Metabolic changes during acute phase reaction

The acute phase response results in a large number of changes, distant from the site or sites of inflammation and involving many organ systems. It mainly focuses on the orchestrated accelerated hepatic production of specific acute phase proteins (Moshage, 1997;Gabay and Kushner, 1999). Other responses include increased gluconeogenesis and mobilization of amino acids to the liver. Hypoferremia and hypozincemia are also among features of the APR (Liuzzi et al., 2005). Extensive metabolic changes also include suppression of pathways for cholesterol, fatty acid and phospholipid synthesis (Yoo and Desiderio, 2003).

2.3 Acute phase proteins (APP)

APR encompasses intertwined effects of inflammation and global tissue damage. It involves a complex interplay of interacting physiological and inflammatory elements working at multiple levels, resulting in the positive or negative regulation of various acute phase proteins (Lagoa et al., 2006). An acute phase protein (APP) has been defined as one whose plasma concentration increases (positive acute phase proteins) or decreases (negative acute phase proteins) by at least 25 percent during inflammation (Morley and Kushner, 1982). APPs are synthesized almost exclusively in the liver and are mostly glycosylated. They serve important functions in restoring homeostasis after infection or inflammation. These include homeostatic functions, microbial and phagocytic functions, antithrombotic properties and antiproteolytic actions which are important to contain protease activity at sites of inflammation only (Ramadori et al., 2009).

Acute phase proteins can be divided into two groups:

1. Type I acute phase proteins include SAA (serum amyloid A), CRP (C-reactive protein; human), complement C3, haptoglobin (rat), and α1-acid glycoprotein. They are induced by interleukin-6 (IL-6), interleukin-1 (IL-1)-like cytokines which comprise IL-1α, IL-1ß, tumour necrosis factor (TNF)-α and TNF- ß.
2. Type II acute phase proteins are induced by IL-6-like cytokines which include IL-6 and its family members LIF (leukaemia inhibitory factor), IL-11, OSM (oncostatin M), CNTF (ciliary neurotrophic factor) and CT-1 (cardiotrophin-1). Type II proteins include fibrinogen, haptoglobin (human), α1-antichymotrypsin, α1-antitrypsin, and α2-macroglobulin (rat).

In general, IL-6-like cytokines synergize with IL-1-like cytokines in the induction of type I acute phase proteins, whereas IL-1-like cytokines have no effect on or even inhibit the induction of type II acute phase proteins (Moshage, 1997).

2.4 Cytokines are the core mediators of acute phase reaction

Cytokines are the imperative mediators of acute phase reaction. They are intercellular signalling molecules produced by activated immune cells (phagocytes). Most cytokines have multiple sources, multiple targets, and multiple functions. They operate both as initiators of linear signal cascades and as a part of complex interacting networks in regulating the production of acute phase proteins (Siewert et al., 2004). They can function either locally in a paracrine or autocrine manner or at sites distant from their sites of production in a manner comparable to the endocrine hormones (Frost et al., 2002). The major cytokines involved in starting and developing the APR are IL1-β, TNF-α, IL-6 and IFN-γ (Barany, 2001). These cytokines promote cellular immunity. Transcription factor NF-κB is regarded as the principal mediator of the inflammatory response and is responsible for the transcription of more than 100 genes including IL1-β, TNF-α and IL-6 during inflammation (Elenkov et al., 2005).

2.5 Iron participates in the conduction of acute phase response

Iron is an essential element to almost all cell types due to its participation in numerous vital biological processes. It is an important factor required for growth and survival of various cell types (Sheikh et al., 2006) and plays a crucial role in oxidative stress as a major catalyst for hydroxyl radical formation via the fenton reaction (Tsukamoto et al., 1999). Under physiological conditions the free form of iron is practically insoluble and potentially toxic. Thus, iron is always found bound to specific ligands to render it both soluble and non-toxic. The toxicity of iron stems from its ability of redox cycling. The release of an electron from ferrous iron, if uncontrolled, may result in the formation of reactive oxygen species (ROS) capable of oxidizing lipids, proteins and DNA (Papanikolaou and Pantopoulos, 2005). However, many catalytic and other biological processes rely on the redox properties of iron; hence, iron must be available in a form which allows it to donate and accept electrons without causing non-specific damage (Graham et al., 2007).

Acute phase reaction interacts with iron metabolism at several levels. Serum iron concentration decreases whereas the liver iron storage pool increases during inflammation, particularly due to increased sequestration of iron by macrophages. This sequestration is in part mediated by initiation of inflammatory cytokine-cascades (Barany, 2001;Oates and Ahmed, 2007). Iron stimulates ROS production in hepatic macrophages and in turn these ROS activate NF-kappa B, a stereotypical inflammatory transcription factor. Conversely, stimulation of hepatic macrophages with an agonist such as LPS induces ROS generation and ROS may initiate intracellular signalling that is dependent on a chelatable pool of iron. Thus, iron acts as a pro-inflammatory effector molecule via selective induction of the intracellular signalling for NF-kappa B activation (Xiong et al., 2004). A critical balance in iron levels is therefore

essential to sustain optimum shield by immune cells during infections to protect against free radical induced cellular damage and promotion of microbial growth, while at the same time providing adequate iron for essential metabolic functions in the body. Iron deficiency reduces the efficiency of immune cells whereas iron overload undermines immune protection and promotes free radical tissue damage (Ward and Conneely, 2004;Cronje et al., 2005).

Studies have shown a correlation between iron concentration and immune cell functions. Cellular iron availability alters the proliferation and activation of lymphocytes and natural killer (NK) cells, modulates proliferation and differentiation of T cells, monocytes and macrophages, interacts with cell-mediated immune effector pathways and modulates cytokine activities (Markel et al., 2007).Neutrophil and macrophage dysfunction has also been noted with low iron levels, as evidenced by deficient nitroblue terazoleum reduction and hydrogen peroxide formation in the respective cell lines of neutrophils and macrophages. In addition, certain DNA machinery components, such as ribonucleotide reductase, are iron-dependent. Iron levels have also been shown to alter the proliferation of T_{H1} and T_{H2} cell-subsets, likely related to the difference in dependence of these cells on transferrin-related iron uptake (Markel et al., 2007). T_{H2} clones possess larger pools of iron susceptible to chelation as compared with T_{H1} cells, making T_{H1} immune pathways more susceptible to changes in ambient iron concentrations (Marx, 2002).

Changes in iron homeostasis accompany infectious or pro-inflammatory insults(Lagan et al., 2008). Mammalian iron homeostasis is maintained through the concerted action of sensory and regulatory networks that modulate the expression of proteins of iron metabolism at the transcriptional and/or post-transcriptional level. The liver expresses a complex range of molecules which regulate iron homeostasis (Crichton et al., 2002). The key modulators of iron homeostasis include hepcidin, haptoglobin, hemopexin, lactoferrin, and ferritin, which promote iron sequestration during inflammatory conditions(Nemeth et al., 2003;Sheikh et al., 2007). On the contrary, proteins that endorse iron accumulation, like transferrin and ferroportin, are suppressed during inflammation. The changes in the expression pattern of these iron regulatory proteins promote a hypoferremic state during acute phase reaction (Ballantyne, 1984). Thus, during the APR the increase in iron-binding plasma proteins represents an active mechanism for the sequestration of excess iron rather than a purely passive defence against the invading pathogens.

2.6 Cytokines regulate the production of acute phase proteins including iron regulating proteins

The cytokines produced during the inflammatory processes are the chief stimulators of acute phase protein production. The patterns of cytokine production and of the APR differ in different inflammatory

conditions. Acute phase changes reflect the presence and intensity of inflammation, and they have long been used as a clinical guide for diagnosis and treatment (Moriconi et al., 2009).

During an acute inflammatory response serum iron has been shown to decrease (Sheikh et al., 2007). Inflammatory hypoferremia is mediated in part by initiation of the inflammatory cytokine cascades. This has also been supported in murine studies, demonstrating that TNF-α injections resulted in significantly lower serum iron levels and higher levels of circulating ferritin (Laftah et al., 2006). In addition, elevated IL-6 has been shown to result in hypoferremia, likely as a mechanism to limit bacterial iron availability. Decreased release of iron from ferritin stores and decreased absorption of iron from the intestine are part of this process (Hoppe et al., 2009). The final hypoferremic response, viewed clinically as the anemia of inflammation or chronic disease, is a result of elevated inflammatory cytokines and decreased anti-inflammatory cytokines (Ganz and Nemeth, 2009). Hypoferremia is a defining component of the anemia of inflammation. Specifically, IL-6, the proinflammatory cytokine responsible for inducing the majority of the acute phase genes, has been shown to induce hepatic production of hepcidin, the major iron regulatory hormone in response to inflammation (Malyszko et al., 2009). Hepcidin then inhibits both iron release by macrophages and absorption of dietary iron from the intestine and thereby produces hypoferremia (Munoz et al., 2009).

2.7 Role of liver in acute phase response and regulation of iron homeostasis

Liver is the main organ of the reticuloendothelial system (Tacke et al., 2009) and performs exceptional metabolic, synthetic and detoxifying functions. The central function of the liver for homeostasis and inflammatory responses is also underscored by its sole anatomical location, allowing continuous blood supply not only from the arterial system (hepatic arteries) but also from the gastrointestinal tract via the portal vein. Circulating blood cells, e.g. from the innate or adaptive immune system are pressed through a network of sinusoids allowing contact to a variety of intrahepatic cell populations, such as parenchymal liver cells (hepatocytes), endothelial cells, liver-resident macrophage (Kupffer cell) or lymphocyte populations, hepatic stellate cells and others (Fig. 1). Communication between these cell types and the regulation of hepatic functions are primarily achieved by cytokines (Racanelli and Rehermann, 2006;Tacke et al., 2009)**.**

An important feature of the APR is the drastically altered protein biosynthetic profile in the liver. The liver is the central organ of the cytokine activity due to the fact that it hosts hepatocytes, which are highly susceptible to the activity of cytokines in a variety of physiological and pathophysiological processes. Moreover, the non-parenchymal cells of the liver, in particular Kupffer cells (KCs), the main population of tissue macrophages, are able to synthesize a variety of cytokines that may act systemically

on other body organs or in a paracrine manner on hepatocytes and other non-parenchymal liver cells and are responsible for the induction of acute phase proteins (APPs).

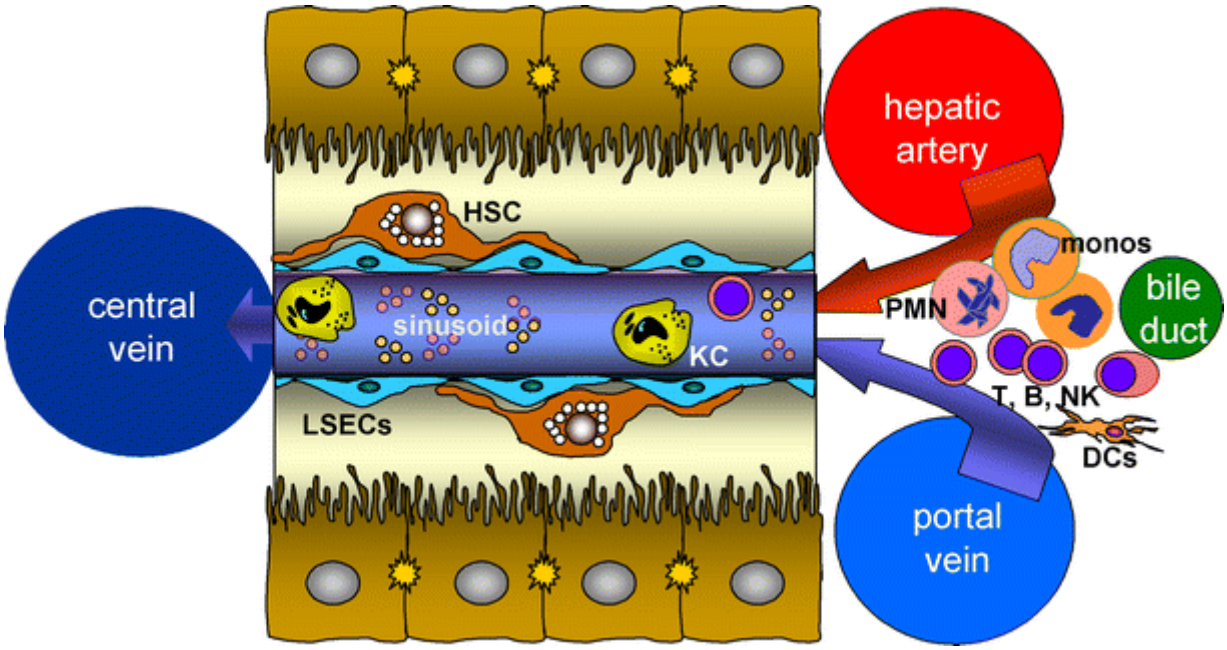


Fig. 1: *Cell populations in liver*. The healthy liver consists of 60–80% hepatocytes; the other cell populations include biliary cells, liver sinusoidal endothelial cells (*LSECs*) lining the liver sinusoids, Kupffer cells (*KC*), and hepatic stellate cells (*HSC*) in the Dissé space between hepatocytes and LSECs. Besides, many immune cells entering from the circulation via hepatic arteries and portal vein branches, including neutrophils (*PMN*), monocytes (*monos*), dendritic cells (*DCs*), and lymphocytes (*T*, *B*, *NK*, and NKT cells) are found in liver (Tacke et al., 2009).

Under normal circumstances the liver synthesizes a characteristic range of plasma proteins at steady concentrations. Most of the APPs are synthesized by hepatocytes which have important functions to perform. Higher plasma levels of APPs are required during the APR following an inflammatory stimulus. Synthesis of the major APPs can increase up to 1000-fold over normal levels. This group of proteins includes serum amyloid A (SAA) and either C-reactive protein (CRP) in humans or its homologue in mice serum amyloid P component (SAP). The concentration of negative APPs (e.g. albumin, transferrin, α-1 lipoprotein) decreases in plasma during APR to allow an increase in the capacity of the liver to synthesize the positive APPs (Ramadori et al., 1985;Ramadori and Christ, 1999).

Liver also performs a central role in iron metabolism and is the major site for iron storage in the body. It expresses a wide range of molecules involved in iron homeostasis (Graham et al., 2007). During APR liver enhances the production of all the major iron regulatory proteins for an effective removal of iron from the serum. Hepatocytes and liver resident macrophages, i.e. Kupffer cells, play an interactive role in this regard (Graham et al., 2007).

2.8 Role of skeletal muscle in the development of acute phase reaction

Liver is the major site of synthesis of serologic and cytoplasmic acute phase proteins. On the other hand, skeletal muscle itself possesses both afferent and efferent arms of the innate immune system and contributes significantly to the development of APR (Frost et al., 2004). The metabolic response to tissue trauma and sepsis results in an accelerated muscle protein catabolism (Hasselgren et al., 1988) and these broken down proteins from the skeletal muscle tissue are converted into amino acids, taken up by the liver and used for augmented acute phase protein synthesis (Sayeed, 2000). Moreover, skeletal muscle is one of the largest organ in the body and acts as an endocrine organ by producing and releasing cytokines (Pedersen and Febbraio, 2008). Muscle derived cytokines not only contribute to the development of systemic acute phase response, but are also able to signal local inflammatory cells invasion into the muscle tissue (Tidball, 2005).

2.9 Heme oxygenase-1 (HO-1)

Heme oxygenase-1, also known as heat shock protein-32, is a non-secretory cytoprotective protein and is involved in the maintenance of normal physiology and repair of pathology (Immenschuh and Ramadori, 2000). Among the positive APPs, crucial role of intracellular HO-1 in maintaining normal body homeostasis during APR has been advocated using HO-1 knockout mice (Poss and Tonegawa, 1997). HO-1 is induced by various stress stimuli to confer cytoprotection against oxidative stress (Poss and Tonegawa, 1997;Pae et al., 2008). It catalyzes heme degradation to produce carbon monoxide, ferrous iron and biliverdin (Willis et al., 1996b). These end products are responsible for most of the biologic activity of HO-1, including anti-inflammatory, anti-apoptotic, anti-oxidative and anti-proliferative effects (Fig. 2) (Tron et al., 2005;Kinderlerer et al., 2009).

Large amounts of free hemeproteins are released during hemolysis or rhabdomyolysis under various pathologic conditions, such as hemorrhage, hematoma, hemoglobinopathies, excessive blood transfusion and muscle injury (Wagener et al., 2001). Excess of free heme released from these hemeproteins under oxidative stress can be a major threat because it can catalyze the formation of reactive oxygen species (ROS) (Takahashi et al., 2004). Heme may also be released from mitochondrial P450 cytochromes and contribute to inflammatory changes and cellular injury associated with oxidative stress (Beri and Chandra, 1993). The importance of heme in inflammatory processes *in vivo* is further emphasized by the rapid increased expression of hemoglobin and heme scavengers, haptoglobin and hemopexin, respectively, in response to inflammation. HO-1 induction during this process may act as a feedback mechanism (Wagener et al., 2001). The overexpression of HO-1 is known to be associated with an early resolution of inflammation (Willis et al., 1996) and protection against heme- and hemoglobin-

mediated toxicity (Wagener et al., 2001). However, the induction of HO-1 is not only limited to catabolism of cellular heme, it is also strongly induced by non-heme stimuli as well during APR (Sheftel et al., 2007).

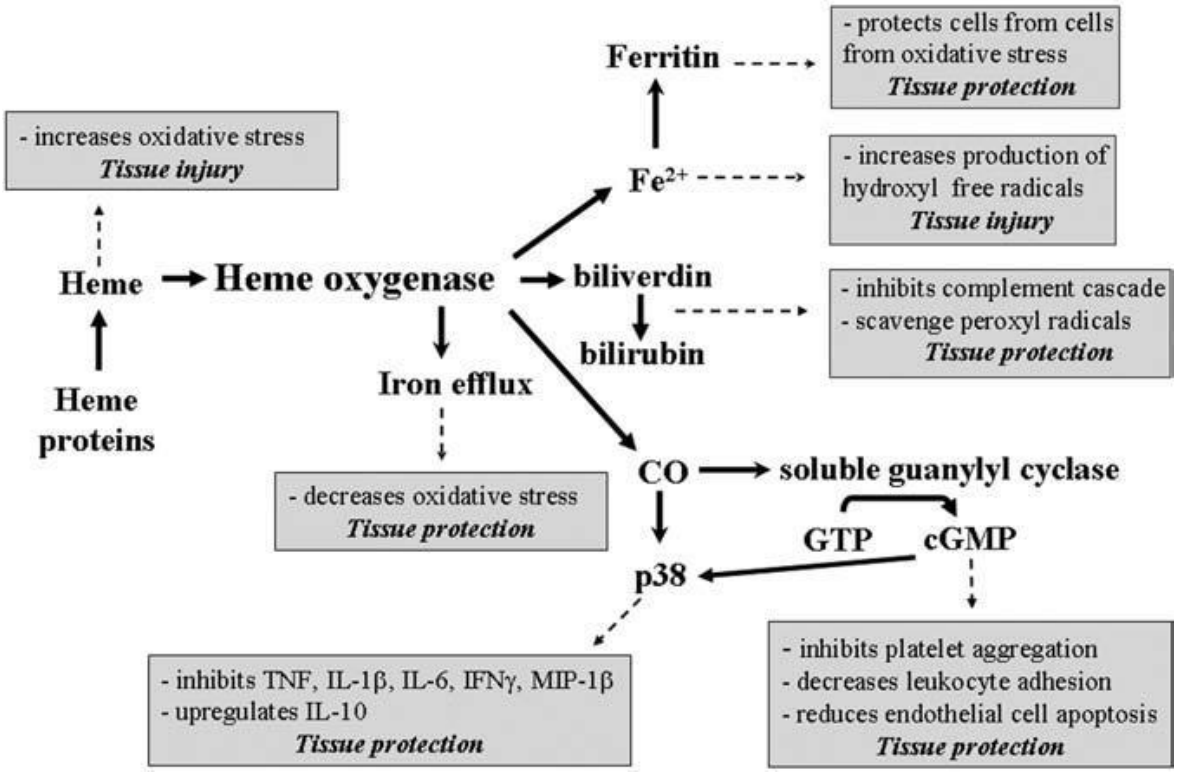


Fig. 2: HO-1 pathway and biologic activities of HO-1 products (Kinderlerer et al., 2009).
CO (carbon monoxide), GTP (Guanosine-5'-triphosphate), cGMP (Cyclic guanosine monophosphate).

HO-1 expression is principally regulated at transcriptional level (Naidu et al., 2008) and transcriptional activation of HO-1 gene is an integral part of the cellular response to oxidative stress (Bauer and Bauer, 2002). Activator protein (AP)-1 is a major transcription factor that can transactivate HO-1 (Karin et al., 1997). The results of several studies have supported that the induction of HO-1 by oxidative stimuli, such as heme (Shan et al., 2004), sodium arsenite, cobalt chloride (Lu et al., 2000) and cobalt protoporphyrin (Shan et al., 2004) is mediated via AP-1 activation. Nuclear factor-κB (NF-κB) has also been implicated in the induction of HO-1 expression in response to different stimuli, such as hemin, cadmium (Chen et al., 2004), and LPS (Wijayanti et al., 2004).

2.10 Lactoferrin (Ltf)

Lactoferrin is an 80 kDa pleiotropic cationic monomeric glycoprotein, belongs to transferrin protein family (Montreuil et al., 1960) and is widely distributed all over the body. It is a key element in host immune system and is involved in the protection against a multitude of activities, including antibacterial, antiviral, antifungal and anti-tumor activities (Actor et al., 2009). Furthermore, lactoferrin sequence

includes a defensin-like peptide, lactoferricin, which shows antimicrobial properties that include iron sequestration, direct lytic activities and impaired binding of microbes to host cells (Markel et al., 2007). The lactoferricin domain of lactoferrin binds with high affinity to the lipid A of bacterial lipopolysaccharide (LPS) and hence defuses its effects (Legrand et al., 2004). Lactoferrin is a major constituent of milk, but it has also been measured in other body fluid including serum (Hallgren et al., 1982).

In the circulation and on mucosal surfaces, lactoferrin is incompletely saturated with iron and therefore can clear iron from the tissues (Griffiths et al., 2004). Reactive oxygen intermediates and other free radicals appear during acute inflammation (Koj, 1998). Lactoferrin can sequester iron at the site of inflammation and therefore prevent free iron from being available to catalyze harmful free radical reactions (Guillen et al., 2002). One unique property of lactoferrin that differentiates it from a closely related protein transferrin is that lactoferrin shows greater affinity for iron at pH values below 4.0 (Vansnick et al., 1974).

Lactoferrin is an important regulator of inflammation and immunity. It can inhibit granulopoiesis, suppress antibody production and regulate natural killer cell activity (Crouch et al., 1992). It can also act as an anti-inflammatory factor by regulating the production of inflammatory cytokines in a similar manner as that of anti-inflammatory cytokines(Yamaguchi et al., 2001). Its receptors have been found on monocytes, macrophages and lymphocytes (Haversen et al., 2002). Mounting evidence implies that lactoferrin binding to target cells impinges on cellular signalling pathways, including mitogen-activated protein kinase (MAPK) and the nuclear factor κB (NFκB). Some reports also mention that lactoferrin enters the nucleus and directly activates target genes (Ward et al., 2005). Lactoferrin released from neutrophils at the site of inflammation interacts with mononuclear cells and suppresses the production of TNF-α and IL-1, the two major pro-inflammatory cytokines (Yamaguchi et al., 2001). However, lactoferrin can also favour the activation, differentiation and proliferation of immune cells, and this promoting activity has been related to a direct effect of lactoferrin on immune cells (Puddu et al., 1998). The site of synthesis of serum lactoferrin is still unknown.

2.11 Exaggerated inflammatory response and IL-6

In cases of prolonged systemic inflammation, APR remains persistently activated and if this inflammatory response is too exaggerated, it will start compromising the healthy tissue, therefore leading towards detrimental consequences (Vodovotz et al., 2006). Nevertheless, if the immune response is suppressed or not sufficient to eliminate the injuring agent, the inflammatory process will progress,

ultimately resulting in death (Chung et al., 2009). Therefore a critical balance between pro- and anti-inflammatory mediators is vital.

In order to avoid the spreading of inflammation, modulation of cytokine release is considered as an appealing strategy (Kobbe et al., 2008), but sharp differences in cytokine release patterns exist in APR resulting from different kinds of injuries (Ayala et al., 1991;Klein et al., 2003). Moreover, due to the inherent nonlinearity of APR, analyzing individual components in isolation may not elucidate how the entire system will behave to the presence or absence of a specific component (Day et al., 2006). However, *in vivo* studies using animal models attempt to reproduce features of human septic and traumatic shock conditions. The most important acute phase cytokines are interleukin-6, interleukin-1ß, TNF-α and interferon-γ. They are mainly released by activated macrophages, but other cells are also able to produce them.

Interleukin (IL)-6 is a pleiotropic cytokine that has important roles in the regulation of the immune response, inflammation and hematopoiesis. It is also known to regulate iron metabolism at a basic level and is the major regulator of hepatic acute phase protein synthesis in response to experimental inflammation by turpentine oil (TO) and LPS(Siewert et al., 2000; Siewert et al., 2004;Sheikh et al., 2007). Progress in the study of IL-6 has increased our understanding of the pathological roles of this cytokine in different diseases and provided key evidence that antagonizing its activities can be used as a therapeutic strategy. Blocking IL-6 actions by use of a humanized antibody, tocilizumab, which targets the IL-6 receptor, has been proven to be therapeutically effective for a number of diseases (Nishimoto, 2006). However, the cost-benefit ratio of the negative to positive effects of blocking an inflammatory mediator varies with the situation (Klein et al., 2003). The role of IL-6 blockade on lactoferrin and HO-1 that constitute the positive healing mechanism during inflammation was the center of current study.

2.12 Aims of the study

2.12.1 Comparison of Ltf and HO-1 gene expression regulation in two acute phase reaction models in mice

Acute phase reaction is a defense response that varies from individual to individual. During different acute inflammatory conditions, the pattern of induction of different acute phase proteins may be different in time and quantity. In order to diagnose and treat a pathological condition, it is very important to understand the regulation of different acute phase proteins and their mediators under different inflammatory conditions. In the current work, we aimed to investigate the regulation of hepatic HO-1 and lactoferrin gene expression regulation in two mouse models of acute phase inflammation.

Sepsis and tissue trauma by turpentine oil injection lead to different activation of intracellular and extracellular mediators in terms of expression pattern and concentration (Klein et al., 2003). Former is a representative animal model for gram negative bacterial infections resulting in activation of liver macrophages whereas the latter, a local sterile tissue necrosis, like in burns or surgical traumas, and liver becomes the target of locally released acute phase cytokines (Yang et al., 2005) While tissue injury elicits a distinct pattern of cytokine release into the blood by the inflammatory cells at the site of injury, endotoxins induce cytokine production by stimulated tissue macrophages at different organ sites, but mainly in the liver itself (Klein et al., 2003;Kobbe et al., 2008). To study lactoferrin and HO-1gene expression changes, we planned to use these two animal models.

2.12.2 Role of IL-6 in the *in vivo* and *in vitro* gene expression regulation of Ltf and HO-1 during acute phase reaction in mice

Regulation of HO-1 gene expression by interleukin-6 via STAT3 under acute phase conditions has been demonstrated (Tron et al., 2006). Accordingly, Yamaji *et al.* (2008) have described a significant reduction of HO-1 mRNA and protein expression in animals receiving CCl_4 intraperitoneal injection after a pre-treatment with anti IL-6 antibodies. Recently, it has also been shown that interleukin-6 may be the major regulatory cytokine of HO-1 in a rat model of acute phase reaction, induced by intramuscular TO-administration(Tron et al., 2005). Similarly, LPS is the key initiating factor in the pathogenesis of septic shock and is a potent inducer of HO-1 (Song et al., 2003). However, because of the complex signalling associations, it is still not clear, to which extent IL-6 is contributing to the regulation of HO-1 gene expression under different inflammatory conditions *in vivo*.

Considering the central role of IL-6 in iron metabolism, we aimed to investigate the role of IL-6 to regulate hepatic HO-1 and Ltf gene expression in IL-6 treated mice. However, the diversity of pro- and anti-inflammatory effects of IL-6 indicate that IL-6 blockade may be useful in one inflammatory setting but harmful or non-beneficial in another setting (Riedemann et al., 2003). Therefore, in order to determine the specific role of IL-6 to regulate hepatic HO-1 and lactoferrin gene expression during APR we aimed to analyze the gene expression regulation of these two iron regulatory genes in IL-6 knockout mice during APR.

2.12.3 Role of other acute phase cytokines to regulate hepatic HO-1 and Ltf expression *in vivo* and *in vitro*

Although IL-6 is a principle mediator of APR, it is not the only cytokine regulating APR and iron metabolism. Therefore, in order to determine the particular role played by other acute phase cytokines to

regulate hepatic HO-1 and Ltf gene expression, we aimed to study the gene regulation of Ltf and HO-1 in liver after intraperitoneal injections of different pro-inflammatory cytokines. These cytokines included TNF-α, IL-1ß and IFN-γ.

2.12.4 Role of cytokines in the development of acute phase response

Dysregulated cytokine actions after liver injury can result in excessive apoptosis, leading towards detrimental consequences. In order to determine the role of different cytokines to induce APR-related inflammation, and in particular to analyze the impact of IL-6 absence on the development of acute phase reaction, we aimed to determine gene expression regulation of serum amyloid A-1, which is a classical marker of acute inflammation, in the liver of mice after different treatments.

2.12.5 Evaluation of Ltf and HO-1 gene expression in skeletal muscle of TO- and LPS-treated wild type and IL-6 knockout mice

Muscle wasting is a common feature of systemic inflammation and malignant diseases. The primary cause is increased proteolysis (Lecker et al., 1999). Skeletal muscle wasting has been associated with elevations in circulating inflammatory cytokines (Petersen et al., 2009). IL-6 is an important messenger in systemic and skeletal muscle metabolism and profoundly alters the amino acid turn over during inflammatory conditions (van Hall et al., 2008). HO-1 and Lactoferrin have protective roles during acute inflammation and might represent the mechanisms for protecting skeletal muscle from excessive catabolism during inflammation. In order to look into their specific roles in controlling skeletal muscle loss during inflammation we aimed to study the pattern of regulation of these two genes in the injured and non-injured muscle of TO- and LPS-treated wild type and IL-6 knockout mice.

2.12.6 In vitro studies

In order to support our *in vivo* studies to examine the role of different cytokines, we planed to determine the regulation of HO-1 and lactoferrin expression by the *in vitro* stimulations of these acute phase cytokines to hepatocytes and hepa 1-6 cells.

3 Materials and Methods

3.1 Materials

All the chemicals used were of analytical grade and were purchased from commercial sources.

3.1.1 Animals

Male IL-6 Knockout mice (B6.129S2-IL6^{tm1Kopf}/J) were purchased from Jackson Laboratories USA, whereas wild type C57Bl6 mice were purchased from Harlan-Winkelmann, Germany. All the animals were of 6-8 weeks of age. Animals were fed standard laboratory chow, given free access to water, and maintained on a 12h: 12h light-dark cycle. Animals were used for the experiments not earlier than one week after arrival. All animals were handled in accordance with the institution's guidelines, the German convention for the protection of animals and the National Institutes of Health guidelines.

