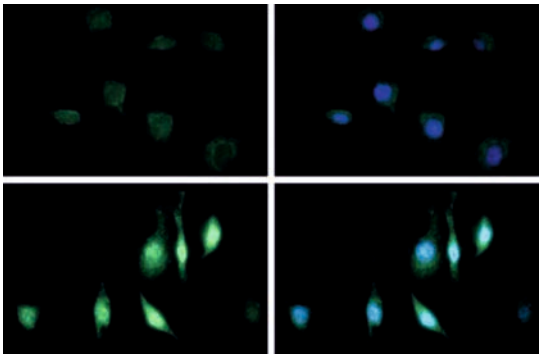
Lipopolysaccharide- and p38 MAPK-mediated signaling of the Heme oxygenase-1 (HO-1) gene in macrophages

VIJITH VIJAYAN



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Date: 14th March 2012 Vijith Vijayan

Place: Giessen, Germany

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1. INTRODUCTION

1.1 Inflammation

Inflammation has been recognized in the field of medicine from the period as early as 30AD when Celsius first described the four basic markers of it: *Rubor* (redness), *Calor* (heat), *Tumor* (swelling) and *Dolor* (pain), to which Virchow in 1870 added the fifth element, *Functio laesa* (loss of function). The term *inflammation* can be defined as a stereotypical reaction of the body against invading pathogens including viruses, bacteria, fungi, protozoa, cell injury or any toxic substance. Inflammation can be *acute* or short-lived which is rather non-specific and *chronic* or persistent where the process is more specific and is dominated by a specific cell type (e.g. neutrophils in chronic airway inflammation).

1.2 Heme-Heme oxygenase System

Heme oxygenase (HO) was first described by Tenhunen et al in 1968 as a distinct enzyme which degrades heme in hepatic microsomes. To date, HOs are the only known enzymes which are responsible for catalyzing the first and rate-limiting step of oxidative breakdown of heme into iron (Fe), carbon monoxide (CO) and biliverdin (Tenhunen et al. 1968). Biliverdin is consecutively converted to bilirubin by the enzyme biliverdin reductase (Kapitulnik and Maines 2009). HO enzymatic activity requires three moles of molecular oxygen (O_2) and NADPH. HOs are ubiquitously expressed in higher eukaryotes. Heme degrading systems similar to the HO system have also been described in lower entities such as bacteria, algae and flies (Ryter et al. 2006).

The heme molecule is a double-edged sword, which on the one hand is required for the biological functions of many heme apoproteins such as hemoglobin, myoglobin and cytochrome c oxidase (Padmanaban et al. 1989; Wijayanti et al. 2004). On the

other hand free heme catalyzes the formation of reactive oxygen species (ROS) through Fenton chemistry and thus leads to oxidative-stress-induced tissue and cellular damage. Therefore, intracellular generation of heme is tightly regulated (Sassa 1996; Ponka 1999; Sassa 2004). Moreover, heme has recently been shown to have potent pro-inflammatory properties (Wagener et al. 2003; Soares et al. 2009). Intravenous administration of heme has been shown to cause experimental inflammation with high leukocyte infiltration. Moreover, Jeney et al have shown that heme-dependent oxidation of low-density lipoproteins is involved in inflammation-mediated tissue damage (Jeney et al. 2002). Hence, the HO system plays a crucial role in regulating the intracellular heme homeostasis.

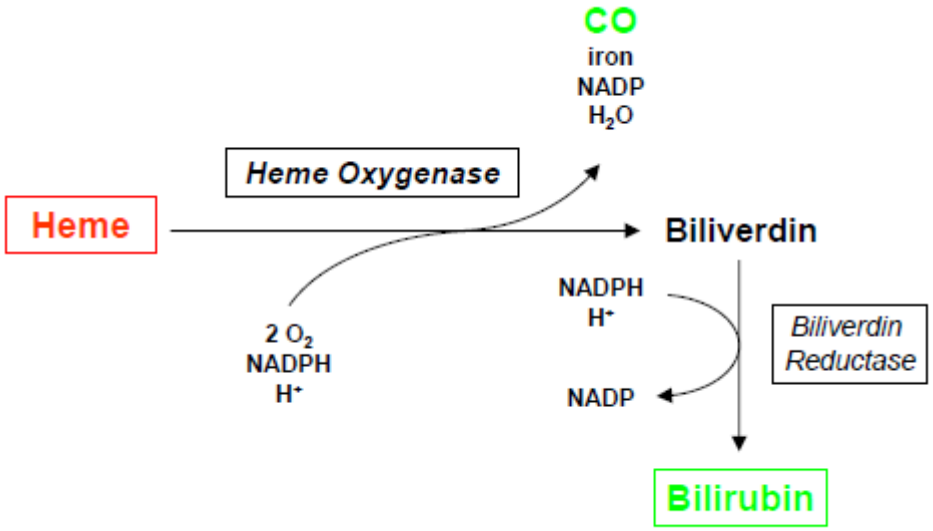


Fig 1: Heme is enzymatically degraded to yield carbon monoxide (CO), iron and biliverdin, which is converted into bilirubin in a coupled reaction (Vijayan et al. 2010).

1.3 Heme oxygenase isoforms

Two isoforms of HO have been characterized: HO-1, which is an inducible isoform of HO and HO-2, which is constitutively expressed. Both enzymes are genetically

distinct; the human HO-1 and HO-2 are localized on chromosomes 22q12 and 16p13.3, respectively. Human HO-1 contains 288 amino acids and has a molecular weight of 32 kDa, whereas HO-2 consists of 316 amino acids with a total molecular weight of 36 kDa. Rat HO-1 and HO-2 share 43% amino acid homology and between species HO-1 in humans and rats have 80% similarity and HO-2 shares 88% similarity. The mouse HO-1 protein contains one cysteine while the other species do not. HO-1 and HO-2 also vary in their tissue expression pattern where HO-1 is found highly expressed in spleen and tissues that degrade senescent red blood cells, including specialized reticulo-endothelial cells of the liver and bone marrow (Maines et al. 1986; Ryter et al. 2006; Abraham and Kappas 2008). HO-2 in turn is highly expressed in the testes and brain (Trakshel et al. 1986). Both HO-1 and-2 contain a COOH-terminal hydrophobic domain, which allows attachment to membranes and has been predominantly found to be localised in the endoplasmic reticulum. Recent studies have, however, reported the localisation of HO-1 in the plasma membrane, mitochondria and nucleus (Kim et al. 2004; Converso et al. 2006; Sacca et al. 2007).

1.4 Heme oxygenase-1

Why do we need two isoforms of an enzyme with the same property if its role is the same? In contrast to HO-2, HO-1 as aforementioned has an inducible gene expression. Strikingly, HO-1 gene expression can be induced not only by its substrate heme, but also by a wide variety of stress-inducing stimuli such as UV, heavy metals, quinones and LPS (Choi and Alam 1996; Immenschuh and Ramadori 2000; Alam et al. 2004). The high inducibility of HO-1 gene expression raised the speculation that this enzyme might be involved in cytoprotection but experimental evidence was lacking. HO-1 was first linked to inflammation by Willis et al in an animal model of carragenin-induced pleurisy, in which specific upregulation of HO

enzyme activity attenuated a complement-dependent inflammation (Willis et al. 1996). In 1997 the first report on a knockout mouse model of HO-1 (Poss and Tonegawa. 1997) marked a new era in the field of HO-1 with a rapid increase in interest for this protein leading to over 4000 publications. To date, only one case of human HO deficiency is reported in the literature (Yachie et al. 1999). Molecular alterations in both the patient and the knockout mouse with HO-1 deficiency will be discussed in the following chapters.

1.5 HO-1 knockout mouse model studies

Poss and Tonegawa (1997) analysed the iron reutilisation properties and observed an increased amount of oxidized proteins and lipid peroxidation levels in the HO-1 knockout mouse with values of 51% and 95% in comparison to normal or heterozygous mice. HO-1 knockout mice showed signs of chronic inflammation with typical histological changes such as fibrosis, high peripheral leukocyte count and elevated infiltration of leukocytes into hepatic and renal tissue. Moreover, cells from these knockout mice were highly sensitive to oxidative-stress induced by heme and H_20_2. Furthermore, the production of ROS and other free radicals upon heme or H_20_2 stimulation was significantly higher in mouse embryonic fibroblasts (MEFs) of HO-1 knockout animals in comparison to wild type cells. Moreover, both heterozygous as well as the wild type mice were more resistant to an endotoxin challenge as compared to knockout mice (Poss and Tonegawa 1997). Hence, HO-1 appeared to have an important function in protecting against LPS-induced toxicity. Similarly, when haemoglobin was administered, HO-1 knockout mice suffered from irreversible renal damage with a 100% mortality rate (Poss and Tonegawa 1997). It is noteworthy that HO-2 the constitutive isoform does not compensate for the loss of HO-1.

Furthermore, Kapturczak and colleagues (2004) explored the immune modulatory properties of HO-1 in HO-1 knockout mice and reported that the regulation of cytokine and chemokine expression was impaired in HO-1 knockout mice. High amounts of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1 were observed in these knock out animals. Although many in vitro studies have linked HO-1 with cytokine regulation this study was the first to show direct immunomodulatory functions of HO-1 (Kapturczak et al. 2004). Similarly, other studies have shown that adaptive immune responses are also impaired in these knockout mice (George et al. 2008; Soares et al. 2009). In contrast, HO-2 knockout mice appear to have an intact immune regulation but exhibit defects in their central and autonomous nervous system (Burnett et al. 1998).

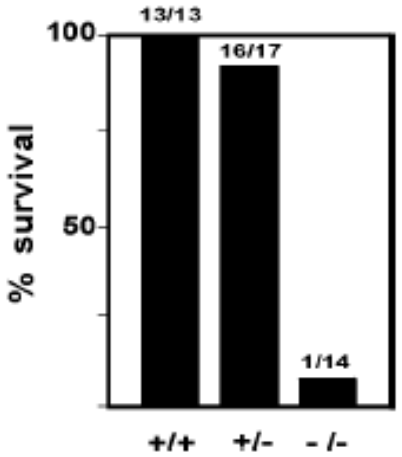


Fig 2: Wild type mice, heterozygous mice and HO-1 knockout mice 6-9 weeks old were challenged with endotoxin. HO-1 knockout mice exhibited high mortality rates (Toss and Ponegawa 1997).

1.6 Human deficiency of HO-1

Only one case of human deficiency of HO-1 has been reported so far (Yachie et al. 1999). Nevertheless studies on this patient contribute to and validate most of the phenotype observed in the HO-1 knockout mouse model. The patient was a 6 year old Japanese boy with growth retardation and delayed motor development.

From the time he was 2 years old he developed hepatomegaly and lymphadenopathy. His peripheral blood exhibited a high concentration of fragmented erythrocytes and showed increased levels of white blood cells with dysmorphic monocytes. One of the clinical features, which led to the diagnosis of a HO-1 gene defect, was the complete absence of the HO-1 enzyme in Kupffer cells in an immunohistochemical staining of hepatic biopsy specimens. Examination of lungs and heart revealed no significant abnormalities, whereas the deficiency of HO-1 led to a heavy damage of the kidney. Electron microscopic analysis of the renal biopsy specimen revealed marked endothelial damage with an unknown deposition in renal glomerular capillary loops. Moreover, the endothelium was found to be detached widely. Infiltration of leukocytes, iron deposition and an increase in adhesion molecules such as ICAM, selectins and other inflammatory markers like von Willebrand factor were observed. Furthermore, absence of HO-1 rendered the lymphoblastoid cell line (LCL) (EBV transformed B cells) from the patient more sensitive to heme-induced oxidative stress. In summary the absence of HO-1 led to an increased pro-inflammatory state in the patient (Yachie et al. 1999; Kawashima et al. 2002).

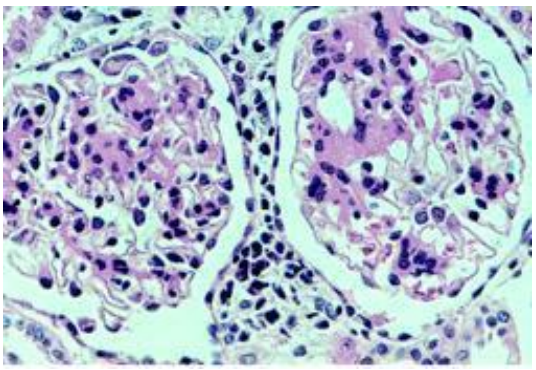


Fig 3: High magnification of the kidney cortex in the human HO-1 deficient patient showing heavy infiltration of leukocytes (Yachie et al. 1999).

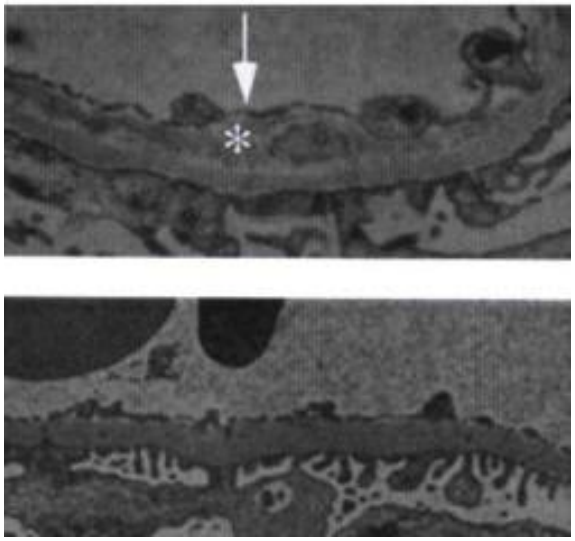


Fig 4: In comparison to the normal intraglomerular endothelium (bottom panel), the endothelium of the HO-1 deficient patient (upper panel) was highly detached (arrow) and exhibited an unknown precipitate between the endothelium and the common basement membrane (Yachie et al. 1999)

1.7 How does HO-1 regulate inflammation?

The mechanism by which HO-1 confers its cytoprotective effects is widely discussed but still not clearly understood. A heavy body of literature suggests a pivotal role for CO and bilirubin, the two enzymatic by-products of HO-1 reaction, in the cytoprotective effects.

1.7.1 Biliverdin and Bilirubin (BR)

Antioxidant properties of bilirubin have been described in the 1980s, before which it was considered only to be a toxic waste product excreted in bile. The antioxidant potential of bilirubin is comparable to the efficacy of alpha-tocopherol, the most efficient antioxidant acting against lipid peroxidation (Kapitulnik 2004; Stocker 2004). Later, an anti-inflammatory function for bilirubin was shown in a microvessel model in which the leukocyte transmigration rate was observed to correlate with the amount of bilirubin produced from HO-1 activity (Hayashi et al. 1999). In a murine asthma model, it was shown that administration of bilirubin significantly reduced the VCAM-1-dependent transmigration of leukocytes which is a mechanism also implicated in the pathogenesis of inflammatory bowel disease (Keshavan et al. 2005). In

another study on a rat model of endotoxemia it was noted that bilirubin treatment significantly blocked the hepatotoxicity seen after endotoxin exposure. Hepatic iNOS expression, serum levels of NO, TNF-α and aminotransferases, the profound markers of endotoxemia were significantly reduced (Wang et al. 2004). Similarly, another independent study reported analogous observations in a mouse model of endotoxemia, in which a single bolus addition of bilirubin rescued the mice from endotoxemia (Kadl et al. 2007). Thus, the protective or anti-inflammatory functions of HO-1 can be in part attributed to bilirubin. However, how exactly bilirubin brings about these anti-inflammatory effects remains an open question.

1.7.2 Carbon monoxide

The last decade has seen a rapid interest in understanding the physiological functions of CO. HO-1-generated CO has been implicated in a variety of physiological functions such as anti-apoptosis, neurotransmission, anti-coagulation, vasodilation and most importantly protection against inflammation. The anti-inflammatory functions of CO have been reviewed in the following references (Ryter et al. 2002; Kim et al. 2006). An initial finding in this context was its ability to downregulate the inflammatory cytokines TNF-α, IL-1 beta, MIP-1 both *in vitro* in cell lines and *in vivo* in mice models challenged with LPS (Otterbein et al. 2000). Similar to bilirubin, CO also blocks the iNOS activity or production of NO but unlike the former does not regulate the protein expression of iNOS (Sawle et al. 2005). Abrogation of TNF-α, IL-6 and IL-1β production by CO is mediated by modulating the MAPKs of which p38 is required for most of its functions. More recently it was shown in a RAW 264.7 murine macrophage cell line and *in vivo* in a mouse model for acute lung injury that CO via production of ROS induces the expression of PPAR-gamma

which is responsible for the downstream blockage of pro-inflammatory genes such as Egr-1 (Bilban et al. 2006). Apart from this, CO also induces the production of cGMP via activating the soluble guanyl cyclase (sGC). CO and bilirubin share similarities in their anti-inflammatory functions. Interestingly, CO and bilirubin appear to form an autocrine loop by which they induce the activity and expression of endogenous HO-1. The possibility that both CO and bilirubin exerts its anti-inflammatory function by inducing the endogenous HO-1 enzymatic activity or a yet undefined function of HO-1 protein cannot be ruled out.

1.7.3 HO-derived iron

The third product of HO enzymatic reaction, iron is an important compound involved in redox-dependent enzyme reactions and bioenergetics. However, presence of free iron inside the cell may lead to the formation of toxic ROS, and therefore specific intracellular protective mechanisms exist to prevent intracellular toxicity. As an example, HO-1-derived iron bind to the intracellular iron storage protein ferritin and is transported outside the cell, thereby protecting the cell from oxidative damage (Ponka 1997). Interestingly, it has been shown in various models that upregulation of ferrittin correlated with the synthesis of HO-1 (Balla et al. 1992; Ryter and Tyrrell 2000). Of note, deficiency of HO-1 in mice (Poss and Tonegawa 1997) and humans (Yachie et al. 1999; Kawashima et al. 2002) has been shown to be related with an increase iron deposition in the liver and kidney.

1.8 How does HO-1 modulate an inflammatory response?

Studies on the role of HO-1 in different cell types have revealed that HO-1 exhibits cell-specific anti-inflammatory roles of which some are discussed in the following.

1.8.1 Role of HO-1 in endothelial cells

Activation of endothelial cells is a crucial step in the process of inflammation because it regulates the influx of leukocyte population, especially that of polymorphonuclear neutrophils. Pro-inflammatory cytokines modulate the gene expression of adhesion molecules such as E-selectin, P-selectin, ICAM-1 and VCAM-1 on endothelial cells, thus facilitating the adhesion and transmigration of leukocytes into the site of injury (Muller 2003; Cook-Mills and Deem 2005; Immenschuh and Schroder 2006). In an *in vivo* rat model it was observed by intravital microscopy that induction of HO-1 in microvessel endothelial cells downregulates the adhesion of leukocytes to these cells during oxidative stress (Hayashi et al. 1999). In another study it was shown that inhibition of HO activity potentiated the heme-induced leukocyte infiltration into the tissues (Wagener et al. 2001). Recently, it was shown that the TNF-α-mediated upregulation of the adhesion molecules E-selectin and V-CAM1 was downregulated by overexpressed HO-1 through inhibiting the activation of NF-κB (Seldon et al. 2007). Thus, the major role for HO-1 in endothelial cells for counteracting inflammation seems to be achieved by regulating the expression of adhesion molecules on these cells and maintaining them in a less active state. Additionally, HO-1 also protects the endothelial cells from inflammatory damage by turning on an anti-apoptotic signaling cascade. This mechanism involves the degradation of the p38-alpha isoform of the p38 MAPK (Soares et al. 2002; Silva et al. 2006).

1.8.2 Modulation of immunologically active cells by HO-1

Among immunologically active cells, the most prominent model of study for heme oxygenase functions has been the macrophages or monocyte population. Studies in macrophages from HO-1 knockout mice suggested an

important functional role of HO-1 in these cells. HO-1 knock out cells produced high levels of the pro-inflammatory cytokines TNF-α, IL-6 and IL-1beta (Kapturczak et al. 2004). Several independent studies have also reported the negative regulation of HO-1 on the proinflammatory genes cyclooxygenase 2 (COX-2) and inducible nitric oxide (iNOS) in macrophages, in which induction of HO-1 has been shown to block the production of prostaglandins as well as NO after LPS challenge (Lee et al. 2011; Park et al. 2011). Similarly HO-1 has been shown to have an antioxidant role in macrophages (Schulz-Geske et al. 2009).

Another important function of HO-1 containing antigen presenting cells (APCs) seems to be the regulation of regulatory T-cells (Tregs). In a recent study it was shown that Treg cells require the presence of a functional HO-1 in the antigen presenting cells to execute their function of suppressing the effector T cell proliferation. Interestingly absence of HO-1 in Treg cells itself does not affect its suppressive activity *in vitro* (George et al. 2008)

Dendritic cells (DCs) occupy the major population of antigen presenting cells and play an important role in adaptive immune responses by presenting the antigen to the B cells and T cells. A study on HO-1 functions in DCs revealed that immature human and rat DCs express HO-1 significantly which, upon receiving a maturation signal is downregulated. HO-1 if induced by co-treatment of a maturation signal which in this case was LPS with Cobalt protoporphyrin significantly blocked the expression of DC cell surface maturation markers such as MHC class II, CD86 and ICAM-1. Moreover their capacity to stimulate mixed lymphocyte reactions was abrogated. Hence, in DCs HO-1 seems to be one of the critical factors maintaining the balance between immature and mature DCs.