3.1.2 Bacterial strain

NovaBlue Singles™ Competent Cells from Novagen (Germany) were used for bacterial transformations.

3.1.3 Plasmid

Human lactoferrin cDNA in pCMV6-XL5 vector was obtained from Origene (USA) for making lactoferrin Northern probe.

3.2 Methods

3.2.1 Animal treatments and generation of acute inflammatory response

Mainly two types of animal models were used to study HO-1 and lactoferrin gene expression regulation during APR. In the first model, mice were injected with 10ml/kg turpentine oil i.e., 100µl in each of the hind limb muscle. This animal model of localised tissue injury was carried out in IL-6 Knockout mice and their wild type C57Bl6 mice. In the second animal model, mice were intraperitoneally injected with 50µg of LPS from *E.coli* (Sigma Aldrich). This animal model of sepsis was also performed in IL-6 knockout mice and wild type C57Bl6 mice. Control animals received no treatment. Animals were sacrificed at different time points from 0h to 24h after treatment, using pentobarbital sodium anaesthesia

(Hallbergmoos, Germany). Livers and skeletal muscles were excised, rinsed shortly in physiological saline solution and snap frozen in liquid nitrogen and stored at -80 ºC.

Animal Strain	**Treatment**	**Treatment time points**	**Total animals per Test series**	**Control**
Male wild type C57BL/6 J	TO	2h, 4h, 6h, 12h, 24h	20	4
	LPS		20	
Male IL-6 Knockout mice (B6.129S2-IL6^{tm1Kopf}/J)	TO		20	4
	LPS		20	

In another series of experiments, wild type mice were injected intraperitoneally with 1000 units of murine IL-6 or 2µg of TNF-α, or 2µg of mouse recombinant lL-1ß. Another group of mice was injected with recombinant murine IFN-γ. Control animals received no treatment. The livers of the cytokine-treated animals were excised at different time points till 48 hours, snap frozen in liquid nitrogen and stored at -80 ºC.

Animal Strain	**Treatment**	**Treatment time points**	**Total animals per Test series**	**Control**
Male wild type C57BL/6 J	Interleukin-6	2h, 6h, 12h, 24h	12	8
	TNF-α	1h, 3h, 6h, 12h, 24h, 36h, 48h	21	
	IL-1ß	1h, 3h, 6h, 12h, 24h, 36h	12	
	IFN-γ	0.5h, 1h,2h, 4h, 6h, 12h, 24h, 36h, 48h	22	

3.2.2 Blood samples

Blood was collected from inferior vena cava in serum collecting tubes, allowed to clot overnight at 4ºC and centrifuged for 20 minutes at 4000xg. Serum was collected and stored at -20C.

3.2.3 Stock solutions

All solutions were prepared as described in ‘‘Basic methods in Molecular Biology’’ by Leonard G. Davis (1986).

3.2.4 Methods of cell biology

3.2.4.1 Isolation of mouse hepatocytes

Hepatocytes were isolated from male C57BL/6J mice livers by circulating perfusion with collagenase essentially as described previously (Chua et al., 2004). The liver was perfused in non-recirculative mode through the portal vein with 150-200ml CO_2-enriched preperfusion medium at a flow rate of 10 ml/minute for 5 minutes so that the liver was free from blood. To break down components of extracellular matrix, the liver was then perfused in recirculative mode with 0.01% collagenase Type I for 5 minutes at a rate of 12 ml/minute.

After perfusion, the liver was excised and transferred into a sterile glass beaker filled with Dulbecco modified Eagle medium containing 10% fetal calf serum, 1% penicillin/streptomycin, 1% L-glutamin, 10nM dexamethasone and 10nM insulin. Glisson's capsule, i. e. collagen tissue around the liver, was carefully removed and discarded. To obtain a cell suspension, the tissue was disrupted mechanically using sterile forceps. Connective tissue and remainder of the liver capsule as well as big cell aggregates were removed by filtration of the primary cell suspension through a nylon mesh (pore size 79µm). Non-parenchymal cells and cell debris were removed by centrifugation (200x*g*, 2minute, 4°C) in wash medium. After centrifugation, hepatocytes were suspended in Dulbecco modified Eagle medium with additives.

3.2.4.2 Media and solutions for hepatocyte preparation and culture

All media and solutions for cell culture were prepared in double distilled water, were further purified by sterile filtration and stored at 4°C. All solutions were prepared no more than one day before the cell isolation.

Preperfusion medium

	For 1 l	Final concentration
EGTA	95.1 mg	0.25 mM
Krebs-Ringer stock solution	to 1 l	

Collagenase perfusion medium

	For 100 ml	Final concentration
HEPES	360 mg	15 mM
$CaCl_2 \times 2H_2O$	58.8 mg	4 mM
Collagenase	50 mg	
Krebs-Ringer stock solution	to 100 ml	

The medium was prepared directly prior to isolation, equilibrated with carbogen for 30 minutes and finally sterile filtered.

Wash medium

	For 1 l	Final concentration
HEPES/NaOH pH 7.4	4.77 g	20 mM
NaCl	7.00 g	120 mM
KCl	0.36 g	4.8 mM
$MgSO_4 \times 7H_2O$	0.30 g	1.2 mM
KH_2PO_4	0.16 g	1.2 mM
Bovine serum albumin	4.00 g	0.4%
ddH_2O	to 1 l	

3.2.4.3 IL-6 treatment of primary culture of mouse hepatocytes

Primary mouse hepatocytes were treated with various concentrations of murine IL-6 on the next day after plating. The medium was changed 6h prior to treatment; the stimuli were diluted to the required concentrations in the culture medium and added directly to the culture dishes by pipetting. The same amount of normal culture medium was pipetted to the dishes with cells which served later as experimental controls. To stop the culture, dishes with cells were taken from the incubator; the cells were washed with phosphate buffered saline, pH 7.4, and frozen at –80°C for subsequent RNA or protein isolation. Alternatively, cells were subjected to lysis immediately after washing.

3.2.4.4 Culture and stimulation of murine Hepa 1-6 cells

Murine hepatoma cells Hepa 1-6 were grown in DMEM medium containing L-Glu, P/S and FCS. Cells were plated in the Petri-dishes for 2-3 nights till 80-100% confluence, and were then treated with IFN-γ (murine), TNF-α (murine), IL-1ß (murine) and IL-6 (murine) in a time dependent manner and were harvested after 3h, 6h, 12h and 24h of stimulation. Cytokines (100ng/ml) were mixed in the medium in 15ml falcon tubes under the hood. Cells were gently washed twice with sterilised PBS. The medium in the plates was replaced with the cytokines containing medium. In control the medium was changed with a new normal medium. Medium was collected at the end of treatment time and the cells were snap-frozen in liquid nitrogen and stored in freezer.

3.2.5 Methods of molecular biology

3.2.5.1 Transformation of *E. coli*

The competent bacteria (100µl) were thawed on ice. Next, 100ng of plasmid DNA was added directly to the competent cells and the mixture was incubated on ice for 30 minutes. Cells were subjected to heat shock by incubating at 42°C for 30 seconds and were subsequently incubated on ice for 2 minutes. Afterwards, 300µl of SOC medium was added to the cells followed by 40 minutes incubation at 37°C under continuous shaking at 225 rpm. After chilling on ice, transformed cells (50µl) were spread over LB-ampicillin agar dishes and incubated for 18 h at 37°C.

3.2.5.1.1 Purification of plasmid DNA

A single colony of transformed competent *E. coli* was picked from LB-ampicillin agar dish and was inoculated into 2 ml of ampicillin-containing LB medium, followed by incubation for 12 h at 37°C with vigorous shaking at 300 rpm. Afterwards, 850µl aliquot of bacterial suspension was mixed with 150µl of sterile 87% glycerol and stored at – 80°C as a bacterial stock, other portion of this starter culture was diluted 1:1000 in LB medium, i.e. 100µl of bacterial suspension was inoculated into 100 ml of LB medium and bacteria were grown at 37°C for 12-16 h with vigorous shaking at 300 rpm to a density of approximately $3\text{-}4\times10^9$ cells per ml, which corresponds to OD600 of 1-1.5. The bacterial cells were harvested by centrifugation at 6,000 *g* for 15 minutes at 4°C. Purification of plasmid DNA from transformed bacteria was performed using 'EndoFree™ Plasmid Maxi Kit' (Qiagen).

The DNA pellet obtained was dissolved in endotoxin-free TE buffer.The integrity and purity of the obtained plasmid DNA was controlled by agarose gel analysis.

3.2.5.2 Real-time polymerase chain reaction

3.2.5.2.1 Reverse transcription

The cDNA was generated by reverse transcription of 1µg of total RNA with 100nM of dNTPs, 50pM of primer oligo $(dT)_{15}$, 200U of moloney-murineleukemia virus reverse transcriptase (M-MLV RT), 16 U of protector RNase inhibitor, 1x RT buffer and 2.5µl of 0.1M DTT for 1h at 40°C. Gene expression of murine lactoferrin, hemeoxygenase-1, IL-6, TNF-α, IL-1ß, IFN-γ, SAA-1 and of house keeping genes

GAPDH and ß-actin was analyzed using Platinum SYBR® Green qPCR mix (Invitrogen). Primer sequences used are given in Table 1.

3.2.5.2.2 Thermal cycler amplification program

The amplification was performed at 50°C for 2 minutes, 95°C for 2 minutes, 95°C for 15 seconds to 60°C for 30 seconds for 45 cycles in an ABI prism 7000 sequence detection system. All samples were assayed in duplicate. The PCR amplification program was followed by dissociation curve protocol for controlling the specificity of the PCR products. Curves of amplification were analyzed to measure the Ct value in the linear range of the amplification. The results were normalized to the housekeeping gene and fold change expression was calculated using Ct values by Prism Graph Pad 4 software.

3.2.5.3 Primers designing

Primers for different genes were designed using the program "Primer3 Input (version 0.4.0)" and the gene bank data (http://www.ncbi.nlm.nih.gov). All the primer sets used for real-time PCR are listed in Table 1.

Table 1: Primer sequences used for real-time PCR analysis.

	Forward Primer	**Reverse Primer**
Mouse HO-1	TTACCTTCCCGAACATCGAC	TCCTCTGTCAGCATCACCTG
Mouse ß-actin	ATTGTTACCAACTGGGACGACATG	CGAAGTCTAGAGCAACATAGCACA
Mouse TNF-α	CAAACCACCAAGTGGAGGAG	GTGGGTGAGGAGCACGTAGT
Mouse IL-6	TTCCATCCAGTTGCCTTCTTGG	TTCTCATTTCCACGATTTCCCAG
Mouse IL-1ß	TACAGGCTCCGAGATGAACA	AGGCCACAGGTATTTTGTCG
Mouse IFN-γ	GCGTCATTGAATCACACCTG	TGAGCTCATTGAATGCTTGG
Mouse SAA-1	ATTTGTTCACGAGGCTTTCC	TTTTCTCAGCAGCCCAGACT
Mouse GAPDH	AGAACATCATCCCTGCATCC	CACATTGGGGGTAGGAACAC
Mouse Lactoferrin	CGAAGCACGAATGACAAAGA	ACAAAGCCAATGGCAGACTC

3.2.5.4 Isolation of total RNA

3.2.5.4.1 RNA isolation procedure using silica columns

Isolation of total RNA from cultured murine hepatocytes and hepa 1-6 cells was performed using the NucleoSpin® RNAII kit (Macherey-Nagel) in accordance with the manufacturer's protocol for cultured animal cells. The RNA obtained was eluted with 60µl of RNase free H_2O.

3.2.5.4.2 Isolation of RNA by density-gradient ultracentrifugation

Total RNA from liver and the skeletal muscle was isolated by means of guanidine isothiocyanate extraction, cesium chloride density-gradient ultracentrifugation and ethanol precipitation according to method of Chirgwin *et al.* (1979).

About 100mg of frozen tissue was homogenized with ultra-turrax TP 18/10 homogenizer 3 times for 10 seconds each in 3ml of ice-cold GITC buffer with freshly added antifoam A (Sigma). The homogenates were centrifuged for 10 minutes at 3,500xg in a Rotixa/RP centrifuge (Hettich) at 4°C to pellet connective tissue and large cell debris.

To prepare the gradient, 2ml of CsCl buffer was poured into 5ml polyallomer ultracentrifuge tubes (6 per preparation). The cleared guanidine lysed samples were carefully layered on top of the CsCl buffer. The samples were centrifuged overnight (21h) at 35,000xg in a Kontron TST55 rotor at 20°C. The supernatants were carefully removed by aspiration and the transparent gelatin-like RNA pellets were gently washed (preserving undisturbed) with 200µl of 70% ethanol at room temperature. Each pellet was resuspended in 200µl of RNase-free water by pipetting and transferred into sterile 1.5ml Eppendorf tube.

The RNA was precipitated with 450µl of 100% ethanol in the presence of sodium acetate, pH 5.4 (20 µl of 2 M solution per pellet) overnight at –20°C. The RNA precipitates were centrifuged for 30 minutes at 12,000xg in an Eppendorf bench-top centrifuge at 4°C to get RNA pellet. Supernatants were discarded and pellets were washed with 200µl of ice-cold 70% ethanol to remove all traces of sodium acetate. The RNA precipitates were centrifuged as described above, the supernatants were discarded and the pellets were dried for 30 minutes at room temperature. The RNA pellets were reconstituted in 100µl of RNase-free water.

3.2.5.5 Northern blot analysis

3.2.5.5.1 Preparation of Northern probe by amplification of DNA with conventional PCR

To prepare the Northern probe for GAPDH, SAA-1, HO-1 and transferrin, PCR amplification was performed using the Go Taq ® Green master mix (Promega, Mannheim, Germany), using specific primers, as mentioned in Table 1. The cDNA was denatured at high temperature (Stage 1: 2 minute incubation at 95°C to ensure complete denaturation of the target DNA). It was followed by repetitive cycles of denaturation, annealing and extension for 45 repeats (stage 2: denaturation for 30 seconds at 95°C, annealing for 30 seconds at 60°C and extension for 1 minute at 72°C). Final extension of the DNA was performed in stage 3 at 72°C for 5 minutes. Finally, the PCR product was stored at 4°C for infinity.

Go Taq ® Green master mix also contains blue and yellow dyes that allow monitoring the progress during electrophoresis. To verify the specificity and purity of the PCR product, the size of the product was determined by agarose gel electrophoresis. For this purpose, an aliquot of 10µl from the PCR reaction mix was loaded on 1% agarose gel made in 1x TAE and 5-8µl of ethidium bromide (10 mg/ml).

3.2.5.5.2 RNA filter preparation and DNA probe labelling

Total RNA (5-10µg per sample) was size fractionated in 1% agarose-formaldehyde gels by electrophoresis, transferred to nylon membranes (Amersham Pharmacia Biotech, Freiburg, Germany) by using the capillary transfer systems and cross-linked by using ultraviolet light. The DNA probe was labelled with 40–60mCi of (α-32P)-labelled deoxy-cytidine-triphosphate (3000Ci/mmol) by nick translation using a Nick Translation Kit (Invitrogen) or by random primed DNA labelling, using NE Blot® Kit (New England Biolabs). The probes were purified from non-incorporated radiolabeled nucleotides on NICK columns prepacked with Sephadexs G-50 (Amersham Pharmacia Biotech).

3.2.5.5.3 Hybridization

The RNA membrane was pre-hybridized for 2 h with 12ml of QuikHyb® hybridization solution at 68°C to prevent unspecific binding. The labelled DNA and salmon sperm DNA were mixed and the mixture was denatured for 5 minutes at 95°C and chilled on ice for 3-5 minutes. The probe was centrifuged shortly and mixed with QuikHyb® solution in the hybridization tube. The hybridization was carried out for 2h at 68°C in a hybridization oven. The volume of the radiolabelled DNA used for an appropriate volume of QuickHyb (Stratagene, TX, USA) solution was calculated as follows:

$$V_{[\alpha\text{-}^{32}P]_{DNA}} = (V_{quick\ hyb} \times 1{,}000{,}000) / \text{ß-radioactivity of cDNA in 1µl}$$

$$V_{salmon\ sperm\ DNA} = 2\times V_{[\alpha\text{-}^{32}P]\ DNA}$$

3.2.5.5.4 Washing

After hybridization, the membrane was rinsed twice in the hybridization tube with 30ml of 2×SSC/0.1% SDS. Then 2x SSC with 0.1% SDS was used for 10 minutes at room temperature and 0.1x SSC/0.1% SDS for two washings at 55°C in the hybridization oven. Afterwards, the membrane was transferred to a plastic tray and further washed in 2×SSC/0.1% SDS with shaking at room temperature. The radioactivity was controlled to 100cpm by the washing steps. The membrane was packed in a cling film, placed in an X-ray film cassette and exposed to X-ray film, which was developed afterwards.

3.2.6 Biochemical methods

3.2.6.1 Protein extraction from liver tissue and cultured hepatocytes

3.2.6.1.1 Preparation of tissue homogenates

All steps were performed at 4°C to prevent proteolytic degradation of the proteins. About 100mg of frozen tissue was homogenized with Ultra-Turrax TP 18/10 model homogenizer 3 times for 10 seconds each in 10 volumes of 50 mM Tris-HCl buffer, pH 7.4, containing 150mM NaCl, 1 mM EDTA, 1% Triton X-100, 1mM PMSF, 1mM benzamidine, 1µg/ml leupeptin, 10µM chymostatin, 1µg/ml antipain, 1µg/ml pepstatin A. Crude homogenates were passed 5 times through a 22G injection cannula connected to a syringe. To pellet the nuclei and other suspended material, crude homogenates were centrifuged for 5 minutes at 10,000x*g* (4°C) and the protein concentration of supernatants was determined using the BCA protein assay reagent kit (Pierce, Bonn, Germany). Prepared homogenates were dispensed in aliquots and stored at −20°C until use.

3.2.6.1.2 Preparation of cell lysates

All steps of the procedure were performed at 4°C to prevent proteolytic degradation of the proteins. The cells frozen in the culture dishes were thawed on ice. 1x ice-cold lysis buffer, supplemented with protease inhibitors was added to the cells (360µl per 6 cm dish), followed by incubation on ice for 15 minutes. Afterwards, the cells were scraped with a disposable scraper, transferred to new tubes and passed 5 times through a 22G injection cannula connected to a syringe. The lysates were then centrifuged for 5 minutes at 10,000xg (4°C) and the protein concentration of supernatants was determined by BCA method using the kit from Pierce. Prepared lysates were aliquoted and stored at −20°C until use.

3.2.6.2 Western blot analysis

Protein samples were denatured in electrophoresis buffer (50 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate, 10% glycerol, 50 μg/ml bromphenol blue, 2% β-mercaptoethanol) at 95°C for 10 minutes and 30-50µg of total protein was subjected to gel electrophoresis using NuPAGE® Novex 4-12% Bis-Tris Gels (Invitrogen), using XCell SureLock® Mini-Cell (Invitrogen). The Rainbow™ coloured-protein-markers (Amersham Pharmacia Biotech) were used as molecular weight standards. The proteins from gel were shifted to a nitrocellulose membrane using XCell II™ Blot (invitrogen).

The membranes were blocked in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) and 5% nonfat dry milk at 4°C overnight and processed for immunodetection using a rabbit polyclonal antiserum against HO-1 or Ltf (in TBS-T with 0.5% milk) for 2 h at room temperature. After a subsequent washing step, the membrane was incubated with a peroxidase-conjugated secondary antibody. Visualization of immunoreactive bands was performed using Amersham™ ECL™ Western blotting detection reagent and the signal was detected by short exposure to X-ray film (Fuji, Duesseldorf, Germany). To ensure equal loading of proteins, the blots were stripped and reprobed with anti-β-actin antibody (1:5000 dilution in TBS-T with 0.5% milk; 1 h, room temperature).

Primary antibodies were used in the following dilutions:

Antibody	Used dilution
anti-HO-1 rabbit polyclonal antiserum	1:1000
anti-Ltf rabbit polyclonal antiserum	1:5000
anti-β-actin mouse monoclonal antibody	1:3000

Secondary HRP-conjugated antibodies were used in the following dilution:

Antibody	Used dilution
Donkey anti-rabbit whole Ig	1:5000
Rabbit anti-mouse Ig	1:5000

3.2.6.3 Nuclear protein isolation and gel mobility shift assay

Nuclear proteins were isolated as described by Essani *et al.* (1996). Briefly, liver tissue (0.1-0,2g) was homogenized in 1ml of buffer A (10mM Hepes-KOH, pH 7.9, 1.5mM MgCl2, 10mM KCl, 0.5mM DTT, 0.4mM PMSF, 1mM NaVO3, 2µg/ml pepstatin and 2µg/ml apoprotinin) with a Potter homogenizer.

These homogenates were then centrifuged at 600xg for 10 minutes at 4ºC and the pellet was resuspended in 1ml of buffer B (Buffer A with 0,1% triton X-100). This mixture was incubated for 10 minutes on ice and then centrifuged at 850xg for 10 minutes at 4ºC. The supernatant was discarded and the nuclear pellet was washed again with buffer A, as before, and then resuspended in buffer C (20mM Hepes-KOH, pH7.9, 1.5mM MgCl2, 0.42mM NaCl, 0.2mM EDTA, 20% glycerol, 0.5mM DTT, 0.4mM PMSF, 1mM NaVO3, pepstatin and apoprotinin as above) and was incubated at 4 °C for 30-60 minutes with gentle agitation. Nuclear debris was pelleted by centrifugation at 12000xg for 15 minutes and supernatant-containing nuclear proteins were stored at −80 °C until use. DNA consensus sequence for AP-1 and NFκB and Gel shift core assay system from Promega were used according to manufacturers' instruction to study DNA binding activity of these two transcription factors.

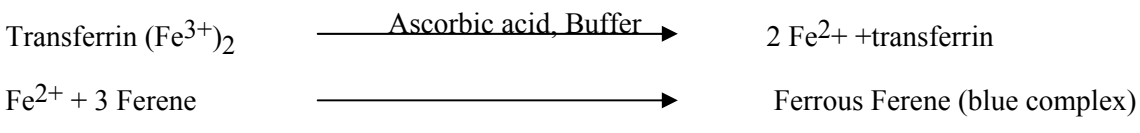
3.2.6.4 Enzyme-linked immunosorbent assay (ELISA)

For detection of cytokines in serum, Quantikine® IL-1ß and TNF-α ELISA kits from R&D systems were used. For serum IL-6 a sandwitch ELISA kit from Pierce Biotechnology was used. To measure the changes of lactoferrin concentration in serum, a sandwich enzyme immunoassay (Uscn Life Science Inc. Wuhan) for murine lactoferrin was used. Samples were processed according to the manufacturer's instructions. Samples consisted of sera from TO- and LPS-treated wild type and IL-6 knockout mice at different time points after the induction of acute phase reaction.

3.2.6.5 Iron measurement

3.2.6.5.1 Principle

Iron bound to transferrin is released in an acidic medium as ferric iron and is then reduced to ferrous iron in the presence of ascorbic acid. Ferrous iron forms a blue complex with ferene. The absorbance at 595 nm is directly proportional to the iron concentration.



Reagents

			Final concentration
R1:	Acetate buffer	pH 4.5	800 mM/l
	Thiourea		90 mM/l

R2:	Ascorbic acid	45 mM/l	
	Ferene	0.6 mM/l	
	Thiourea	20mM/l	
Standard (Should be protected from light)		100µg/dl	(17.9 µM/l)

3.2.6.5.2 Assay Procedure

To determine the serum iron level, 100 µl of the serum sample along with 1000µl of the reagent 1 was taken in the reaction tube. For blank 100µl of dd H_2O was used. The sample and reagent 1 was mixed thoroughly, incubated for 5 minutes and read the absorbance (A1). After that 250µl of the reagent 2 was added, mixed thoroughly and the absorbance (A2) was read after 10 minutes of incubation.

3.2.6.5.3 Calculations

$$\Delta A = [(A2- 0.82\ A1)\ sample/Std.] – [(A2-0.82A1)\ blank]$$

The factor 0.82 compensates the decrease of the absorbance by addition of reagent 2. The factor is calculated as follows:

(Sample +R1)/Total volume.

This compensation is necessary as a high sample volume is used.

$$\text{Iron [µg/dl]} = \Delta A\ \text{Sample}/\Delta A\ \text{Std/Cal} \times \text{Conc. Std. /Cal [µg/dl]}$$

$$\text{Conversion factor Iron [µg/dl]} \times 0.1791\ [\mu M/l]$$

3.2.6.5.4 Measuring range

The test has been developed to determine iron concentrations within a measuring range from 5-1000µg/dl (0.9-179µM/l). When values exceed this value samples should be diluted 1 + 2 with NaCl solution (9 g/l) and the results multiplied by 3.

3.2.6.5.5 Specificity and sensitivity

The test was specific and no interference was observed by conjugated and free bilirubin up to 60 mg/dl, hemoglobin up to 100 mg/dl, lipemia up to 2000 mg/dl triglycerides, and copper up to 200µg/dl. The lower limit of detection for the test is 2µg/dl (0.4µM/l).

3.2.7 Safety measures

All operations with genetically modified organisms and plasmid DNA were performed in accordance to the ‘‘Gentechnikgesetz’’ of 1990 and to the rules prescribed by the ‘‘Gentechnik-Sicherheitsverordnung’’ of 1990. Ethidium bromide, formaldehyde, DEPC and other chemicals deleterious for the environment when used in the course of the work were carefully managed and disposed properly in accordance with institutional guidelines. All the operations with radioactive chemicals were performed in a radioactivity class II laboratory and the radioactive waste was disposed off according to the institutional instructions.

4 Results

4.1 Analysis of magnitude of induction of acute phase response

In order to evaluate the magnitude of induction of APR in IL-6 knockout (KO) mice in comparison to their respective wild type counterparts, we used measurement of SAA-1 gene expression as a positive marker for generation of acute phase response. In TO-treated wild type mice (Fig. 3) significant augmentation of hepatic SAA-1 mRNA was seen at 4h ($P<0.0001$) and 12h (0.0005), whereas in case of TO-treated IL-6 knockout mice (Fig. 8) significant increase of SAA-1 mRNA was seen at 4h (18.65 ± 4.17 fold change, P= 0.0241) and 24h (4.35 ± 0.97 fold change, P= 0.0409) after TO-Injection. However, the interesting difference between wild type and IL-6 knockout mice was, that in knockout mice at 4h was the peak augmentation of SAA-1 messenger RNA and then it started to decline, but in wild type mice although statistically non-significant, increased hepatic SAA-1 mRNA levels still could be seen even at 24h (171.00 ± 56.36 fold change). In LPS treated wild type mice (Fig. 4) statistically significant intensification of hepatic SAA-1 mRNA was observed at 4h (80.23 ± 23.49 fold change, P=0.0433), 12h (35.91 ± 4.03 fold change, P= 0.0032) and 24h (40.80 ± 4.36 fold change, P=0.0028), whereas in LPS-treated IL-6 knockout mice (Fig. 9) significant increase of SAA-1 gene expression was evident at 4h (265.71 ± 80.78 fold change, P= 0.0465) and 6h (137.63 ± 11.52 fold change, P= 0.0013). Considering the fold change and the pattern of decline of SAA-1 messenger in livers of LPS treated animals at different time points, it appeared that the absence of IL-6 resulted in a delayed resolution of inflammation in knockout mice (150.89 ± 59.78 fold change) as compared to their wild type equivalents(40.80 ± 4.36 fold change).

4.2 The pattern of changes in hepatic HO-1 gene expression during APR is dependent on the nature of insult

RT-PCR analysis revealed a significant upregulation of hepatic HO-1 gene expression after TO (Fig. 3) or LPS (Fig. 4) induced acute phase reactions in wild type mice. However, the augmentation pattern of HO-1 mRNA was different in both types of acute phase responses. LPS injection resulted in an immediate peak of HO-1 mRNA at 2h (30.65 ± 8.66 fold change, P= 0.0209), whereas TO-injection caused a significant augmentation of HO-1 mRNA at 2h (32.72 ± 0.99; $P<0.0001$), but the peak was seen at 4h (57.33 ± 3.8 fold change, $P<0.0001$).

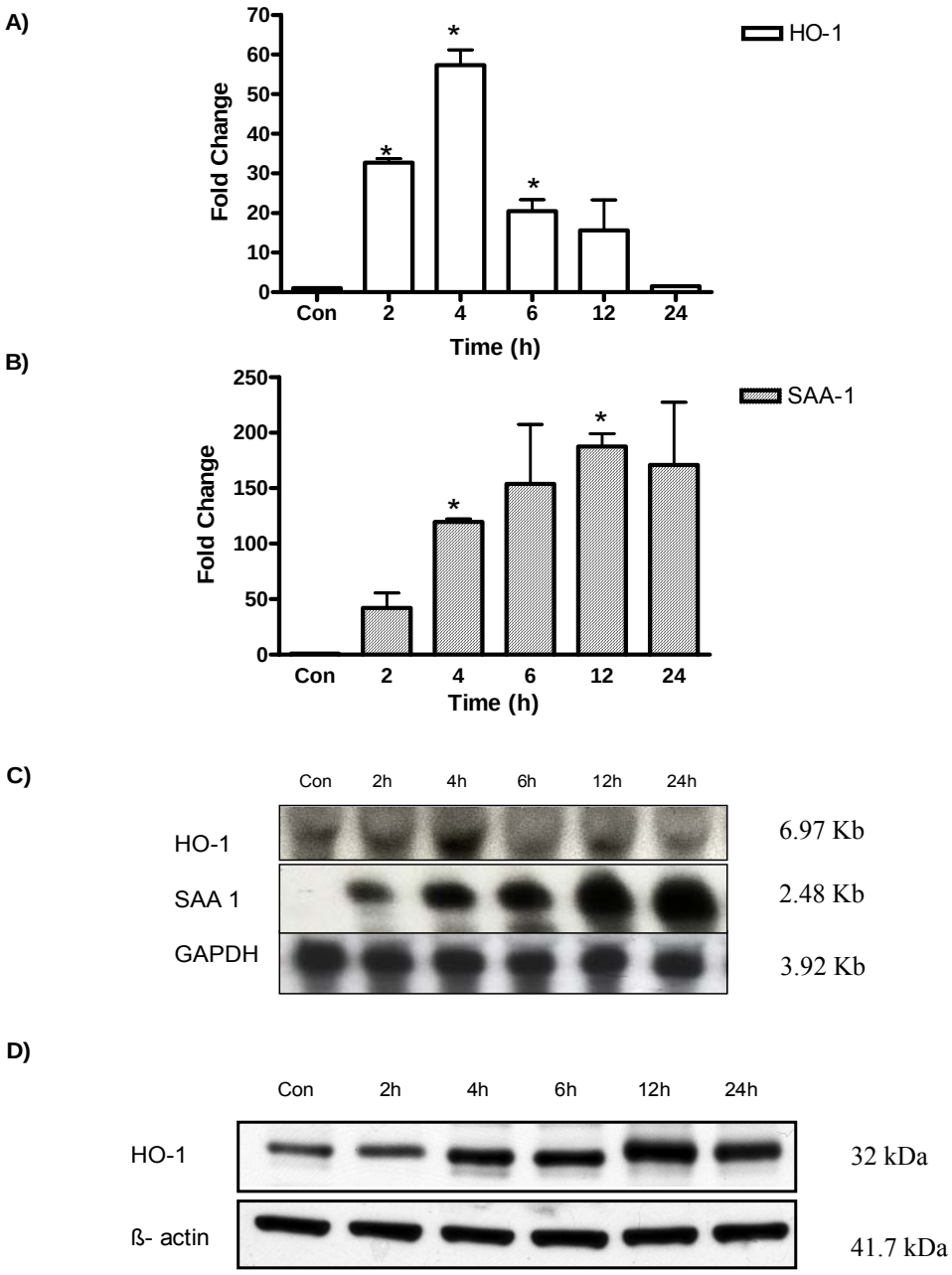


Figure 3:

HO-1 and SAA-1 gene expression regulation in the livers of TO- treated wild type mice: Wild type C57Bl6 male mice were injected with 10ml/kg turpentine oil (TO), i.e. 100µl in each of the hind limb muscle to induce localised tissue injury. HO-1**(A)** and serum amyloid A-1 **(B)** mRNA expression analysis by quantitative RT-PCR in the liver of TO-treated mice (results represent the mean value± s.e.m. **P*<0.05, analyzed by *t*-test; *n*=4 animals per time point). **(C)** Northern blot analysis of the same mRNA shows the mRNA identification by size. **(D)** Western blot analysis of HO-1-protein in total liver homogenates (representative of one animal per time point).

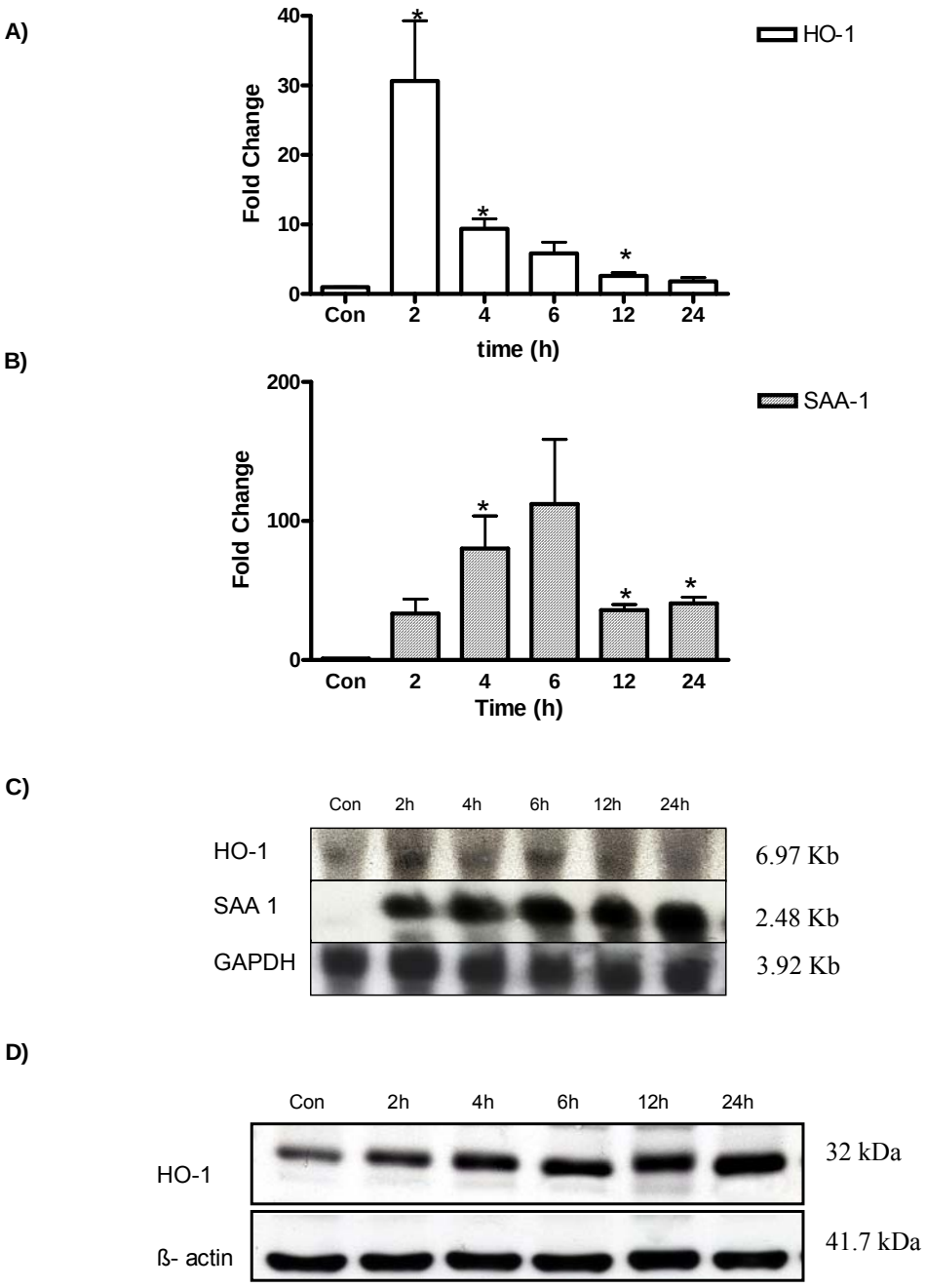


Figure 4:

Hepatic HO-1 and SAA-1 gene expression analysis in LPS-treated wild type mice: Wild type C57Bl6 male mice were intraperitoneally injected with 50µg of LPS from *E.coli* to produce systemic inflammation. Control animals received no treatment. HO-1**(A)** and serum amyloid A-1**(B)** mRNA expression analysis by quantitative RT-PCR in the livers of LPS treated mice (results represent the mean value± s.e.m. (*P<0.05, analyzed by t-test; n=4 animals per time point). **(C)** Northern blot analysis shows the specific mRNA identity by size. **(D)** Protein expression analysis of HO-1 in total liver homogenates by Western blot (representative of one animal per time point).

4.3 IL-6 upregulates hepatic HO-1 gene expression

In order to confirm that IL-6 induces hepatic HO-1 mRNA, we treated mice intraperitoneally with recombinant IL-6 (Fig. 5). A statistically significant induction of HO-1 at 2h (1.44 ± 0.045 fold change, P<0.01) was observed. In IL-6 treated murine hepatocytes also, HO-1 was induced (Fig. 6). 100mg and 1000ng were the doses of IL-6 that induced statistically significant induction of HO-1 in the mouse hepatocytes.

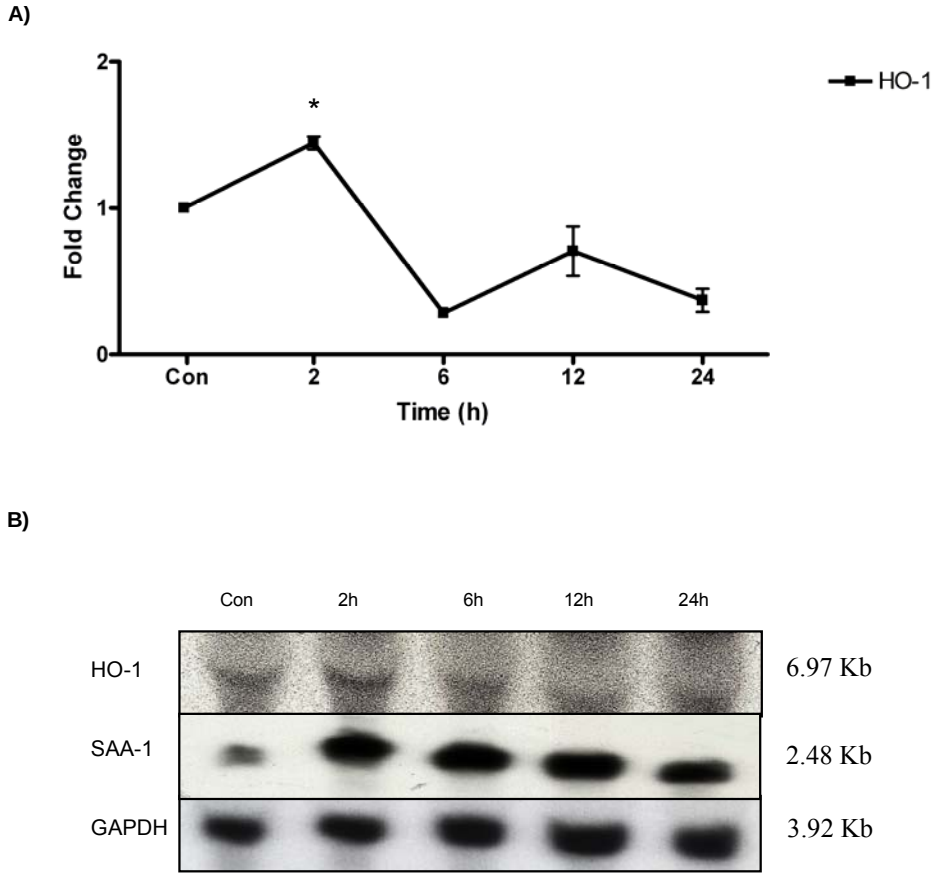


Figure 5:
Effect of IL-6 on HO-1 gene expression: Mice were injected intraperitonealy 1000 units of interleukin 6 to analyze the time dependent kinetics of hepatic HO-1 **(A)** HO-1 gene expression was analyzed by quantitative RT-PCR (Results represent the mean value± s.e.m. (**P*<0.05, analyzed by *t*-test; n= 2-3 animals per time point). **(B)** Northern blot analysis of HO-1 and SAA-1 (representative of one animal per time point).

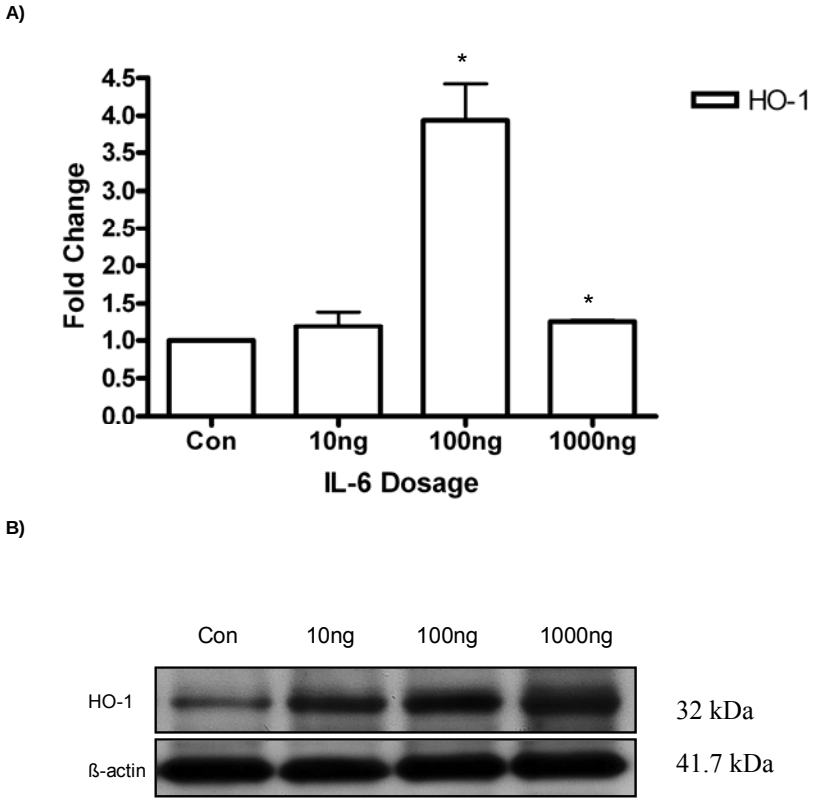


Figure 6:
HO-1 in IL-6 treated mice hepatocytes: Murine hepatocytes were treated with different doses of murine IL-6 and were harvested after 6h **(A)** Quantitative RT-PCR analysis of HO-1 mRNA induction in mouse hepatocytes (results represent the mean value± s.e.m. (*P<0.05, analyzed by t-test; n=3 replicates per time point). **(B)** Western blot analysis of HO-1 protein expression in mouse hepatocytes.

4.4 Serum interleukin-6 closely correlates with hepatic HO-1 induction

The concentration of IL-6 in the sera of TO- and LPS-treated wild type mice increased instantaneously (Fig. 7). In TO-treated animals, however, the statistically significant upregulation, as analyzed by t-test, was seen only at 4h (P=0.0276), 12h (P= 0.0094) and 24h (P=0.0269) after TO-injection, whereas in LPS-treated wild type animals statistically significant increase of IL-6 amount was observed at 2h (P=0.0119), 4h (P=0.0098), 6h (P=0.0095) and 12h (P=0.0012) time points after injection.

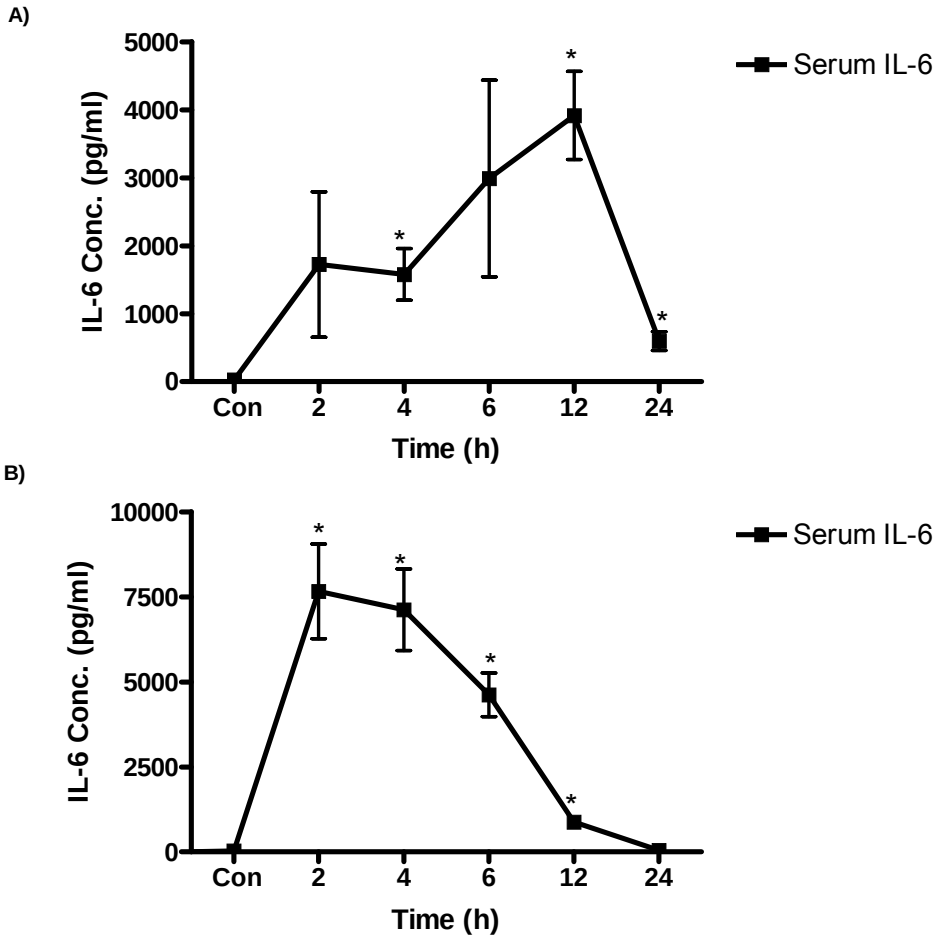


Figure 7:
Serum interleukin-6 concentration analyzed by ELISA **(A)** TO-treated C57Bl6 male mice. **(B)** LPS-treated C57Bl6 male mice (results represent the mean value± s.e.m. (**P*<0.05, analyzed by *t*-test; *n*=4 animals per time point).

4.5 HO-1 induction in IL-6 knockout mice

HO-1 was upregulated at mRNA level after TO-treatment of IL-6 knockout mice (Fig. 8). However, unlike wild type mice, statistically significant induction of HO-1 in TO-treated IL-6 knockout mice was seen only at 2h (2.39 ± 0.29 fold change, P=0.0182). In LPS-treated IL-6 knockout mice also, HO-1 mRNA expression increased (Fig. 9) and significant changes were seen at 2h (P=0.0217), 4h (P=0.0191) and 24h (P=0.0127) post LPS injection.

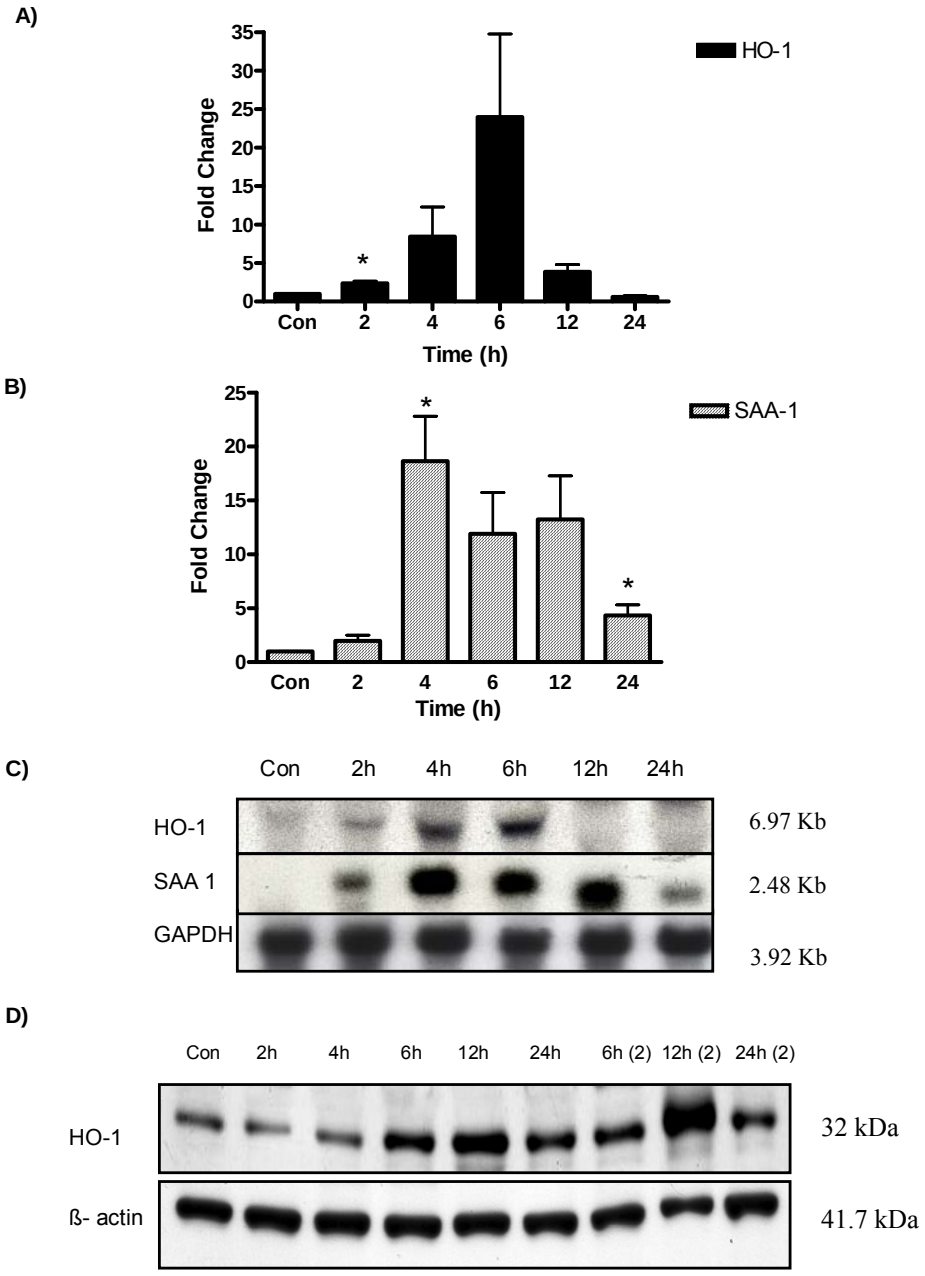


Figure 8:
HO-1 and SAA-1 mRNA expression in TO-treated IL-6 knockout mice liver: Male IL-6 knockout mice (B6.129S2-IL6^{tm1Kopf}/J) were injected 100µl turpentine oil in each of the hind limb muscle. Control animals received no treatment. HO-1**(A)** and SAA-1**(B)** mRNA expression analysis by quantitative RT-PCR in the livers of TO-treated IL-6 knockout mice (results represent the mean value± s.e.m. (*P<0.05, analyzed by t-test; n=4 animals per time point). **(C)** Northern blot analysis to identify the specific transcripts by size. **(D)** Western blot analysis of HO-1 from total liver protein.

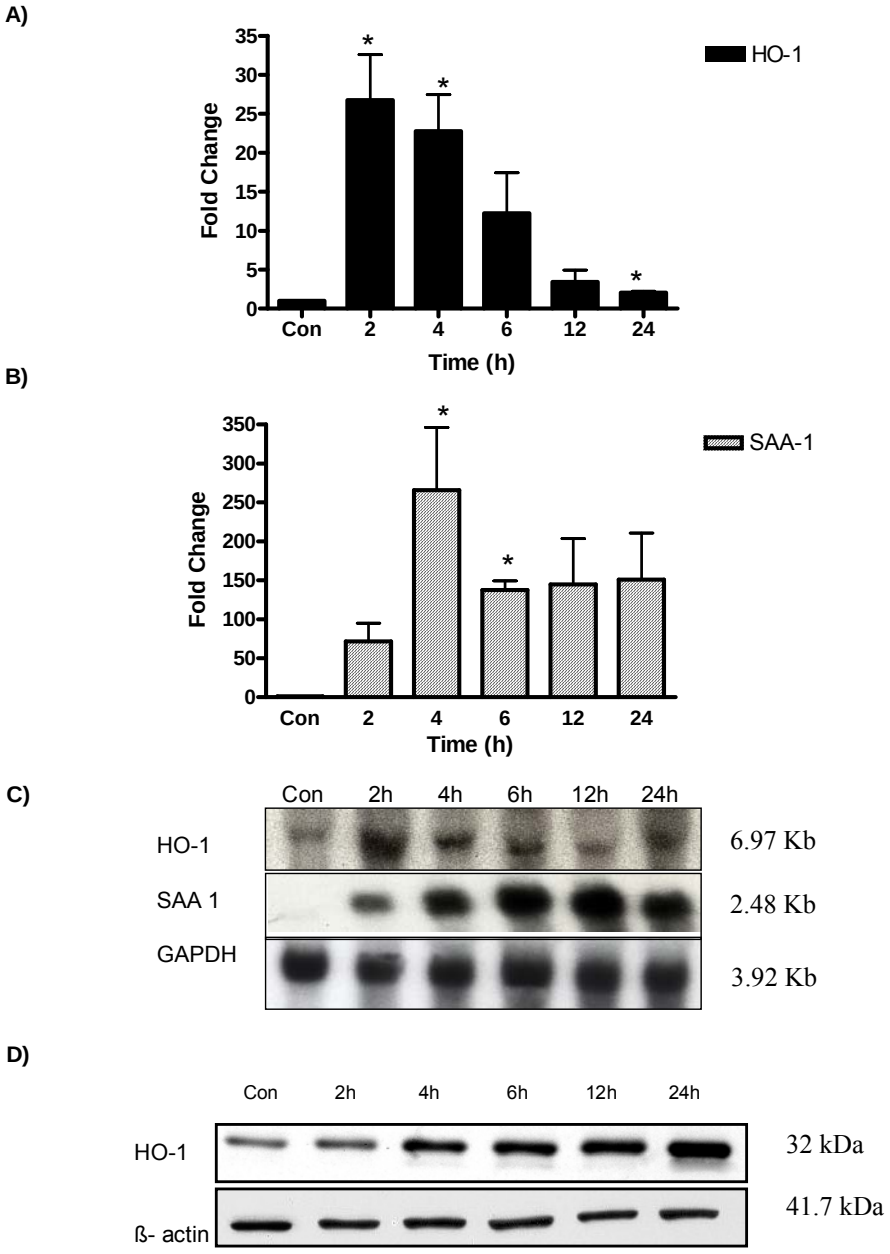


Figure 9:

HO-1 and SAA-1 gene expression examination in LPS-treated IL-6 knockout mice liver: Male IL-6 knockout mice (B6.129S2-IL6^{tm1Kopf}/J) were intraperitoneally injected with 50µg of LPS to induce systemic inflammatory response. Control animals received no treatment. HO-1**(A)** and SAA-1**(B)** mRNA expression analysis by quantitative RT-PCR in the liver of LPS-treated mice (results represent the mean value± s.e.m. (*P<0.05, analyzed by t-test; n=4 animals per time popint). **(C** Northern blot shows the specific mRNA, as identified by size. **(D)** Western blot analysis of HO-1 protein in total liver homogenates (representative of one animal per time point).

4.6 TNF-α and IL-1ß concentrations in serum of TO-treated wild type and IL-6 knockout mice

Overall, the serum levels of TNF-α and IL-1ß are almost comparable in TO-treated wild type and IL-6 knockout mice (Fig. 10). In wild type animals the amount of IL-1ß increased significantly at 2h (P= 0.0002), 6h (P= 0.0346), 12h (P= 0.0229) and 24h (P<0.0001), whereas no significant increase was seen for TNF-α in the sera of these animals. In fact, a significant decrease of TNF-α concentration was seen at 24h (P= 0.0053). However, no significant changes were observed in serum TNF-α levels in TO-treated IL-6 knockout mice, but significant increase of IL-1ß was evident at 2h (P= 0.0085)and 6h(P= 0.0239).

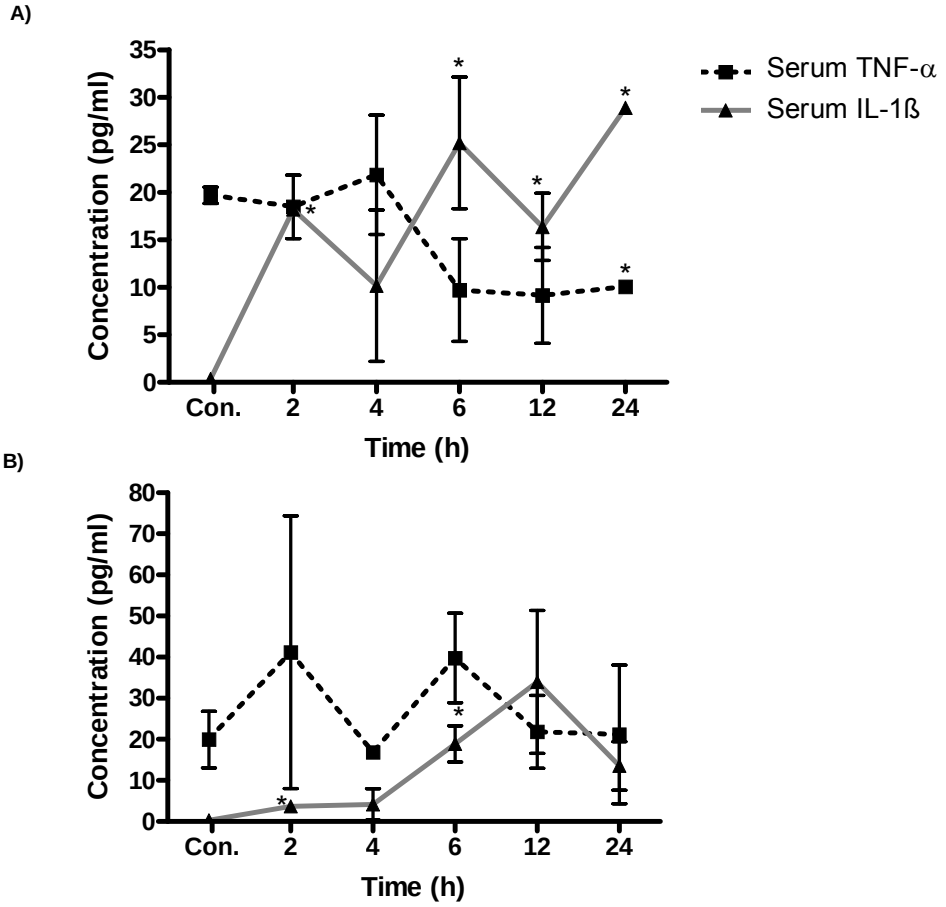


Figure 10:

Alterations of serum TNF-α and IL-1ß concentrations of TO-treated mice: the two cytokines were measured in the serum of animals using ELISA kits from R&D (**A)** Serum TNF-α and IL-1ß in TO treated wild type mice. **(B)** Serum TNF-α and IL-1ß in TO treated IL-6 knockout mice (results represent the mean value± s.e.m. (**P*<0.05, analyzed by *t*-test; *n*=4 animals per time point).

4.7 Changes in TNF-α and IL-1ß serum levels in LPS-treated wild type and IL-6 knockout mice

In LPS treated wild type animals TNF-α peaked at 6h in serum, although it was statistically non-significant, a significant upregulation of serum TNF-α was seen at 4h (P= 0.0088) in these animals, whereas in LPS-treated IL-6 knockout mice the peak in serum TNF-α occurred at 2h (P= 0.0356) post LPS injection and it was statistically significant (Fig. 11). Changes in serum TNF-α in these knockout mice were statistically significant also at 4h (P= 0.0073) and 12h (P= 0.0049) as well. In control animals IL-1ß was not detectable in the sera of both wild type and IL-6 knockout mice. But a significant upregulation in IL-1ß concentration was seen in the sera of both wild type (P= 0.0425) and IL-6 knockout mice (P = 0.0474) at 4h. However, in the sera of LPS treated wild type mice, IL-1ß declined to basal level at 12h whereas it was significantly high at 6h (P= 0.0047)and 12h (P= 0.0499) time points in knockout mice and returned to basal level at 24h(Fig. 11).