This function of HO-1 as proposed by the authors could be used to modulate functions of DCs in inflammatory disorders (Chauveau et al. 2005).

1.9 Signaling mechanisms that regulate HO-1

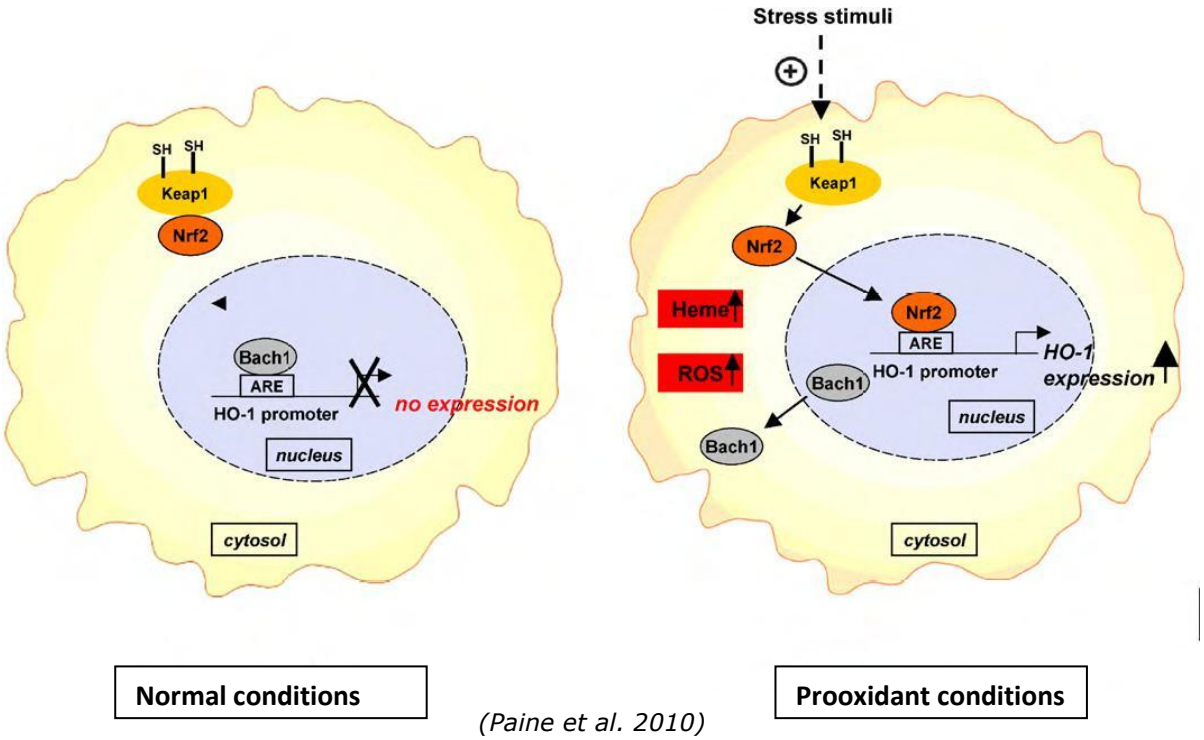
HO-1 is primarily regulated at the transcriptional level by multiple cis-acting regulatory elements (REs) present in the HO-1 gene promoter. The most studied of these REs are the two upstream enhancer regions termed E1 and E2 (Alam 1994; Alam et al. 1994). E1 and E2 contain multiple copies of anti-oxidant response elements (AREs) which have been shown to be involved in the specific upregulation of HO-1 in response to various stimuli (Nguyen et al. 2003). Interestingly, the human HO-1 gene contains a GT-microsatellite polymorphism in the proximal promoter region, which has been shown to be of major biological significance. Individuals carrying HO-1 gene allele with lower number of GT repeats have been associated with higher inducibility of HO-1 gene and seem to be protected against various diseases such as cardiovascular disorders (Yamada et al. 2000). NF-E2 related factor 2 (Nrf2), AP-1 and NF-κB are thought to be the key transcriptional factors involved in the regulation of HO-1 gene expression and the findings are briefly discussed below.

1.9.1 Keap1/ Nrf2 system

Nrf2 is a redox-dependent transcription factor which belongs to Cap'n'Collar (CNC) family of leucine-rich zipper proteins. Under basal conditions Nrf2 is bound to the cytoplasmic protein Kelch-like ECH-associated protein1 (Keap1) which mediates the proteosomal degradation of Nrf2. Under stress conditions, Keap1 is modified leading to the release of Nrf2, which translocates to the nucleus (Itoh et al. 1999; Kobayashi and Yamamoto 2005; Kimura et al. 2007; Kaspar et al. 2009). Once in the nucleus Nrf2 forms heterodimers with small maf proteins and binds to anti-oxidant response elements in the promoter of

various phase II detoxifying enzymes, such as glutathione transferases and NADPH: oxidoreductase (Motohashi et al. 2002).

A noteworthy feature of Nrf2 signaling with respect to the specific up-regulation of HO-1 gene is the interplay between Nrf2 and the transcriptional repressor Bach1. Under basal conditions Bach1 is bound to ARE-elements on HO-1 promoter. But when stimulated with heme, Bach1 dissociates from HO-1 promoter and is rapidly transported to the cytoplasm allowing the incoming Nrf2 to bind to the Bach1-free anti-oxidant response element and thus activate the transcription of HO-1 gene (Ogawa et al. 2001). Similar mechanisms have been reported for other stimuli, such as sodium arsenite (Reichard et al. 2008).



(Paine et al. 2010)

Fig 4: Regulatory interplay between the transcription factors Nrf2 and Bach1 on the gene expression of HO-1. Under normal conditions Bach1 acts as a repressor of the HO-1 gene by binding to the AREs of the HO-1 promoter. When cellular heme levels are high Bach1 dissociates from the promoter and translocate to the cytoplasm. At the same time Nrf2 translocate to the nucleus and bind to AREs and activate the transcription of HO-1 gene (Paine et al. 2010)

1.9.2 NF-κB

The NF-κB/Rel family of transcription factors consist of many proteins factors which form homodimers and heterodimers among themselves to drive the gene expression of a plethora of pro-inflammatory genes such as cytokines, adhesion molecules and anti-oxidant proteins (Gilmore 2006; Perkins and Gilmore 2006). The classical activation pathway of NF-κB has been studied extensively. Under basal conditions NF-κB is maintained in the cytoplasm by the NF-κB inhibitor protein, IκB. Upon receiving an activation signal IκB is phosphorylated and undergoes degradation whereby NF-κB is released. NF-κB translocates to the nucleus where it binds to the NF-κB binding elements on the gene promoter and induces the gene expression (Ghosh and Hayden 2008).

The role for NF-κB in regulating HO-1 gene expression has been discussed controversially. Although NF-κB inhibitors have been shown to block HO-1 induction by various stimuli such as LPS, only two studies have so far reported the presence of functional NF-κB binding elements in the HO-1 promoter. In one study it was shown that TPA-induction of HO-1 is regulated by a proximal NF-κB site in the rat HO-1 promoter. Another independent study reported that nitric oxide synthase upregulates HO-1 via a NF-κB-dependent pathway and is regulated by a NF-κB element in the mouse HO-1 promoter (Naidu et al. 2008; Li et al. 2009).

1.9.3 AP-1

Alam and colleagues have reported that the transcription factor AP-1 is important for the induction of mouse HO-1 (Alam and Den 1992; Alam et al. 1995). AP-1 belongs to a family of transcription factors which is involved in various cellular processes including stress responses (Karin 1995; Hess et al.

2004). Elucidating the role of AP-1 in HO-1 gene expression is highly complex due to the fact that the AP-1 binding site (TGATGCA) is a part of the HO-1 ARE sequence (TGCTGAGTCA). Most likely there is a cross-talk between Nrf2 and AP-1 family of transcription factors and this notion has been supported by the recent finding that c-jun in association with Nrf2 induce the expression of the ARE-regulated genes NADPH:quinone oxido reductase and glutamate-cysteine ligase (Alam et al. 1999; Levy et al. 2009).

In summary, multiple transcription factors may act co-ordinately to upregulate HO-1 gene expression during stress conditions.

1.10 Role of kinases in HO-1 signaling

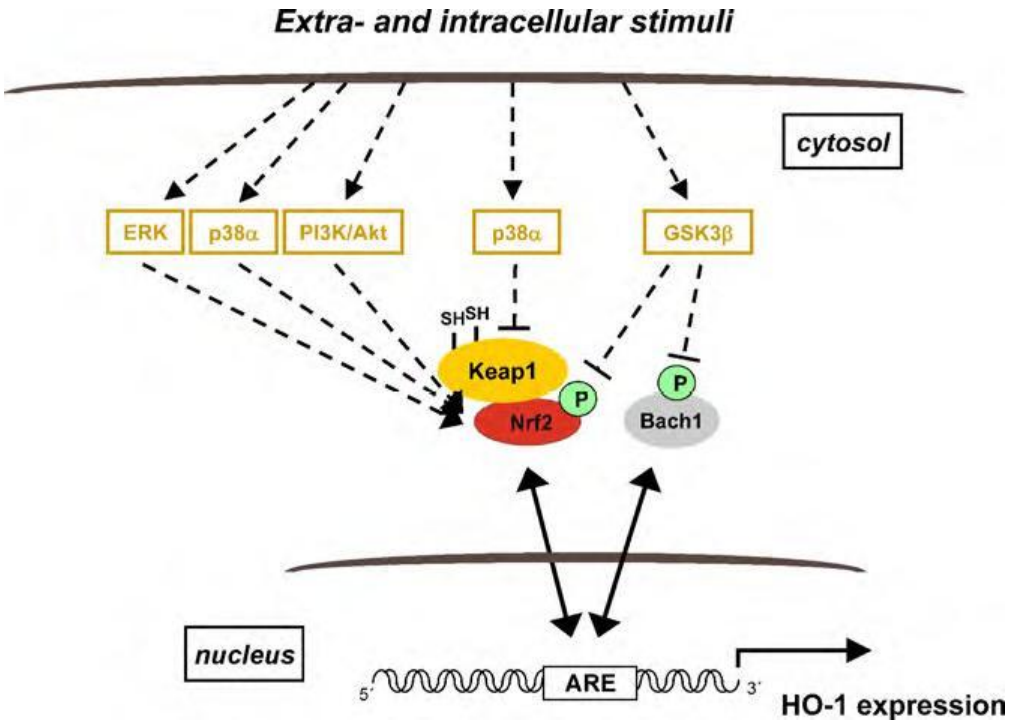
Generally, transcription factors are regulated by intracellular signaling cascades, which are controlled by the activation of various kinase and phosphatase modules and the redox state of the cell. Due to space limitations in this thesis the role of these kinases are not described in detail. Hence, only the mitogen activated protein kinases (MAPK), which have been explored in my work, specifically the role of p38 MAPK for the regulation of HO-1 gene expression are highlighted.

1.10.1 The MAPK pathway

Activation of MAPKs has been shown to play a central role in the regulation of HO-1 gene expression (Alam and Cook 2007). There are three major subfamilies of MAPKs: p38 MAPK, extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) (Kyriakis and Avruch 2001). ERK is shown to be mainly involved in the activation of signaling pathways induced by growth factors and hormones, whereas JNK and p38 MAPK are considered to be activated under stress conditions (Kyriakis and Avruch 2001; Wagner and Nebreda 2009).

Activation of p38 MAPK has been shown to be associated with antioxidant and anti-inflammatory responses. Several studies indicate that blocking the activity of p38 MAPK either by chemical inhibitors or by gene silencing blocks the protein induction of HO-1 in response to various stress-stimuli (Cook-Mills and Deem 2005; Alam and Cook 2007). It is noteworthy that the different isoforms of p38 MAPK have opposing roles in the regulation of HO-1, which has been demonstrated for sodium arsenite and LPS induced expression of HO-1 (Kietzmann et al. 2003; Wijayanti et al. 2004).

Apart from MAPKs other kinases such as JAK kinase (Zhang et al. 2006) and PI3 kinase (Martin et al. 2004) also have a role in the regulation of HO-1. It is important to note that the regulation of HO-1 by kinases is stimuli-specific and in some cases cell-type specific (Paine et al. 2010).



(Paine et al. 2010)

Fig 5: Signaling cascades that lead to the activation of Nrf2 regulating HO-1 gene expression (Paine et al. 2010).

1.11 LPS signaling of HO-1

LPS, a cell wall component of gram negative bacteria is a potent inducer of inflammation and has been extensively used to induce and study sepsis in mouse models. LPS signaling in cells is mediated by the CD14 receptor in association with the TLR4 receptor present on the cell surface. TLR4 activation is followed by activation of a series of adaptor molecules that lead to the phosphorylation and degradation of IκB and subsequently to the release of the transcription factor NF-κB (Zhu and Mohan 2010). LPS has been previously shown to induce the expression of HO-1 in various cell types. Camhi and co-workers (1995) described that LPS-induced HO-1 upregulation in murine macrophages is primarily mediated by the transcription factor AP-1 (Camhi et al. 1995). Independently, others have demonstrated that the transcription factor Ets2 is crucial for the promoter regulation of mouse HO-1 (Chung et al. 2005). Previous studies from the Immenschuh laboratory have indicated a role for NO, NF-κB and p38 MAPK in the LPS-induced upregulation of HO-1 in macrophages (Immenschuh et al. 1999; Wijayanti et al. 2004). The signaling mechanisms that lead to the induction of HO-1 are regulated by multiple factors and are highly complex.

Noteworthy, increased HO-1 activity has been shown to exert inhibitory effects on the intracellular signaling initiated by TLR4 activation (Nakahira et al. 2006). This regulatory interplay between TLR4 and HO-1 seems to form a negative feedback loop, in which HO-1 activation controls the excessive activation of macrophages by LPS. This feedback circuit might be

of major significance for the regulation of inflammatory responses. Understanding this complex mechanism in detail will help us develop new therapeutical strategies to treat inflammatory conditions such as sepsis.

1.12 Bruton's Tyrosine kinase

Bruton's tyrosine kinase (Btk) is a member of the Tec family of non-receptor tyrosine kinases and is found to be expressed in all cells of the hematopoietic lineage except for plasma cells and T cells. The defining feature of this kinase is the presence of a pleckstrin homology (PH) domain at the N-terminus. Naturally occurring mutations on this kinase, that make it non-functional were identified initially in humans in a rare genetic disorder called X-linked agammaglobulinemia (XLA) (Vetrie et al. 1993), which is characterized by a deficiency of mature circulating B-cells and immunoglobulins. In murine models, a single point mutation (R28C) in the mouse Btk gene or targeted disruption of the Btk gene is associated with a similar phenotype and is termed XID syndrome (Rawlings et al. 1993; Khan et al. 1995).

In addition to the PH domain Btk also contains three Src homology domains (SH1, SH2 and SH3) and the Tec homology domain (TH) that is conserved in all the members of the Tec family except for Etk/Bmx. Btk is versatile in its interaction with other proteins. Moreover, the presence of these domains makes it possible for Btk to form protein-protein as well as protein-lipid interactions. Under basal conditions, the SH3 domain and the TH domain form an intramolecular interaction which folds Btk in a "closed" inactivated form. Upon receiving an activation signal, the protein undergoes

a conformational change and is then translocated to the membrane via its PH domain (Mano 1999).

Initial studies mainly focused on the role of this kinase in B cells which indicated that Btk is crucial for their function and development (Satterthwaite and Witte 2000). Recently, it has been shown that Btk plays a major role in TLR-induced innate immune responses evoked in macrophages (Jefferies and O'Neill. 2004). Specifically, inhibition of Btk in macrophages caused an impaired TLR4-induced gene expression of central pro- and anti-inflammatory cytokines such as TNF-α, IL-1 and IL-10 (Horwood et al. 2003; Doyle et al. 2005; Horwood et al. 2006; Koprulu and Ellmeier 2009). Moreover, Btk is required for appropriate phagocytic function and for the survival of mononuclear phagocytes (Schmidt et al. 2006; Jongstra-Bilen et al. 2008).

1.12.1 Regulation of HO-1 gene expression in mononuclear phagocytes

Mononuclear phagocytes (monocytes, macrophages) are immune cells which play an important role in modulating an inflammatory response. On the one hand they play a crucial role in the initiation of an inflammatory response via secretion of pro-inflammatory cytokines, such as IL-1, IL-6 and TNF-α and by the intracellular generation of ROS, which aids in killing the phagocytosed pathogen. On the other hand mononuclear phagocytes promote resolution of inflammation by phagocytosing apoptotic cells and by the secretion of the anti-inflammatory cytokines IL-10 and TGF-β. Recent evidence suggests that the immunomodulatory functions of HO-1 in mononuclear phagocytes may be

involved in the regulation of an inflammatory immune response. The prototypical inflammatory stimulus LPS, which initiates signaling by binding to TLR4, has been shown to induce HO-1 in macrophages.

1.13 Aims of the study

1.13.1 Part A

TLR-induced signaling mechanisms activate diverse signaling molecules. Recently it was shown that Btk, a kinase which is crucial for the development and effector functions of B-cells, is involved in the TLR-induced signaling of cytokines. Signaling pathways are highly complex and many signaling molecules that have been shown to be involved in cytokine regulation have also been implicated in the regulation of HO-1 gene. In the first part of this thesis, therefore it should be analyzed whether Btk would also be involved in the TLR-induced regulation of the HO-1 gene. Furthermore, downstream signaling events to the HO-1 promoter should be analyzed and the role of ROS and Nrf2 should be evaluated in this regulatory process.

1.13.2 Part B

In the second part of this thesis the role of p38 MAPK in the regulation of HO-1 should be analyzed. Activation of p38 MAPK has been shown to be crucial for the induction of HO-1 gene expression in response to various stimuli including LPS. Furthermore, our group noticed an opposing effect of p38 MAPK on HO-1 gene expression during conditions when under the absence of an external stimulus, chemical inhibitors of p38 MAPK were used. Therefore, in this thesis it should be analyzed which functional role p38 MAPK

exerts on the regulation of the HO-1 gene. Moreover, the role of p38 MAPK in the presence and absence of external stimuli should be compared and the downstream signaling pathways explored. Finally, the cross-talk between p38 MAPK and the other MAPKs such as JNK and ERK should be evaluated.

2 Materials and Methods

2.1 Materials

2.1.1 Experimental animals

Specific pathogen free (SPF) C57Bl/6J mice 8-12 weeks old were purchased for experimental purposes from Charles River Laboratories (Sulzfeld, Germany). Btk knockout mice and their wildtype littermates were a generous gift from Dr. Cornelia Brunner (Dept. of Biochemistry, Ulm University Medical School). They had free access to food and water and were kept under standardized environmental conditions (12h light/dark cycle, 23°C ± 1°C and 55% ± 1% relative humidity).