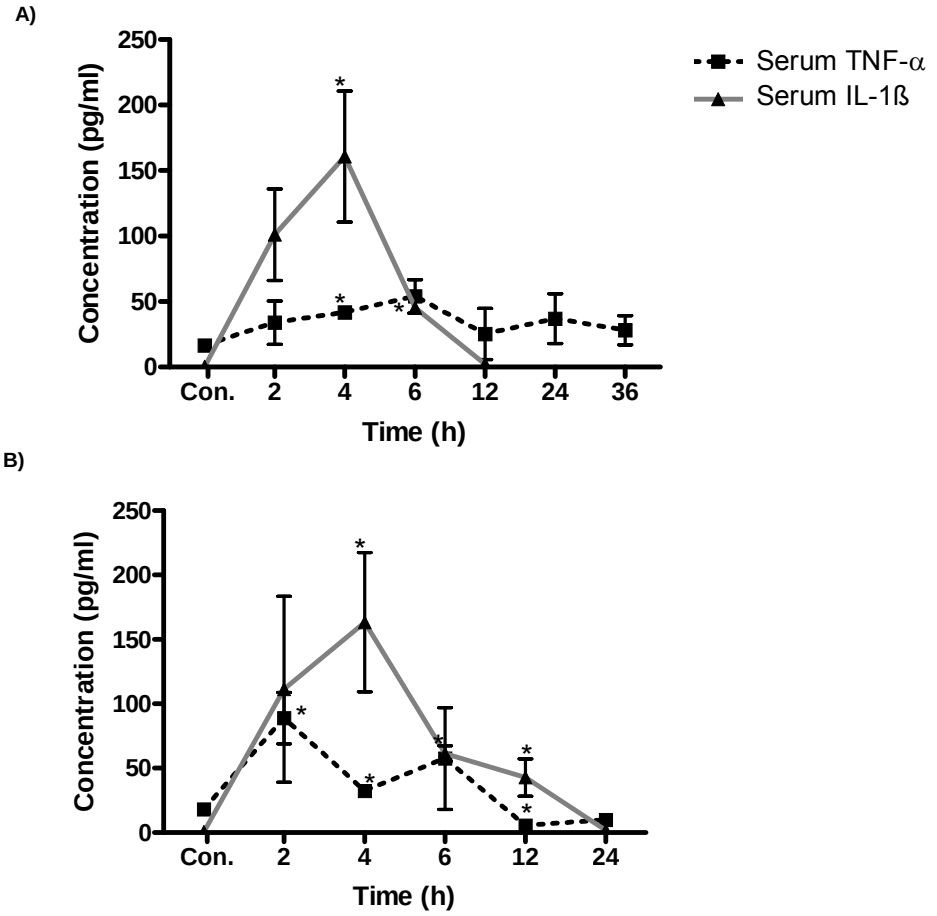


Figure 11:
TNF-α and IL-1ß concentrations in the sera of LPS treated wild type and IL-6 knockout mice: **(A)** Serum TNF-α and IL-1ß concentrations in LPS-treated wild type mice (**B)** Changes in the amount of TNF-α and IL-1ß in the sera of LPS treated IL-6 knockout mice (results represent the mean value± s.e.m. **P*<0.05, analyzed by *t*-test; *n*=4 animals per time point).

4.8 Determination of STAT-3 phosphorylation in the liver of TO- and LPS-treated mice

IL-6 regulates HO-1 gene expression via STAT-3. In order to estimate the possible involvement of STAT-3 in the regulation of HO-1 gene expression in IL-6 knockout mice, STAT-3 phosphorylation was determined in the hepatic nuclear extracts of TO- and LPS-treated IL-6 knockout mice using a specific antibody and was compared to their wild type counterparts (Fig. 12). It was interesting to note that STAT-3 followed more or less the same phosphorylation pattern in both wild type and IL-6 knockout mice in both types of acute phase response, however as seen in Western blot, the magnitude of phosphorylation appeared to be less in knockout mice when compared with their respective wild type animals using same amount of nuclear protein.

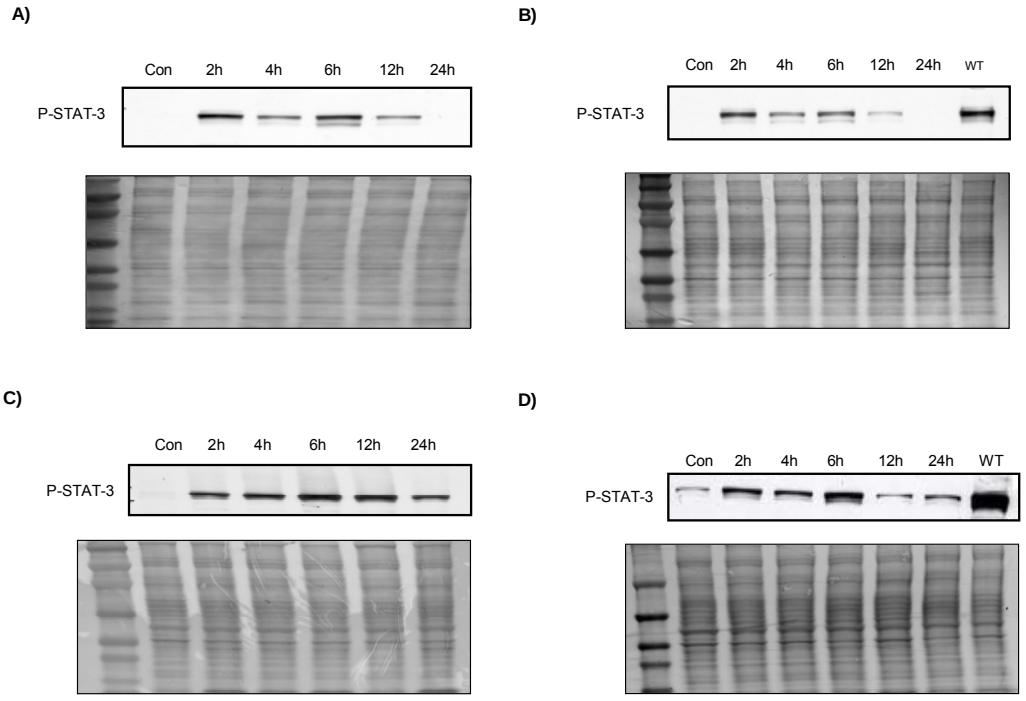


Figure 12:
Hepatic STAT-3 phosphorylation analysis by Western blotting: STAT3 phosphorylation was analyzed by Western blot analysis with the hepatic nuclear extract of LPS- and TO-treated mice using a specific antibody that recognises only the phosphorylated form of STAT3. **(A)** STAT-3 phosphorylation analysis in the nuclear extract of LPS treated wild type mice. **(B)** Western blot analysis of phosphorylated form of STAT-3 in the nuclear extract of LPS-treated IL-6 knockout mouse liver. **(C)** STAT-3 phosphorylation was analyzed in the hepatic nuclear extract of TO treated wild type mice. **(D)** Western blot analysis of phosphorylated STAT-3 in the nuclear extract of TO-treated IL-6 knockout mouse liver.

4.9 Distinct AP-1 and NF-κB DNA binding activity in liver of IL-6 knockout mice in two models of acute phase reaction

AP-1 and NF-κB are the two distinctive transcription factors, well known to regulate inflammatory processes, and are also involved in the positive regulation of HO-1 gene expression regulation during APR. Their DNA binding activity was analyzed in the hepatic nuclear extracts of TO and LPS-treated IL-6 knockout mice (Fig 13& 14). In TO-treated animals AP-1 binding activity was high at 6h and 12h, whereas NFκB peaked at 24h. However, in LPS-treated animals NFκB peaked at 2h, whereas AP-1 binding to consensus DNA sequence started to increase at 2h, and it was maximum at 12h.

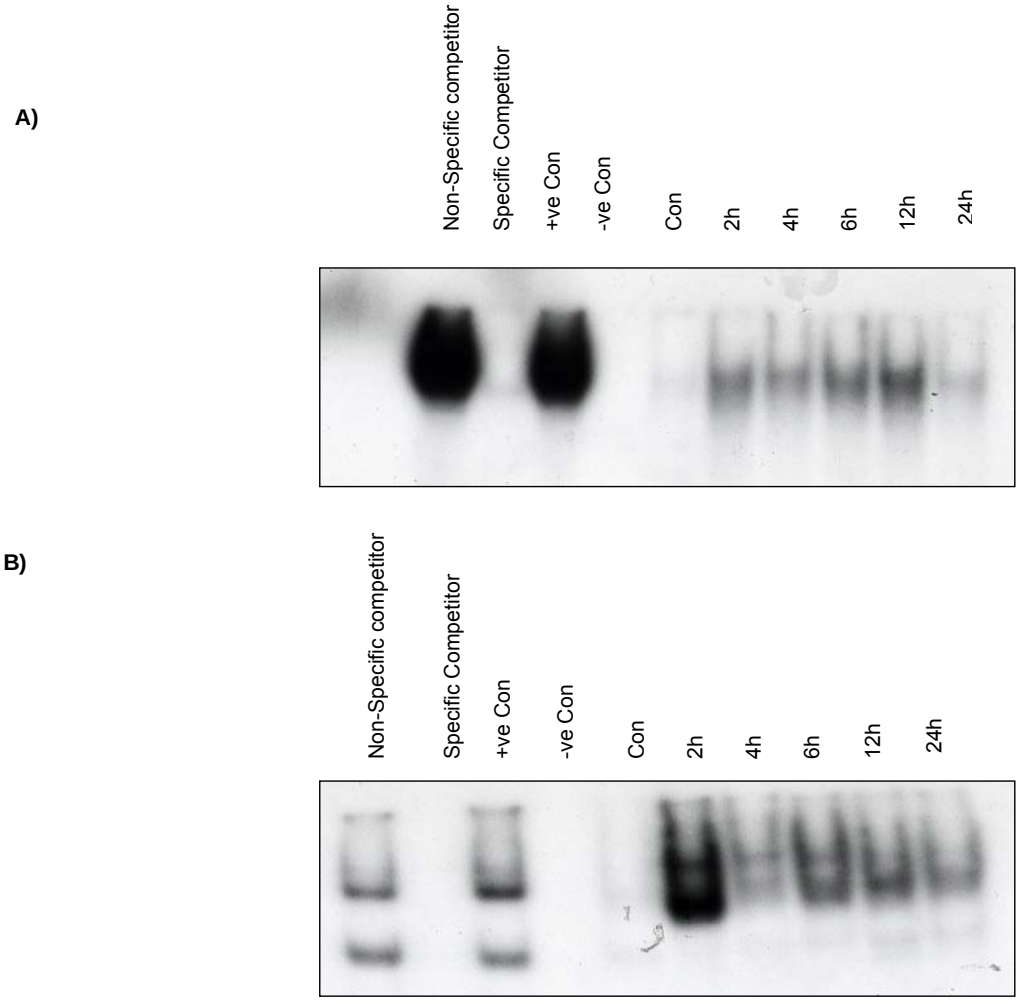


Figure 13:
DNA binding activity of Activator Protein 1 (AP-1) and nuclear factor kappa-light-chain-enhancer of activated B cells(NF-κB) by gel mobility shift analysis: **(A)** AP-1 DNA binding activity was analyzed in the hepatic nuclear extracts of LPS treated IL-6 knockout mice using consensus oligos for AP-1 **(B)** DNA binding activity of NF-κB was analyzed in the hepatic nuclear extract of LPS treated IL-6 knockout mice using consensus oligos for NF-κB.

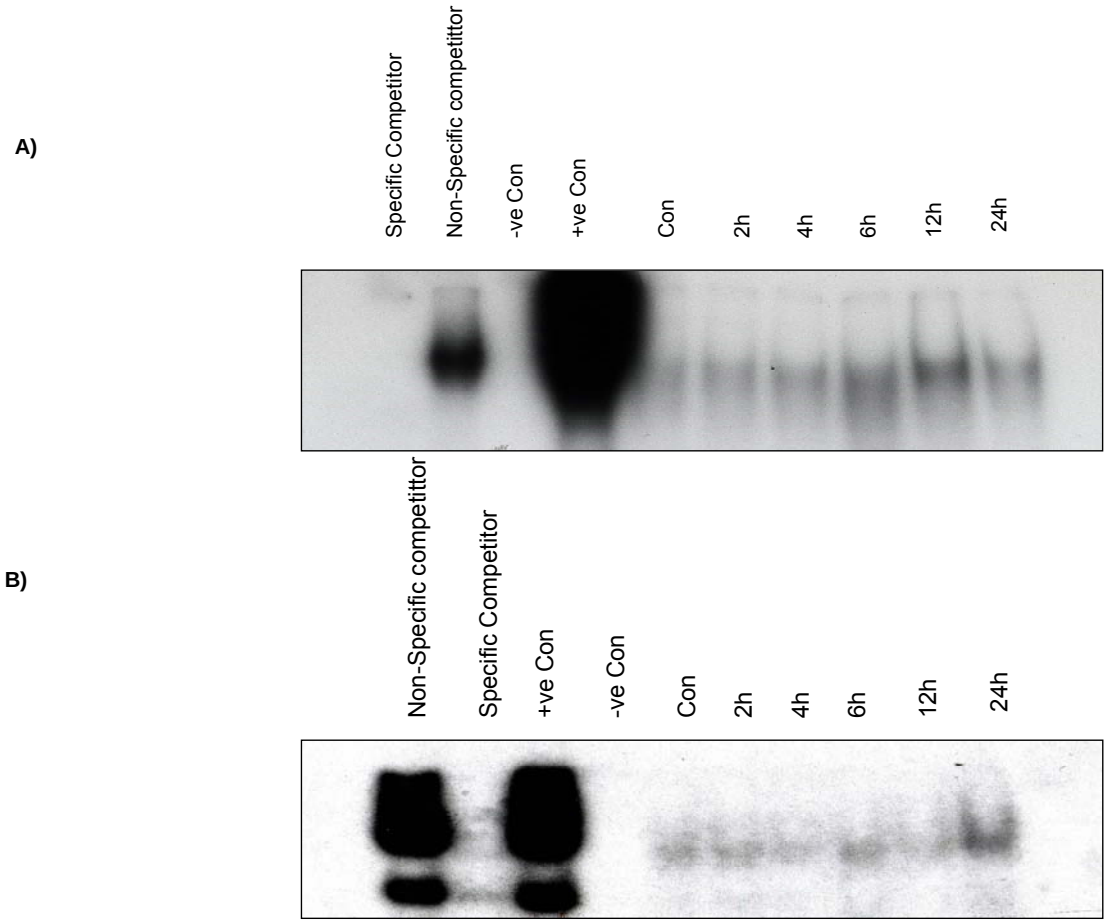


Figure 14:
Activator Protein 1 (AP-1) and nuclear factor kappa-light-chain-enhancer of activated B cells(NF-κB) DNA binding activity by electro mobility shift analysis: **(A)** AP-1 DNA binding activity was analyzed in the hepatic nuclear extract of TO treated IL-6 knockout mice using consensus oligos for AP-1 **(B)** DNA binding activity of NF-κB was analyzed in the hepatic nuclear extract of TO treated IL-6 knockout mice using consensus oligos for NF-κB.

4.10 Changes in cytokine gene expression in the liver and muscle of TO- and LPS-treated mice

The serum levels of various acute phase cytokines changed in the two models of acute phase reaction. In order to determine the source of these cytokines and to analyze the effects of TO- and LPS-treatments on liver in our animal models, we analyzed the gene expression of IL-6, IL-1ß, IFN-γ gamma and TNF-α in the liver of these animals by real time PCR. In TO-treated (Fig. 15) wild type mice, IL-6

expression increased in liver. TNF-α mRNA expression levels did not increase significantly in both wild type and IL-6 knockout mice after TO-treatment. IFN-γ also remained almost unchanged. However, when we analyzed IL-1ß, an increase was seen in the liver of wild type mice, but no increase, rather a decrease, was seen in IL-6 knockout mice, but serum ELISA indicated an increase in IL-1ß levels in these animals. Therefore, in order to determine the source of serum IL-1ß we checked the cytokine-mRNA expression at the site of injury i.e., TO-injected hind limb muscle (Fig. 16). Significant increase in IL-1ß gene expression levels were seen by RT-PCR in the TO-injected hind limb muscles of both wild type and IL-6 knockout mice.

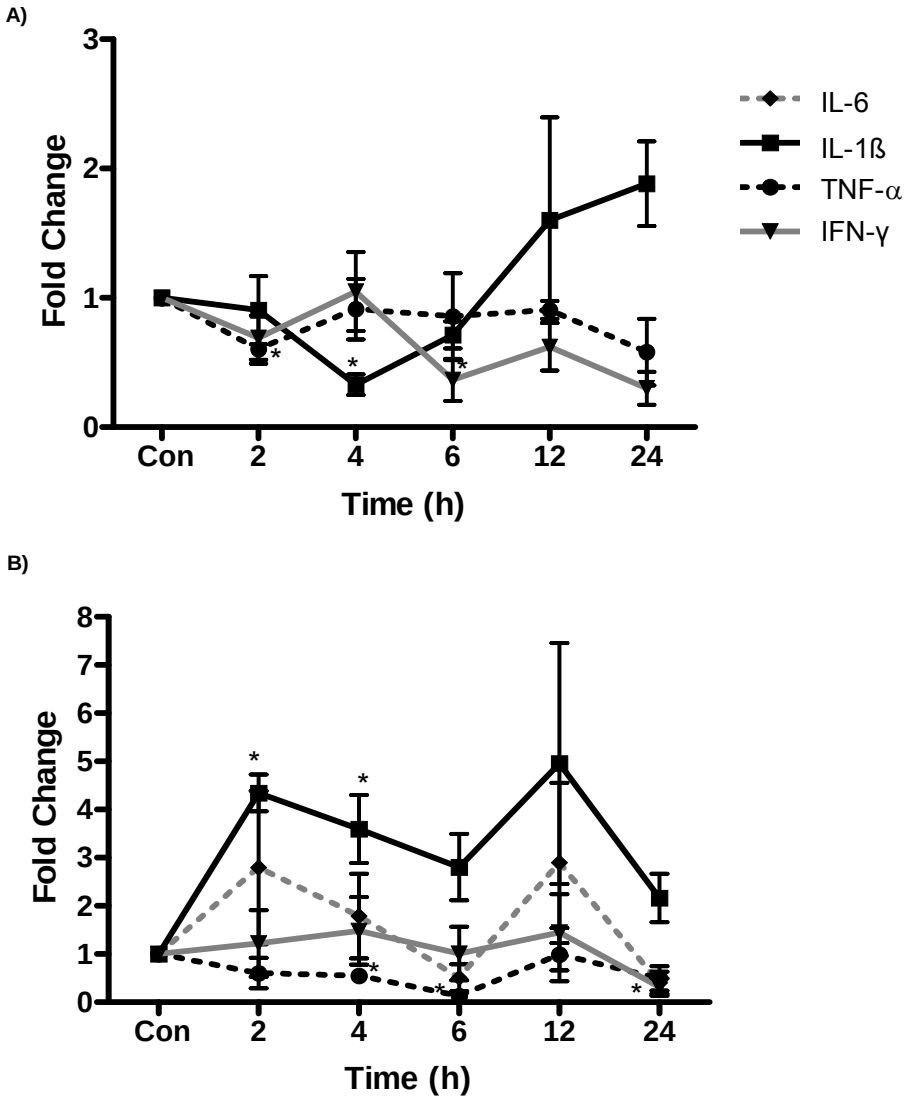


Figure 15:

Pro-inflammatory cytokines mRNA expression analysis by quantitative real time PCR in the liver of TO-treated IL-6 knockout mice **(A)**, and their wild type equivalents **(B)**: (results represent the mean value± s.e.m. (**P*<0.05, analyzed by *t*-test; *n*=4 animals per time point).

In the LPS-treated animals a significant upregulation of all these pro-inflammatory cytokines was seen, and these changes in hepatic cytokines' expression levels were very well in accordance with the serum levels of the cytokines in these animals (Fig. 17). However, in order to compare the TO-treated injured muscle with the skeletal muscle from LPS-treated animals, we analyzed the cytokines' gene expression in the skeletal muscle of LPS-treated wild type and IL-6 knockout mice. It was interesting to note that the cytokines' mRNA expression levels were significantly augmented in the non-injured hind limb muscle of LPS-treated animals. The magnitude of upregulation of TNF-α and IFN-γ was comparable in wild type and IL-6 knockout mice. However, in case of IL-1ß a much higher and more prolonged upregulation of mRNA was seen in IL-6 treated mice (Fig. 18).

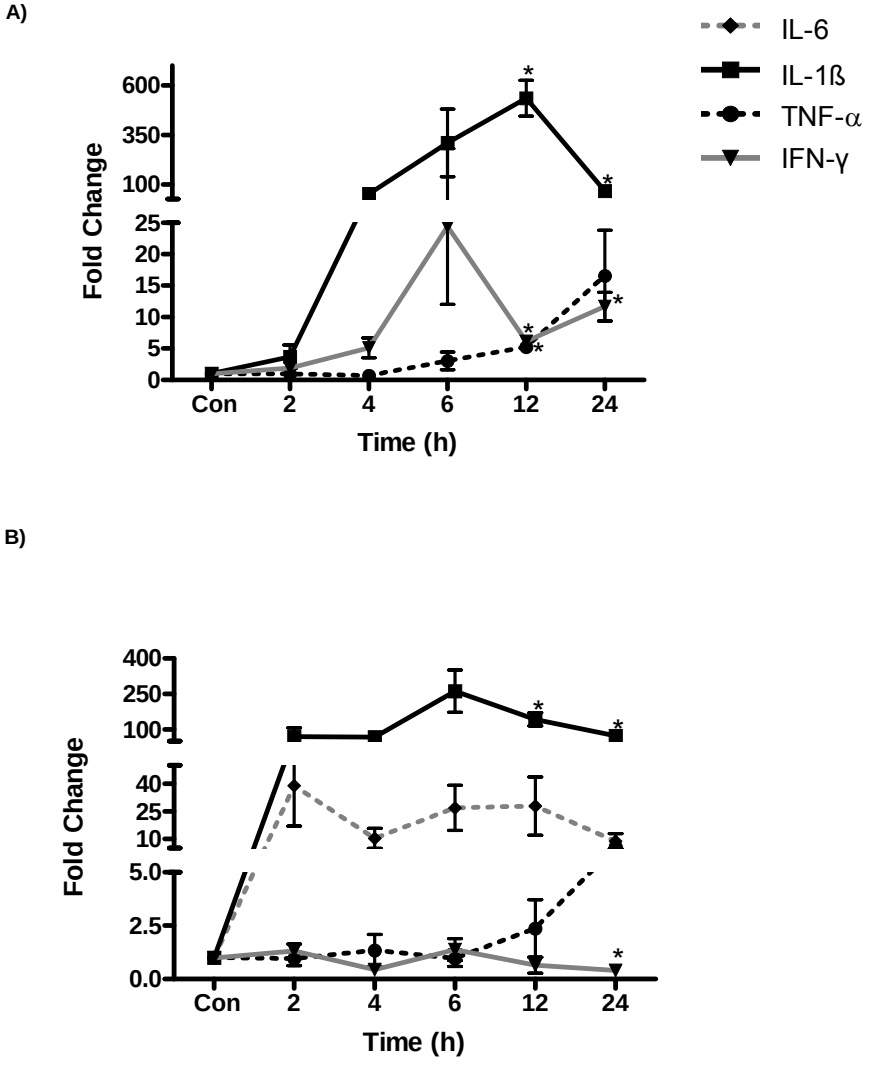
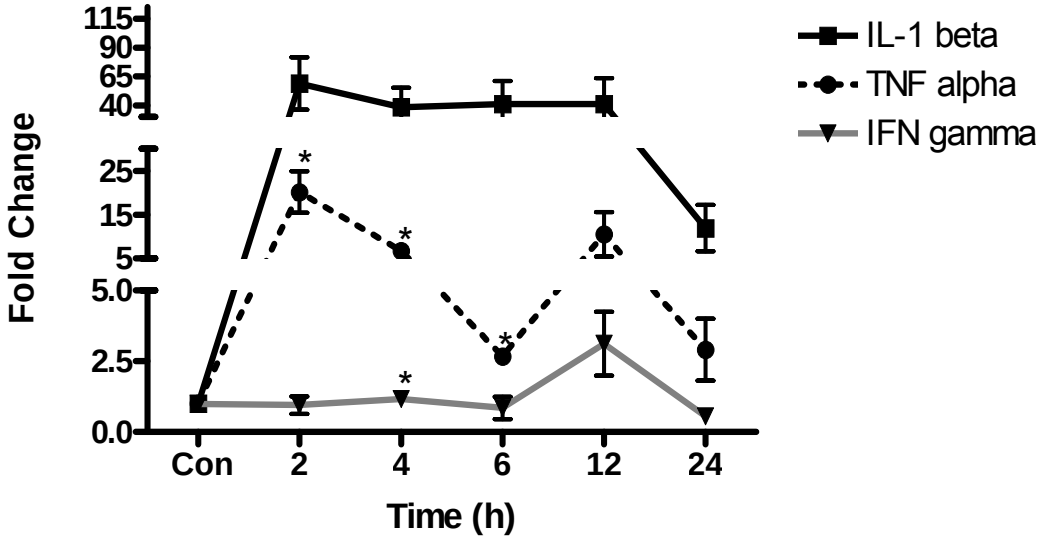


Figure 16:
Gene expression analysis of various pro-inflammatory cytokines in the hind limb muscle by quantitative RT-PCR: TO-treated IL-6 knockout mice **(A),** and their wild type equivalents **(B)**. (Results represent the mean value± s.e.m. (**P*<0.05, analyzed by *t*-test; *n*=4 animals per time point).



B)

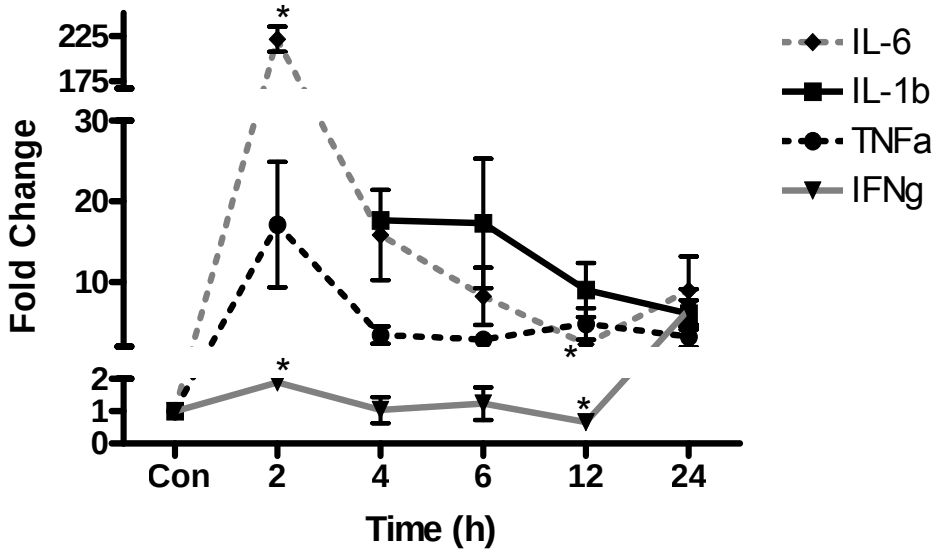
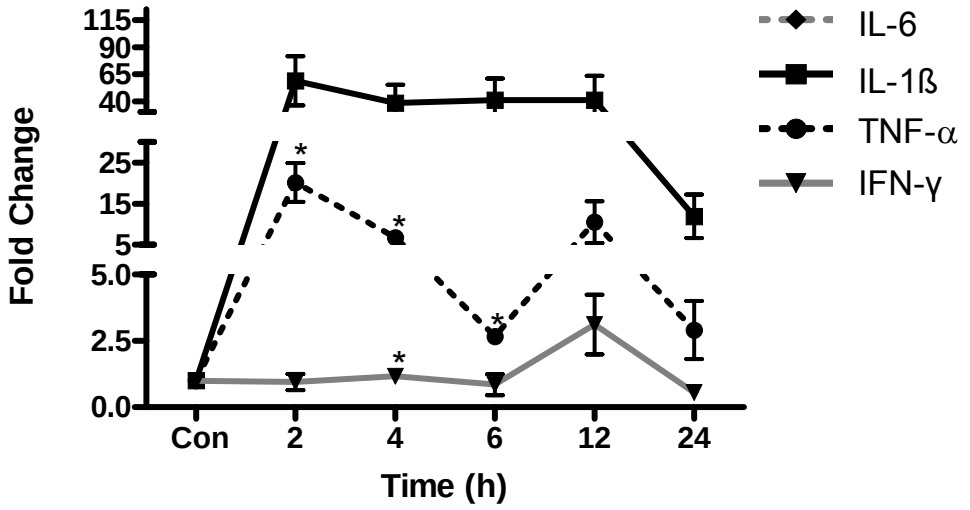


Figure 17:

Changes in hepatic gene expression of various pro-inflammatory cytokines in LPS treated IL-6 knockout mice by quantitative RT-PCR **(A)**, and their wild type counterparts**(B),** analyzed by real time PCR (results represent the mean value± s.e.m. **P*<0.05, analyzed by *t*-test; *n*=4 animals per time point).

A)



B)

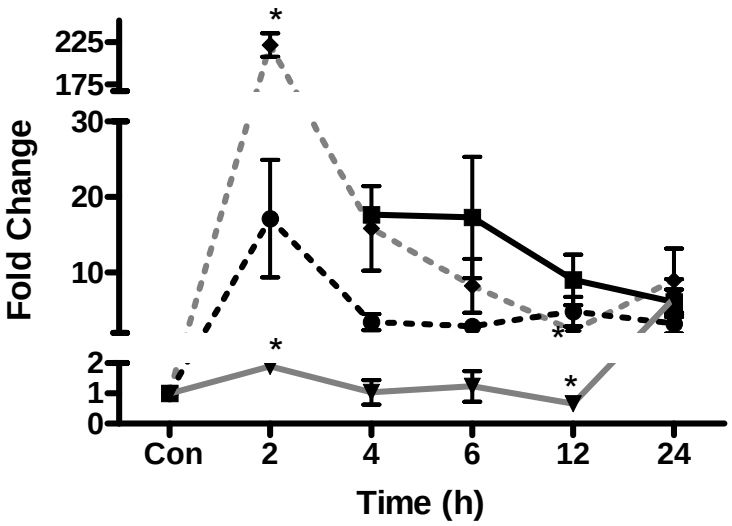


Figure 18:
Various pro-inflammatory cytokines mRNA expression inspection by quantitative RT-PCR in the hind limb muscles of LPS treated IL-6 knockout mice **(A)**, and their wild type counterparts**(B)** (results represent the mean value± s.e.m. **P*<0.05, analyzed by *t*-test; *n*=4 animals per time point).

4.11 Hepatic HO-1 gene expression upregulation by IL-1ß, TNF-α and IFN-γ

In order to analyze the direct effects of different other cytokines on hepatic HO-1 gene expression, mice were intraperitoneally injected with these cytokines. The results from these experiments indicate that TNF-α (Fig. 19), IL-1ß (Fig. 20), IFN-γ (Fig. 21) increased hepatic HO-1 gene expression.

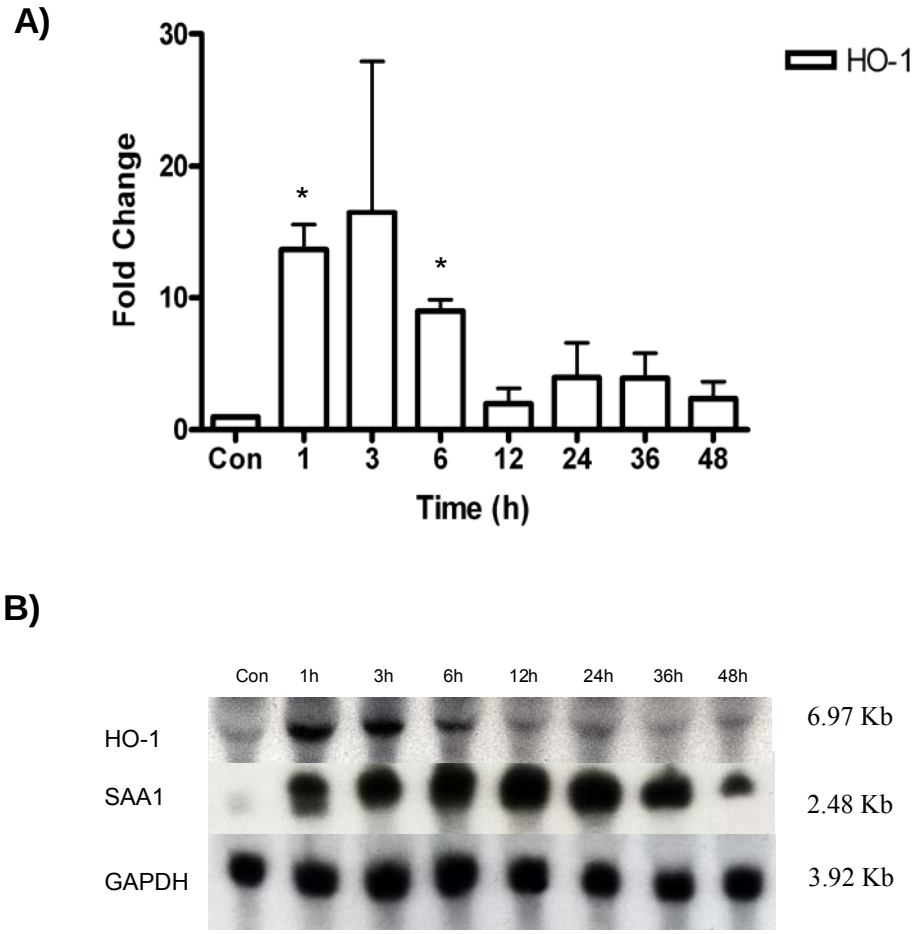


Figure 19:
Time-dependent effect of TNF-α on hepatic HO-1 mRNA expression: Mice were injected intraperitonealy with 2µg TNF-α to evaluate the time-kinetics of hepatic HO-1 gene expression after TNF-α injection. Livers were taken at different time points up to 48h. **(A)** Hepatic HO-1 mRNA expression analysis after TNF-α injection in a time dependent manner by real time quantitative PCR (Results represent the mean value± s.e.m. **P*<0.05, analyzed by *t*-test; *n*=3 animals per time point). **(B)** Northern blot analysis of HO-1 and SAA-1**.**

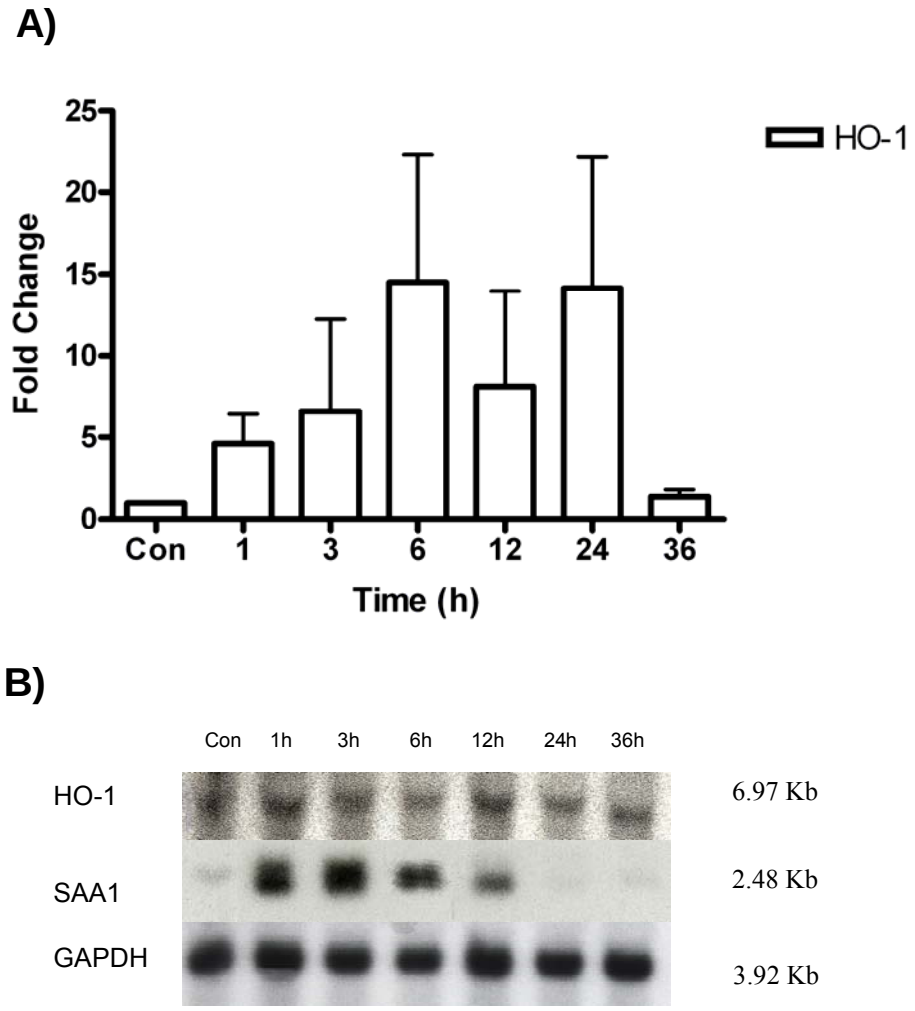


Figure 20:
Effect of IL-1 beta on hepatic HO-1 gene expression: Mice were injected intraperitonealy with 2µg interleukin 1 beta. Livers were excised at different time points after injection and quickly stored in liquid nitrogen. Quantitative real time PCR (results represent the mean value± s.e.m. **P*<0.05, analyzed by *t*-test; *n*=2 animals per time point) **(A)** and Northern blot **(B)** analysis of hepatic HO-1 and SAA-1 mRNA.

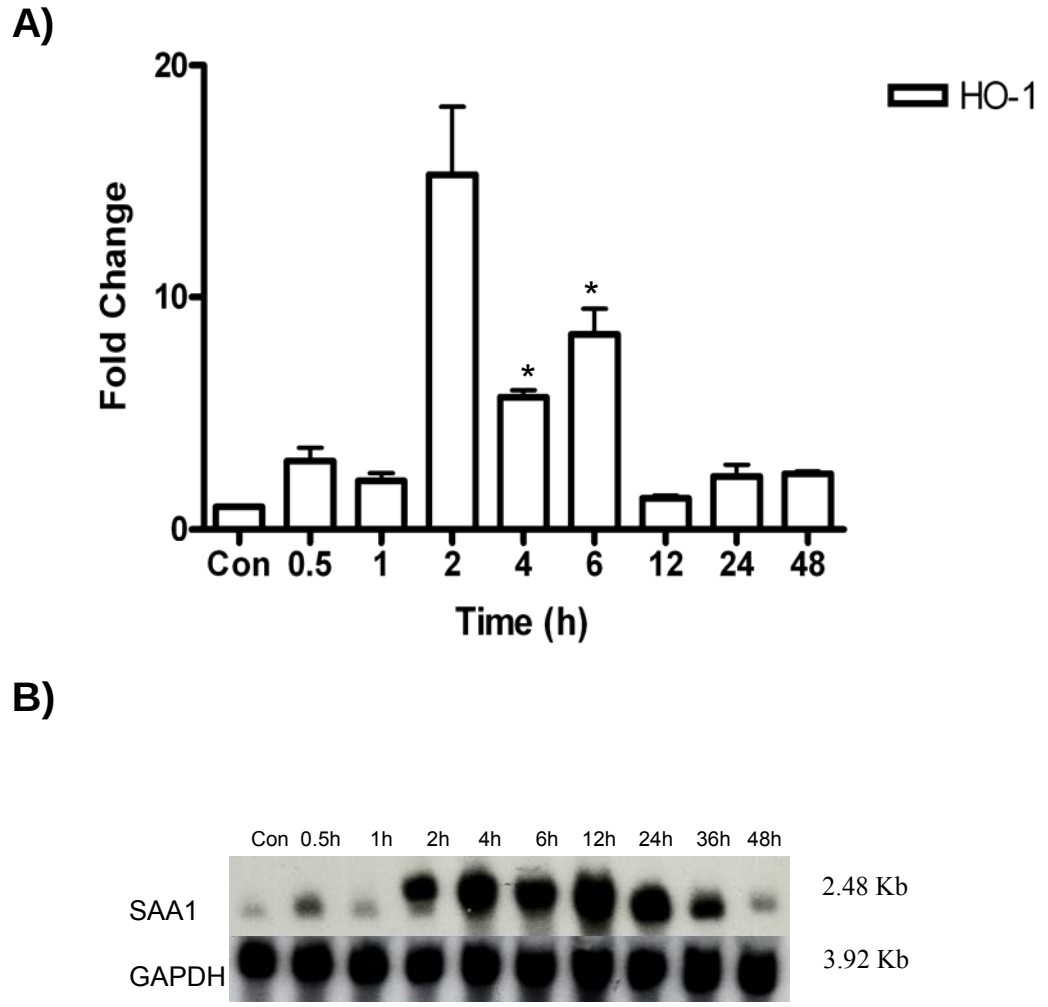


Figure 21:
Regulation of hepatic HO-1 by IFN gamma: A group of mice was treated with IFNγ in a time dependent manner from 1h till 48h. Northern blot **(B)** and quantitative real time PCR (results represent the mean value± s.e.m. **P*<0.05, analyzed by *t*-test; *n*=2-3 animals per time point) **(A)** analysis show the changes in hepatic HO-1 and SAA-1 mRNA expression in these animals.

4.12 HO-1 gene expression analysis in the skeletal muscles of TO- and LPS-treated wild type and IL-6 knockout mice

HO-1 mRNA was upregulated in the injured muscle of TO-treated wild type and IL-6 knockout mice (Fig. 22); however, the statistical significance was seen only at 24h in wild type mice. Although non-significant, but it appeared that the induction of HO-1 mRNA was delayed in the injured muscle of IL-6 knockout mice, and the extent of upregulation of HO-1 mRNA was higher in wild type mice. In LPS treated animals (Fig. 23), HO-1 gene expression increased, but significant upregulation was seen only at 12h in wild type mice and at 24h in IL-6 knockout mice. HO-1 gene expression was determined in the non-injured muscle of TO-treated wild type and knockout mice, and it was interesting to note that APR

resulted in an increase of HO-1 mRNA in the non-injured muscle of these TO-treated animals (Fig.23). This increase was statistically significant only in wild type mice. In TO-treated IL-6 knockout mice, the increase of HO-1 mRNA was not significant.

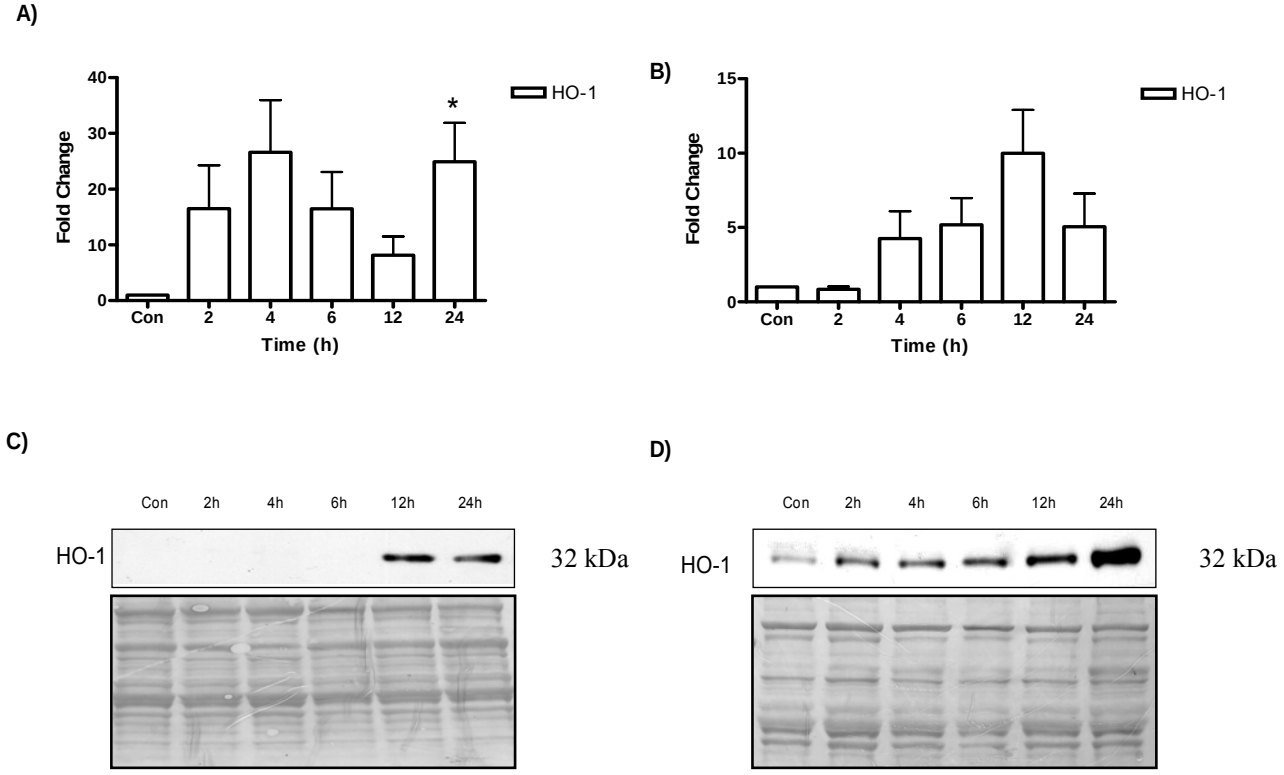


Figure 22:
HO-1 gene expression analysis by quantitative RT-PC, in the hind limb muscle of TO- and LPS-treated mice: **(A)** HO-1 mRNA in the injured hind limb muscle of TO-treated wild type mice. **(B)** HO-1 mRNA expression analysis in the injured muscle of hind limb of TO-treated IL-6 knockout mice (results represent the mean value± s.e.m. **P*<0.05, analyzed by *t*-test; *n*=4 animals per time point). **(C)** HO-1 protein expression analysis in injured hind limb muscle of TO-treated wild type mice by Western blotting **(D)** Western blot analysis of HO-1 in the injured muscle of hind limb of TO-treated IL-6 knockout mice.

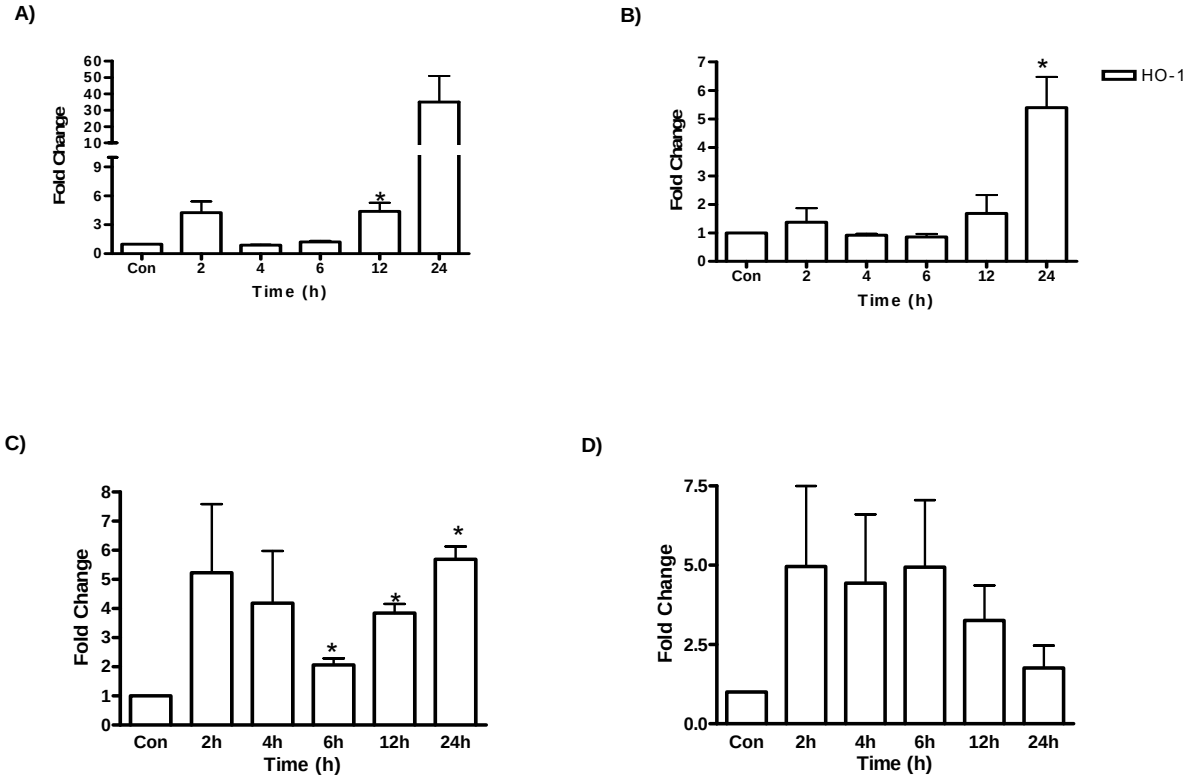


Figure 23:
HO-1 gene expression analysis by quantitative RT-PCR in the non-injured muscle of LPS- and TO-treated mice: **(A)** HO-1 mRNA expression changes in the hind limb muscle of LPS treated wild type mice. **(B)** Changes in HO-1 gene expression in the hind limb muscle of LPS treated IL-6 knockout mice. **(C)** HO-1 mRNA expression in non-injured muscle of TO-treated wild type mice **(D).** HO-1 mRNA expression in non-injured muscle of TO-treated IL-6 knockout mice (results represent the mean value± s.e.m. *P<0.05, analyzed by t-test; n=4 animals per time point).

4.13 Lactoferrin gene expression in liver

Acute phase inflammation resulted in an increase of hepatic lactoferrin mRNA expression, and the magnitude and duration of this upregulation was found to depend on the causative stimuli of APR. In TO-treated animals a significant increase in lactoferrin gene expression in liver was seen at 6h (P= 0.0139) and 12h (P= 0.0220) and the level of mRNA was still rising at 24h (Fig 24). However, in LPS-treated wild type animals an immediate increase of lactoferrin mRNA at 2h (P<0.0001) was observed in the liver that peaked at 6h (P<0.0001) and then returned to basal level at 24h (Fig. 25). The increase in lactoferrin mRNA was followed by an equivalent increase in protein synthesis. As for transferrin, it increased slightly but statistically significant at 4h (P=0.0235) in TO-treated animals and then its expression declined. In the livers of LPS-treated animals also its expression increased slightly but it was not significant, however in these animals a significant decrease of transferrin mRNA was seen at 6h (P=0.0122), 12h (P=0.0145) and 24h (P=0.0463) (Fig. 24 &25).

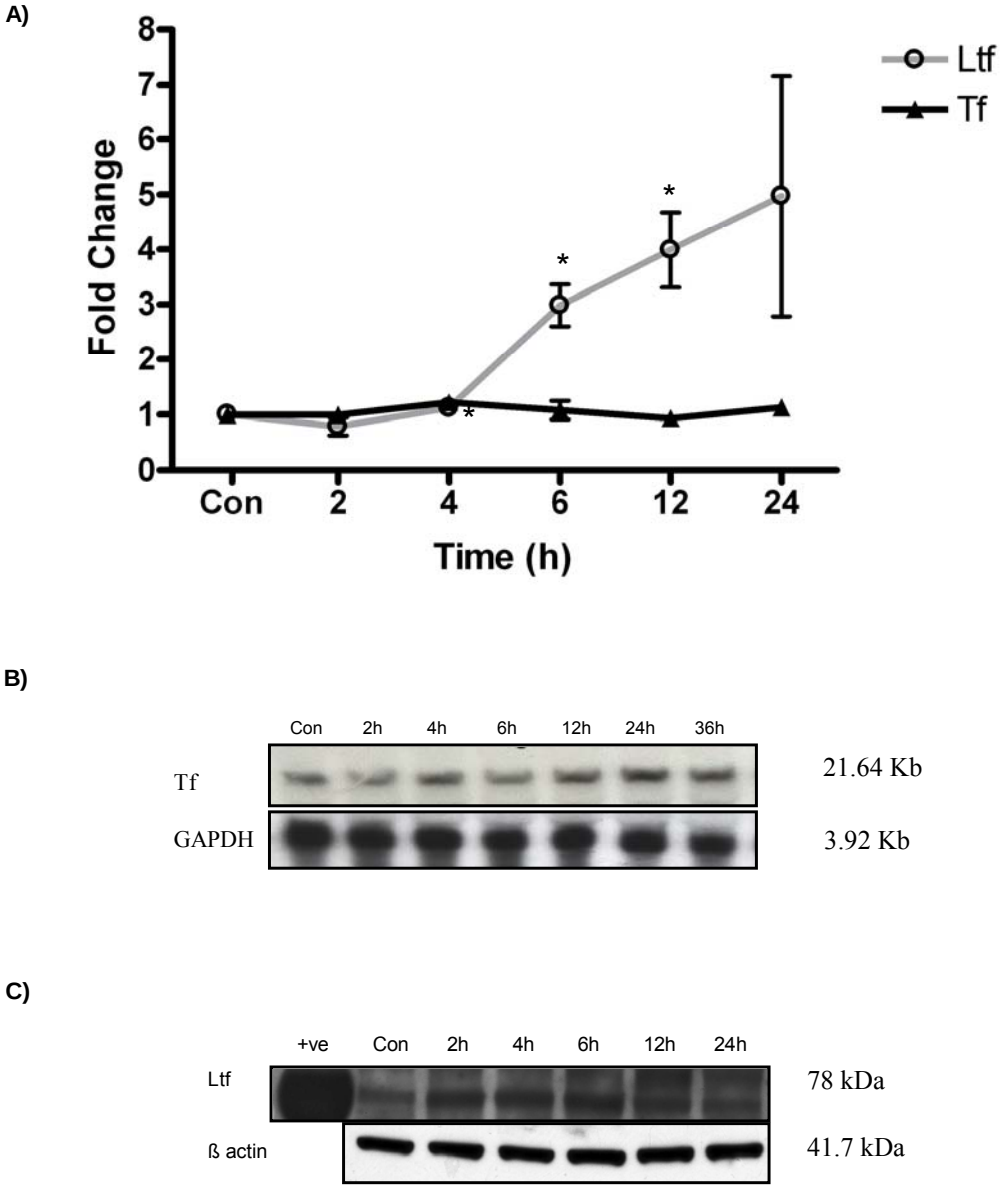
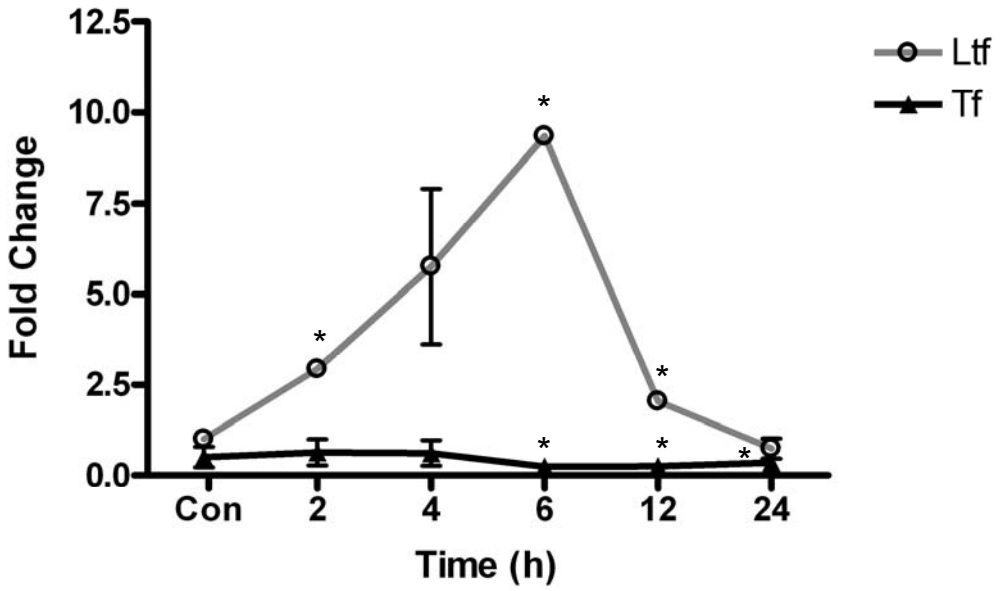


Figure 24:
Ltf and Tf gene expression regulation in the liver of TO- treated wild type mice: Wild type C57Bl6 male mice were injected with 10ml/kg turpentine oil (TO) i.e., 100µl in each of the hind limb muscle to induce localised tissue injury. **(A)** RNA expression analysis of lactoferrin and transferring by quantitative RT- PCR (results represent the mean value± s.e.m. **P*<0.05, analyzed by *t*-test; *n*=4 animals per time point) (**B)** Northern blot analysis to study transferrin mRNA expression (representative of one series of animals). **(C)** Western blot analysis of lactoferrin in total liver homogenate (representative of one animal per time point).

A)



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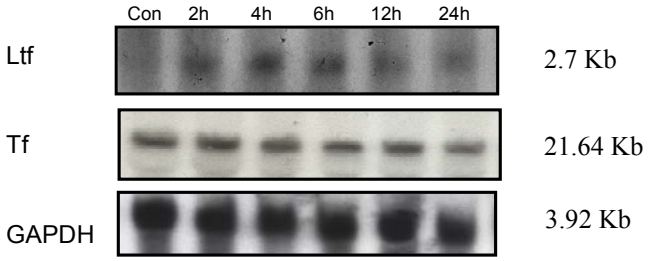


C)

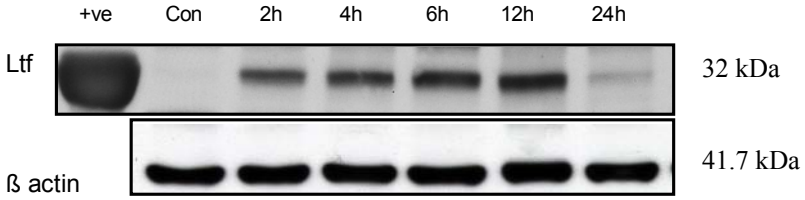


Figure 25:
Hepatic lactoferrin and transferrin gene expression analysis in LPS-treated wild type mice: Wild type C57Bl6 male mice were intraperitoneally injected 50µg of LPS from *E.coli* to produce systemic inflammation. Control animals received no treatment. **(A)** Ltf and Tf mRNA expression analysis by quantitative RT-PCR in the liver of LPS-treated mice (Results represent the mean value± s.e.m. (**P*<0.05, analyzed by *t*-test; *n*=4 animals per time point). **(B)** Northern blot analysis to study mRNA expression of Ltf and Tf (representative of one series of animals). **(C)** Protein expression analysis of lactoferrin in total liver homogenate by Western blot (representative of one series of animals).

4.14 IL-6 induced lactoferrin gene expression in liver

In order to determine the role of IL-6 to induce lactoferrin gene expression, mice were intraperitoneally injected with IL-6 (Fig. 26). IL-6 induced significant expression of lactoferrin mRNA and of the protein at 2h (P=0.0314) in the liver of these mice.

Hepatocytes are the major cell population in liver and play a major role in the induction of acute phase response. In order to determine if the hepatocytes are able to produce lactoferrin under the influence of IL-6, murine hepatocytes were treated with different doses of IL-6 (Fig. 27). IL-6 significantly upregulated lactoferrin gene expression in the primary hepatocytes' cell culture in a dose dependent manner (Fig. 27). Similar results were obtained by treating murine hepatic carcinoma cell line hepa 1-6 with different doses of IL-6 (Fig. 28). Hepa 1-6 cells showed significant increase of lactoferrin mRNA with 0.1ng (P=0.0004), 100ng (P=0.0011), 500ng (P<0.0001) and 1000ng (P<0.0001) of IL-6. The changes in mRNA levels were followed by equivalent changes in protein expression in these cells (Fig. 28).

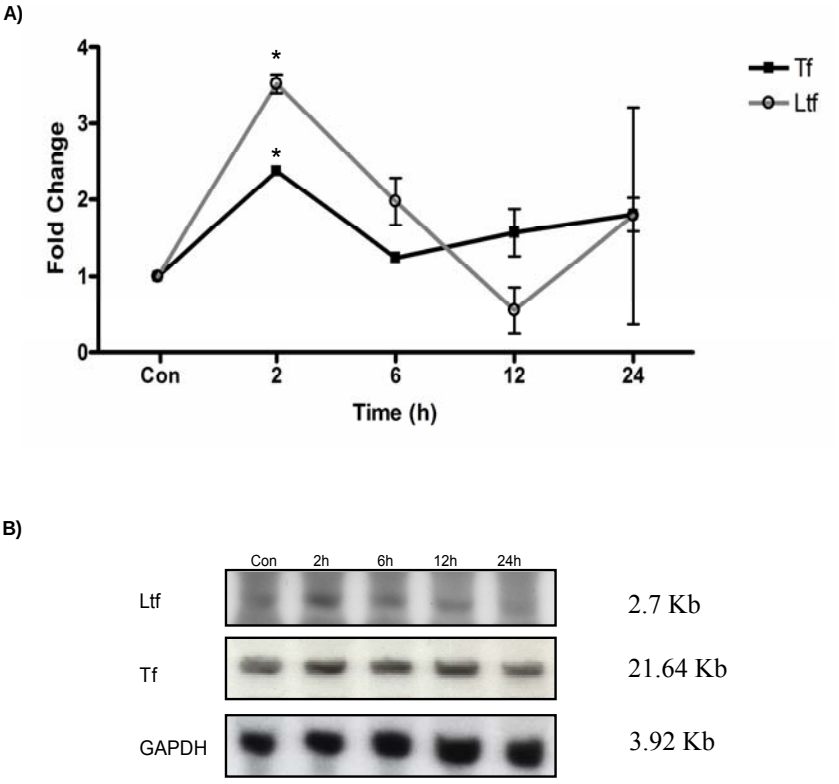


Figure 26:

Effect of IL-6 on Ltf gene expression: Mice were injected intraperitonealy with 1000 units of interleukin 6 to analyze the time dependent kinetics of hepatic Ltf expression **(A)** Ltf gene expression was analyzed by quantitative RT-PCR (results represent the mean value± s.e.m. (**P*<0.05, analyzed by *t*-test; n= 2-3 animals per time point). **(B)** Northern blot analysis of Ltf and Tf (representative of one series).

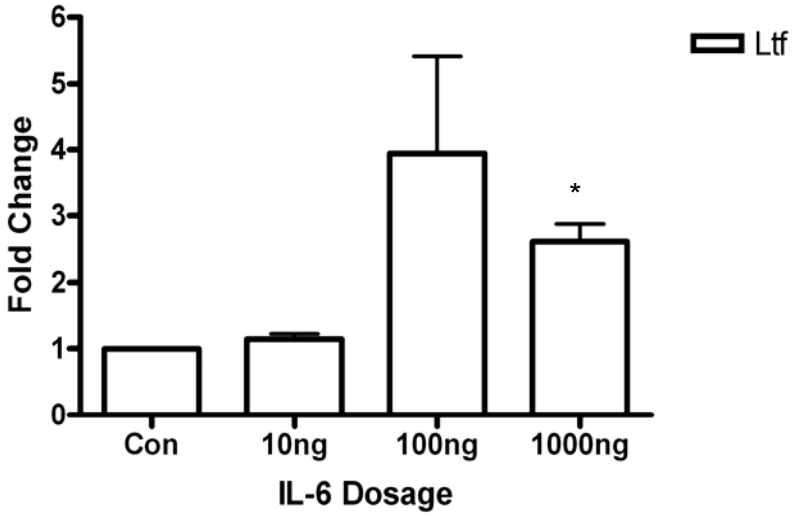


Figure 27:
IL-6 dependent regulation of Ltf mRNA: Murine hepatocytes were treated with varying doses of IL-6 and were harvested after 6 hours. Quantitative RT-PCR analysis of Ltf mRNA induction in mouse hepatocytes (results represent the mean value± s.e.m. (**P*<0.05, analyzed by *t*-test; *n*=3 replicates).