2.1.2 Laboratory instruments

Table 1. All laboratory instruments used for experiments in this thesis are listed with notice of corresponding suppliers:

Instruments	Company name
AGFA Horizon Ultra Colour Scanner	AGFA, Mortsel, Belgium
Biocell A10 water system	Milli Q-Millipore, Schwalbach, Germany
Biofuge Fresco	Heraeus, Hanau, Germany
Biofuge Pico	Heraeus, Hanau, Germany
Bio-Rad electrophoresis apparatus	Bio-Rad, Heidelberg, Germany
Dish washing machine	Miele, Gütersloh, Germany
Cary 50 Bio-UV-visible spectrophotometer	Varian, Darmstadt, Germany
Gel-Doc 2000 gel documentation system	Bio-Rad, Heidelberg, Germany
Hera cell 240 incubator	Heraeus, Hanau, Germany
Hera safe, clean bench	Heraeus, Hanau, Germany
Ice machine, Scotsman AF-100	Scotsman Ice Systems, Vernon Hills, IL, USA

Leica DMRD fluorescence microscope	Leica, Bensheim, Germany
Leica DC 480 camera	Leica, Bensheim, Germany
Microwave oven	LG, Willich, Germany
Mini-Protean 3 cell gel chamber	Bio-Rad, Heidelberg, Germany
Multifuge 3 SR centrifuge	Heraeus, Hanau, Germany
pH meter	IKA, Weilheim, Germany
Pipettes 2.5 µl, 10 µl, 20 µl, 100 µl, 200 µl and 1000 µl.	Eppendorf, Hamburg, Germany
Power supply - 200, 300 and 3000 Xi	Bio-Rad, Heidelberg, Germany
Pressure/Vacuum Autoclave FVA/3	Fedegari, Albuzzano, Italy
Pump Drive PD 5001	Heidolph Instruments, Schwabach, Germany
Sorvall Evolution RC centrifuge	Kendro, NC, USA
SmartspecTM 3000 spectrophotometer	Bio-Rad, Heidelberg, Germany
Thermo plate	Medax, Kiel, Germany
Trans-Blot SD semidry transfer cell	Bio-Rad, Heidelberg, Germany
Trimmer TM60	Reichert, Wolfratshausen, Germany
TRIO-thermoblock	Biometra, Göttingen, Germany
Ultra balance LA120 S	Sartorius, Göttingen, Germany
Vortex M10	VWR International, Darmstadt, Germany
Water bath shaker GFL 1083	GFL, Burgwedel, Germany

2.1.3 General materials and culture media

Table 2. General materials and culture media used in this thesis are listed with notice of corresponding suppliers:

General materials and culture media	**Company name**
BioMax MR-films	Kodak, Stuttgart, Germany
Cover slips 12mm diameter	Menzel-Gläser, Braunschweig, Germany

Culture dish (35mmx10mm)	BD Biosciences, Heidelberg, Germany
Culture dish (100mmx20mm)	BD Biosciences, Heidelberg, Germany
Filter tips and canules	Braun, Melsungen, Germany
Dulbecco's modified Eagle's medium (DMEM)	PAA, Pasching, Austria
Molecular weight markers (DNA, RNA)	Fermentas, St.Leon-Rot, Germany
RPMI medium	PAA, Pasching, Austria
Multi-well cell culture plates (12 wells)	BD Biosciences, Heidelberg, Germany
Oligo(dT)12-18 primer	Invitrogen, Heidelberg, Germany
PVDF membranes	Millipore, Schwalbach, Germany
qRT-PCR primers (see table 14)	Operon, Cologne, Germany

2.1.4 Proteins and enzymes

Table 3. Proteins and enzymes used in this thesis here are listed with notice of corresponding suppliers:

Proteins and enzymes	**Company name**
Bovine serum albumin (BSA)	Roth, Karlsruhe, Germany
Fetal calf serum (FCS Gold Heat Inactivated)	PAA, Pasching, Austria
Immunostar-alkaline phosphatase	Bio-Rad, Heidelberg, Germany
Milk powder	Roth, Karlsruhe, Germany
Precision Plus protein standards, dual color	Bio-Rad, Heidelberg, Germany
Precision Plus protein standards, unstained	Bio-Rad, Heidelberg, Germany
Primary antibodies (see table 15)	Various companies see table 15
Secondary antibodies (see table 16)	Various companies see table 16
SuperScript II reverse transcriptase	Invitrogen, Karlsruhe, Germany
Taq DNA polymerase	Eppendorf, Hamburg, Germany
Accutase	PAA, Pasching, Austria

2.1.5 Chemicals

Table 4. List of chemicals and drugs used in this study with notice of corresponding suppliers:

Chemicals	Company name
Acrylamide	Roth, Karlsruhe, Germany
Agarose LE	Roche, Grenzach-Wyhlen, Germany
Ampicillin	Difco, Detroit, MI, USA
Bradford reagent	Sigma, Steinheim, Germany
Bromophenol blue	Riedel-de-Haën, Seelze, Germany
Cell culture lysis reagent 5X (CCLR)	Promega, Madison, WI, USA
Dimethylsulfoxide (DMSO)	Sigma, Steinheim, Germany
Ethanol	Riedel-de-Haën, Seelze, Germany
Ethidium bromide	Fluka, Neu-Ulm, Germany
Ethylene diamine tetraacetic acid (EDTA)	Fluka, Neu-Ulm, Germany
Glycine	Roth, Karlsruhe, Germany
Glycerol	Sigma, Steinheim, Germany
β-Glycerolphosphate	Sigma, Steinheim, Germany
H_2-dichlorofuorescein-diacetate (H_2-DCFDA)	Invitrogen, Karlsruhe, Germany
Luciferase assay systems	Promega, Madison, WI, USA
Mowiol 4-88	Polysciences, Eppelheim, Germany
N-Propyl-gallate	Sigma, Steinheim, Germany
Paraformaldehyde (PFA)	Sigma, Steinheim, Germany
Penicillin/Streptomycin	PAN Biotech, Aidenbach, Germany
Phenylmethanesulfonyl fluoride (PMSF)	Serva, Heidelberg, Germany
Ponceau S	Serva, Heidelberg, Germany
Rotiphorese Gel 30	Roth, Karlsruhe, Germany
RNaseZap	Sigma, Steinheim, Germany
Sodium carbonate	Merck, Darmstadt, Germany

Sodium chloride	Roth, Karlsruhe, Germany
Sodium hydrogen carbonate	Merck, Darmstadt, Germany
Sodium hydroxide	Merck, Darmstadt, Germany
Sucrose	Merck, Darmstadt, Germany
Sodium dodecyl sulphate (SDS)	Sigma, Steinheim, Germany
Tetramethylethylenediamine (TEMED)	Roth, Karlsruhe, Germany
Trans^{IT}-LT1 transfection reagent	Mirus Bio LLC
Trishydroxymethylaminomethane (Tris)	Merck, Darmstadt, Germany
Triton X-100	Sigma, Steinheim, Germany
Trypan blue	Sigma, Steinheim, Germany
Tween 20	Fluka, Steinheim, Germany

All other standard reagents if not indicated were purchased from Sigma.

2.1.6 Kits

Table 5. List of kits used in this study with notice of corresponding suppliers:

Kits	**Company name**
Dual luciferase kit	Promega, Madison, WI, USA
Nitrite detection kit	Promega, Madison, WI, USA
PCR kit	Qiagen, Hilden, Germany
QIAGEN Plasmid midi kits	Qiagen, Hilden, Germany
RNeasy kit	Qiagen, Hilden, Germany
RT-PCR kit	Invitrogen, Karlsruhe, Germany

2.1.7 Inhibitors and Ligands

Table 6. List of used kits in this study with notice of corresponding suppliers:

Ligands, Inhibitors	**Company name**
Flagellin (TLR5 ligand)	Qiagen, Hilden, Germany
LFM-A13 (Btk inhibitor)	Calbiochem, La Jolla, CA, USA

Lipotechoic acid (TLR2 ligand)	Invivogen, San Diego, CA, USA
LPS (B14: E.coli)	Invivogen, San Diego, CA, USA
LPS (pure) (TLR4 ligand)	Invivogen, San Diego, CA, USA
N-acetylcysteine (ROS scavenger)	Roth, Karlstuhe, Germany
ODN2395 (TLR9 ligand)	Invivogen, San Diego, CA, USA
PD98059 (ERK MAPK inhibitor)	Calbiochem, La Jolla, CA, USA
PMA (PKC activator)	Invitrogen, Karlsruhe, Germany
Poly I:C (TLR3 ligand)	Invivogen, San Diego, CA, USA
R837 (TLR7 ligand)	Invivogen, San Diego, CA, USA
SB202190 (p38 MAPK inhibitor)	Calbiochem, La Jolla, CA, USA
SB203580 (p38 MAPK inhibitor)	Calbiochem, La Jolla, CA, USA
SP600125 (JNK MAPK inhibitor)	Calbiochem, La Jolla, CA, USA

2.1.8 Bacterial strains, cell lines and plasmid constructs

Table 7. List of cell lines and bacterial strains used in this study with notice of corresponding suppliers:

E.coli strains and cell lines	**Company name**
Nrf2 MEF mouse embryonic fibroblasts	Dr. Larry Higgins (University of Dundee, UK)
p38 MEF mouse embryonic fibroblasts	Dr. Angel R. Nebreda (Spanish National Cancer Center, Madrid, Spain)
RapidTrans™ competent *E.coli*	Active Motive, Rixensart, Belgium
RAW 264.7 mouse macrophage cell line	ATCC, Manassas, VA, USA

Table 8. List of plasmids used in this study with notice of corresponding suppliers:

Plasmids	**Company name**
BtkDNK430R, BtkDNR28C	Dr. Sarah Doyle (Trinity College, Dublin, Ireland)

pAP-1-luc	Dr. Craig A. Hauser (The Burnham Institute, La Jolla, CA)
pARE-luc	Dr. William E. Fahl (University of Wisconsin, Madison, WI, USA)
pcDNA3.1	Stratagene, La Jolla, CA, USA
pE2-luc	Dr. Jawed Alam (Alton Ochsner Medical Center, New Orleans, LS)
pGL3 basic, pGL2 basic	Promega, Madison, WI, USA
pHO-4045-luc	Dr. Mark A. Perrella (Harvard Medical School, Boston, MS, USA)
pTNF-585-luc	Dr. Gordon Duff (University of Sheffield, Sheffield, UK)
Gal4 plasmid system (pFR-luc, pFA-CHOP, pFC2-dbd) PathDetect CHOP trans-Reporting system)	Stratagene, La Jolla, CA, USA

2.1.9 Buffers and solutions

Table 9. Solutions for immunofluorescence:

Fixative solution	4% PFA in 1X PBS (150mM NaCl, 13.1mM K_2HPO_4 , 5mM KH_2PO_4), pH 7.4
Glycine (1%)	1g Glycine in 100 ml of 1X PBS buffer
Glycine (1%) + Trition X-100 (0.3%)	1g Glycine in 100 ml of 1X PBS buffer + 0.3 ml Triton X-100
Blocking buffer- 1% PBSA + 0,05% Tween 20	To 2g BSA add 200 ml of 1X PBS and 100 µl of Tween 20
Mowiol 4-88 solution	Overnight stirring of 16.7 % Mowiol 4-88 (w/v) + 80 ml of 1X PBS, add 40 ml of glycerol, stir again overnight; centrifuge at 15,000 g for 1h and take off the supernatant and store at -20°C
Anti-fading agent (2.5%)	2.5g N-propyl-gallate in 50 ml of PBS and add 50 ml of glycerol
Mounting medium	3 parts of Mowiol 4-88 + 1 part of anti-fading agent

Table 10. Solutions for isolation of proteins for SDS PAGE and Western blotting:

Cell lysis buffer (1X)	50mM Tris +150mM Nacl +1% Triton-X-100 (pH 7.4). Before use 10% protease inhibitor cocktail was added
Resolving gel buffer A	1.5M Tris-HCl, pH 8.8 + 0.4% SDS
Stacking gel buffer B	0.5M Tris-HCl, pH 6.8 + 0.4% SDS
Resolving gel (12%) (for 4 SDS-PAGE gels)	8 ml of 30% acrylamide + 10 ml of buffer A + 2 ml of ddH_2O + 15 µl of TEMED + 130 µl of 10% APS
Stacking gel (for 4 SDS-PAGE gels)	1.25 ml of 30% acrylamide + 5 ml of buffer B + 5 ml of DH_2O + 15 µl of TEMED + 130 µl of 10% APS
10X Sample buffer	3.55 ml ddH_2O + 1.25 ml 0.5M Tris-HCl, pH 6.8 + 2.5 ml 50% (w/v) glycerol + 2.0 ml 10% (w/v) SDS + 0.05% bromophenol blue. Before use, add 50 ml β-mercaptoethanol
10X Electrophoresis buffer	250mM Tris + 2M glycin + 1% SDS
20X Transfer buffer	Bis-Tris-HCl buffered (pH 6.4) for transfer of proteins from polyacrylamide gel to PVDF membrane; NuPAGE transfer buffer, Invitrogen, Heidelberg, Germany
10X TBS	0.1M Tris + 0.15M NaCl in 1000 ml of ddH_2O, adjust to pH 8.0
10% Blocking buffer	10g fat free milk powder in 100 ml of ddH_2O
5% BSA	5g BSA in 100 ml 1X TBST +0.05% Tween 20, pH 8.0
1X Washing buffer (TBST)	10mM Tris/HCl, 0.15M NaCl, 0.05% Tween 20, pH 8.0
Ponceau S solution	0.1% (w/v) Ponceau S in 5% (v/v) acetic acid

Table 11. Solutions for molecular biology:

Transfer buffer 10X (TAE)	40mM Tris base + 20mM acetic acid + 1mM EDTA, pH 7.6
RNA-loading dye (10 ml)	16 µl saturated aqueous bromophenol blue, 80 µl 500mM EDTA (pH8.0), 720 µl 37% formaldehyde, 4 ml 10X gel buffer, then fill up to 10 ml with ddH_20
10X RNA transfer buffer	200mM MOPS, 50mM sodium acetate, 10mM EDTA, pH 7.0
1X Formaldehyde gel	100 ml 10X RNA transfer buffer + 20 ml 37% formaldehyde + 880 ml ddH_20
LB medium	0.17 M sodium chloride + 1% Trypton + 0.5% Yeast extract, pH 7.0

LB-Agar	LB medium + 1 g/50 ml Agar + 100 µg/ml Ampicillin
Plasmid mini-prep-Solution 1	50mM glucose + 25mM Tris-Cl (pH-8.0)+ 10mM EDTA + fresh 0.33 µg/µl RNAse A
Plasmid mini-prep-Solution 2	1% SDS in 0.2 N NaOH
Plasmid mini-prep-Solution 3	3M potassium acetate + 11.5% glacial acetic acid
6X-Agarose loading dye	0.025% (w/v) bromophenol blue without xylene cyanol + 30% (v/v) glycerol

2.1.10 Primers

The inventoried realtime rtPCR primers were ordered from Applied Biosystems. The catalogue numbers and the company provided names of the primers used are mentioned in the Table below.

Table 12. List of Primers used in this study

Gene symbols	**Catalogue number**	**Company provided primer name**
Gene of Interest		
Cycloxygenase 2 (COX-2)	Cat. # 4331182	Mm03294838_g1
Heme oxygenase-1 (HO-1)	Cat. # 4331182	Mm00516005_m1
NADPH quinone oxidoreductase 1 (NQO1)	Cat. # 4331182	Mm01253561_m1
Loading control		
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	Cat. # 4331182	Mm99999915_g1
28s ribosomal rna (28SrRNA)	Cat. # 4331182	Mm03682676_s1

2.1.11 Antibodies

Table 13 and 14 depict the list of primary and secondary antibodies, which were used for immunofluorescence and for Western blots.

Table 13. List of primary antibodies used in this study

Antigens	Species AB raised in	Dilution (IF)	Dilution(WB)	Supplier
Cycloxygenase -2 (COX-2)	Rabbit, polyclonal	-	1:1,000	New England Biolabs GmbH, Frankfurt Am main, Germany, Cat. no: 9331
Extracellular signal regulated kinase mitogen activated protein kinase (ERK MAPK)	Rabbit, polyclonal		1:1'000	Cell Signaling, Beverly, MA, USA, Cat. No:9102
Phosphorylated form of extracellular signal regulated kinase mitogen activated protein kinase (phosphor-ERK MAPK)	Rabbit, polyclonal		1:1'000	Cell Signaling, Beverly, MA, USA, Cat. No:9101
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), rabbit	Mouse, monoclonal		1:10,000	HyTest Ltd, Turku, Finland, Cat. no: 5G4
Heme oxygenase 1 (HO-1)	Rabbit, polyclonal	1:4,000	1:2,500	Assay Designs, Inc. Michigan, USA,Cat.no:SPA-895
Inhibitor of NF-kB-alpha (IκB-α)	Rabbit, polyclonal		1:500	Cell Signaling, Beverly, MA, USA, Cat. No:9242
Inhibitor of NF-kB-alpha (Phospho IκB-α)	Rabbit, monoclonal		1:1,000	Cell Signaling, Beverly, MA, USA, Cat. No:2859
Nuclear factor-erythroid-2 related factor 2 (Nrf2)	Rabbit, polyclonal	1:500	1:500	Santa Cruz Biotechnology Inc., Heidelberg, Germany, Cat. no:sc-13032
p38 mitogen activated protein kinase (p38 MAPK)	Rabbit, polyclonal		1:1,000	Cell Signaling, Beverly, MA, USA, Cat. No:9212
Phosphorylated form of p38 mitogen activated protein kinase (Phospho-p38 MAPK)	Rabbit, polyclonal	-	1:1,000	Cell Signaling, Beverly, MA, USA, Cat. No:9211

Table 14. List of secondary antibodies used in this study

Secondary detection system used	**Host**	**Method**	**Dilution**	**Supplier**
Anti-mouse IgG alkaline phosphatase	Goat	WB	1:20,000	Sigma, Steinheim, Germany. Cat. no: A3562
Anti-rabbit IgG alkaline phosphatase	Goat	WB	1:20,000	Sigma, Steinheim, Germany. Cat. no: A3562
Anti-rabbit-IgG Alexa Fluor 488	Donkey	IF	1:600	Molecular Probes/Invitrogen, Cat. no: A21206

2.2 Methods

2.2.1 Cell culture

RAW264.7 cells, MEF from p38$\alpha^{-/-}$ mice and MEF from Nrf2$^{-/-}$ mice were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin. All cell cultures were kept under air/CO_2 (19:1) at 100% humidity.

2.2.2 Transformation and preparation of plasmid DNA

The reaction tube containing the competent E.coli cells were removed from -80^{o} C storage and placed on ice to thaw. 1 µg of the plasmid DNA was mixed with the thawed cells by gently tapping the tube and was incubated on ice immediately for 30 min. The cells in the transformation reaction tube were subjected to a heatshock at 42 ^{o}C in a waterbath for exactly 30 s and the tube was placed thereafter quickly on ice for 2 min. 250 µl of LB medium were added to the tube and the cells were incubated at 37 ^{o}C for 1 h with shaking at 225-250rpm. Using a sterile spreader, 20-200 µl of transformation mixture was plated out on a pre-warmed LB agar plate. Plates were inverted and incubated overnight at 37 ^{o}C.

2.2.3 Mini and midi preparation of plasmid DNA

A single bacterial colony was inoculated into 3 ml of selective LB medium and was grown for 12 h with vigorous shaking. 700 µl of culture bacteria were mixed with 300 µl of 50% glycerol and stored at -80^{o}C as frozen stock. The plasmid preparation was done with a modified alkaline lysis protocol of Qiagen (Qiagen Miniprep plasmid kit). Shortly, 1 ml of the bacterial suspension was centrifuged at 13,000 rpm for 30 s. The pellet was resuspended in 150 µl of solution 1 and incubated at room temperature (RT) for 5 min after which 150 µl of solution 2

was added to the mixture and incubated further for 5 min. To this mixture 150 µl of solution 3 was added, incubated for 5 min at RT and then centrifuged for 5 min. 1 ml cold ethanol (100%) was added to the supernatant, vortexed and incubated for 10 min at RT and centrifuged for 10 min. The pellet was washed with 1 ml ethanol (70%) and centrifuged at maximum speed for 2 min. The pellet obtained was air-dried. 20-30 µl of 10mM Tris-Cl (pH-8.0) was added to the pellet and incubated for 2 min at RT.

For amplification of plasmids, plasmid preparations were performed with Qiagen plasmid Midi or Maxi kits according to the manufacturer's instructions. The concentration of plasmid DNA was calculated by measuring the absorbance at 260 nm and 280 nm.

Agarose gels (1-2%) were used for analyzing the isolated plasmid DNA. Briefly, the agarose (10 mg/ml) was melted in 1X TAE buffer using a microwave oven at 500W. Ethidium bromide was added to the solution at a dilution of 1:10,000 and then poured into a casting platform and the gel was allowed to set. DNA samples were mixed with 5 µl of 6x loading buffer before loading into the wells. The voltage of electrophoresis was set to 10 V/cm. The intercalated ethidium bromide in the DNA was visualized by placing the gel on a UV light source.

2.2.4 Transfection and luciferase assay

Cells were seeded in 6-well plates 24 h prior to transfection at a density of $5x10^5$ cells/well. The lipofection solution was prepared by adding 2 µl of Trans IT® LT-1 transfection reagent to 110 µl of DMEM serum free medium (per well) and incubated for 15min at RT. To this mixture 1 µg of the reporter plasmid was added mixed gently and further incubated for another 15min at RT. The medium in the wells were replaced by adding fresh medium (2ml/well). 100 µl of the

DNA-lipid complex was added drop by drop to each well containing cells in the presence of medium with 10% FBS. The plates were swirled gently to ensure even dispersal of the complex and the plates were cultured for 24 h before cell harvest or treated with various stimuli as indicated.

For co-transfection experiments 1 µg of expression vector plasmid was added together along with the reporter plasmid. For these experiments the amount of Trans IT transfection reagent used per well was increased to 4 µl.

2.2.4.1 Luciferase activity assay

2.2.4.1.1 Preparation of cell lysates

1 volume of the luciferase cell culture lysis reagent (CCLR) (5X) was mixed with 4 volumes of sterile water to prepare the working concentration 1X. The growth medium from the transfected culture cells was removed carefully and rinsed with 1X PBS. 100-200 µl of 1X CCLR reagent was added to each well and the plate was shaked several times to ensure complete coverage of cells with lysis buffer. Cells were scrapped from the culture dishes and transferred to an eppendorf tube. The tube was placed on ice vortexes for a short period of 15 s and then centrifuged at 13,000 g for 30 s at RT. The cell lists were used for the luciferase assay directly.

2.2.4.1.2 Luciferase assay

Prepared luciferase assay reagent was mixed well and equilibrated at RT before use. The illuminometer was first primed with luciferase assay reagent. The illuminometer was programmed to perform a 2s measurement delay followed by a 10s measurement read for luciferase enzyme activity. 50 µl of the sample was dispensed into illuminometer tubes. The reading time was adjusted to work in a linear range in the respective experiment. Relative light units of luciferase activity were normalised to mg protein values of the samples.

2.2.5 Isolation and culture of primary alveolar macrophages

Alveolar macrophages were isolated as previously described (Zhang et al. 2008). Briefly, after cervical dislocation of the mice, skin of the cervical region was cut open and the trachea exposed surgically. A canula was inserted into the trachea and with a 1 ml syringe, 0.5 ml of prewarmed DPBS saline containing 0.5mM EDTA were instillated into the trachea, withdrawn and the lavage was collected in a 5 ml conical centrifuge tube. The instillation and lavage steps were repeated 10 times and the collected lavages thereafter centrifuged for 10 min at 400g. The cells were counted and $1x10^5$ cells were plated in each well of a 24 well plates containing coverslips. After 4 h the medium was removed to get pure macrophage cultures and replaced with fresh medium.

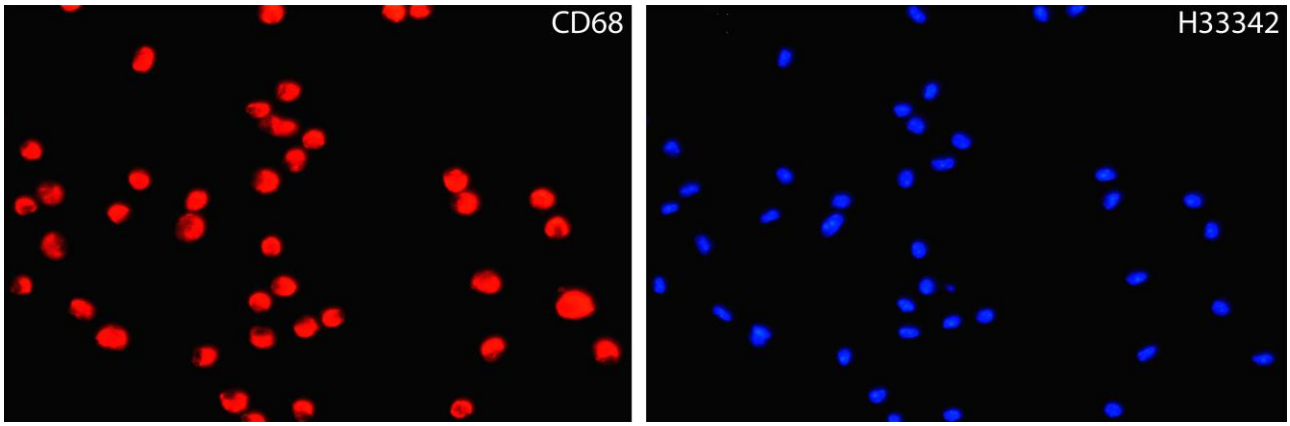


Fig6. Primary alveolar macrophages were labelled with the macrophage marker CD68 to examine the purity of the macrophage culture**.** More than 95% of cells were positive for CD68 suggesting a high purity of the alveolar macrophage preparation.

2.2.6 Indirect immunofluorescence

Cells were plated on coverslips and allowed to grow overnight. 12 h after treatment with LPS or LPS+LFMA13, the coverslips were rinsed with PBS (pH

7.4) and fixed with 4% paraformaldehyde (PFA) in PBS for 20min at RT. Thereafter, the cells were washed three times with PBS. Then the coverslips containing the cells were incubated for 10min in PBS containing 1% glycine and for an additional 10min in PBS containing 1% glycine and 0.3% Triton X-100 for permeabilization. After washing with PBS, the blocking step to block nonspecific protein binding sites was performed by incubating the cells for 30min in PBS containing 1% BSA and 0.05% Tween 20. After blocking, the coverslips were incubated with primary antibodies overnight at 4°C in a moist chamber, followed by extensive washing with PBS (3α5min) and incubation with secondary antibodies for 1h at RT. Nuclei were counterstained with Hoechst 33342 (2 µg/ml) (Vijayan et al. 2011).

2.2.7 Flowcytometric Analysis of ROS

MEFs: dihydroethidium (DHE) dye (Invitrogen) was used to detect the generation of intracellular ROS. Upon accumulation of ROS, DHE is cleaved to form the fluorescent byproduct ethidium, which was detected with a flow cytometer. Cells were first incubated in the presence or absence of SB 202190 (10 µM) for 1 h, followed by further incubation with 5 µM DHE for 20 min. After washing, the levels of ROS were determined with a FACScalibur flow cytometer. DHE-detectable (FL2-H) fluorescent signals were displayed as histograms (Naidu et al. 2009).

RAW264.7 cells: intracellular generation of ROS was detected with dichlorodihydrofluorescein diacetate (H_2-DCFDA) dye (Invitrogen). Fluorescent ROS-modified H_2DCFDA byproduct dichlorofluorescein, which is produced after oxidation and cleavage by cellular esterases, was detected with a flow cytometer. After pre-incubation with LFM-A13 for 1 h followed by addition of LPS

for another 9 h, $1x10^6$ cells were incubated at 37°C for 30 min in medium containing 15 μM H2-DCFDA. Cells were thereafter washed with PBS and the levels of ROS-oxidized DCFDA were determined with a FACSCalibur flow cytometer. In NAC experiments, cells were first treated with LPS for 9 h followed by 1h NAC treatment. DCF-detectable (FL1-H) fluorescent signals were displayed as histograms (not shown). Ratios of signals *versus* control data were calculated using mean fluorescence intensity (Vijayan et al. 2011).

2.2.8 Nitrite Assay

Griess reagent assay kit from Promega was used to detect nitrite levels in the samples. Briefly, the medium from treated and untreated cells were collected and centrifuged. 50μl of sulfinilamide solution was added to 50 μl of the sample medium in a 96 well plate and incubated for 10 min followed by addition of 50μl of N-(1-naphthyl)-ethylenediamine dihydrochloride. The plate was further incubated for 10 min and the absorbance was read at 550nm. The nitrite level in each sample was calculated from a standard generated with sodium nitrite (0-300 μM) (Vijayan et al. 2011).

2.2.9 Methylthiazole tetrazolium (MTT) assay

The effect of heme treatment on the viability of RAW264.7 macrophages was evaluated by the MTT assay (Sigma). Briefly, after pretreatment with LFM-A13 for 1 h cells were incubated with heme for additional 14 h. Cells were then washed, incubated with 0.5 mg/ml MTT in Dulbecco's modified Eagle's medium (without phenol red) for 2 h. The MTT-transformed formazan crystals formed in viable metabolic cells were dissolved in dimethyl sulfoxide and absorbance read at 570 nm using a spectrophotometer. The percentage of cell viability was

calculated by the following formula: A_{570} of treated cell/A_{570} of non-treated cells x 100 (Vijayan et al. 2011).

2.2.10 RNA expression analysis by realtime-RT-PCR

2.2.10.1 RNA isolation

Total RNA isolation from cells was performed using the RNeasy Mini Kit from Qiagen. Homogenization of cells in RLT buffer was performed by passing the lysate through a 22G needle attached to a 1 ml syringe several times. RNA extraction was carried out with the Qiagen RNeasy Mini kit according to the manufacturer's protocol. The isolated RNA was redissolved in RNase free water and stored at -80°C till used. The quantity of the isolated RNA was measured by optical density measurements using a Bio-Rad spectrophotometer. The integrity of the RNA was analyzed by visualization of ethidium bromide-stained intact 28S, 18S and 5S ribosomal RNA bands in 1% denaturing formaldehyde agarose gels.

2.2.10.2 DNase I digestion

Before the samples were used for reverse transcription, a DNase I digestion step was performed to remove residual genomic DNA in the RNA preparation. To the prepared DNase digestion mixture, 1 µg of the RNA sample was added and incubated for 15min at RT after which the DNase I was inactivated by the addition of 1 µl of 25mM EDTA followed by heating for 10min at 65°C.

DNase digestion of RNA samples

RNA sample	1 µg
10X DNase I reaction buffer	1 µl
DNase I, amplification grade, 1U/µl	1 µl
DEPC-treated water	to 10 µl

2.2.10.3 Reverse transcription

0.5 µg oligo (dT) 12-18 primers and 10mM dNTP mixtures were used along with total RNA (0.5 - 1 µg) for reverse-transcription into cDNA as described below.

Oligo(dT)12-18 (500 µg/ml)	1 µl
RNA (1 µg)	~ 10 µl
dNTP mixture (10mM each)	1 µl
Sterile distilled water	to 12 µl

The denaturation step was done by heating the RNA mixture to 65°C for 5min followed by immediate incubation on ice. Thereafter, 4 µl of 5X first strand buffer, 2 µl of 0.1M DTT and 1 µl of RNaseOUT™ (40U/µl) were added to the reaction mixture.The reaction mixture was mixed thoroughly and incubated at 42°C for 2min. 1 µl of SuperScript™ II Reverse Transcriptase (200U) was added to the mixture and the reaction tubes were incubated at 42°C for 50min for reverse transcription. Finally, the samples were heated at 70°C for 15min for inactivation of the reaction.

Amplification was performed using TaqMan Gene Expression Master Mix on a StepOnePlus™ Real-Time PCR System (Applied Biosystems) according to the manufacturer's instructions. Thermal cycling was performed at 95°C for 10 min followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. The constitutively expressed gene, 28srRNA was used as a control for normalization of cDNA levels. The *ΔΔ*CT method was used to semiquantify mRNA levels, according to the manufacturer's protocol.

2.2.11 Western Blot analysis

2.2.11.1 Isolation of whole cell lysates

After washing cell cultures twice with 1XPBS, 150 µl of cell lysis buffer was added to each well. Cells were scraped thoroughly using a rubber policeman and transferred to eppendorff tubes. The tubes were kept on ice for 30 min with vortexing at intervals. The tubes were then centrifuged for 30 min at maximum speed for 30 min at 4^{o}c after which supernatant was collected and stored at -20 ^{o}c.

2.2.11.2 Preparation of Western blots

Concentration of the protein samples were determined by the Bradford method using BSA as a standard (Bradford 1976). The protein samples (30-50 µg) were separated on 12% SDS polyacrylamide gels and then transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were incubated at RT with shaking in Tris-buffered saline (TBS) containing 10% nonfat milk powder and 0.05% Tween-20 for 1h to block nonspecific protein-binding sites. Thereafter, the membranes were incubated with the primary antibody overnight at 4°C or for 1h at RT. Following the primary antibody incubation, the membranes were washed 3 times and then incubated with alkaline phosphatase-conjugated secondary antibody for 1h at RT. Immun-Star^{TM} AP substrate from Bio-Rad (Munchen, Germany) was used to detect the alkaline phosphatase activity and the blots were exposed to Kodak Biomax MR Films. Blots were scanned using an AGFA scanner. All Western blot experiments were performed and analyzed at least three times.

2.2.12 Statistical Analysis

Each result is a representative of at least three experiments. Student's paired t test was done to compare the control cells with the treated cells and the data is represented as average +/- standard mean error (SEM). P values of 0.05 or less were considered significant.

3 RESULTS

The results of this thesis are divided into two parts (Chapter I and Chapter II) that were published in the Journal of Immunology and the pubmed Ids of the following are (I) PMID: 21677132 and (II) PMID: 19454702

(I) **Vijayan, V.**, E. Baumgart-Vogt, S. Naidu, G. Qian and S. Immenschuh **(2011)**. "Bruton's tyrosine kinase is required for TLR-dependent heme oxygenase-1 gene activation via Nrf2 in macrophages." J Immunol **187**(2): 817-27.

(II) Naidu, S., **V. Vijayan**, S. Santoso, T. Kietzmann and S. Immenschuh **(2009)**. "Inhibition and genetic deficiency of p38 MAPK up-regulates heme oxygenase-1 gene expression via Nrf2." J Immunol **182**(11): 7048-57

PART-I: Btk-mediated regulation of the HO-1 gene

3.1 LPS-dependent HO-1 gene induction is reduced by the pharmacological Btk inhibitor LFM-A13

LPS, a proinflammatory mediator induce signaling in macrophages by binding to TLR4. It has been shown earlier that LPS upregulates HO-1 gene expression in mononuclear phagocytes such as monocytes and macrophages (Camhi et al. 1995; Immenschuh et al. 1999; Horwood et al. 2003; Wijayanti et al. 2004; Rushworth et al. 2005; Schmidt et al. 2006). Herein, we determined the effect of LFM-A13, an inhibitor of Btk which belongs to the tec family of kinases, on LPS-dependent HO-1 induction in the mouse macrophage cell line RAW264.7. Btk has previously been shown to differentially regulate gene expression in myeloid/macrophage cells (for a review see (Koprulu and Ellmeier 2009). RAW264.7 cells are known to express Btk (Jongstra-Bilen et al. 2008) and pretreatment with LFM-A13 blocked the LPS-induced protein expression of HO-1. LPS-dependent COX-2 induction which is another early-induced proinflammatory gene in macrophages was not significantly affected by LFM-A13 (Fig. 7A) treatment. Real-time RT-PCR studies revealed that pretreatment of RAW264.7 cells with LFM-A13 markedly downregulated the LPS-induced HO-1 mRNA expression (Fig. 7B). In a previous study LFM-A13 was shown to inhibit JAK2

which belongs to the family of Janus kinases. To rule out the possibility that, the observed effect of LFM-A13 on LPS-dependent HO-1 induction is due to JAK2 inhibition, we used a specific JAK2 inhibitor AG490 in RAW264.7 cells. However, addition of AG490 already, led to a significant upregulation of HO-1 gene expression in dose-dependent manner (Fig. 7C).

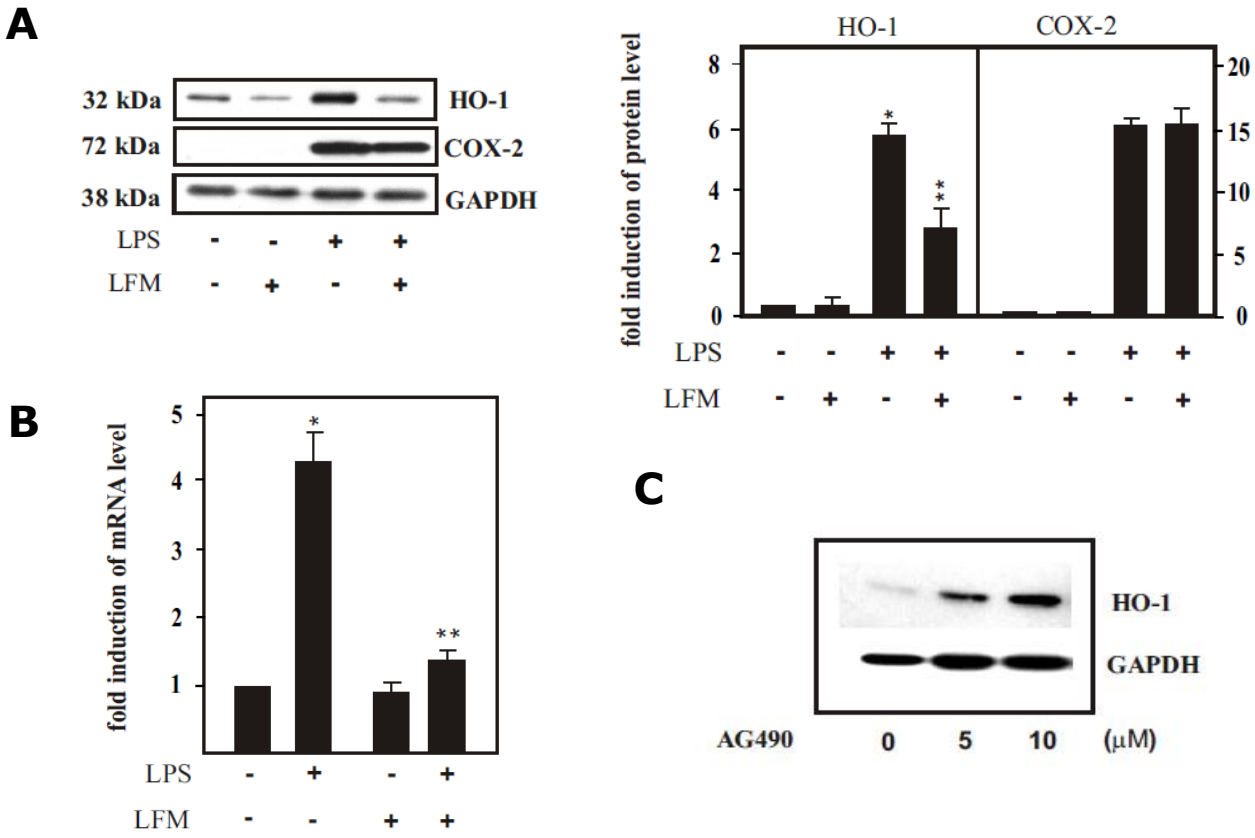


Figure 7: Effect of LFM-A13 on LPS-dependent induction of HO-1 gene expression in cell cultures of RAW264.7 cells and mouse alveolar macrophages

RAW264.7 cells and mouse alveolar macrophages were cultured as described under *Materials and Methods*. A: RAW264.7 cells were pretreated with LFM-A13 (100 μM) for 1 h, after which incubation was continued with or without LPS (1 μg/ml) for another 18 h, as indicated. Total protein (30 μg) was subjected to Western blot analysis and sequentially probed with Antibodies against HO-1, COX-2 and GAPDH. Autoradiographic signals were visualized and quantified as described under *Materials and Methods*. Values ± SEM represent the fold induction of HO-1 normalized to GAPDH from three independent experiments. B: RAW264.7 cells were treated with LFM-A13 (100 μM) for 1 h, after which treatment was continued with or without LPS (1 μg/ml) for another 12 h, as indicated. One μg of RNA was used for real-time RT-PCR analysis with HO-1 and GAPDH primers as described in *Materials and Methods.* C: RAW264.7 cells were treated with AG490 at the indicated concentrations for 12 h (result not shown in Vijayan et al, 2011). Total protein (30 μg) was subjected to Western blot analysis and sequentially probed with antibodies against HO-1 and GAPDH. Statistics: Student's *t-test* for paired values: *: significant differences treatment *versus* control; **: LPS *versus* LPS+LFM-A13, $p \leq 0.05$. [published as Figure number 1 A and 1 B in Vijayan et al, 2011]

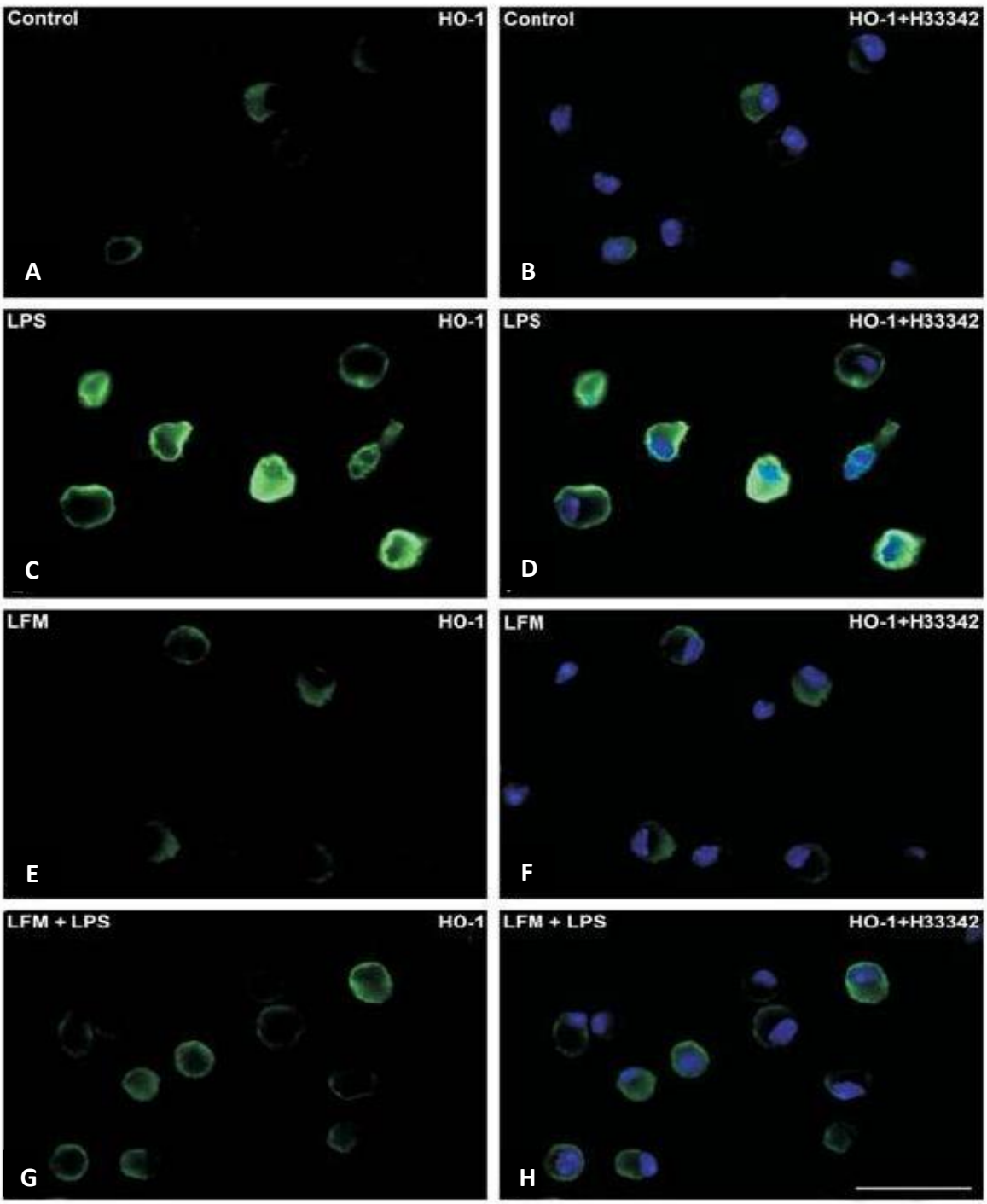


Figure 8: Effect of LFM-A13 on LPS-mediated induction of HO-1 gene expression in cell cultures of mouse alveolar macrophages.