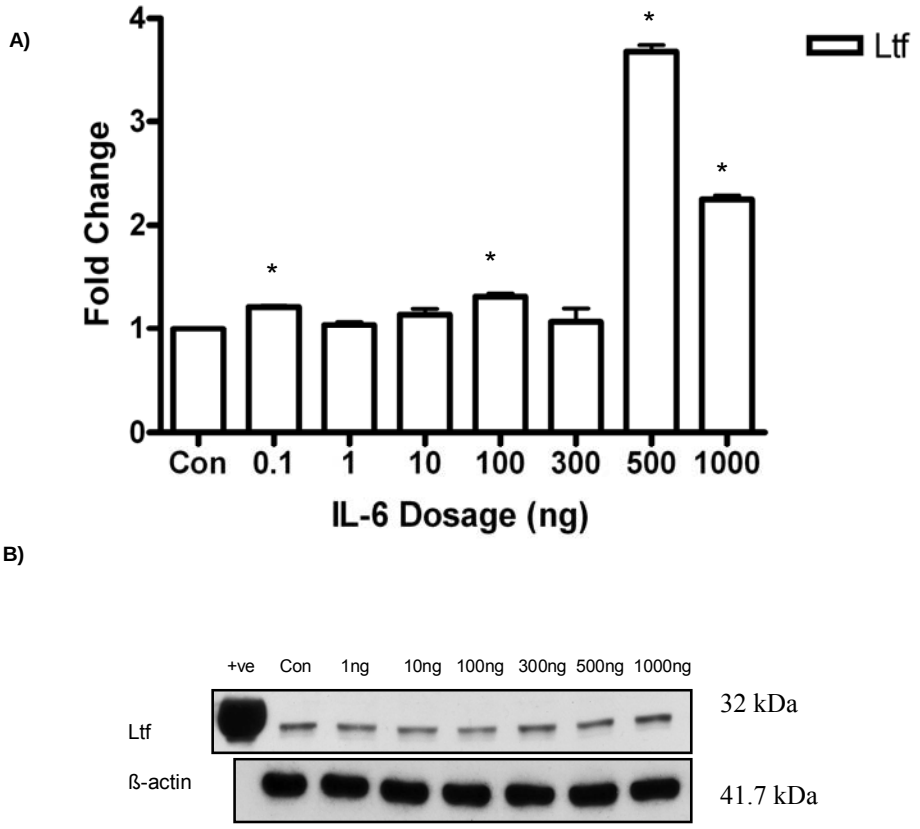


Fig 28:
IL-6 treated hepa 1-6 cells. **(A)** Ltf gene expression analysis by quantitative RT-PCR (results represent the mean value± s.e.m. (**P*<0.05, analyzed by *t*-test; *n*=3 replicates). **(B)** Ltf gene expression analysis by Western blot.

4.15 Changes in lactoferrin gene expression in the liver of IL-6 knockout mice after TO- or LPS-injection

IL-6 boosts lactoferrin gene expression and might be the major regulator of Ltf gene expression. However, under certain pathological conditions blocking IL-6 might prove helpful in controlling the inflammation, but lactoferrin itself is part of the protective mechanisms of the immune system and is able to reduce the severe impacts of oxidative stress at the molecular level. Therefore, blocking of IL-6 might also result in the inhibition of induction of Ltf during acute phase reaction and thereby rendering the individuals more vulnerable to deleterious impacts of inflammatory response. In order to determine if IL-6 is the only inducer of lactoferrin in liver during APR and to further explore the specific role of IL-6 to regulate Ltf gene expression, we induced APR in IL-6 knockout mice with TO and LPS just like the wild type counterparts. Lactoferrin was induced in the liver of these animals even in the absence of IL-6, indicating that IL-6 is not the only inducer of hepatic lactoferrin during APR. In TO-treated IL-6 knockout mice magnitude of lactoferrin mRNA expression was comparable to that observed in their wild type counterparts. However an earlier decline of Ltf mRNA was seen in the knockout mice at 24h ($P=0.0351$) after the peak expression at 12h ($P=0.0049$) was revealed. mRNA expression changes were followed by equivalent adjustments of lactoferrin expression at protein level in the liver of TO-treated IL-6 knockout mice (Fig. 29). Transferrin showed a slight but significant decrease at 2h ($P=0.0015$), but otherwise its levels in liver remained almost the same during the time after TO-treatment. Furthermore, in LPS-treated IL-6 knockout mice (Fig. 30) the magnitude of upregulation of lactoferrin expression was much higher as compared to their wild type equivalents, and unlike LPS-treated wild type mice, in the knockout animals LPS resulted in an immediate increase in hepatic lactoferrin expression, although it was statistically non-significant. However, as seen in the wild type mice, LPS injection resulted in a decline of transferrin mRNA expression in the liver of IL-6 knockout mice. The statistically significant changes were seen at 4h ($P<0.0001$), 6h ($P=0.0024$) and 12h ($P=0.0005$).

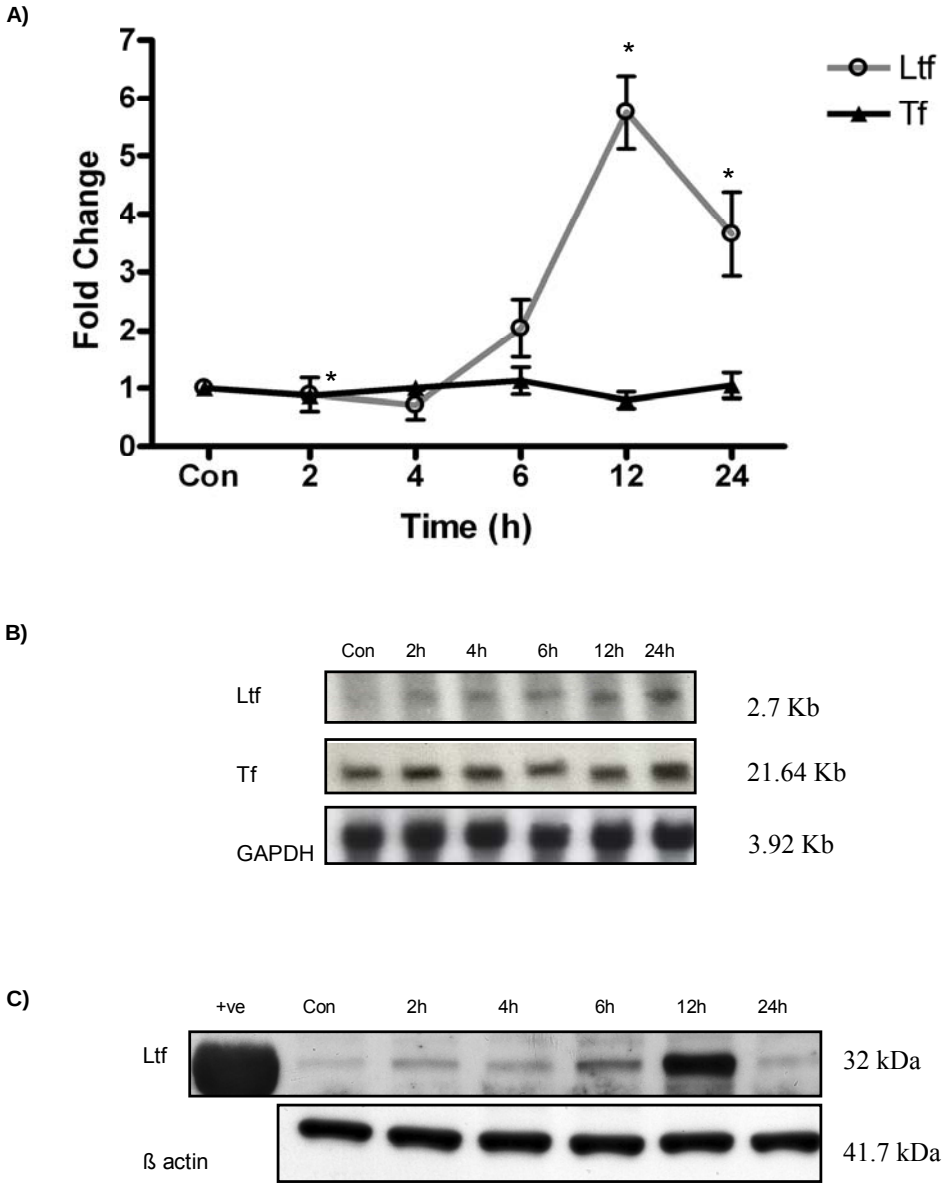


Figure 29:
Ltf and Tf mRNA expression in TO-treated IL-6 knockout mouse liver: Male IL-6 knockout mice (B6.129S2-IL6^{tm1Kopf}/J) were injected with 100µl turpentine oil in each of the hind limb muscle. Control animals received no treatment. **(A)** Ltf and Tf mRNA expression analysis by quantitative RT-PCR in the livers of TO treated IL-6 knockout mice (results represent the mean value± s.e.m. (**P*<0.05, analyzed by *t*-test; *n*=4 animals per time point). **(B)** Northern blot to analyze hepatic mRNA expression of Ltf and Tf (representative of one series of animals). **(C)** Western-blot analysis of Ltf from total liver protein.

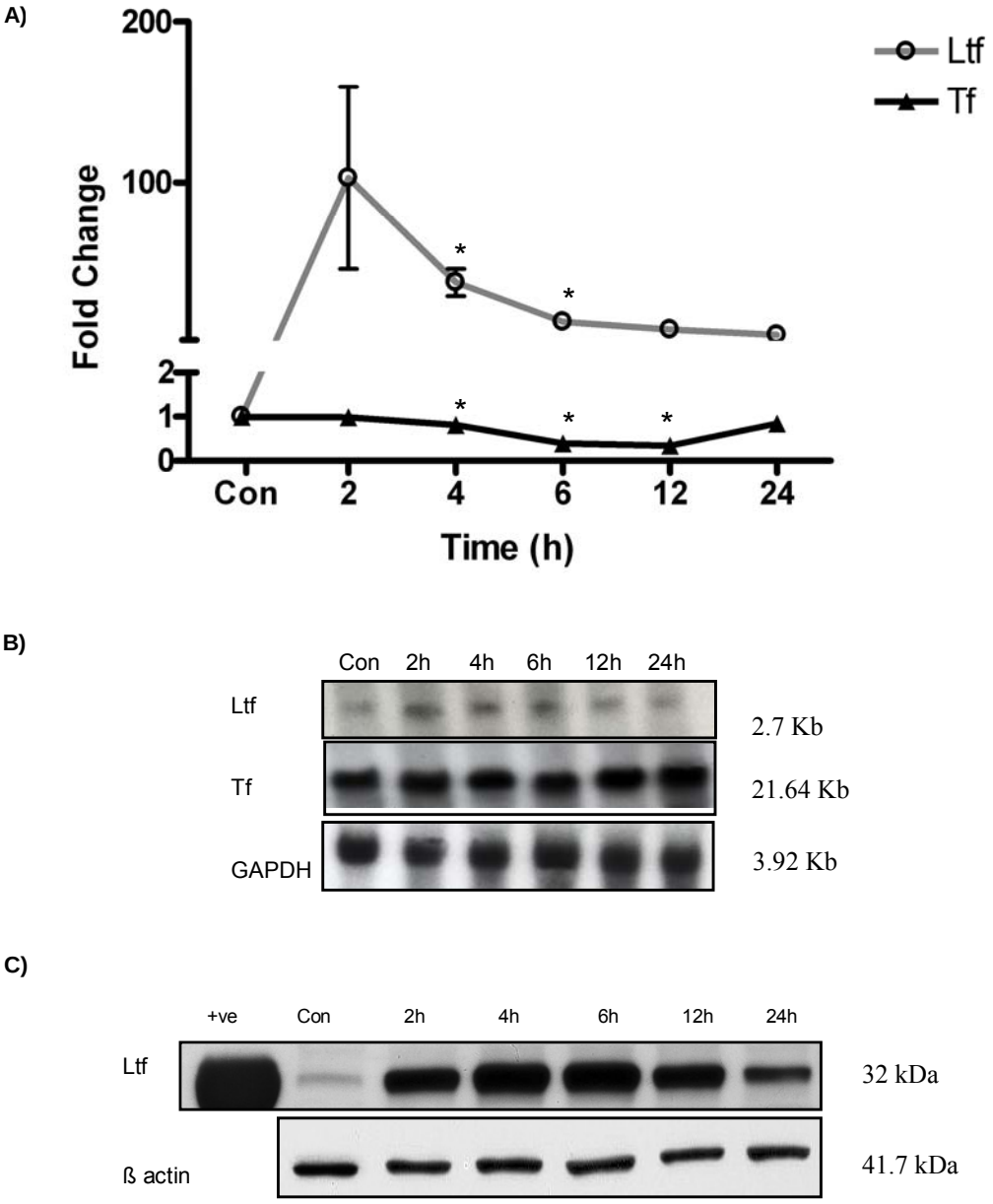


Figure 30:
Ltf and Tf gene expression examination in LPS-treated IL-6 knockout mouse liver: Male IL-6 knockout mice (B6.129S2-IL6^{tm1Kopf}/J) were intraperitoneally injected with 50µg of LPS to induce systemic inflammatory response. Control animals received no treatment. **(A)** Ltf and Tf mRNA expression analysis by quantitative RT-PCR in the liver of LPS-treated IL-6 knockout mice (results represent the mean value± s.e.m. (**P*<0.05, analyzed by *t*-test; *n*=4 animals per time point). **(B)** Northern blot to study mRNA expression of Ltf and Tf in liver (representative of one series of animals). **(C)** Western blot analysis of Ltf in total liver homogenate (representative of one series of animals).

4.16 Serum lactoferrin levels

Lactoferrin is a secretory protein. Therefore, lactoferrin levels in the sera of TO- and LPS-treated animals were determined using an ELISA kit. In TO-treated wild type animals there was an immediate increase in serum lactoferrin levels 2h and 4h (P=0.0397), which was followed by a statistically non-significant drop in serum Ltf levels and then at 24h (P=0.0394) again a significant increase was seen in serum lactoferrin levels. However, in the sera of TO-treated IL-6 knockout mice, although slight increases were seen in serum lactoferrin levels in the initial hours of APR, but a statistically significant increase of serum lactoferrin was seen at 12h (P=0.0015) only, followed by a decline (Fig. 31). On the other hand, LPS induced an immediate increase in serum Ltf levels in wild type mice starting at 2h (P=0.0344), peaked at 4h (P=0.0023), and then started declining at 6h (P=0.0023), 12h (P=0.0030) and 24h (P=0.0027) very constantly. LPS-treated IL-6 knockout mice also showed statistically significant upregulation of serum lactoferrin levels, immediately after the i.p. LPS injection (Fig. 32).

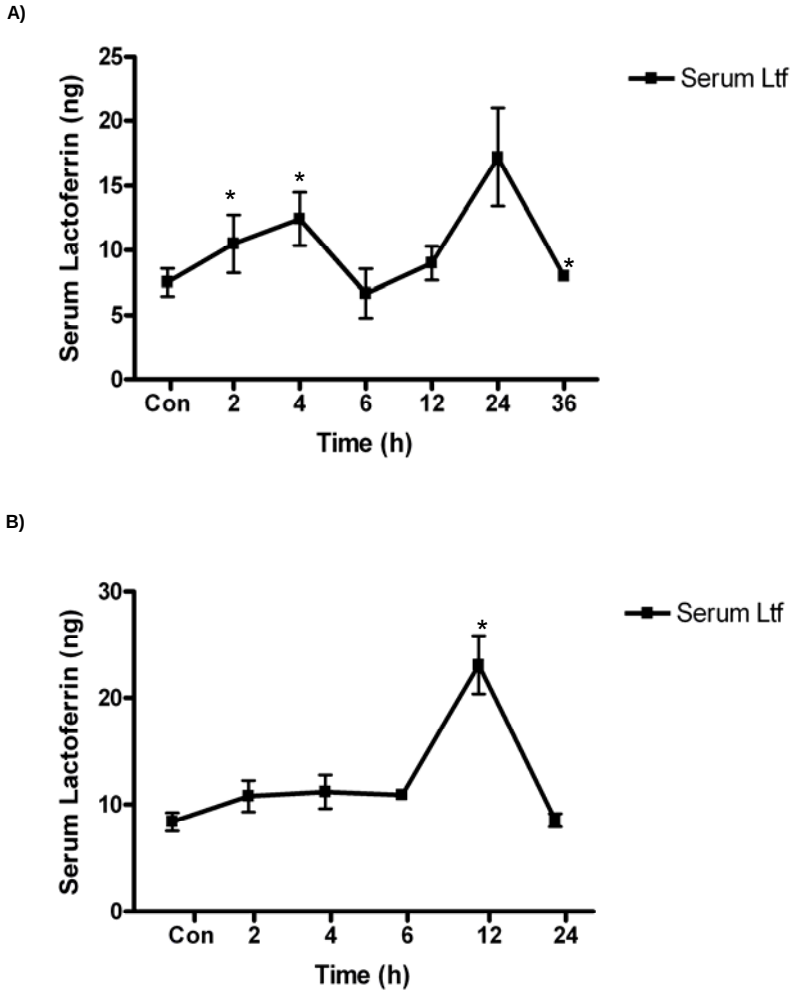


Figure 31:
Serum lactoferrin concentration analyzed by an ELISA **(A)** TO-treated C57Bl6 male mice. **(B)** TO-treated male IL-6 knockout mice (results represent the mean value± s.e.m. (**P*<0.05, analyzed by *t*-test; *n*=4 animals per time point).

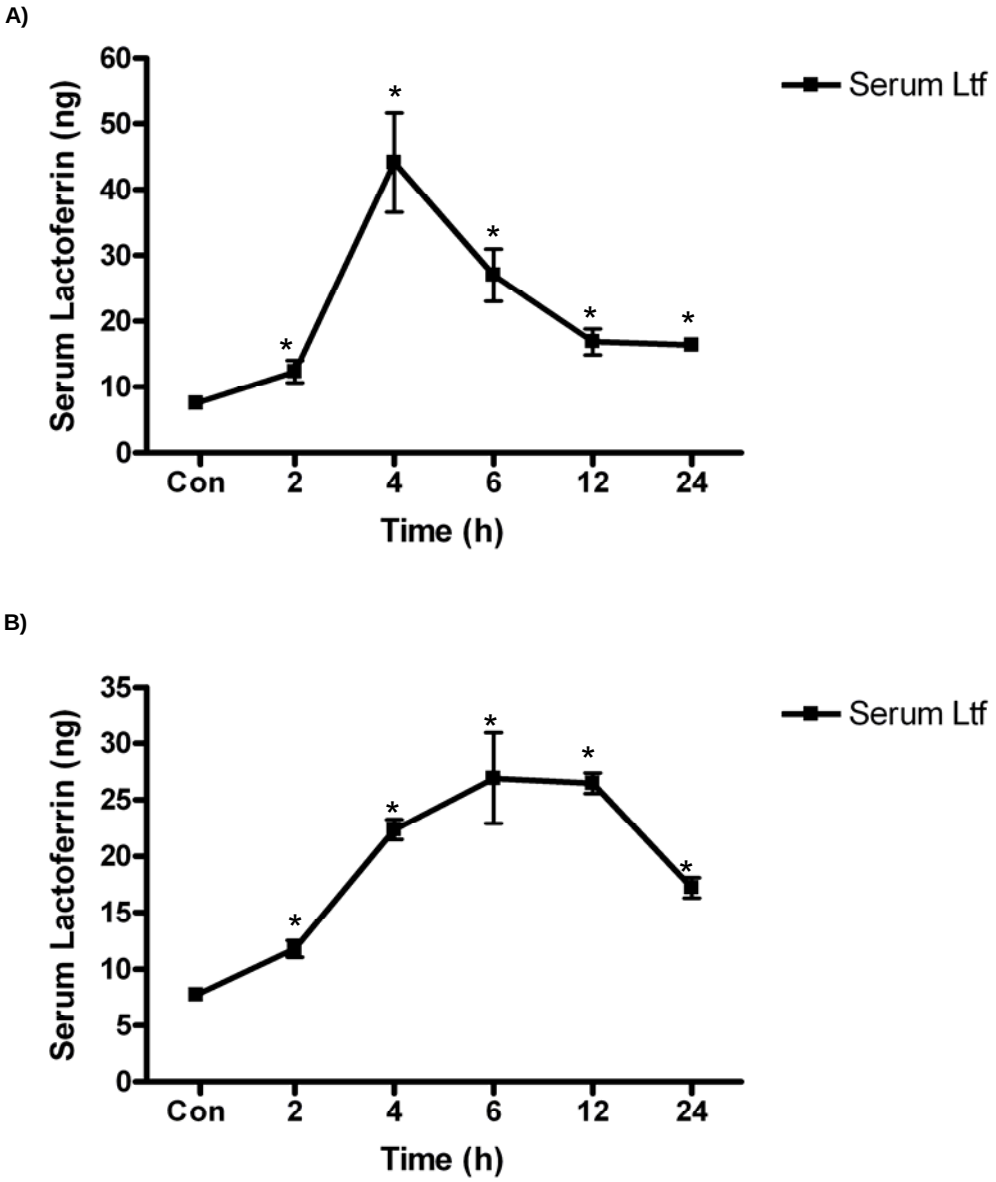


Figure 32:
Serum lactoferrin concentration analyzed by ELISA: **(A)** LPS-treated C57Bl6 male mice. **(B)** LPS-treated male IL-6 knockout mice (results represent the mean value± s.e.m. (**P*<0.05, analyzed by *t*-test; *n*=4 animals per time point).

4.17 Role of different cytokines in lactoferrin gene expression regulation

After evaluating that although IL-6 enhances hepatic Ltf gene expression, but is not the only inflammatory signal that regulates Ltf gene expression during acute phase reaction, we analyzed how other acute phase cytokines affect lactoferrin gene expression in liver.

Intraperitoneal injection of TNF-α enhanced Ltf gene expression in liver (Fig. 33). Statistically significant increase of Ltf mRNA was seen 3h and 6h (P=0.0054) post TNF-α injection. In the same animals, after TNF-α injection, hepatic transferrin mRNA increased significantly at 0.5h, but then was continuously down regulated, although this decline was not statistically significant. Similarly, TNF-α treatment to hepa 1-6 cells resulted in a significant increase of Ltf mRNA at 3h (P=0.0083), 6h (P=0.0022) and 12h (P=0.0358) (Fig. 36A).

IL-1ß treatment of mice could not increase lactoferrin mRNA statistically significant in mouse liver (Fig. 34), neither could the *in vitro* stimulation of IL-1ß to hepa 1-6 cells (Fig. 36B).

Significant increase of hepatic Ltf mRNA was observed after 0.5h (P=0.0216), 1h (P=0.0337) and 2h (P=0.0055) post intraperitoneal injection of IFN-γ (Fig. 35). Likewise, IFN-γ treatment to hepa 1-6 cells resulted in a statistically prominent increase of Ltf mRNA at 3h (P= 0.0276), 6h (P= 0.0002) and 12h (P<0.0001) (Fig. 36C).

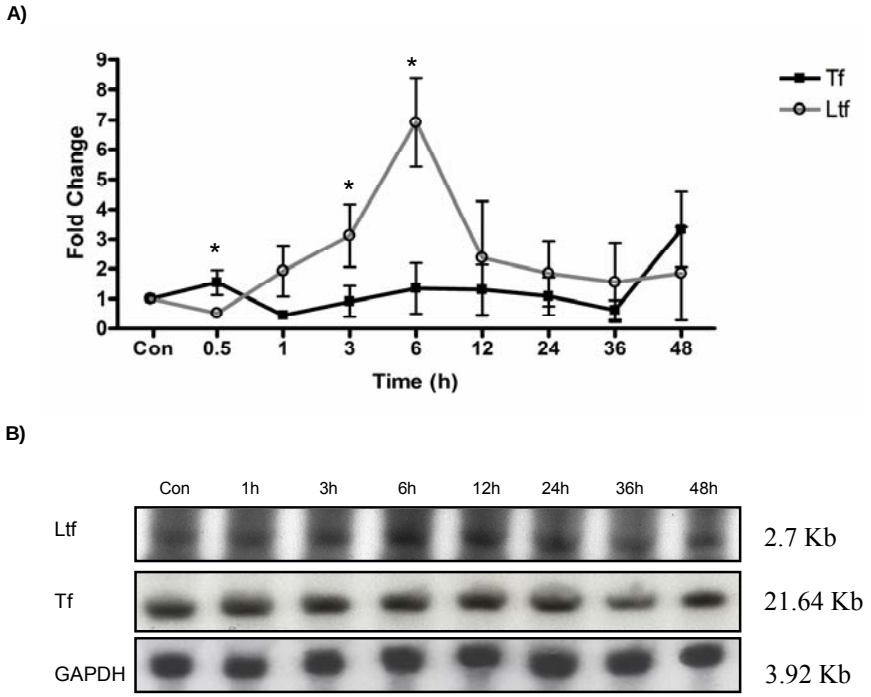


Figure 33:
Time-dependent effect of TNF-α on hepatic Ltf and Tf mRNA expression: mice were injected intraperitonealy with 2µg TNF-α to evaluate the time-kinetics of hepatic Ltf and Tf gene expression after TNF-α injection. Livers were taken at different time points up to 48 hours. **(A)** Hepatic Ltf and Tf mRNA expression analysis after TNF-α injection by quantitative real time PCR (Results represent the mean value± s.e.m. **P*<0.05, analyzed by *t*-test; *n*=3 animals per time point). **(B)** Northern blot analysis of Ltf and Tf**.**

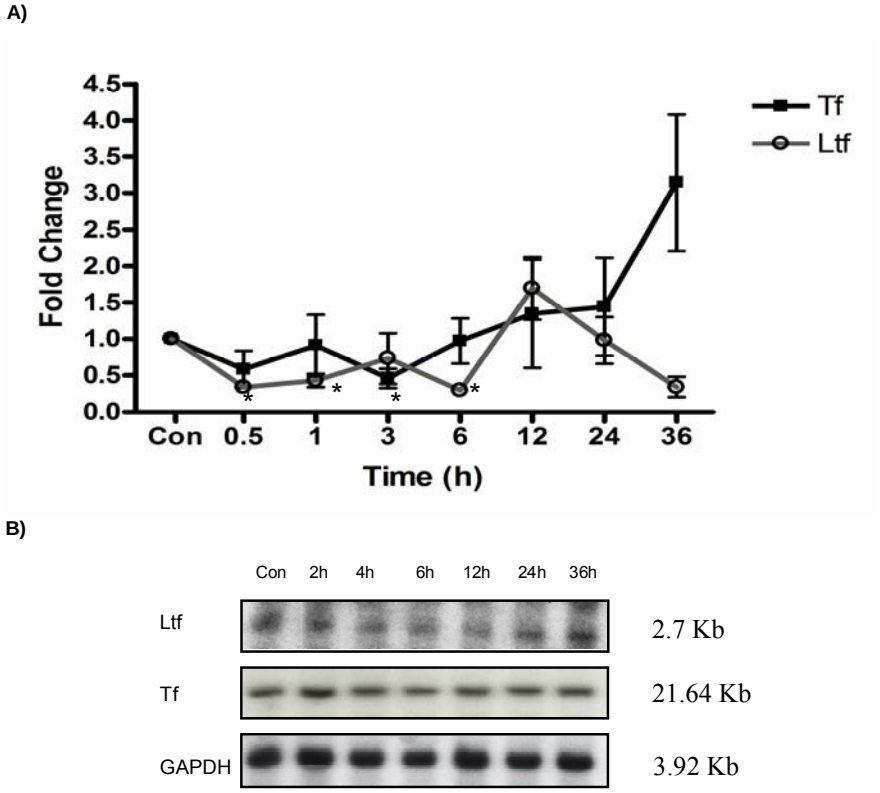


Figure 34:
Effect of IL-1ß on hepatic Ltf gene expression: Mice were injected intraperitonealy with 2µg interleukin 1ß. Livers were excised at different time points after injection and quickly stored in liquid nitrogen. **(A)** Quantitative real time PCR analysis of hepatic Ltf and Tf mRNA (results represent the mean value± s.e.m. **P*<0.05, analyzed by *t*-test; *n*=2 animals per time point) and **(B)** Northern blot analysis of hepatic Ltf and Tf mRNA.

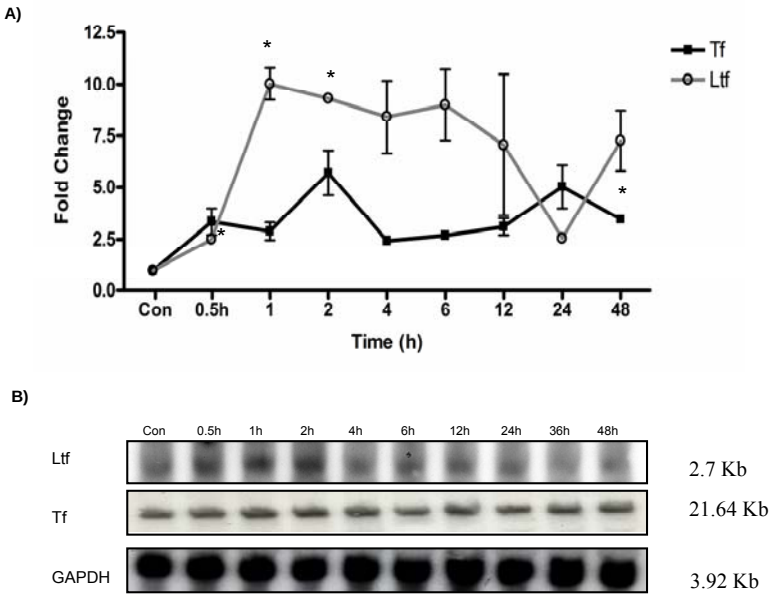


Figure 35:
Regulation of hepatic Ltf mRNA expression by IFN-γ: A group of mice was treated with IFN-γ in a time dependent manner from 1h till 48h. Northern blot **(B)** and quantitative real time PCR (Results represent the mean value± s.e.m. **P*<0.05, analyzed by *t*-test; *n*=2-3 animals per time point) **(A)** analysis show the changes in hepatic Ltf and Tf mRNA expression in these animals.