A-H: mouse alveolar macrophages were pretreated with LFM-A13 (100 μM) for 1 h, after which treatment was continued with or without LPS (1 μg/ml) for another 12 h, as indicated. Cells were then subjected to IF staining using a HO-1 antibody at a dilution of 1:4,000 and nuclei were visualized with Hoechst 33342 dye as described in *Materials and Methods*. LFM: LFM-A13; H33342: Hoechst 33342. Bar = 560 μm. [Published as figure number 1 C-J in Vijayan et al, 2011]

In the following, we investigated the effect of LFM-A13 on LPS-mediated HO-1 induction in cell cultures of primary mouse alveolar macrophages. As determined by immunofluorescence (IF) studies, LPS stimulation of alveolar macrophages caused a significant increase of HO-1 protein expression. The upregulation of HO-1 by LPS was attenuated when cells were pretreated with LFM-A13 (Fig. 8A-H). In conclusion, the data indicate that specific inhibition of Btk with LFM-A13 markedly reduces LPS-dependent induction of HO-1

3.2 Induction of HO-1 upregulation is blocked in alveolar macrophages from Btk$^{-/-}$ mice

To further investigate the role of Btk, we compared the effect of LPS on HO-1 induction in cell cultures of alveolar macrophages from Btk$^{-/-}$ mice and thier wildtype littermates. In contrast to a pronounced induction of HO-1 gene expression in LPS-treated macrophages from wild type mice, only a weak staining for HO-1 protein was observed in cells from Btk knockout mice in response to LPS (Fig. 9A-H). In addition, studies with real time rtPCR studies showed a marked upregulation of HO-1 mRNA levels upon LPS stimulation in cell cultures of alveolar macrophages from wild type mice, but not in those from Btk deficient mice (Fig. 10). The data confirm that Btk plays a major regulatory role in LPS-dependent induction of HO-1 gene expression in primary murine macrophages.

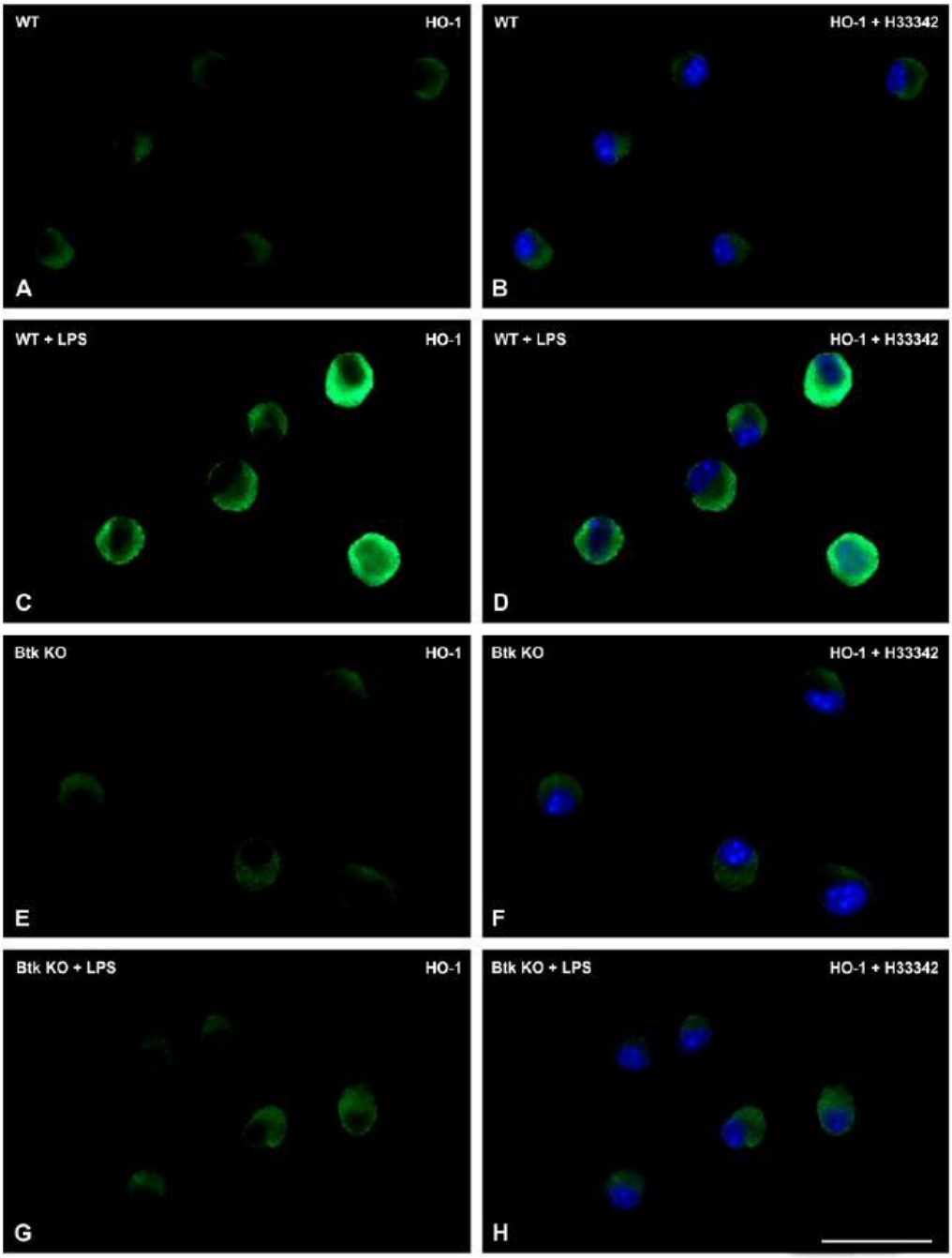


Figure 9: LPS-dependent induction of HO-1 is blocked in alveolar macrophages from Btk$^{-/-}$ mice.

A-H: cell cultures of alveolar macrophages from Btk$^{-/-}$ mice and from their wild-type littermates were incubated with or without LPS (1 μg/ml) for 12 h. Cells were then subjected to IF staining with a HO-1 antibody at a dilution of 1:4,000 and nuclei were visualized with Hoechst 33342 dye. Btk ko: Btk knockout; H33342: Hoechst 33342; WT, wild-type. Bar = 560 μm. [Published as figure number 2 A-H in Vijayan et al, 2011]

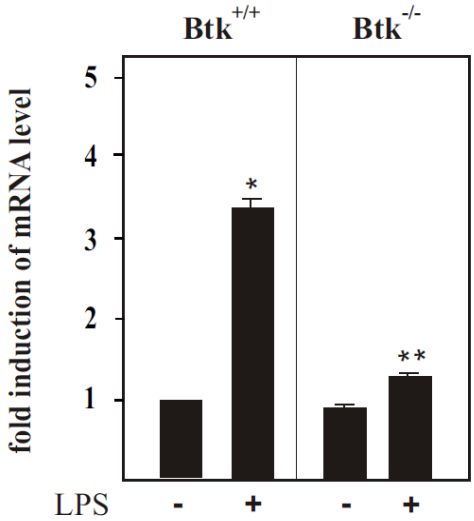


Figure 10: LPS-dependent induction of HO-1 is blocked in alveolar macrophages from Btk$^{-/-}$ mice.

Mouse alveolar macrophages were treated with LPS (1 µg/ml) for 12 h and the RNA was isolated for real-time RT-PCR analysis using the primers for HO-1 and GAPDH as described in *Materials and Methods*. Statistics: Student's *t-test* for paired values: *: significant differences treatment *versus* control, **: LPS treatment in Btk$^{+/+}$ *versus* LPS treatment in Btk$^{-/-}$ $p \leq 0.05$. [Published as figure number 2I in Vijayan et al, 2011]

3.3 Btk-dependent induction of HO-1 by LPS is mediated via a transcriptional mechanism

HO-1 expression by most stimuli is mainly regulated at the transcriptional level (Choi and Alam 1996; Paine et al. 2010). Luciferase gene reporter studies were performed to further elucidate the mechanisms by which Btk mediate LPS-dependent induction of HO-1. RAW264.7 cells were transiently transfected with a reporter gene construct containing the proximal 4,045 bp of the mouse HO-1 gene promoter (pHO-4045-luc) and then treated with LFM-A13 prior to LPS exposure. Luciferase activity of pHO-4045-luc was induced in response to LPS and pretreatment with LFM-A13 significantly reduced the luciferase activity of the pHO-4045-luc (Fig. 11A). By contrast, LPS-dependent upregulation of the luciferase activity of pTNF-585-luc, a plasmid containing the proximal 585 bp of the TNF-α gene promoter region, was not affected by LFM-A13 treatment (Fig. 11A).

A major target of Btk-mediated TLR4 signaling is the transcription factor NF-κB (Jefferies and O'Neill 2004). Since NF-κB has previously been shown to regulate HO-1 gene expression (Wijayanti et al. 2004; Naidu et al. 2008), we compared the effect of LFM-A13 on the classical pathway of NF-κB activation, that occurs via the phosphorylation of the NF-κB inhibitory protein IκB subunit, followed by the degradation of IκB. LPS treatment led to phosphorylation and degradation of IκB in RAW264.7 cells and pretreatment with LFM-A13 did not affect the phosphorylation and degradation of IκB (Fig. 11B).

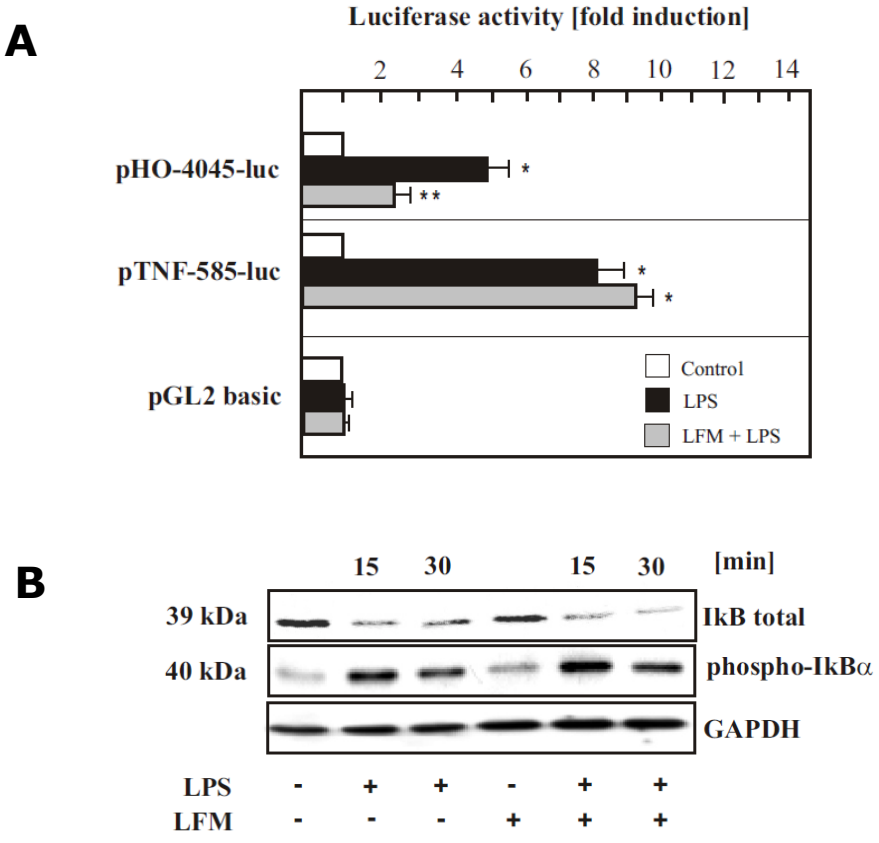


Figure 11: Btk is required for LPS-dependent induction of mouse HO-1 promoter activity.

A: RAW264.7 cells were transfected with the indicated luciferase reporter gene vectors as described in *Materials and Methods*. 24 h after transfection, cells were treated with LFM-A13 (100 µM) for 1 h before incubation was continued with or without LPS (1 µg/ml) for 18 h. Cell extracts were collected and assayed for luciferase activity. Statistics: Student's *t-test* for paired values: *: significant differences treatment *versus* control; **: LPS *versus* LPS+LFM-A13, $p \leq 0.05$. B: RAW264.7 cells were treated with or without LFM-A13 (100 µM) for 1 h followed by treatment with

LPS (1 µg/ml) for the indicated times. Western blot analysis was performed with antibodies to phospho-IκB-alpha, total IκB and GAPDH as described in Fig. 7. [Published as figure number 3A and 3B in Vijayan et al, 2011]

In the following, we cotransfected the pHO-4045-luc construct and an expression vector with the dominant negative Btk mutant BtkDN-K430R into RAW264.7 cells (Doyle et al. 2005). As shown in Fig. 12, overexpression of dominant negative Btk downregulated the LPS-induced luciferase activity of the pHO-4045-luc construct in comparison to cells, which have been co-transfected with an empty control vector (Fig. 12). A similar inhibitory effect was also observed in cotransfection studies with the dominant-negative mutant BtkDN-R28C (data not shown). Taken together, the data indicate that Btk regulates HO-1 induction via a transcriptional mechanism that is independent of NF-κB.

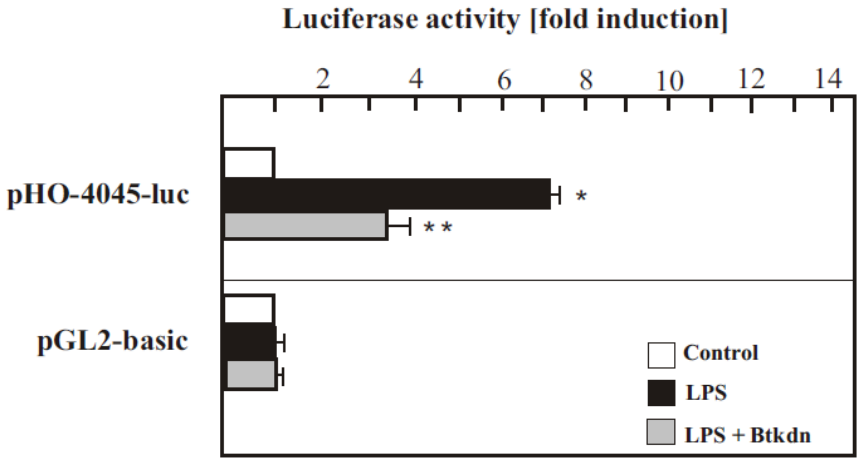


Figure 12: The dominant negative form of Btk blocked LPS-dependent induction of the mouse HO-1 promoter activity.

RAW264.7 cells were co-transfected with the mouse HO-1 luciferase reporter vector pHO-4045-luc or pGL2-basic together with the expression vector BtkDN-K430R or empty control vector (pcDNA3.1), as indicated. 24 h after transfection cells were treated with or without LPS (1 µg/ml) for another 18 h. Cell extracts were collected and assayed for luciferase activity. Statistics: Student's *t-test* for paired values: *: significant differences treatment *versus* control; **: LPS *versus* LPS+LFM-A13, $p \leq 0.05$. LFM: LFM-A13, Btkdn: Btk-DNK430R. [Published as figure number 3C in Vijayan et al, 2011]

3.4 Nrf2 plays a role in Btk-mediated induction of HO-1 by LPS

Nrf2, is a key transcriptional regulator of the antioxidant response and mediates the inducible expression of HO-1 and also that of other phase II detoxification and antioxidant genes such as NADPH quinone oxidoreducatse (NQO1) and glutathione S-transferase (GST) (Kobayashi and Yamamoto 2005; Kensler et al. 2007). Nrf2, once activated, translocates to the nucleus and binds to the promoter of target genes. Therefore, we performed immunofluorescence studies to monitor Nrf2 translocation. As shown in Fig. 13, upon treatment with LPS, the immunofluorescence signal in the nuclear areas markedly increased, indicating significant translocation of Nrf2 from the cytoplasm into the nucleus of RAW264.7 cells (Fig. 13A-D). However, pretreatment of RAW264.7 cell with LFM-A13 strongly inhibited the LPS-induced nuclear accumulation of Nrf2 (Fig. 13E-H).

To examine the functional relevance of the nuclear translocation of Nrf2, we also performed luciferase gene reporter assays with a reporter vector containing the antioxidant response element (ARE) (pARE-luc), which is the target sequence of Nrf2. Treatment with LPS caused a marked increase in the luciferase activity of this construct and pretreatment with LFM-A13 significantly blocked this induction (Fig. 14A). This impaired activation of Nrf2 might not only be relevant to HO-1, because LFM-A13 also reduced the LPS-induced mRNA levels of the Nrf2 target gene NADPH quinone oxidoreductase (NQO1) (Fig. 14B). Taken together, these results indicate that activation of Nrf2 is involved in Btk-dependent upregulation of HO-1 by LPS and this Btk-Nrf2-mediated signaling pathway might also regulate other LPS-induced antioxidant genes.

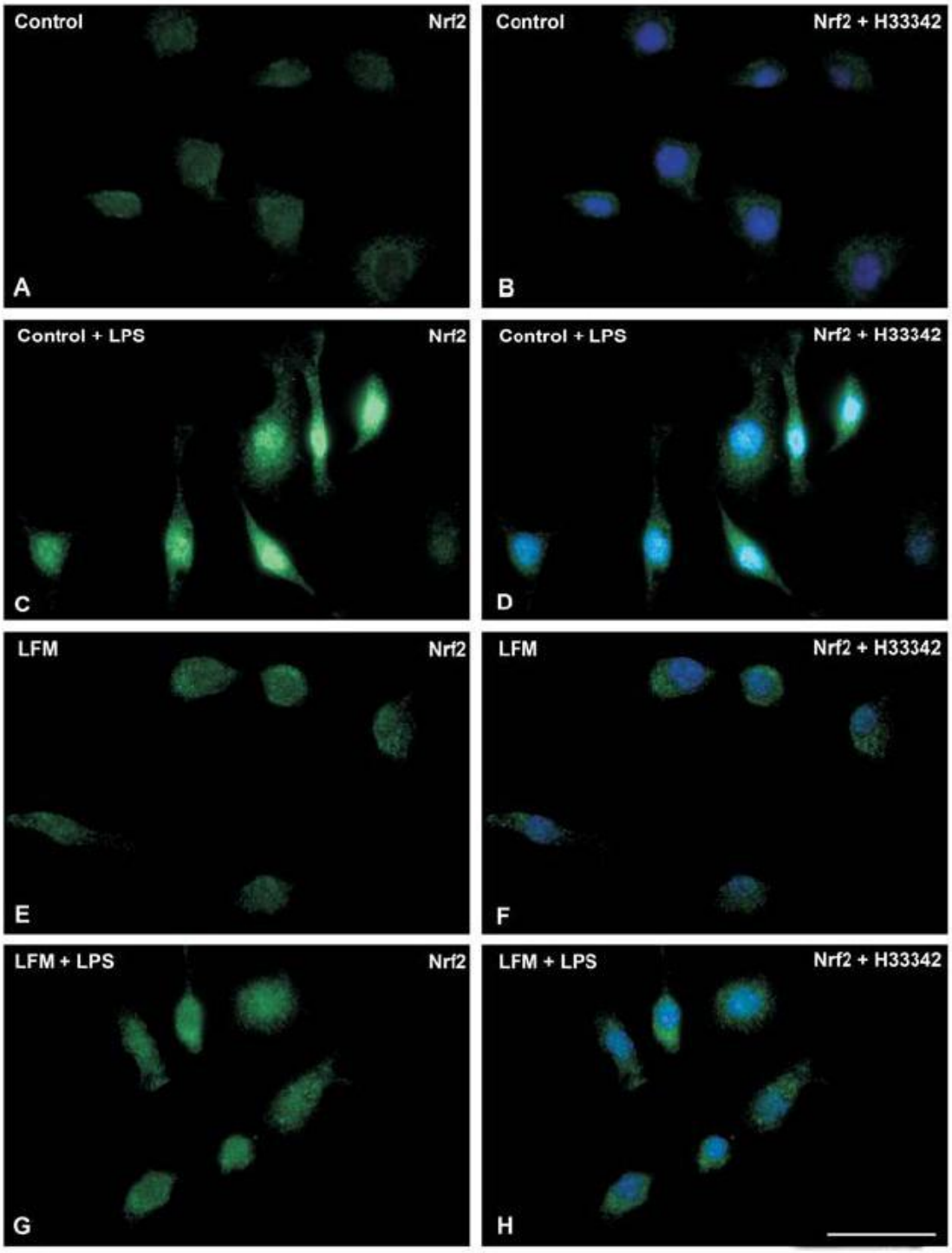


Figure 13: Nuclear translocation of Nrf2 in response to LPS is downregulated by LFM-A13.

A-H: RAW264.7 cells were pretreated with LFM-A13 (100 µM) for 1 h, after which incubation was continued with or without LPS (1 µg/ml) for another 9 h, as indicated. Cells were then subjected to immunofluorescence staining using an Nrf2 antibody at a dilution of 1:500, and nuclei were visualized with Hoechst 33342 dye as described in *Materials and Methods*. LFM: LFM-A13; H33342: Hoechst 33342. Bar = 560 µm. [Published as figure number 4 A-H in Vijayan et al, 2011]

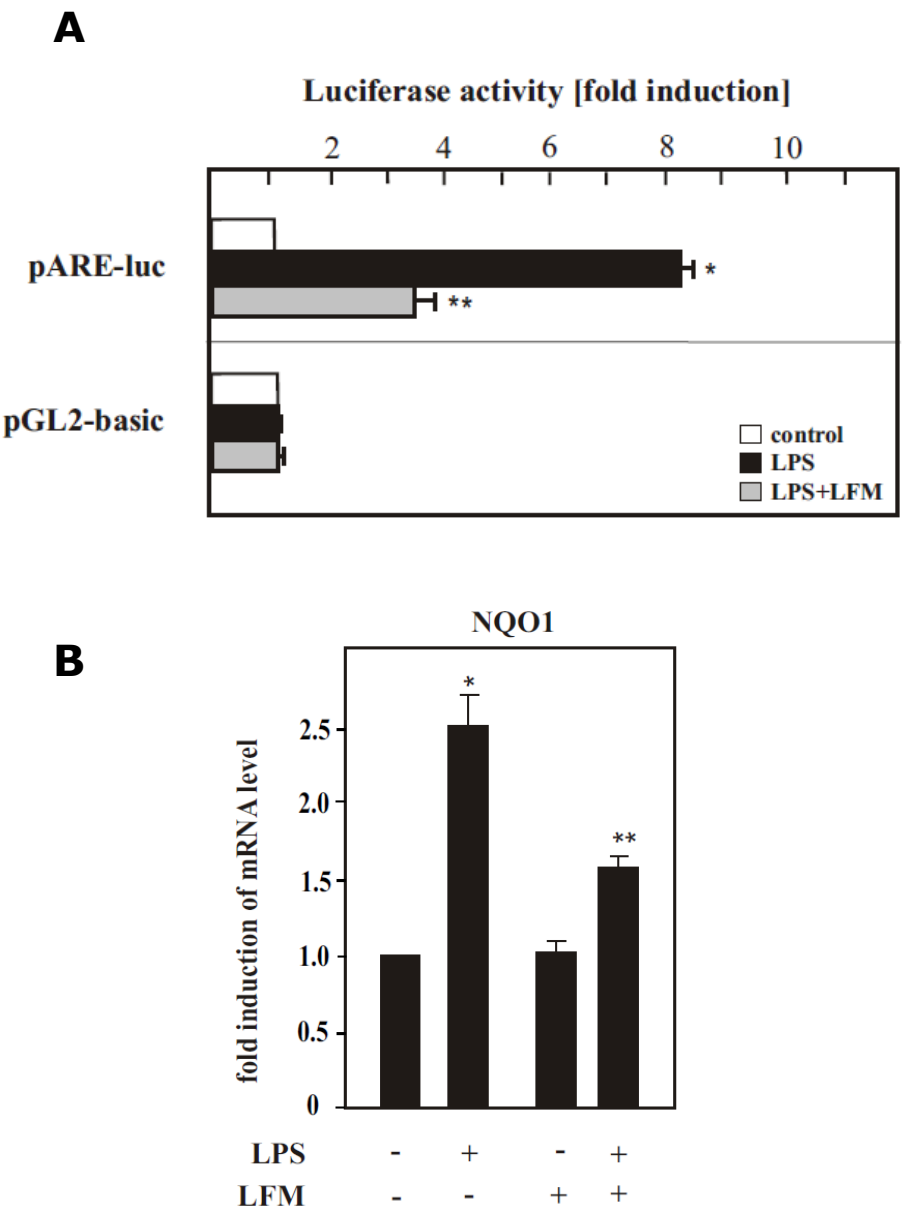


Figure 14: LPS-dependent induction of an ARE-regulated reporter gene construct is inhibited by LFM-A13.

A: RAW264.7 cells were transfected with the indicated luciferase reporter gene vectors as described in *Materials and Methods*. 24 h after transfection, cells were pretreated with LFM-A13 (100 µM) for 1 h, after which incubation was continued with or without LPS (1 µg/ml) for another 18 h, as indicated. Cell extracts were collected and assayed for luciferase activity. B: RAW264.7 cells were pretreated with LFM- A13 (100 µM) for 1 h, after which treatment was continued with or without LPS (1 µg/ml) for another 12 h, as indicated. One µg of RNA was used for real-time RT-PCR analysis with NQO1 and GAPDH primers as described in *Materials and Methods*. Statistics: Student's *t-test* for paired values: *: significant differences treatment *versus* control; **: LPS *versus* LPS+LFM-A13, $p \leq 0.05$. LFM: LFM-A13. [Published as figure number 5A and 5B in Vijayan et al, 2011]

3.5 LPS-dependent production of ROS is involved in Btk-mediated upregulation of HO-1 in macrophages

The lack of Btk has previously been shown to cause an attenuated production of ROS and NO in immunostimulated mononuclear phagocytes from Btk-deficient mice (Mangla et al. 2004). Since Nrf2-dependent induction of HO-1 is known to be regulated by prooxidant cellular conditions, we investigated the possibility that cellular levels of ROS might be affected by the activation of Btk. To this end we determined cellular levels of ROS in response to LPS in RAW264.7 cells with and without pharmacological inhibition of Btk by LFM-A13. As determined with a flow cytometry-based assay, LPS upregulated the production of ROS and pretreatment with LFM-A13 blocked the LPS-induced production of ROS and addition of the anti-oxidant N-Acetyl cysteine (NAC) attenuated the production of LPS-induced ROS (Fig. 15A). We also examined the LPS-induced levels of NO in RAW264.7 cells after pre-treatment with the Btk inhibitor LFM-A13 by measuring the amount of nitrite levels in the cell culture media. As expected, LPS markedly induced the amount of nitrite in the supernatant, which was blocked by pretreatment with LFM-A13. Addition of the specific iNOS inhibitor 1,400W abolished the LPS-induced production of NO (Fig. 15B). Moreover, LFM-A13 also blocked the LPS-induced gene expression of iNOS (Fig. 15C). Taken together these results indicate that inhibition of Btk impairs the LPS-induced production of ROS and that of NO in RAW264.7 cells. Finally, to evaluate the role of ROS and NO in LPS-induced activation of Nrf2, RAW264.7 cells were transfected with pARE-luc and were pretreated with NAC or 1,400W before exposure to LPS. LPS-induced luciferase activity of pARE-luc was markedly reduced in these cells by (NAC), but not by pretreatment with 1400W (Fig. 15D). Furthermore, LPS-dependent induction of HO-1 was blocked when the cells were pretreated with

NAC. In summary, Btk-dependent HO-1 activation via Nrf2 is mediated via the production of ROS in LPS-stimulated macrophages.

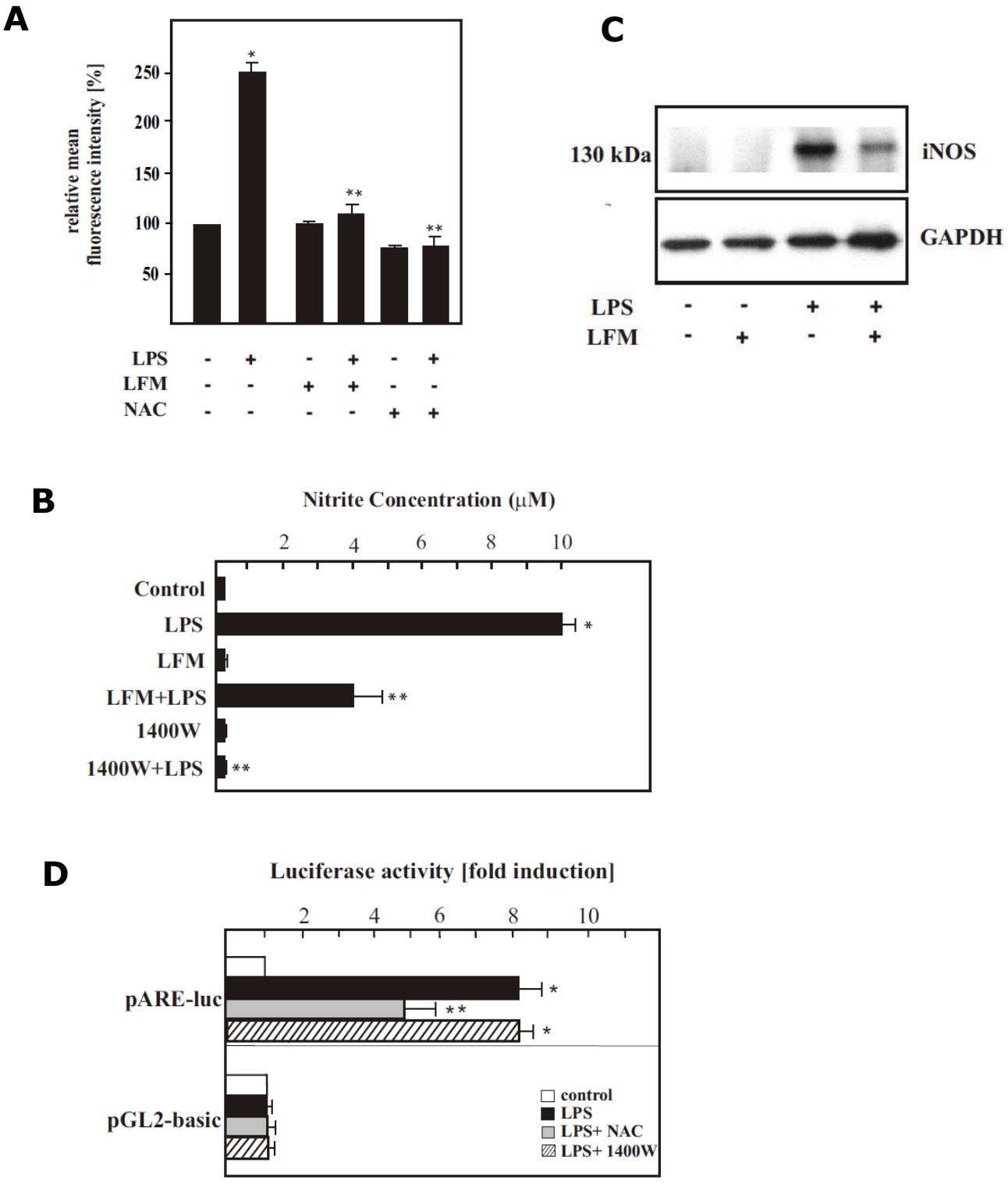


Figure 15: Role of ROS in Btk-mediated induction of HO-1 by LPS.

A: RAW264.7 cells were treated for 9 h with LFM-A13 (100 μM) and LPS (1 μg/ml) alone or in combination, as indicated. After 9 h of incubation NAC (25 mM) was added and treatment was continued for 1 h, as indicated. Cells were washed, incubated with H2DCFDA dye for 30 min and

analyzed by flow cytometry as described in *Materials and Methods*. Numbers indicated represent mean fluorescence intensity relative to control cells from at least three independent experiments SEM. Statistics: Student's *t-test* for paired values: *: significant differences treatment *versus* control; **: LPS *versus* LPS+LFM-A13; ***: LPS *versus* LPS+NAC, $p \leq 0.05$. B: RAW264.7 cells were pretreated with or without LFM-A13 (100 µM) for 1 h or 1400W (500 µM) for 30 min, after which incubation was continued with LPS (1 µg/ml) for 18 h, as indicated. The supernatant was collected and assayed for nitrite as described in *Materials and Methods*. Statistics: Student's *t-test* for paired values: *: significant differences treatment *versus* control; **: LPS *versus* LPS+LFM-A13; ***: LPS *versus* LPS+1400W, $p \leq 0.05$. C: RAW264.7 cells were pretreated with LFM-A13 (100 µM) for 1 h followed by treatment with LPS (1 µg/ml) for another 12 h. Total protein (30 µg) was subjected to Western blot analysis and was sequentially probed with antibodies against iNOS and GAPDH. D: RAW264.7 cells were transfected with the indicated luciferase reporter gene vectors as described before. 24 h after transfection, cells were pretreated with NAC (25 mM) or 1400W (500 µM) for 30 min followed by incubation with or without LPS (1 µg/ml) for another 18 h. Cell extracts were collected and assayed for luciferase activity. Statistics: Student's *t-test* for paired values: *: significant differences treatment *versus* control; **: LPS *versus* LPS+NAC, $p \leq 0.05$. LFM: LFM-A13. [Published as figure number 6A-D in Vijayan et al, 2011]

3.6 Btk mediates HO-1 induction in macrophages upon stimulation with various TLR ligands

Btk has previously been shown to be involved in signaling by other TLRs, such as TLR3, TLR7 and TLR9 in myeloid cells (Doyle et al. 2007; Taneichi et al. 2008). As shown in Fig. 16, treatment with both TLR7 and TLR9 ligands (R837 and ODN2395) upregulated HO-1 expression. However, pretreatment with LFM-A13 largely reduced this induction (Fig. 16). Next, we also examined whether TLR7 and TLR9 ligands (R837 and ODN2395) would activate pARE-luc construct in transiently transfected RAW264.7 cells. Both TLR7 and TLR9 ligands up-regulated reporter gene activity of pARE-luc, but the level of induction was lower as compared to TLR4 activation by LPS (Fig. 17A). Similar to the regulation of HO-1 protein, pretreatment with LFM-A13 reduced the induction of pARE-luc activity in response to TLR7 and TLR9 ligands, respectively.

In the following, we also investigated whether gene expression of HO-1 by other TLRs such as TLR2, TLR3, TLR5 and TLR6 is also mediated via Btk. RAW264.7 cells were treated with the TLR-ligands LTA (TLR2/6), polyI:C (TLR3) and flagellin (TLR5) in the presence or absence of treatment with LFM-A13 and HO-1 expression was determined by real-time RT-PCR. As shown in Figure 17B, only the TLR2 ligand LTA upregulated HO-1 mRNA expression and the level of

induction was similar to that of treatment with LPS. Pretreatment with LFM-A13 completely blocked this induction. By contrast, both polyI:C and flagellin failed to induce HO-1 gene expression (Fig. 17B). In summary, our findings suggest that HO-1 upregulation by activation of TLRs 2, 4, 6, 7 and 9 is mediated via Btk in macrophages.

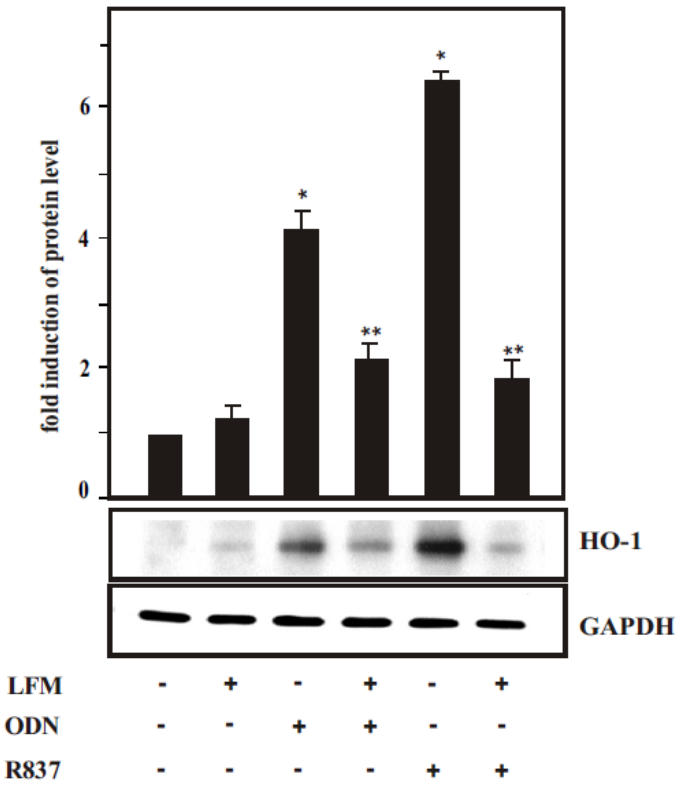


Figure 16: LFM-A13 inhibits HO-1 induction RAW264.7 cells that are stimulated by different TLR ligands.

RAW 264.7 cells were pretreated with LFM-A13 (100 µM) for 1 h and incubation was continued with or without R848 (3 µg/ml) or ODN2395 (3 µg/ml) for another 18 h, as indicated. Total protein (30 µg) was subjected to Western blot analysis and was sequentially probed with antibodies against HO-1 and GAPDH. Autoradiographic signals were visualized and quantified as described in *Materials and Methods.* Statistics, Student's *t-test* for paired values: *: significant differences treatment *versus* control; **: LPS/R848/ODN2395 *versus* LPS/R848/ODN2395+LFM-A13, $p \leq 0.05$. LFM: LFM-A13; ODN: ODN 2395. [Published as figure number 7A in Vijayan et al, 2011]

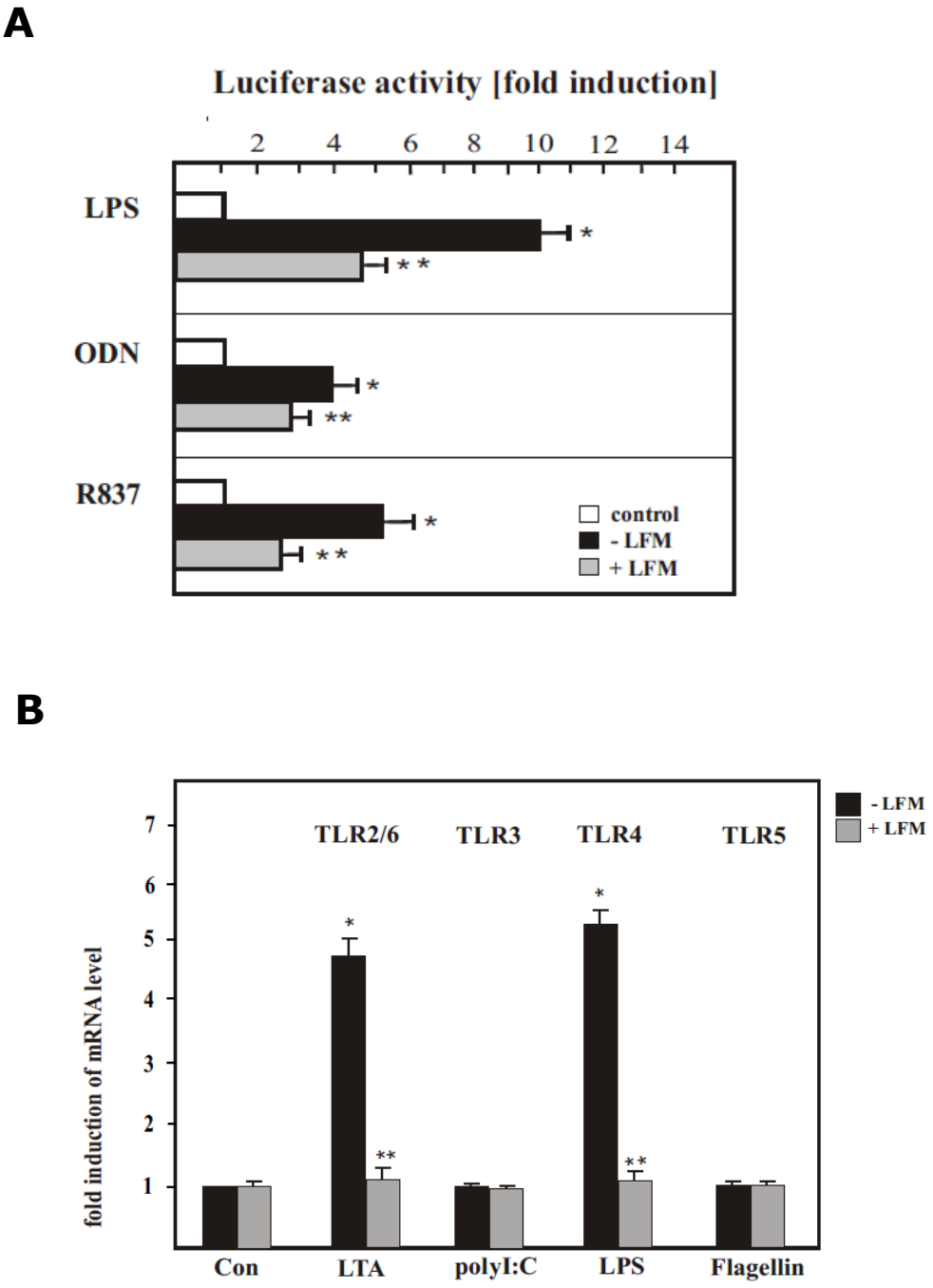


Figure 17: LFM-A13 inhibits HO-1 induction RAW264.7 cells that are stimulated by various TLR ligands.