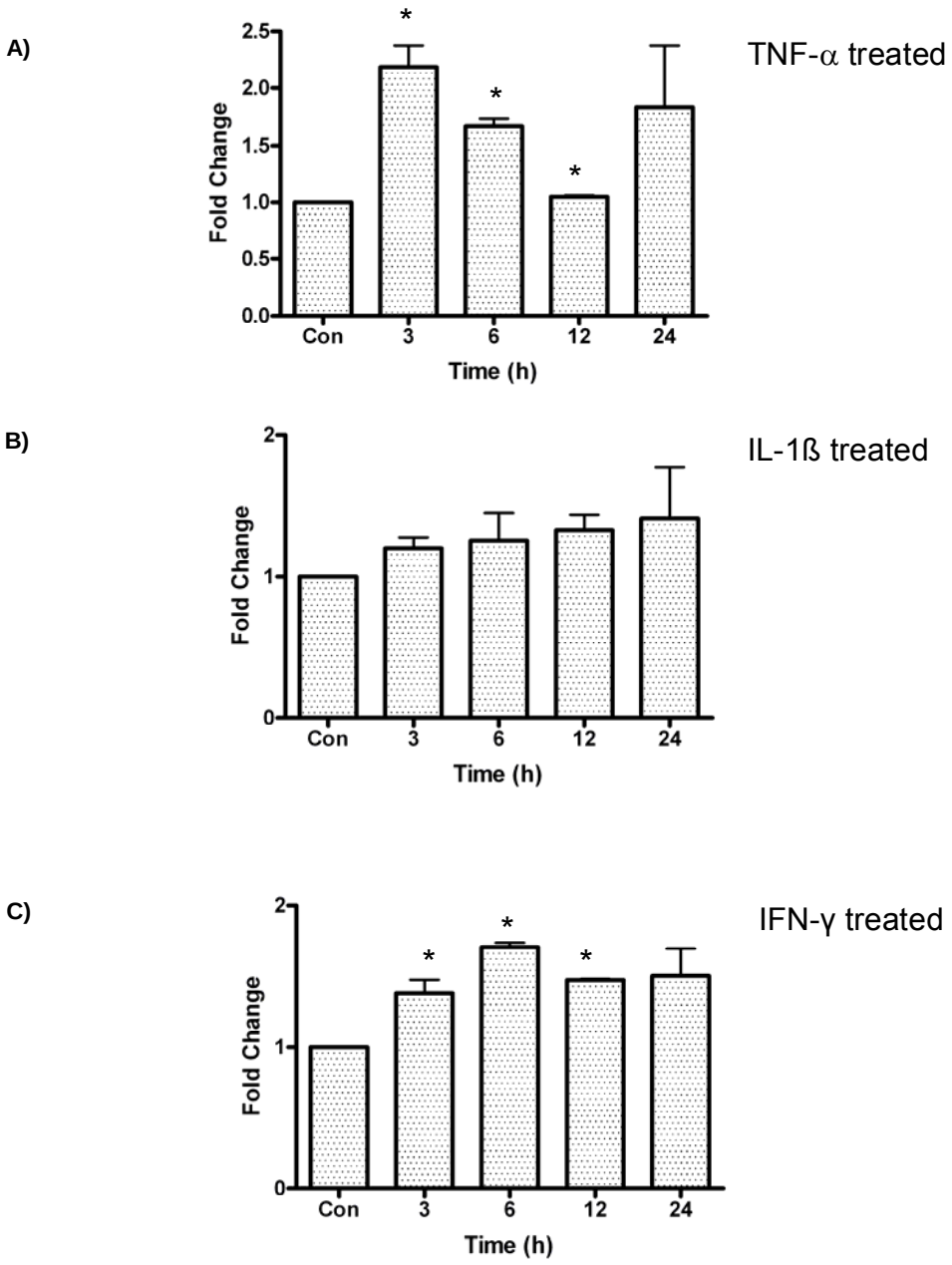


Figure 36:
Ltf gene expression analysis by quantitative RT-PCR in cytokines treated heap 1-6 cells: Cells were treated with 100ng of each cytokine for different durations: **(A)** Ltf expression in TNF-α treated hepa 1-6 cells. **(B)** Ltf expression in IL-1ß treated hepa 1-6 cells **(C)** Ltf expression in IFN-γ treated hepa 1-6 cells (Results represent the mean value± s.e.m. *P<0.05, analyzed by t-test; n=3 repeats)

4.18 Lactoferrin expression in the injured and non-injured skeletal muscle after TO- and LPS-injection

Ltf mRNA increased in the injured TO-injected skeletal muscle of WT and KO mice. However, at protein level a prominent difference in lactoferrin expression was observed in the TO-injected injured muscle of wild type and IL-6 knockout mice. In wild type animals, TO-injection resulted in a peak Ltf expression at protein level at 6h and then it declined to basal level till 24h. However, in the injured muscle of IL-6 knockout mice an early increase in Ltf expression at 2h was seen at protein level, and till 24h lactoferrin protein expression was still increasing in the injured muscle of these knockout mice (Fig. 37).

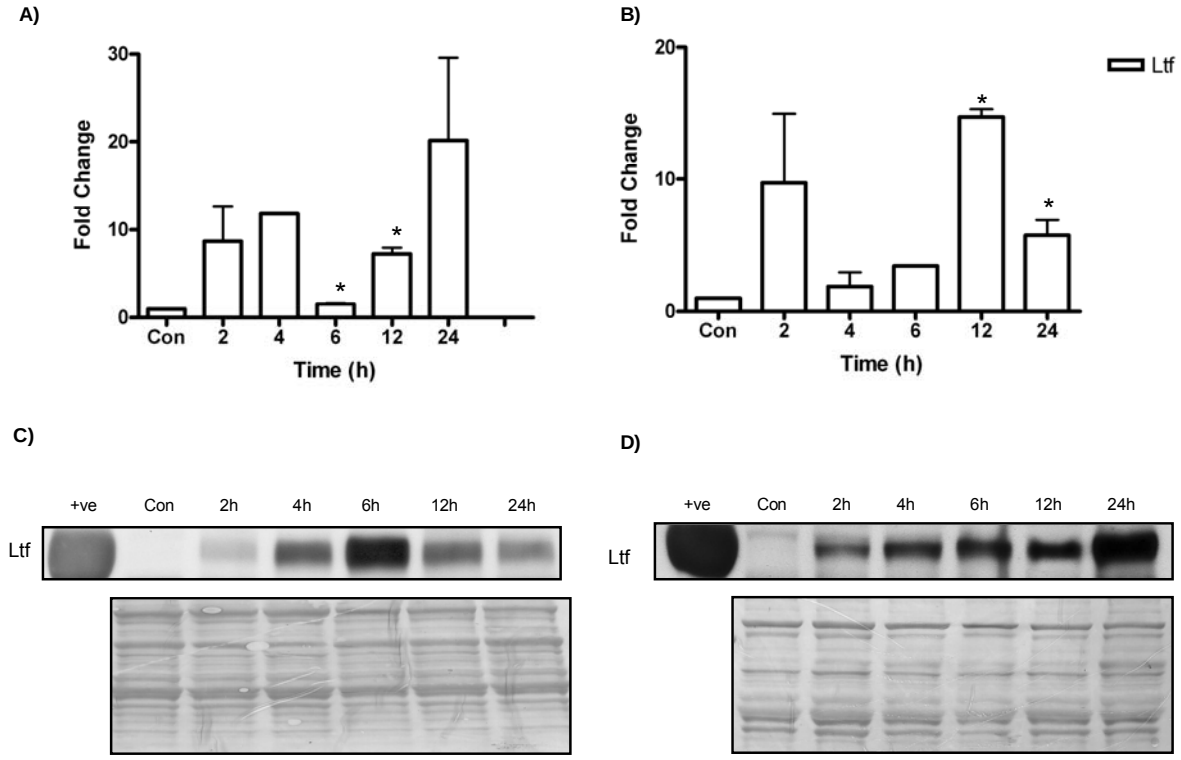


Figure 37:
Ltf gene expression analysis in the hind limb muscle of TO- and LPS-treated mice: **(A)** Ltf mRNA in the injured hind limb muscle of TO-treated wild type mice by quantitative PCR. **(B)** Ltf mRNA analysis by quantitative PCR in the injured muscle of hind limb of TO-treated IL-6 knockout mice (results represent the mean value± s.e.m. **P*<0.05, analyzed by *t*-test; *n*=4 animals per time point). **(C)** Ltf expression at protein level in injured hind limb muscle of TO-treated wild type mice. **(D)** Western blot analysis of Ltf in the injured muscle of hind limb of TO-treated IL-6 knockout mice.

In the non-injured muscles of TO and LPS-treated mice, Ltf mRNA expression was analyzed by RT-PCR. A statistically significant down regulation of Ltf mRNA was seen in the skeletal muscle of LPS-treated IL-6 KO mice, however no significant decrease was observed in their wild type counterparts. On the other hand, in the non-injured muscles of TO-treated IL-6 KO mice a significant upregulation was seen 12h post TO injection, but their wild type counterparts showed a down regulation of Ltf mRNA immediately after TO injection that was statistically significant (Fig. 38).

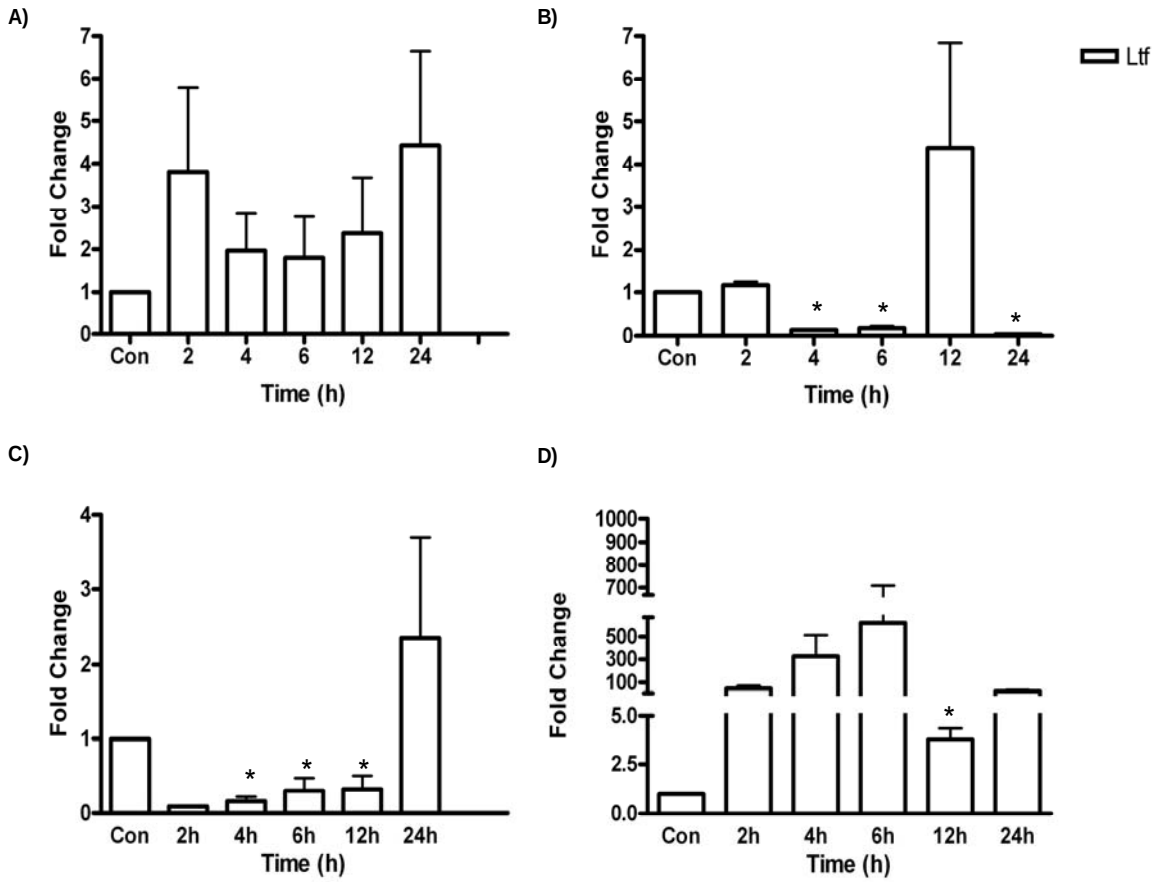


Figure 38:
Ltf gene expression analysis by quantitative RT-PCR, in the non-injured muscle of LPS- and TO-treated mice: **(A)** Ltf mRNA expression changes in the hind limb muscle of LPS treated wild type mice. **(B)** Changes in Ltf gene expression in the hind limb muscle of LPS-treated IL-6 knockout mice. **(C)** Ltf mRNA expression in non-injured muscle of TO-treated wild type mice **(D).** Ltf mRNA expression in non-injured muscle of TO-treated IL-6 knockout mice (results represent the mean value± s.e.m. **P*<0.05, analyzed by *t*-test; *n*=4 animals per time point).

4.19 Serum and hepatic changes in iron concentration

Figures 39 & 40 show changes in hepatic and serum iron concentrations in TO- and LPS-treated wild type and IL-6 knockout mice. Both treatments i.e. TO and LPS, resulted in a decrease of serum iron levels, and at the same time an increase of hepatic iron concentration in wild type and IL-6 knockout mice. Decrease in serum iron was inversely proportional to hepatic iron increase.

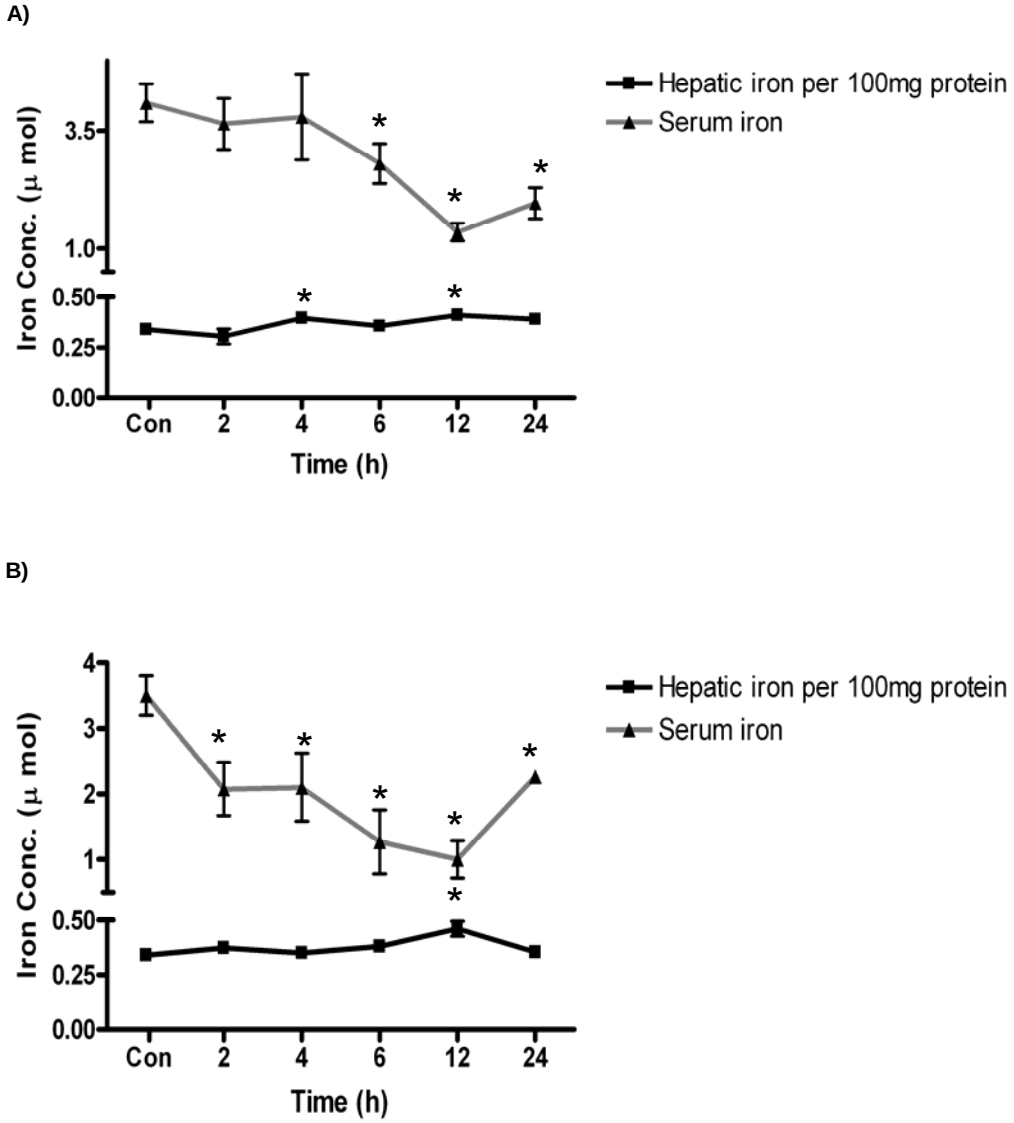
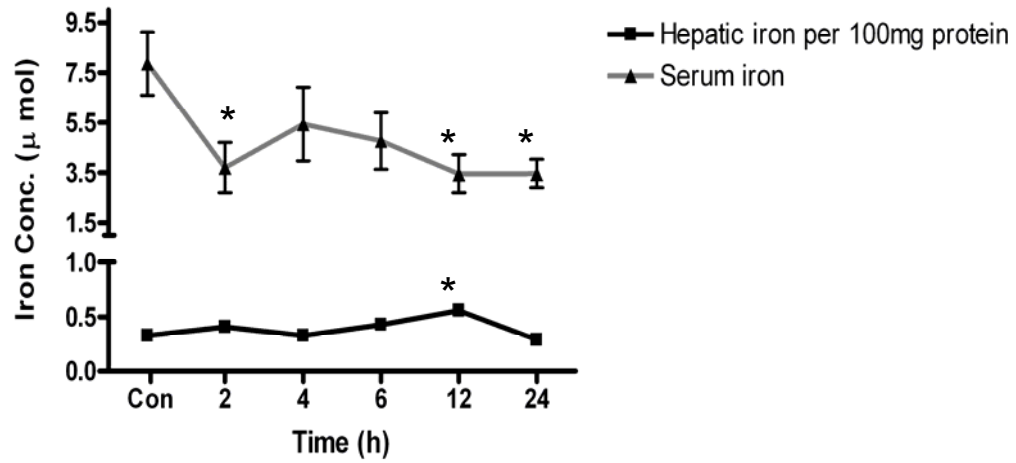


Figure 39:
Iron measurement in liver and sera of wild type mice: **(A)** Hepatic and serum iron concentration in TO-treated wild type mice **(B)** Hepatic and serum iron concentration in LPS-treated wild type mice (results represent the mean value± s.e.m. **P*<0.05, analyzed by *t*-test; *n*=4 animals per time point).

A)



B)

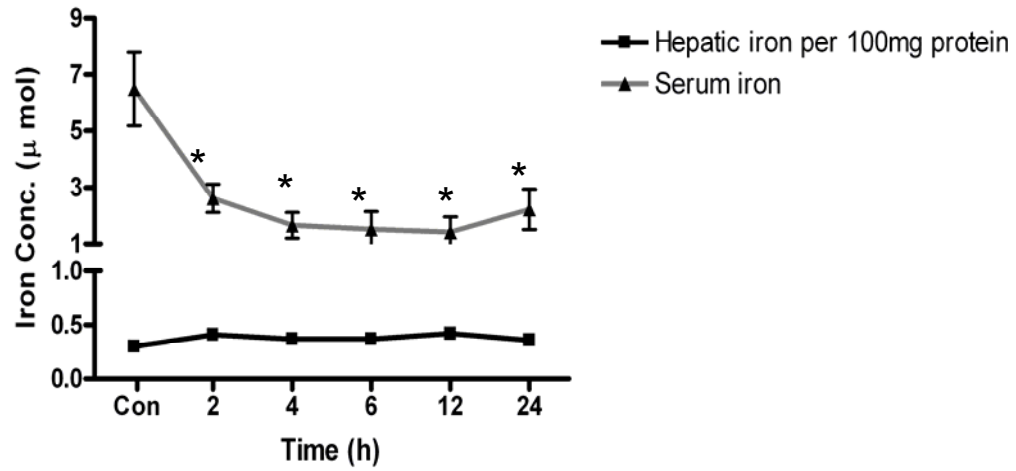


Figure 40:
Iron measurement in liver and sera of IL-6 knockout mice*:* **(A)** Hepatic and serum iron concentration in TO-treated IL-6 knockout mice **(B)** Hepatic and serum iron concentration in LPS-treated IL-6 knockout mice (Results represent the mean value± s.e.m. **P*<0.05, analyzed by *t*-test; *n*=4 animals per time point)

5 Discussion

The current study targeted HO-1 and Ltf gene expression regulation in two animal models of acute phase reaction. Liver and skeletal muscles were the organs of interest. The whole inflammatory process is primarily based on cytokines' signalling cascades, which not only induce pro-inflammatory pathways but also provoke anti-inflammatory mechanisms to reduce the spread of inflammatory noxae. To treat inflammation related pathologies, it is best to understand the role of different individual inflammatory signalling molecules in mounting and resolving the whole acute inflammatory reaction. Keeping this in mind, cytokines' dependent gene expression regulation of HO-1 and Ltf were studied in the current work. Both, HO-1 and Ltf play an important role in iron metabolism and are important cytoprotective molecules, possibly having a great potential for being used as therapeutic targets for protection against inflammatory processes and oxidative tissue damage.

5.1 Induction of inflammatory response

Serum amyloid A (SAA) is the archetypal vertebrate acute phase protein. SAA-1 is the major isotype of all acute phase SAAs (Xu et al., 2006). All the four cytokines, i.e. IL-1ß, IL-6, TNF-α and IFN-γ induced SAA-1 gene expression very prominently in liver, indicating the induction of an APR in these animals. TNF-α dependent induction of SAA has previously been reported in mouse liver (Ramadori et al., 1988) and human hepatocytes (Yap et al., 1991). Ramadori *et al.* (1988) have also shown the induction of SAA gene expression in mouse liver after IL-1ß and IL-6 treatment.

Many cytokines are capable of exerting both positive and negative effects, depending on which cell they interact with and the context of interaction. Whereas the pro-inflammatory cytokines (e.g. TNF-α , IL-1ß) mostly have up-regulating effects on aspects of inflammation and acute phase response, the anti-inflammatory cytokines have in general down regulating effects (Jensen and Whitehead, 1998). Interleukin-6, a pleiotropic cytokine with a wide range of biological activities and produced by various cell types (Fig. 41) (Naka et al., 2002), has both pro- and anti-inflammatory properties. SAA is readily induced by IL-1ß or TNF-α, and each of these cytokines can synergize with IL-6 to dramatically increase SAA production at mRNA and protein level (Ganapathi et al., 1988;1991). However, it is also known that IL-6 suppresses the production of IL-1ß and TNF-α and thereby acting as an anti-inflammatory cytokine (Schindler et al., 1990;Xing et al., 1998).

HO-1 and lactoferrin are part of the protective mechanism during oxidative stress, and IL-6 is involved in the induction of both. In the current investigation we studied the gene expression regulation of Ltf and HO-1 in TO- and LPS-treated IL-6 knockout mice. Some previous investigators have indicated the abrogated inflammatory response in IL-6 knockout mice during APR (Kopf et al., 1994;Gomez et al.,

2006). To assess the induction and resolution of APR, we analyzed serum amyloid A-1 expression in the liver of TO- or LPS-treated IL-6 knockout mice and compared them to their wild type equivalents. In TO-treated knockout animals mRNA of SAA-1 was increased lesser as compared to their wild type counterparts and it started declining earlier i.e. only after 12 h. As IL-6 is the principal mediator of TO induced APR (Knittel et al., 1997), the earlier resolution of inflammatory response in IL-6 knockout mice fits well in this model. On the contrary, in LPS treated animals, absence of IL-6 resulted in an increased hepatic SAA-1 gene expression, and therefore indicating an amplified inflammatory response in LPS-treated knockout mice. LPS is known to be the major mediator of endotoxic shock (Immenschuh et al., 1999), and pro-inflammatory cytokine TNF-α alpha is the principal mediator of LPS-induced hepatic APR (Wijayanti et al., 2004). IL-6 exerts some restraining effects on TNF-α expression (Ricchetti et al., 2004). This might explain the enhanced inflammatory response in this model of APR in IL-6 knockout mice, where TNF-α probably exacerbates the inflammatory response due to lack of protective effect of IL-6.

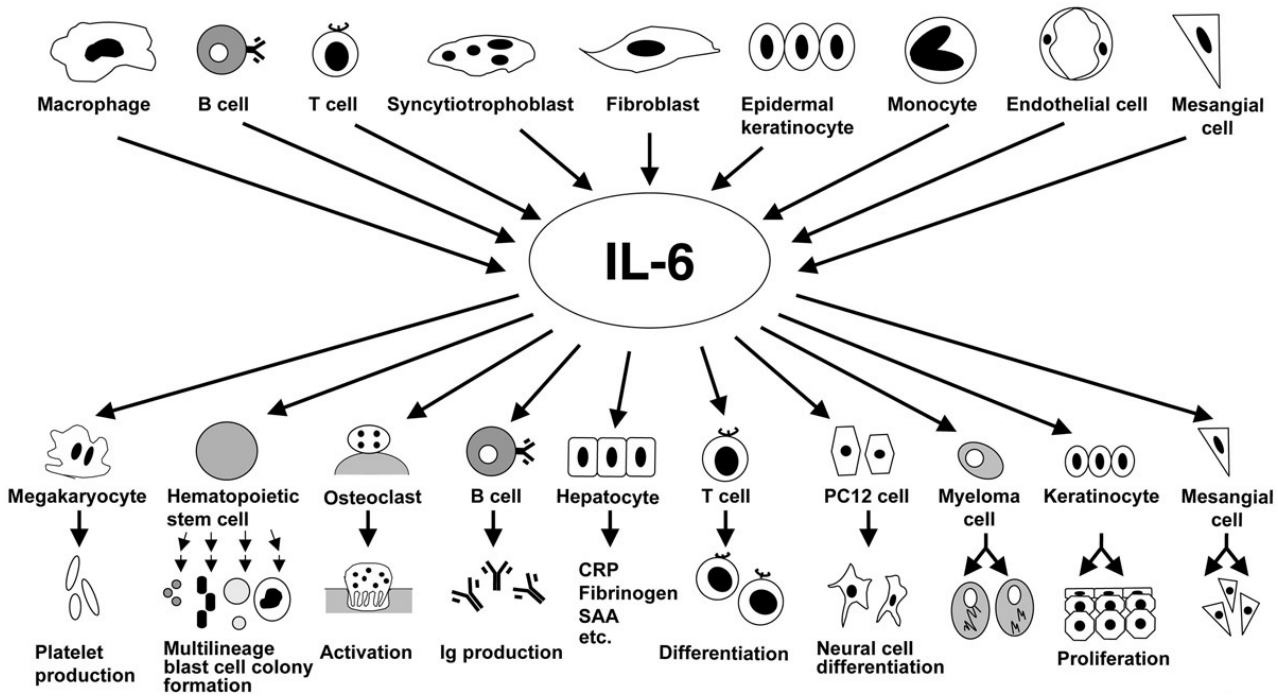


Figure 41: *IL-6 producing cells and biological activities of IL-6*: IL-6 is produced by lymphoid and nonlymphoid cells (top of figure) and has a wide range of biological activities on various target cells (bottom of figure) (Naka et al., 2002).

Iron concentration is another index of assessing inflammation. In this work we also measured serum iron levels and iron concentration in the liver of the animals treated with TO or LPS, and we found an inverse correlation between the decrease of serum iron concentration and the increase of liver iron concentration.

5.2 Heme oxygenase-1

5.2.1 HO-1 upregulation in two models of acute phase reaction in mice

Liver is the main organ that reacts to any kind of acute phase stimuli (Sheikh et al., 2006). In the current study, hepatic HO-1 mRNA was upregulated in wild type mice after TO and LPS treatment. This upregulation of HO-1 transcript in our mice models was quite in accordance with previous APR studies in rat and mouse (Lee and Chau, 2002;Tron et al., 2005;Chang et al., 2006). But as different models of APR are characterised by divergent inflammatory mediators and altered pattern of acute phase proteins' synthesis (Tron et al., 2005), the present comparison of the two APR models shows that the magnitude of increase of HO-1 mRNA and protein synthesis is different in the two mouse APR models. LPS resulted in an immediate increase at 2h, followed by a decline of hepatic HO-1 mRNA, whereas HO-1 gene expression in liver peaked at 4h after TO-treatment. The rise in mRNA was followed by an increased production of the protein. This rise in hepatic HO-1 protein was apparent only after 2h of LPS injection whereas in case of TO-treatment a clear increase of the peptide was evident after 4h. Although the mRNA of HO-1 declined to basal level in both mouse models of APR, the amplification of protein synthesis was still prominently visible even at 24h after the induction of APR in both the mice models. However, this can be explained by the fact that HO-1 mRNA has a half-life of about 3h, whereas the protein has a half-life of about 15-21h (Srivastava et al., 1993). Hepatic HO-1 gene expression regulation has previously been studied by Lyoumi *et al* (1998) and Tron *et al* (2005) in a TO-induced acute inflammation model in rat. Lyoumi *et al* (1998) studied the hepatic HO-1 gene expression in LPS-induced APR in rat as well. But in both models of APR by Lyoumi *et al.* (1998), HO-1 gene expression was studied only at mRNA level using RT-PCR. Tron *et al.* (2005) studied protein expression in liver of TO-injected rats. In the current investigation we studied HO-1 gene expression in both animal models at mRNA as well as at protein level.

At mRNA level, our results indicate that after TO or LPS injection hepatic HO-1 induction was slightly earlier in mouse than in rat at mRNA level. However, the results from Lyoumi *et al* (1998) support the current results in our mouse models in the pattern of induction of hepatic HO-1 mRNA. The magnitude of upregulation of hepatic HO-1 mRNA was higher after TO-injection as compared to LPS injection, and it was true for both, rat from Lyoumi and mouse from the current study. At protein level, however, our results differ from the results of Tron *et al.* (2005), who showed a start of increase of HO-1 peptide at 2h which was still rising at 48h in rat liver. However, in our mouse model of TO-treatment HO-1 peptide seemed to peak at 12h and then it started declining. In another study, Song *et al.* (2003) has shown an increase in hepatic HO-1 at protein level after treating mice with LPS, but their study differed from ours in the sense that we used a single dose of LPS and then studied its effects on hepatic HO-1 expression at different time points till 24h. Song *et al.* (2003), on the other hand, used intravenous injection unlike our intraperitoneal treatment. Besides, to study time kinetics of HO-1 in liver after LPS

treatment, they injected 1, 2 or 3 doses of LPS every 24h. Therefore, the overall scenario in which they performed the experiments is different from our experimental protocols. However, both studies conclude that LPS augments HO-1 in liver.

5.2.2 Role of IL-6 in the development of APR and in the induction of hepatic HO-1

In some previous studies, Tron *et al.* have advocated the central role of IL-6 in gene expression regulation of HO-1 (Tron et al., 2005;Tron et al., 2006). In the current study, serum IL-6 levels were significantly amplified and were found to be closely correlated to HO-1 expression in the liver of wild type mouse. Besides, in IL-6 treated murine hepatocytes and hepa 1-6 cells, HO-1 expression also increased at mRNA and protein levels. These data are quite in accordance with the results obtained by Tron *et al. in vivo* and *in vitro*. The serum IL-6 levels were, however, still high even after the reduction of hepatic HO-1 mRNA. This phenomenon might be explained by the known fact that IL-6 induces down regulation of its cognate receptor, IL-6R, *in vitro* (Hoffmann et al., 1994). Moreover, it has been demonstrated that IL-6R mRNA expression in rat liver is markedly decreased 6-12h after TO administration (Geisterfer et al., 1993). Therefore, it is likely that continuously high levels of serum IL-6 during APR subsequently lead to downregulation of the IL-6R, thus abolishing the action of IL-6 in the liver when it is not required anymore.

In order to analyze the fundamental role of IL-6 in the regulation of HO-1 gene expression during APR, we utilized TO and LPS treatment in IL-6 deficient mice. In knockout mice, the constitutive expression of HO-1 was higher than in their wild type counterparts. The magnitude of HO-1 expression upregulation was comparable in two models of APR in knockout mice, but was lower than their wild type counterparts. However, at protein level TO treatment in the absence of IL-6 resulted in a delayed increase of HO-1 protein in liver, i.e. at 6h, but it peaked at 12h like in wild type mice and started to decline at 24h. Same delay was observed in LPS treated IL-6 knockout mice. Instead of 2h, as was seen in the LPS-treated wild type mice, HO-1 peptide started to increase at 4h in knockout mice, but like their wild type counterparts reached at peak expression at 24h. These data indicate a mandatory role of IL-6 for an early translation of HO-1 in both models of APR. This kind of study in IL-6 knockout mice has not been reported by any other group so far.

5.2.3 Role of other acute phase cytokines to induce HO-1 in liver during APR

Using different kinds of experimental approaches, several groups have demonstrated that in addition to IL-6, HO-1 gene expression is also regulated by other pro-inflammatory cytokines like TNF-α, IL-1ß and IFN-γ, as well (Song et al., 2003; Malaguarnera et al., 2005;Nairz et al., 2008). In spite of the

similarities in general signs and symptoms, different *in vivo* models of the APR represent diverse patterns of acute phase mediators and plasma proteins release during the inflammatory process (Ramadori and Christ, 1999). For example, an LPS model is characterized by rapid circulatory increase of various pro-inflammatory cytokines like IL-6, IL-1β, and TNF-α, all at the same time, causing systemic inflammation. The variety of mediators in this model makes it difficult to define the role of individual cytokines in the induction of certain proteins, particularly in liver. To determine the role of individual cytokines to induce HO-1 gene expression in the liver, we treated mice with different individual cytokines, like IL-6, IL-1ß, TNF-α and IFN-γ. All these cytokines in our *in vivo* mouse models increased hepatic HO-1 mRNA. In order to assess the relative contribution of these cytokines to induce hepatic HO-1 mRNA in our models of APR in IL-6 knockout mice, we analyzed the gene expression of these cytokines in the liver and muscle and compared them to that of their wild type equivalents. All these cytokines were sufficiently upregulated in the liver of LPS-treated knockout mice, whereas in TO-treated animals the amplification of gene expression of these cytokines in the liver was not significant. However, serum ELISA indicated an increase in the levels of TNF-α and IL-1ß in these animals that was comparable to their wild type counterparts. Therefore in order to locate the source, we quantified the mRNA expression of these cytokines at the site of injury, i.e. TO-injected-injured-muscle. All these cytokines were sufficiently upregulated in the injured muscle of IL-6 knockout mice and closely correlated to serum cytokine levels. Considering the time kinetics, IL-1ß levels in serum and in injured muscle correlated more to hepatic HO-1 gene expression, as compared to TNF-α in TO-treated IL-6 knockout mice and thus indicating the possibility of compensation of IL-6 by IL-1ß to regulate HO-1 gene expression in this animal model, whereas in LPS-treated knockout mice serum levels of both IL-1ß and TNF-α started to increase concurrently at the start of APR and were closely associated to hepatic HO-1 mRNA expression level.

5.2.4 Involvement of various transcription factors to regulate hepatic HO-1 gene expression

Various cytokines induce HO-1 gene expression by activating different sets of transcription factors. IL-6 signalling is involved in the major regulation of a variety of acute phase proteins in hepatocytes and is predominantly mediated by the latent transcription factor signal transducer and activator of transcription 3 (STAT3) (Yoo et al., 2002;Grivennikov et al., 2009). STAT3 is essential not only for the induction of inflammatory response by the liver but is also involved in several anti-inflammatory activities. Cytokines other than interleukin-6 that are able to promote STAT3 phosphorylation include oncostatin M, leukaemia inhibitory factor, leptin, IL-12, interferons, IL-10, GCSF and different growth factors (Maritano et al., 2004;Sakamori et al., 2007). STAT-3 phosphorylation is crucial for HO-1 gene expression regulation. STAT-3 inhibition results in non-expression of HO-1 mRNA (Ricchetti et al., 2004). Phosphorylation and activation of STAT3 in IL-6 knockout mice has been reported in a chronic liver injury model after CCL4 injection (Rio et al., 2008). The result of current work

demonstrate for the first time that hepatic STAT3 is activated during APR even in the absence of IL-6, and the pattern of activation/ phosphorylation is completely dependent on the nature of inflammatory stimuli. This observation led us to deduce that STAT3 might also be involved in the augmentation of hepatic HO-1 mRNA expression in our animal models of IL-6 knockout mice. But STAT3 is not the only transcription factor involved in the regulation of HO-1 gene expression. Depending on the intensity and duration of stimuli, HO-1 activation involves various transcription factors, that themselves are regulated by various other inflammatory cytokines. Among these transcription factors, AP-1 and NFκB are the major transcription factors that can regulate HO-1 gene expression (Prawan et al., 2005). Both of these transcription factors are known to actively regulate various sets of genes under different stress conditions and are themselves regulated by various inflammatory cytokines directly or by various interacting inflammatory signalling cascades. Irrelevant to which cytokine plays a major role, we analyzed the DNA binding activity of AP-1 and NFκB that are well known to regulate HO-1 gene expression by these cytokines.

In TO-treated IL-6 knockout mice, DNA binding activity of AP-1 started to intensify at 4h and peaked at 12h that is more relevant to HO-1 mRNA expression than NFκB, which showed a delayed DNA binding activity at 24h time point. But another picture was seen in LPS treated knockout animals, where the early upregulation of HO-1 at 2h closely correlated to increased NFκB DNA binding activity at this time point. Considering the time kinetics to give it a mechanistic rationale, AP-1 seemed to be a little irrelevant to regulate hepatic HO-1 mRNA expression in LPS-treated IL-6 knockout mice, although it is known to regulate the gene expression of HO-1 after LPS-treatment in wild type mice (Wijayanti et al., 2004).

5.2.5 HO-1 gene expression in skeletal muscle in two models of acute phase reaction

HO-1 was upregulated at mRNA and protein level in the TO-treated injured muscle of both wild type and IL-6 knockout mice. These transcriptional changes support previously published data in rat injured muscle (Tron et al., 2005)**.** Similarly, Vesely *et al.* (1998) showed HO-1 induction in skeletal muscle by hemin and sodium nitroprusside. Nonetheless, to our knowledge, at translational level no such study in injured muscle tissue of wild type and IL-6 knockout mice during APR has been reported so far. Elevated levels of HO-1 can significantly inhibit apoptosis in several cell types, and therefore its induction in muscle tissue represents a defensive response to ensure muscle cell survival during atrophy (Hunter et al., 2001). Comparison of HO-1 mRNA levels in the non-injured muscle of TO- and LPS-treated animals revealed that inspite of an increase of various pro-inflammatory cytokines in the non-injured muscle of LPS treated animals, there were no dramatic changes in HO-1 mRNA expression in the earlier time points but a significant increase of HO-1 mRNA was seen at 12h and 24h. Moreover the magnitude of increase

of HO-1 mRNA in the muscle of knockout mice was much less than in their respective wild type counterparts. HO-1 production in diaphragmatic-muscle after LPS treatment has previously been reported by Taille *et al.* (2001), and this group has also shown an increase of HO-1 protein expression at 24h in the diaphragm. However, no comparative study describing HO-1 expression regulation in the skeletal muscle under different kinds of stress conditions is available so far. Contrary to LPS, an early increase of HO-1 mRNA was seen in the non-injured muscle of TO-treated animals, although it was statistically not significant in IL-6 knockout mice. The increased expression of HO-1 represents an antioxidant response by the production of the antioxidant bilirubin and the removal of prooxidant heme (Kondo et al., 1992).

5.3 Lactoferrin

Lactoferrin holds a very basic position in immune response. Diverse physiological functions have been ascribed to lactoferrin, including regulation of cellular growth and differentiation, intestinal iron homeostasis, host defence during inflammations and protection against different types of cancer. Some of these functions clearly involve iron binding properties of lactoferrin, whereas in others lactoferrin appears to involve iron independent mechanisms (Ward and Conneely, 2004). The current work focused mainly on lactoferrin gene expression regulation under acute phase conditions rather than its physiological functions.

5.3.1 Hepatic lactoferrin gene expression regulation during acute phase conditions

Lactoferrin mRNA expression was upregulated in the liver of TO- and LPS-treated wild type animals. However, as both the animal models represent two different scenarios of acute phase inflammation, the pattern of regulation of hepatic Ltf gene expression was clearly dissimilar in the two models. In TO-treated animals an increase was seen at 4h and the level of mRNA was still rising at 24h. However, in LPS treated animals an immediate increase of lactoferrin mRNA at 2h was observed in the liver, and this increase returned to basal level at 24h. This is very typical for this animal model, i.e. an urgent increase of acute phase proteins followed by a quick return to normal levels, but in case of TO injection, where there is no direct liver injury and liver is only the target of locally released cytokines from the injured muscle, lactoferrin gene expression was still upregulated in liver. Lactoferrin is a key element in the mediation of immune response and is known for its coordinated interaction between innate and adaptive immune response. Moreover, lactoferrin is involved in the reduction of inflammatory response and is able to reduce the severe impacts of oxidative stress at molecular level (Actor et al., 2009). To our knowledge, lactoferrin gene expression in various animal models of acute phase response has not been reported so far.

TO- and LPS-treatment result in oxidative stress and induction of lactoferrin after these treatments can be a key step towards the resolution of this inflammatory stress. The results of the current study lead us to deduce that lactoferrin gene expression is highly dependent on the nature of insult that the defence system of the body faces. In case of TO-induced localised tissue injury, the magnitude of upregulation of hepatic lactoferrin expression was lesser than that of LPS-induced hepatic injury. The increase in mRNA levels of hepatic lactoferrin was followed by equivalent changes in hepatic lactoferrin gene expression at protein levels. And at protein level also it was very clearly observed that LPS-treatment is a more potent inducer of lactoferrin peptide as compared to TO-treatment.

For comparison we used transferrin gene expression in liver. Transferrin is the major known iron transporter in the body. Tf carries iron from the intestine, reticuloendothelial system and liver parenchymal cells to all proliferating cells in the body. Diferric Tf interacts with the TfR1 and is internalized by receptor-mediated endocytosis (Frazer and Anderson, 2005). It was very evidently seen that when lactoferrin gene expression increased, transferrin expression decreased in the liver of LPS-treated animals. Same applies in TO-treated animals. TO-injection resulted in a slight upregulation followed by a down regulation of Tf gene expression. Sheikh *et al.* (2007) have also reported a similar pattern of Tf in the liver of rat after TO-treatment. In our experiments, at the same time points when Tf was decreasing lactoferrin expression increased. Furthermore, when these changes in transferrin and lactoferrin gene expression were occurring, serum iron was decreasing, but with an increase in hepatic iron pool. Therefore lactoferrin fits well in the situation, because under oxidative stress, when reactive oxygen species (ROS) are generated, they probably result in the drop of pH, and at low pH, transferrin is unable to bind iron effectively and therefore lactoferrin is increased in order to assist chelation of iron and to avoid spread of oxidative stress generated by free iron. Cairo *et al.* (1994) showed an early upregulation of transferrin with a subsequent down regulation in the liver when the iron was depleted from the circulation .

Lactoferrin is a secretory protein (Brock, 1995). However, no previous study has reported its secretion from hepatic cells. In order to determine that hepatic cells secrete it or not, we did Ltf immunoprecipitation from two hepatic carcinoma cell lines. These results proved that hepatic cells are also able to secrete lactoferrin into serum, and when in serum, lactoferrin can be easily transported to other inflammatory cells in different organs, thus giving it a broader range of action. There, this lactoferrin interacts with the mononuclear cells and suppresses the production of different pro-inflammatory cytokines like TNF-α, IL1-ß and IL-6, and therefore tends to smooth the effects of the inflammatory cytokines at the site of infection or injury (Crouch et al., 1992). Suppression of inflammation by lactoferrin has also been reported in other studies. Peen *et al.* (1998) reported that intravenously administered lactoferrin to rat specifically accumulated in the kupffer cells. Yamaguchi *et al.* (2001) also signified the role of Kupffer cells as the site of action for lactoferrin in LPS-treated mice. Their study

indicated that if the Kupffer cells were suppressed by gadolinium chloride the levels of TNF-α were significantly reduced and so were the serum ALT levels thus indicating lesser liver injury. They got the same results with LPS treatment when the mice were intravenously treated with lactoferrin before. Besides this, in their *in vitro* and *ex vivo* experiments they clearly demonstrated that lactoferrin administered before LPS treatment significantly diminished TNF-α expression by Kuppfer cells due to its direct interaction with these cells. These results are also supported by another study where carrageenan induced TNF-α and IL-6 production was suppressed by cultured spleenocytes when the animals were orally treated with lactoferrin (Zimecki et al., 1998). Choe and Lee(1999) have also shown the same type of results with the mouse monocyte cell line Raw 264.7 and with human monocytic cells, i.e. THP-1 cells. They also studied the capability of lactoferrin to induce TNF-α in these cells, and their results indicate that lactoferrin itself does not have a good potential as an inducer of TNF-α and NO. They also analyzed if lactoferrin could induce NO production in aortic vascular smooth muscle cells, but the results were negative. Therefore, the control of lactoferrin gene expression during oxidative stress can prove to be a promising strategy in clinical practices.

The serum lactoferrin increased significantly after TO- and LPS-injections, however, as seen with hepatic Ltf mRNA and protein expression, the pattern of increase of serum Ltf was different in the two models. In TO-treated wild type animals there was an immediate increase in serum lactoferrin levels. This immediate increase after TO-injection might be due to the invading neutrophils into the TO-injected muscle. After a sudden increase, there was a drop in serum Ltf levels and then at 24h again an increase was seen. This second increase in Ltf concentration in serum might represent the lactoferrin released from the liver that also peaked at 24h. On the other hand, although LPS also induced an immediate increase in serum Ltf levels at 2h, but this increase was more pronounced than that after TO-treatment to wild type animals, and in these animals lactoferrin steadily kept on rising till 4h and then started to decline afterwards very constantly. The increase in serum lactoferrin closely correlated to hepatic lactoferrin expression in these animals. Only a single peak of serum lactoferrin was seen in LPS-treated animals. This lead us deduce that liver is the major source of serum lactoferrin in the LPS treated animals, and neutrophils which are the major cell population that invade liver after the LPS-induced direct injury, might be the main source of this serum lactoferrin in these animals.

5.3.2 Lactoferrin gene expression regulation by interleukin-6

IL-6 is the principal mediator of acute phase response (Stenvinkel et al., 2002) as well as a key player in the regulation of iron metabolism (Marques et al., 2009). In order to determine the role of IL-6 to induce lactoferrin gene expression, we injected mice intraperitoneally with IL-6. IL-6 induced significant expression of lactoferrin in the liver of these mice. Hepatocytes are the major cell population in liver and play a major role in the conduction of APR (Sheikh et al., 2006). In order to determine whether

hepatocytes are able to produce lactoferrin under the influence of IL-6, we treated murine hepatocytes with different doses of IL-6. IL-6 significantly induced lactoferrin in the primary cell culture of murine hepatocytes. Furthermore, we treated murine hepatic carcinoma cell line hepa 1-6 with different doses of IL-6, and the data from this experiment also confirmed the previous results of lactoferrin induction by IL-6 in murine liver and hepatocytes. The changes in mRNA levels were followed by an equivalent increase in protein expression in hepa 1-6 cell line.

To understand the role of IL-6 in regulating iron metabolism by Ltf during acute phase response has remained one of our main focuses in the course of this investigation. Therefore, to further explore the specific role of IL-6 to regulate Ltf gene expression, we treated IL-6 knockout mice with TO and LPS just like the wild type mice. In TO-treated IL-6 knockout mice lactoferrin expression started to increase at 6h and was comparable to their wild type counterparts, peaked at 12h, but unlike the wild type mice, Ltf mRNA started to decline at 24h which was also followed by lactoferrin expression at protein level in the livers of these TO-treated IL-6 knockout mice. The early decrease in hepatic lactoferrin expression can be a direct effect of absence of IL-6, or may be due to decreased inflammation and early resolution of inflammatory process, as shown by Saa-1 expression in TO-treated-IL-6 knockout mice. Transferrin showed a slight decrease at 2h, but otherwise its levels in liver remained almost the same during the whole treatment time in these knockout animals after TO-treatment. On the other hand, in LPS-treated IL-6 knockout mice, a different picture was seen. The magnitude of upregulation of lactoferrin expression after LPS treatment was much higher in the liver of knockout mice as compared to their wild type equivalents. Secondly, unlike LPS-treated wild type mice, in the knockout animals LPS resulted in an immediate peak in hepatic lactoferrin expression. This might be due to the fact that IL-6 suppresses the impact of TNF-α (Xing et al., 1998), which is another major regulator of hepatic APR after LPS treatment. And therefore, in the absence of IL-6, TNF-α resulted in a higher gene expression of lactoferrin. But if this is the case, then this also indicates that TNF-α might be the more potent inducer of Ltf. The same early increase in lactoferrin expression in LPS treated IL-6 knock mice liver could also be seen at protein level as well.

At serum level also, IL-6 knockout mice behaved differently than their wild type equivalents. In the sera of TO-treated IL-6 knockout animals, only one peak of lactoferrin was seen at 12h unlike the biphasic increase in their wild type counterparts. And in LPS-treated animals serum lactoferrin levels increased like in wild type mice, but the decrease in serum concentration of lactoferrin was delayed in these animals. The overall picture, therefore, leads us to deduce that under different acute inflammatory conditions IL-6 plays an important role to regulate lactoferrin gene expression. However, it is still to be determined whether this change in lactoferrin gene expression is due to the direct effect of IL-6 absence in the knockout mice or is an indirect consequence of altered pattern of inflammation in general in these animals.

5.3.3 Lactoferrin gene expression regulation by TNF-α

TNF-α was found to upregulate hepatic Ltf gene expression in the *in vivo* experiments. In order to support the *in vivo* data, *in vitro* experiments were performed. TNF-α increased lactoferrin gene expression in the *in vitro* experiments as well. TNF-α serum levels did not increase significantly in TO-treated wild type and IL-6 knockout mice, indicating that it is not the major player in localised-tissue-injury-induced acute phase response. However, in case of LPS-treatment TNF-α is the major signalling molecule. TNF-α increased the expression of lactoferrin gene in the *in vivo* and *in vitro* experiments. Therefore there is a great possibility that in case of LPS treatment, TNF-α might be the major inducer of lactoferrin gene. And as indicated previously, IL-6 inhibits TNF-α expression. So in IL-6 knockout mice the magnitude of increase of TNF-α was higher after LPS-treatment, and so was lactoferrin expression. Furthermore, in LPS treated wild type and IL-6 knockout mice the time kinetics of serum TNF-α concentration were closely correlating to hepatic Ltf expression.

5.3.4 IL-1ß dependent lactoferrin gene expression regulation

IL-1ß is another important regulator of acute phase response (Plackett et al., 2007) and of iron metabolism (Inamura et al., 2005). Serum levels of IL-1ß rose after both TO- and LPS-treatment. In order to analyze the impact of IL-1ß on hepatic lactoferrin gene expression, we injected mice intraperitoneally with recombinant IL-1ß and also treated mouse hepa 1-6 cells with recombinant murine IL-1ß. Our *in vitro* and *in vivo* result indicated that IL-1ß might not be among the signalling molecules that are regulating lactoferrin gene expression, as we saw no significant changes in lactoferrin gene expression in the liver of IL-1ß-treated mice or in IL-1ß-treated hepa 1-6 cells.

5.3.5 Hepatic lactoferrin gene expression regulation by IFN-γ

Gamma interferon (IFN-γ) is an important cytokine in the host defence and induces a variety of physiologically significant responses that contribute to immunity (Shtrichman and Samuel, 2001). IFN-γ mRNA expression did not change significantly in the liver of TO- and LPS-treated wild type mice. It also remained unchanged in TO-treated IL-6 knockout mice, but IFN-γ gene expression increased significantly in the liver of LPS-treated IL-6 knockout mice. Treatment of animals and hepa 1-6 cells with IFN-γ resulted in an upregulation of lactoferrin RNA. This might explain the continuous increased levels of serum lactoferrin in LPS-treated IL-6 knockout mice. In LPS-treated animals multiple signalling pathways are activated at the same time. Although TNF-α is an important inducer of acute phase proteins after LPS-treatment, but considering the time kinetics of serum TNF-*α,* there were no big differences in LPS-treated wild type and IL-6 knockout mice. However, in LPS-treated IL-6 knockout animals continuously elevated levels of serum lactoferrin could be due to changed IFN-γ expression in these animals. Because of limited amount of mouse serum, it was not possible to measure serum IFN-γ levels in the current study, but the

possibilities of involvement of some other unknown pathways to regulate Ltf gene expression in these animals still remain.

5.3.6 Lactoferrin gene expression regulation in the injured skeletal muscle of TO-treated animals

In TO-injected skeletal muscle, lactoferrin expression was markedly different in wild type and IL-6 knockout mice. In wild type mice Ltf expression peaked at 6h and then declined, however, in knockout mice' injured muscles it kept on increasing till 24h. In injured muscle inflammatory cells like neutrophils may be the main source of lactoferrin after TO-injection. The elevated levels of Ltf in the injured muscle of TO-treated IL-6 knockout mice indicate that probably due to lack of anti-inflammatory effects of IL-6 in the late time points of acute inflammation, inflammatory cells keep on entering the injured muscle. The role of lactoferrin in this scenario can be both as anti-bacterial agent and as an iron chelator.

5.4 Concluding Discussion

Under inflammatory conditions innate immune system activates both pro- and anti-inflammatory signalling pathways. Therefore on one hand it tends to get rid of the inflammatory stimuli, but on the other hand at the same time, it tries to protect the body from excessive damage induced by pathogenic factors or by pro-inflammatory agents. To control the immune system from lending excessive damage to the organism, inflammatory signals' manipulation can prove to be a secure approach for the treatment of a number of diseases. Among these signals, IL-6 holds a key position. It is considered as the emergency cytokine of the innate immune system to initiate the development of APR. The anti-inflammatory role of this cytokine has not been discussed in detail so far, neither the effects of this cytokine on the gene regulation of acute phase proteins that are involved in the resolution of acute phase inflammation. Under certain pathological conditions, blocking IL-6 might prove helpful in controlling the inflammation by avoiding the activation of genes that aggravate the inflammatory conditions, but on the other hand it must be taken into consideration that this blockade should not affect adversely the healing mechanisms of the organism. The results of the current work suggest that absence of IL-6 during tissue trauma or certain bacterial infection does not result in the obstruction of HO-1 and Ltf, the two genes that are not only regulated by IL-6, but can prove to be the major therapeutic targets to control inflammation. Furthermore, STAT-3, that is a main transcription factor in IL-6 related signalling, was still active, indicating the proper activation of different downstream signal. Among different other inflammatory cytokines that induced HO-1 expression individually also, i.e. TNF-α, IL-1ß and IFN-γ were sufficiently upregulated in serum to create an APR, although as shown by SAA-1, these were not sufficient to maintain the inflammation after localised tissue injury in the absence of IL-6, but an exaggerated inflammatory response was seen in the LPS-treated knockout animals due to the lack of protective anti-inflammatory effects of IL-6. The overall

picture indicates that blocking IL-6 in both the inflammatory conditions, i.e. sepsis and tissue trauma might prove to be useful to control inflammation to a certain extent, but it might prove to be more promising in case of tissue trauma. However, in both cases, blocking IL-6 does not abolish the protection by HO-1

As for lactoferrin, it proved to behave like a positive acute phase protein that is probably tending to compensate for the lack of activity of transferrin to chelate iron under acidic conditions of oxidative stress. Lactoferrin gene expression upregulation was also dependent on the nature of inflammatory stimuli. Therefore its expression was more pronounced after LPS-treatment than TO-injection mediated acute phase response. Both animal models have their distinct pattern of regulation of acute phase mediators. Among these mediators, when studied individually, TNF-α, IL-6 and IFN-γ appeared to be involved in the induction of Ltf whereas IL-1ß seemed to be irrelevant to affect this arm of the innate immunity.

The eventual objective of research aimed at understanding inflammatory process is to come up with strategies to control inflammation in situations that are harmful to patients or to stimulate the immune system where it is compromised. Targeting of HO-1 and lactoferrin for therapeutic benefits is getting widely acknowledged. Therefore, a better understanding of HO-1 and Ltf gene expression regulation during APR will lead towards the development of improved and more sophisticated therapies.

6 Summary

Iron is indispensable to living organisms. Iron homeostasis is orchestrated both at the cellular and the systemic level, and liver plays a central role in the regulation of iron metabolism. An increasing number of genes associated with hepatic iron transport or regulation have been identified. Heme oxygenase-1(HO-1) and lactoferrin (Ltf) are among these genes known to regulate iron metabolism at cellular and systemic levels, respectively. Heme-oxygenase-1 is an intracellular positive acute phase protein and regulates one of the pathways that hepatic cells utilize to acquire free iron. The cytokines regulating HO-1 gene expression in liver and in other organs like muscle are only partially known. Moreover, lactoferrin serum concentration is known to increase under acute phase conditions, but the site of synthesis in not known.

In the current work we investigated the changes in HO-1 and Ltf gene expression during turpentine oil- and LPS-induced acute phase reaction (APR). Cytokines are the core mediators of APR, and among different acute phase mediators IL-6 is one of the principle signalling molecules. It is known to play a key role in maintaining body iron homeostasis during APR. For this reason we studied the induction of HO-1 and Ltf in IL-6 treated mice, and in addition we generated TO- and LPS-induced APR in IL-6 knockout (KO) mice to determine the effects of IL-6 absence on HO-1 and Ltf-gene expression regulation during APR. In order to evaluate the relative contribution of other acute phase cytokines to regulate HO-1 and Ltf-gene expression, we treated mice with IL-1ß, TNF-α, and IFN-γ. The *in vivo* results were supported by *in vitro* experiments, using the same cytokines in cell culture.

IL-6 augmented the gene expression of HO-1 in IL-6 treated mice, murine hepatocytes and hepa 1-6 cells. Serum IL-6 levels of TO- and LPS-treated wild type mice were elevated and closely correlated to the upregulation of hepatic HO-1 expression in these animals. Hepatic HO-1 gene expression was found to be increased significantly in IL-6 KO mice as well, in both the models of APR. In IL-6 KO mice, however, the constitutive expression of HO-1 in the liver was higher than in their wild type (WT) counterparts. Among other inflammatory signals, TNF-α, IL-1ß and IFN-γ increased HO-1 gene expression *in vivo* and *in vitro*. Serum IL-1ß showed a delayed increase in TO-treated IL-6 KO mice as compared to their WT counter parts, but serum levels of TNF-α did not augment significantly in these animals. In LPS-treated WT and IL-6 KO mice, the serum levels of TNF-α and IL-1ß elevated prominently. We studied also the different transcriptional regulators of HO-1 in the liver of TO- and LPS-treated IL-6 knockout mice. Among these, hepatic STAT3-phosphorylation was observed in both acute phase models in knockout mice. Among other transcriptional regulators of HO-1, AP-1 appeared to be more involved in TO-treated animals whereas NFκB was prominent in LPS treatment. HO-1 was induced also in injured and non-injured muscles of TO- and LPS-treated WT and IL-6 KO mice. However, whereas in TO-treated animals an early increase of HO-1 gene expression was measurable in non-injured

muscle, HO-1 was induced only at later time points in the muscle of intraperitoneally (i.p.) LPS-treated animals.

Lactoferrin was upregulated at mRNA and protein level in the liver of TO- and LPS-treated WT mice, although the pattern of induction was different in both animal models. IL-6 induced lactoferrin gene expression in WT mice, murine hepatocytes and hepa 1-6 cells. Lactoferrin gene expression also increased in the liver of TO- and LPS-treated IL-6 KO mice. The magnitude of upregulation was similar in WT and IL-6 KO mice, but an early decline in Ltf at mRNA and protein level was observed in TO-treated IL-6 KO animals. On the other hand, magnitude of upregulation of Ltf mRNA in the liver of LPS-treated IL-6 KO mice was higher than in the WT mice. Moreover, in TO-injected skeletal muscle also marked differences were seen in Ltf expression between WT and IL-6 KO mice. As compared to WT mice, IL-6 KO mice showed a higher increase and a delayed decline in Ltf expression in the skeletal muscle after TO-injection. Serum measurement of Ltf showed an increase of Ltf that was more prominent after LPS injection than after TO-injection. Besides IL-6, TNF-α and IFN-γ also increased Ltf gene expression *in vivo* and *in vitro*. However, IL-1ß appeared to play no significant role in Ltf gene expression upregulation.

HO-1 remains a positive acute phase protein, not only in liver but also in skeletal muscle under different acute phase conditions even in the absence of IL-6. Besides, the data acquired from the current work not only shows for the first time muscle and liver as two main sources of serum lactoferrin during different acute phase conditions, but also brings to light different acute phase signals regulating Ltf gene expression during acute phase conditions. In addition to Ltf and HO-1, serum iron levels and iron concentration in the liver-tissues of TO- and LPS-treated animals were measured, and an inverse correlation between the decrease of serum iron concentration and the increase of liver iron concentration was found in both the animal models. Increase in liver iron concentration closely correlated to augmented hepatic HO-1 gene expression, which is one of the pathways that hepatic cells utilize to acquire free iron by the breakdown of heme. At the same time, increase in hepatic Ltf gene expression indicates to a cyto-protective pathway that might not only be involved in the chelation of free iron during APR, but also might be assisting in iron transport from serum to liver to reduce the possibility of free iron being available to pathogens in an ongoing APR. Whereas Ltf serum level increase during APR might probably be another protective mechanism of the immune defense system to chelate any free iron on one side or to transport iron into the area of need.

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8 Dedication

To my family

9 Acknowledgements

I want to express my gratitude to Professor Dr. Giuliano Ramadori for the opportunity that he gave me to pursue my doctoral degree in the Department of Gastroenterology and Endocrinology. I deeply appreciate and am very thankful to him for the time that he spent supervising and teaching me.

I am very grateful to Professor Dr. Wolfgang Engel for his constant noble support for my work, and for reviewing and correcting my PhD thesis. I am very thankful to him for the time that he spent for discussing my results.

I am very thankful to Professor Dr. Uwe Groß for being the co-reviewer of my thesis.

My sincere thanks to Dr. Nadeem Sheikh, Dr. Jozsef Dudas and Dr. Rajesh Pannem for their cooperation and practical suggestions.

I deeply appreciate Mr. Pierluigi Ramadori for his cooperativeness, and for the time that we spent for our numerous discussions.

My special thanks to Mrs. Renate Klages who helped me in organizing my work and for her constant and wholehearted support in every aspect.

I am very thankful to Mrs. Sieglinde Zachmann for her constant miscellaneous support.

I thank David, Gesa, and all other colleagues, for providing me a nice and friendly working atmosphere.

My very special thanks to my family for their constant support in all respects.

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10 Curriculum vitae

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