A: RAW 264.7 cells were transfected with the luciferase reporter vector p-ARE-luc as described in *Materials and Methods*. 24 h after transfection, cells were treated with (grey bars) or without (black bars) LFM-A13 (100 µM) for 1 h followed by incubation with LPS (1 µg/ml), R848 (3 µg/ml) or ODN2395 (3 µg/ml) for another 18 h, as indicated. Cell extracts were collected and assayed for luciferase activity. Statistics, Student's *t-test* for paired values: *: significant differences treatment *versus* control; **: LPS/R848/ODN2395 *versus* LPS/R848/ODN2395+LFM-A13, $p \leq 0.05$. LFM: LFM-A13; ODN: ODN 2395. B: RAW264.7 cells were treated with LFM-A13 (100 µM) for 1 h, after which treatment was continued with or without LPS (1 µg/ml), LTA (5 µg/ml), flagellin (10 nM) or poly I:C (25 µg/ml) for another 12 h, as indicated. One µg of RNA was used for real-time RT-PCR analysis as described in *Materials and Methods*. Statistics, Student's *t-test* for paired values: *: significant differences treatment *versus* control; **: LPS/ LTA/ poly I:C/ flagellin *versus* LPS/ LTA/ poly I:C/ flagellin +LFM-A13, $p \leq 0.05$. LFM: LFM-A13. [Published as figure number 7B and 7C in Vijayan et al, 2011]

3.7 Increased sensitivity to heme-induced toxicity after treatment with LFM-A13

Free heme has been shown to be a TLR4 ligand and to play an important role in sepsis-induced inflammatory damage and induction of HO-1 provides protection against heme-mediated toxicity. Hence, we chose to examine the protective effects of Btk mediated HO-1 regulation against heme in our cell culture model. RAW264.7 cells were treated with high concentration of heme in the presence or absence of LFM-A13 and cytotoxicity was measured with an MTT assay. Treatment with high concentration of heme reduced the viability of cells by 30%. Interestingly cells pretreated with LFM-A13 were drastically sensitive to heme treatment with almost 75% increase in the number of dead cells in comparison to the control untreated cells (Fig. 18). The data indicate that specific inhibition of Btk in macrophages renders these cells highly sensitive to heme-induced oxidative stress.

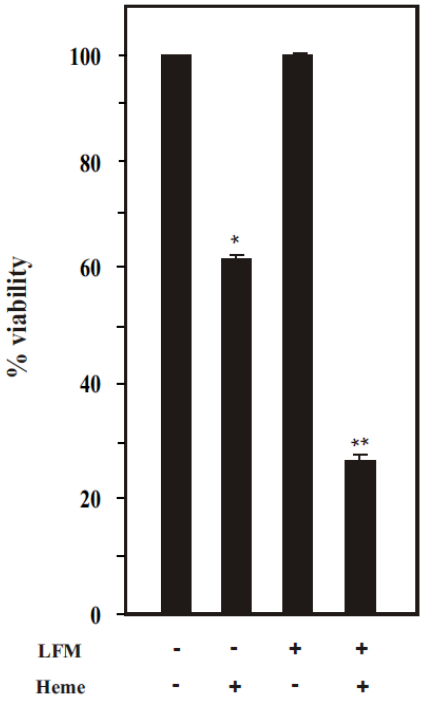


Figure 18: Increased sensitivity to heme-dependent toxicity in LFM-A13-treated RAW264.7 cells

RAW 264.7 cells were pretreated with LFM-A13 (100 μM) for 1 h and incubation was continued with or without heme (30μM) for another 16 h, as indicated. Samples were subjected to MTT assay as described in *Materials and Methods*. Statistics: Student's *t-test* for paired values: *: significant differences treatment *versus* control; **: heme control *versus* heme +LFM-A13, $p \leq 0.05$. LFM: LFM-A13. [Published as figure number 8 in Vijayan et al, 2011]

PART-II: p38 MAPK-mediated regulation of the HO-1 gene

3.8 Pharmacological inhibition and genetic deficiency of p38 MAPK lead to upregulation of HO-1

As reported earlier (Wijayanti et al. 2004; Naidu et al. 2008) treatment with the phorbol ester PMA induced the expression of HO-1 and COX-2 in cell cultures of RAW264.7 macrophages (Fig. 19).

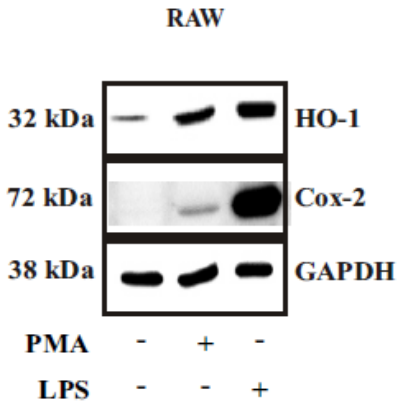
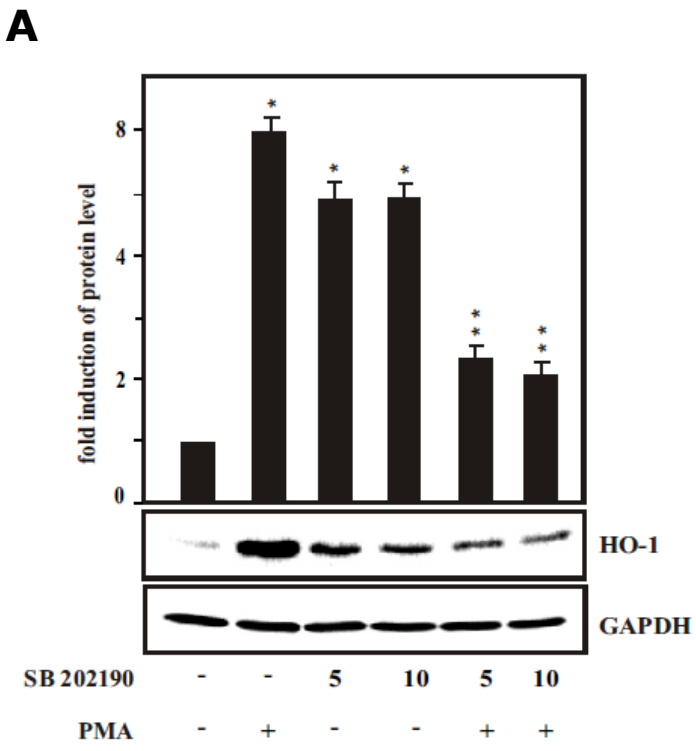


Figure 19: Upregulation of COX-2 and HO-1 gene expression in RAW264.7 cells treated with LPS and PMA.

RAW264.7 cells were treated with or without PMA (0.5 μM) or LPS (1 μg / ml) for 18 h. Total protein (50 μg) was subjected to Western blot analysis and probed sequentially with antibodies against HO-1, Cox-2 and GAPDH. [Published as Figure 1A in Naidu et al, 2009]

Previously, it was reported that p38 MAPK mediate the PMA-dependent induction of HO-1 and COX-2 (Schuette and LaPointe 2000; Chen et al. 2003; Wijayanti et al. 2004; Naidu et al. 2008). As expected, pretreatment of RAW264.7 cells with SB 202190, a specific inhibitor of p38α and p38β MAPK activity, blocked the PMA-dependent induction of HO-1. However, interestingly treatment with this compound alone markedly induced HO-1 (Fig. 20A). Indeed, the following experiments showed that SB202190 upregulated HO-1 in a time dependent manner (Fig. 20B). Similarly a strong induction of HO-1 was also observed when a second p38α inhibitor SB203580 was used (Fig. 20B).

To confirm this unexpected finding we compared PMA-dependent induction of HO-1 and Cox-2 gene expression in cell cultures of MEF, that were genetically deficient for p38α (p38$\alpha^{-/-}$ MEF) in comparison to that in wild-type MEF (p38$\alpha^{+/+}$ MEF). As observed in RAW264.7 macrophages, treatment with PMA upregulated HO-1 and Cox-2 expression in p38$\alpha^{+/+}$ MEF. Whereas in p38$\alpha^{-/-}$ MEF treatment with PMA had little effect on HO-1 and COX-2 expression. It is also noteworthy that the basal level of HO-1 protein expression was markedly higher in p38$\alpha^{-/-}$ MEF in comparison to that of p38$\alpha^{+/+}$ MEF (Fig. 20C). The results indicate that the loss of p38α function causes an increased expression of the HO-1 gene.



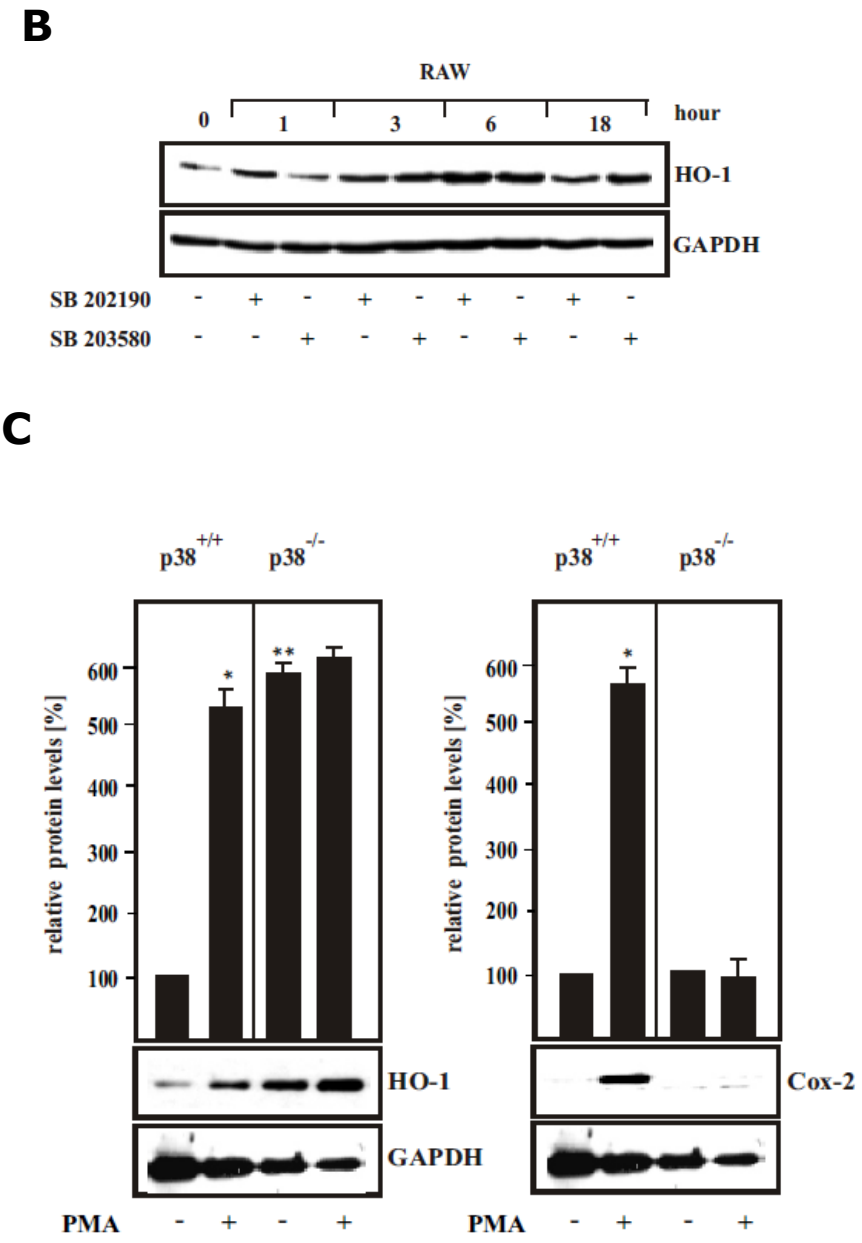


Figure 20: Pharmacological inhibition and genetic deficiency of p38 induce HO-1 gene expression

A: RAW264.7 cells were pretreated for 30 min with SB 202190 (5 and 10 µM), after which treatment was continued for another 6 h with or without PMA (0.5 µM), as indicated. B: RAW264.7 cells were treated with SB 202190 (10 µM), SB 203580 (20 µM) for the times indicated. C: $p38^{+/+}$ and $p38^{-/-}$ MEF were treated for 18 h with or without PMA (0.5 µM), as indicated. Total protein (50 µg) was subjected to Western blot analysis and probed sequentially with antibodies against HO-1, Cox-2 and GAPDH. Autoradiographic signals were visualized and quantified as described under *Materials and Methods*. (A) Values ± SEM represent the fold induction of HO-1 normalized to GAPDH from three independent experiments. (C) Numbers represent the protein expression levels relative to $p38^{+/+}$ MEF normalized to GAPDH from at least three independent experiments ± SEM. Statistics: Student's *t-test* for paired values: *: significant differences treatment *versus* control; **: $p38^{+/+}$ control *versus* $p38^{-/-}$ control, $p \leq 0.05$. RAW: RAW264.7 cells. [Published as figure 1B, 1C and 1F in Naidu et al, 2009]

3.9 Constitutive activation of p38 MAPK in RAW264.7 cells

The findings that p38$\alpha^{-/-}$ MEF possesses an increased expression of HO-1 and pharmacological inhibition of p38 in RAW264.7 cells leads to the upregulation of HO-1 indicate that in the basal state in RAW264.7 cells p38 MAPK may already be active. To test this hypothesis, we determined p38 activity in untreated RAW264.7 cells. As expected, p38 phosphorylation, which is a sign for activation, was detected under basal conditions and treatment with SB 202190 and SB 203580 abrogated this phosphorylation (Fig. 21A). To verify the functionality of p38 MAPK in detail, we performed a cotransfection assay in RAW264.7 cells, in which a luciferase reporter construct containing five binding sites for the yeast transcription factor Gal4 was used along with a construct which express a fusion protein consisting of the Gal4 DNA-binding domain and the transactivation domain of the transcription factor CHOP (pFA-CHOP). Transactivation of CHOP is regulated by p38-dependent phosphorylation (Wang and Ron 1996). The luciferase activity of pFA-CHOP was significantly high in RAW264.7 under basal conditions and treatment with p38 inhibitors blocked this effect (Fig. 21B). Similarly, the luciferase activity of pFA CHOP was higher in p38$^{+/+}$ MEF in comparison to p38$\alpha^{-/-}$ MEF. As observed in RAW264.7 cells, treatement with SB 202190 and SB 203580 significantly blocked the high luciferase activity observed in p38$\alpha^{+/+}$ MEF (Figure 21C). The data demonstrate that p38 MAPK is constitutively active in RAW264.7 monocytic cells.

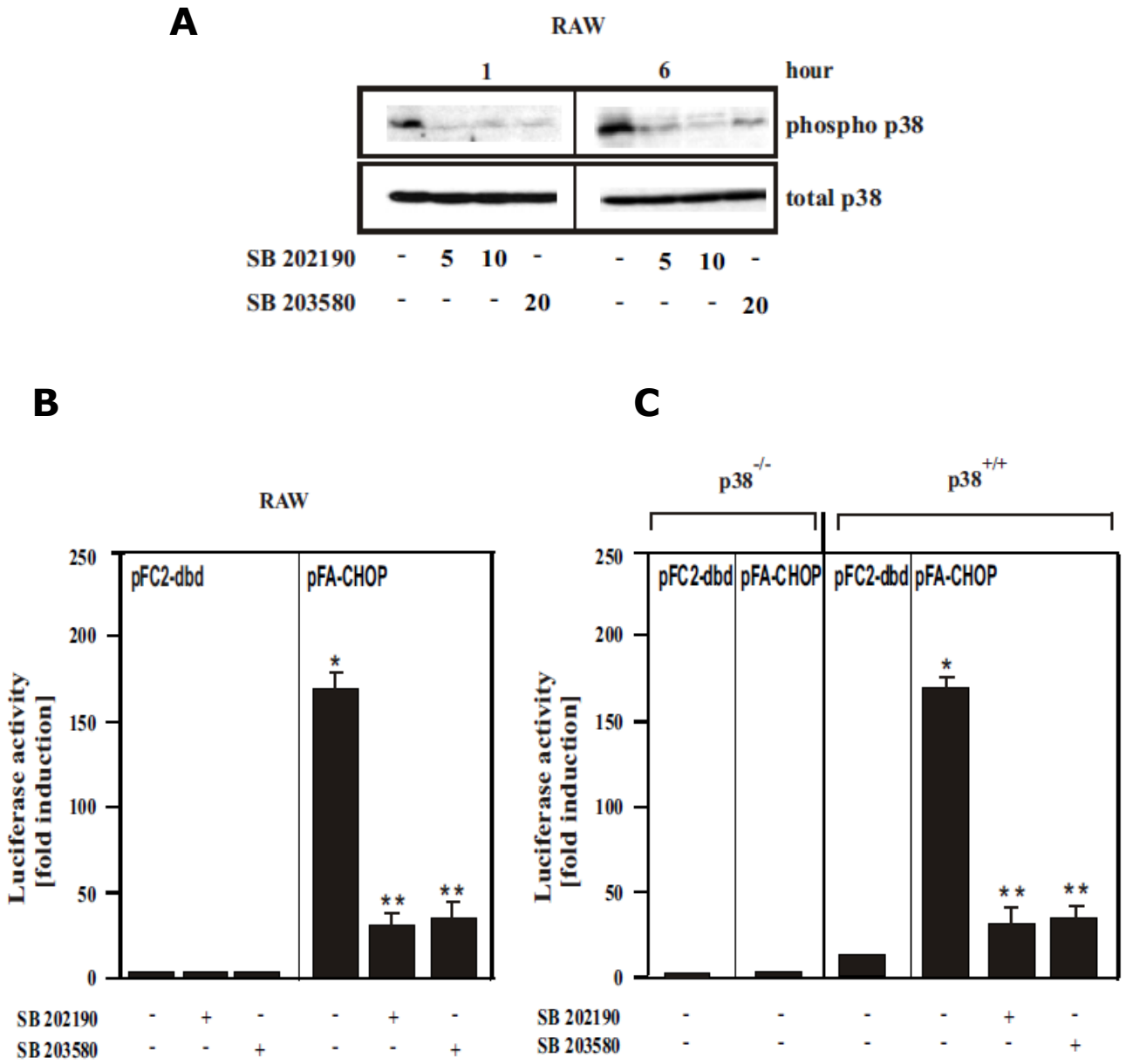


Figure 21: p38 MAPK activity in RAW264.7 and MEF cell cultures

A: RAW264.7 cells were treated with or without SB 202190 and SB 203580 for the times indicated. Total protein (50 µg) was used for Western blot analysis and sequentially probed with antibodies against phospho-specific p38 and total p38 MAPK. The results were reproduced at least three times in independent experiments. B-C: RAW264.7 cells and $p38^{+/+}$, $p38^{-/-}$ MEF were cotransfected with luciferase reporter gene construct pGal4-luc, pFC2-dbd, and pFA-CHOP, as indicated. 24 h after transfection cells were treated with SB 202190 (10 µM) and SB 203580 (20 µM) for 18 h. Cell extracts were assayed for luciferase activity, and the fold-induction relative to the control was determined. Values are means ± SEM from at least three independent experiments with duplicates of each point. Statistics: Student's *t-test* for paired values: *: significant differences pFC2-dbd *versus* pFA- CHOP; **: treatment *versus* control, $p \leq 0.05$. [Published as figure 2A-C in Naidu et al, 2009]

3.10 The HO-1 gene expression is not affected by inhibitors of JNK and ERK

To evaluate the role of MAP Kinases JNK and ERK in the regulation of HO-1 expression under basal conditions, RAW264.7 cells were treated with the JNK specific inhibitor SP 600125, and the ERK specific inhibitor, PD 98059, respectively. Unlike p38 inhibitors, treatment with neither the JNK inhibitor SP 600125 nor the ERK inhibitor PD 98059 induced the expression of the HO-1 gene (Fig. 22).

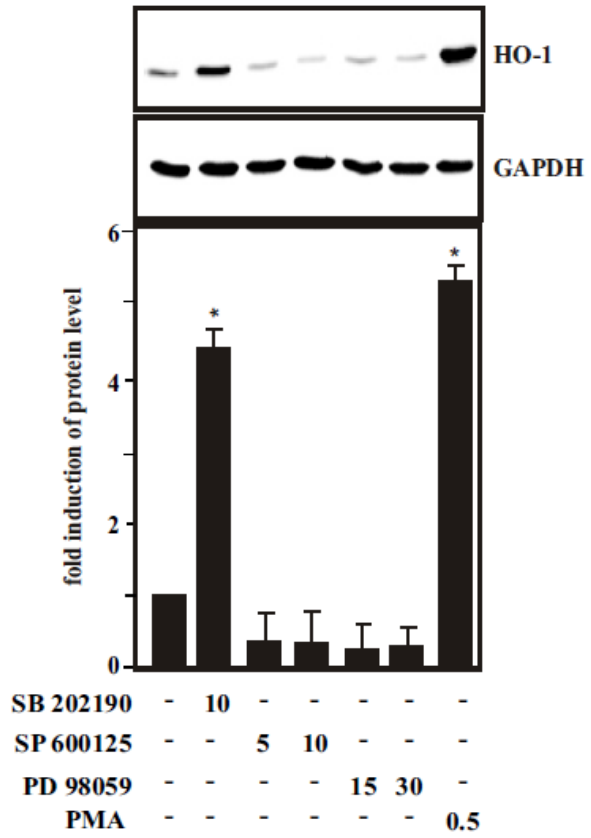


Figure 22: HO-1 gene expression on treatment with JNK and ERK inhibitors

RAW264.7 cells were treated with or without SB 202190, SP 600125, PD 98059 and PMA at the indicated concentrations (μM). Western blot analysis and quantitation was performed as previously described. Values ± SEM represent the fold-induction of HO-1 normalized to GAPDH from three independent experiments. Statistics: Student's *t-test* for paired values: *: significant differences treatment v*ersus* control, $p \leq 0.05$. [Published as figure 3 in Naidu et al, 2009]

3.11 Pharmacological inhibition and genetic deficiency of p38 upregulates HO-1 gene promoter activity

Nrf2, as mentioned earlier is a member of the cap'n'collar family of basic leucine zipper proteins (Motohashi and Yamamoto 2004; Kensler et al. 2007) and plays a major role in the regulation of HO-1 expression by various stimuli (Alam et al. 1999; Ishii et al. 2000). Previously, p38 was reported to be involved in the regulation of Nrf2 activation. However, the precise signaling mechanism is not well understood (Nguyen et al. 2003). The luciferase reporter gene constructs pHO-4045-luc and pE2-luc, both carry the proximal (E1) and distal (E2) enhancer sequences of the mouse HO-1 promoter that have been demonstrated to be the target sites for Nrf2 on the HO-1 promoter (Alam et al. 1999). To further evaluate the relationship between p38 activity and Nrf2 activation, these contructs were tranfected into $p38^{-/-}$ and $p38^{+/+}$ MEF. Since AP-1 is primarily regulated via JNK MAPK, we also transfected $p38^{-/-}$ and $p38^{+/+}$ MEF with the pAP-1-luc construct, which carries three copies of the consensus recognition sequence of AP-1 (Galang et al. 1994), and this was used as a negative control. Basal luciferase activity of the reporter gene construct pHO-4045-luc and pE2-luc was significantly higher in $p38^{-/-}$ MEF in comparison to that in $p38^{+/+}$ MEF. As expected, no difference was observed in the luciferase activity of pAP-1 luc between $p38^{-/-}$ and $p38^{+/+}$ MEF (Fig. 23A). Moreover, luciferase activity of these constructs markedly increased when treated with the p38 inhibitor SB 202190 in RAW264.7 cells (Fig. 23B). Taken together, the data indicates that the transcription factor Nrf2 is involved in p38-mediated regulation of the HO-1 gene.

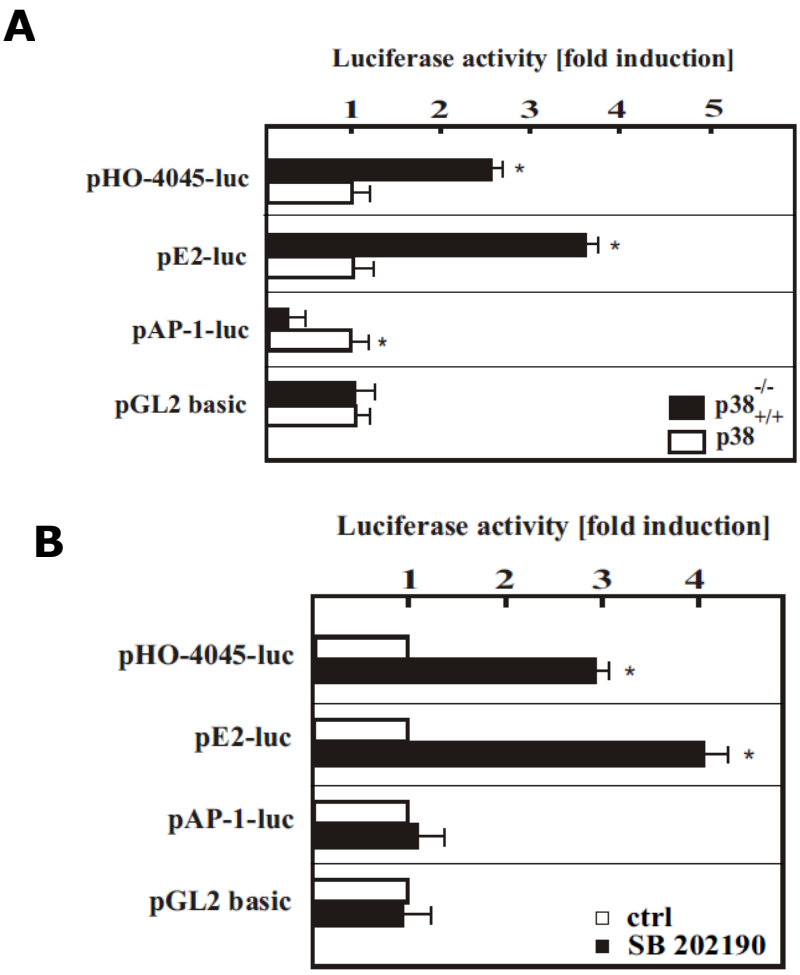


Figure 23: Role of p38 MAPK activity in the regulation of the HO-1 promoter.

A: $p38^{-/-}$ and $p38^{+/+}$ MEF transfected with the indicated reporter gene constructs were assayed for luciferase activity 24 h after transfection. The fold-induction relative to $p38^{+/+}$ was determined. Values are means ± SEM from at least three independent experiments. Student's *t-test* for paired values was used. *: significant differences $p38^{+/+}$ *versus* $p38^{-/-}$, $p \leq 0.05$. B: RAW264.7 cells transfected with the indicated reporter gene constructs were assayed for luciferase acitivity 42 h after transfection. 24 h after transfection cells were treated with or without SB 202190 (10 µM) as indicated for 18 h. The fold- induction relative to the control was determined. Values are means ± SEM from at least three independent experiments. Statistics: Student's *t-test* for paired values: *: significant differences treatment *versus* control, $p \leq 0.05$. [Published as figure 4 in Naidu et al, 2009]

3.12 Inhibitors of p38 do not induce HO-1 gene expression in Nrf2-/- MEF

As another independent strategy to analyze the role of p38 in Nrf2-dependent HO-1 gene regulation, we treated $Nrf2^{-/-}$ and $Nrf2^{+/+}$ MEF with p38 inhibitors. Treatment with either SB 202190 or SB 203580 markedly upregulated HO-1 gene expression in $Nrf2^{+/+}$ MEF but not in $Nrf2^{-/-}$ MEF (Fig. 24).

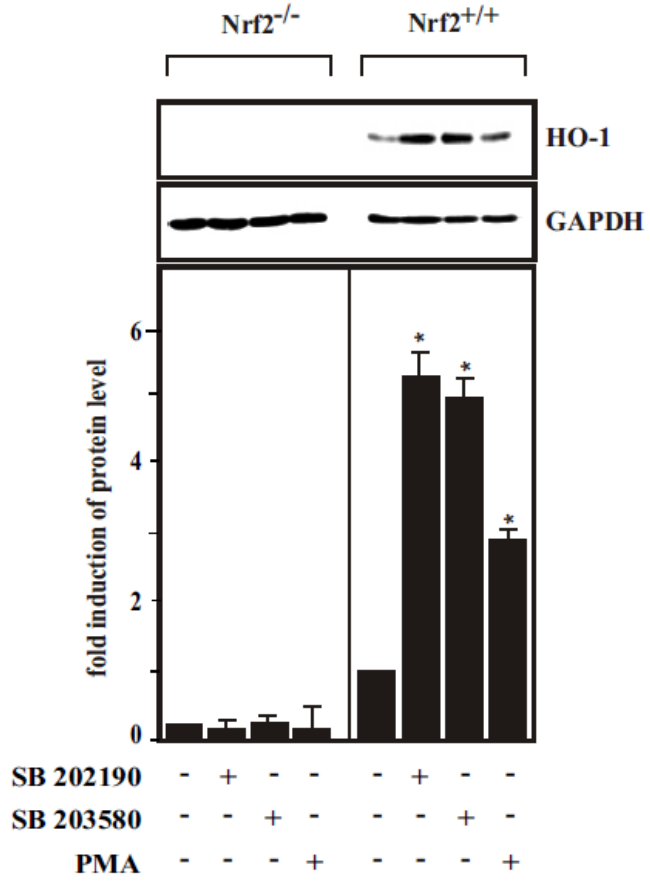


Figure 24: Regulation of the HO-1 gene expression in Nrf2$^{-/-}$ and Nrf2$^{+/+}$ MEF

Nrf2$^{-/-}$ and Nrf2$^{+/+}$ MEF were cultured as described under *Materials and Methods*, and were treated for 18 h with or without SB 202190 (10 μM) , SB 203580 (20 μM) and PMA (0.5 μM). Western blotting and quantitation was performed as described previously in this thesis. Values ± SEM represent the fold-induction of HO-1 normalized to GAPDH from three independent experiments. Statistics: Student's *t-test* for paired values: *: significant differences treatment v*ersus* control, $p \leq 0.05$. [Published as figure 6A in Naidu et al, 2009]

Similarly, when these cells were transfected with the pHO-4045-luc and pE2-luc constructs, the luciferase activity was significantly higher in Nrf2$^{+/+}$ MEF when compared to that in Nrf2$^{-/-}$ MEF (Fig. 25). Moreover, upon treatment with SB 202190 luciferase activity of two HO-1 gene constructs increased in Nrf2$^{+/+}$ MEF whereas no difference was observed in Nrf2$^{-/-}$ MEF (Fig. 25). As expected, the reporter gene activity of the pAP-1-luc construct was not affected by p38 inhibitors (Fig. 25). Taken together, the results suggest that inhibition of p38-dependent regulation of the HO-1 gene expression occurs via an Nrf2-dependent

manner. Taken together, the results suggest that inhibition of p38-dependent regulation of the HO-1 gene expression occurs via an Nrf2-dependent manner.

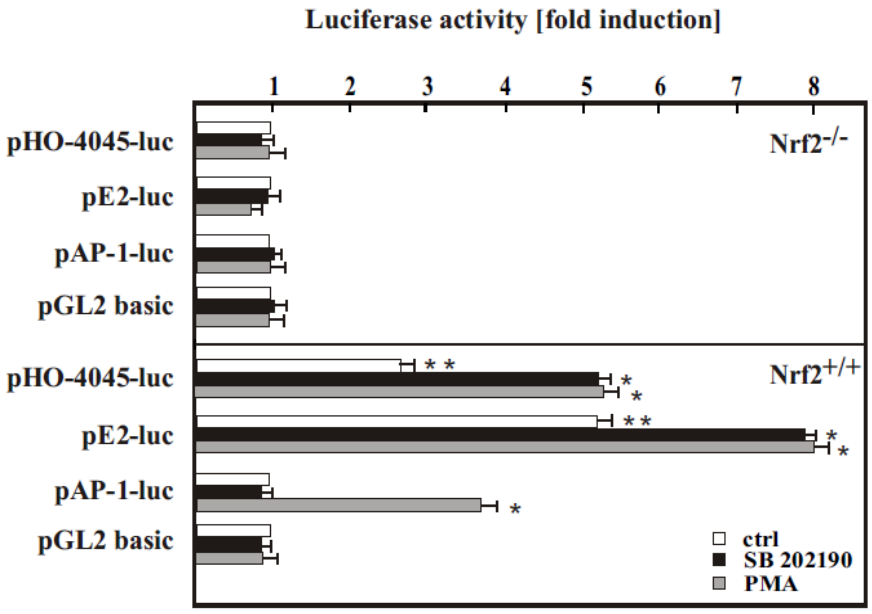


Figure 25: HO-1 promoter activity in $Nrf2^{-/-}$ and $Nrf2^{+/+}$ MEF

$Nrf2^{-/-}$ and $Nrf2^{+/+}$ MEF were transfected with the indicated reporter gene constructs. 24 h after transfection cells were treated with or without SB 202190 (10 μM) and PMA (0.5 μM) for 18 h. The luciferase activity of the different constructs were assayed and determined relative to $Nrf2^{-/-}$ MEF. Values are means ± SEM from at least three independent experiments with duplicates of each point. Statistics: Student's *t-test* for paired values: *: significant differences treatment *versus* control; **: activity of pHO-4045 or pE2-luc in $Nrf2^{-/-}$ MEF *versus* $Nrf2^{+/+}$ MEF, $p \leq 0.05$. [Published as figure 6B in Naidu et al, 2009]

3.13 ERK is involved in HO-1 gene activation via p38 inhibition

Independent studies have reported a role for ERK MAPK in the regulation of the HO-1 gene by numerous stimuli (Immenschuh and Ramadori 2000; Ryter et al. 2006). Signaling pathways induced via MAPKs have been shown to involve cross-talks (Porras et al. 2004) and so we evaluated the effect of p38 MAPK inhibition on the phosphorylation of ERK and whether it plays a role in the regulation of HO-1 gene expression in $p38^{-/-}$ and $p38^{+/+}$ MEF. In comparison to $p38^{+/+}$ MEF, an increased phosphorylation of ERK was observed in $p38^{-/-}$ MEF and

presence or absence of serum did not alter this effect (Fig. 26A). Furthermore, treatment with the specific ERK inhibitor PD 98059 reduced the increase in the protein expression of HO-1 in $p38^{-/-}$ MEF (Fig. 26B). In a second series of experiments, the effects of p38 inhibition on the phosphorylation of ERK in RAW264.7 cells were examined. Addition of p38 inhibitors to cell cultures of RAW264.7 induced the phosphorylation of ERK in these cells (Fig. 26C).

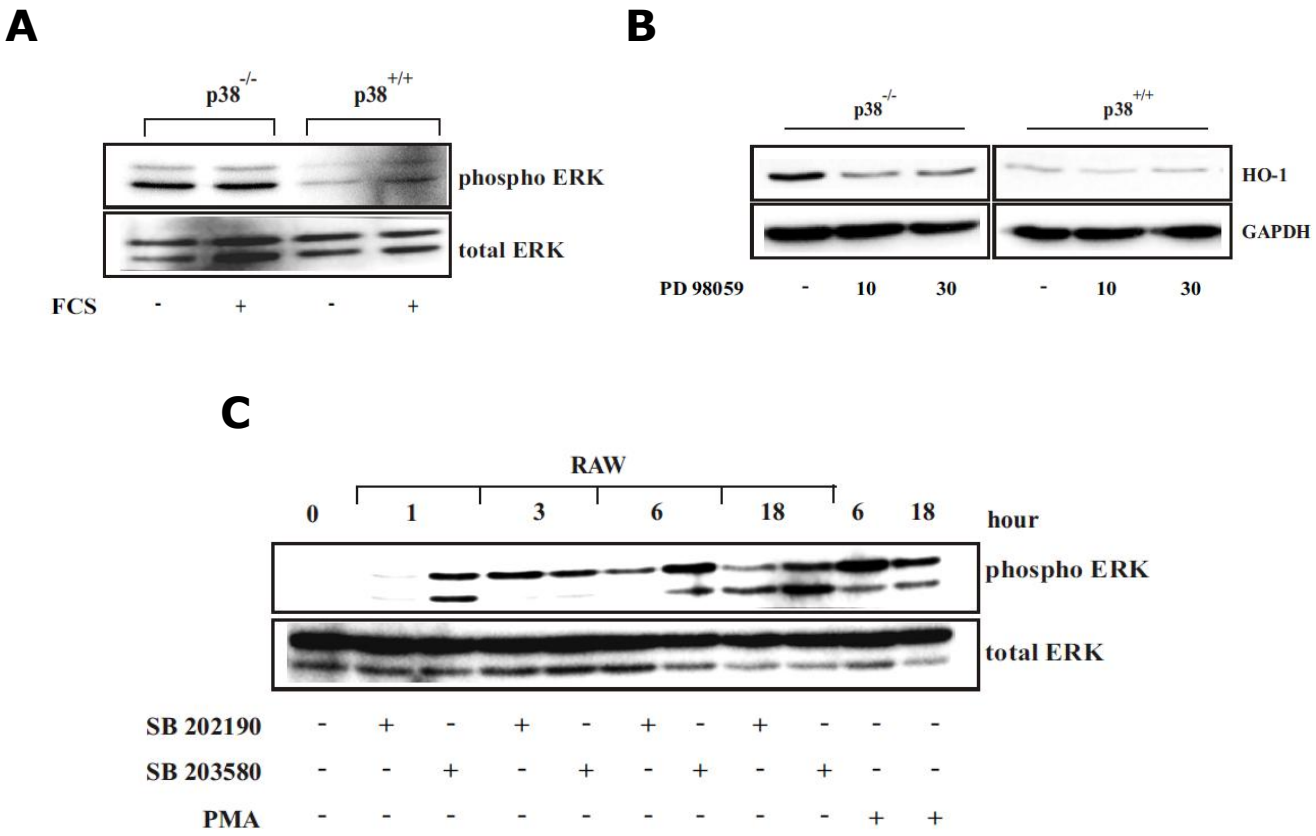


Figure 26: Role of ERK activity in p38-dependent regulation of HO-1 gene expression

A: $p38^{-/-}$ and $p38^{+/+}$ MEF were cultured in the presence or absence of FCS as indicated B: $p38^{-/-}$ and $p38^{+/+}$ MEF were treated for 6 h in the presence or absence of PD98059. C: RAW 264.7 cells were treated with SB 202190 (10 μM), SB 203580 (20 μM) and PMA (0.5 μM), as indicated. Western blot analysis was performed as described previously and sequentially probed with antibodies (A, C) against phosphorylated ERK and total ERK, (B) HO-1 and GAPDH. [Published as figure 8A-C in Naidu et al, 2009]

Subsequent transient transfection studies with the luciferase reporter gene construct pFA-Elk which is specifically regulated via ERK revealed that the

luciferase activity was strongly upregulated in those cells treated with the p38 inhibitors. Similarly, treatment with PMA which is known to induce the acitivity of ERK MAPK also induced the luciferase activity of pFA-Elk (Fig. 27). In summary, the results suggest that deficiency of p38 activity leads to the activation of ERK and may be involved in the regulation of the HO-1 gene expression.

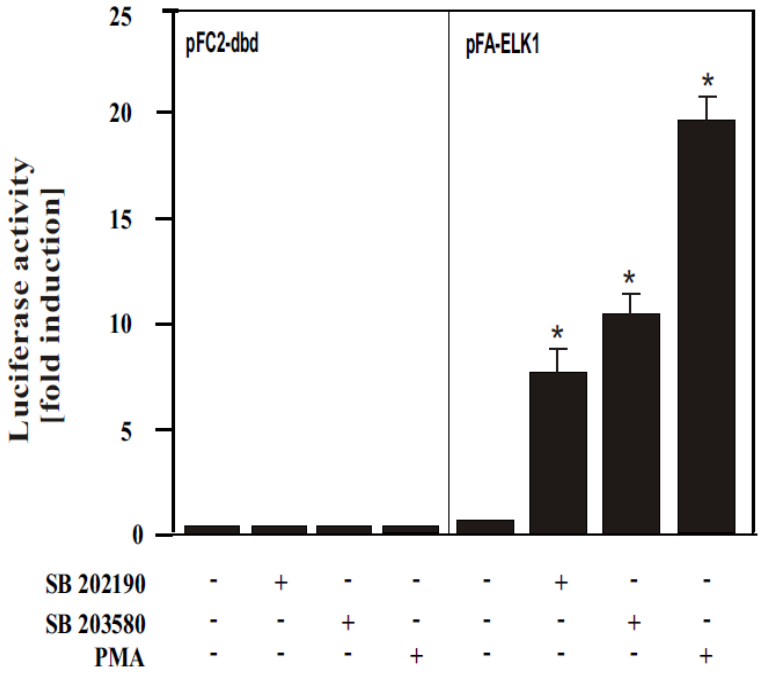


Figure 27: ERK activity in RAW264.7 cells treated with p38 inhibitors

RAW264.7 cells were cotransfected with the luciferase reporter gene constructs pGal4-luc, pFC2-dbd, and pFA-Elk. 24 h after transfection, the cells were treated for 18 h with or without SB 202190 (10 µM), SB 203580 (20 µM) and PMA (0.5 µM). Luciferase activity in the cell extracts was determined. Values are means ± SEM from at least three independent experiments. Statistics: Student's *t-test* for paired values: *: significant differences treatment *versus* control, $p \leq 0.05$. [Published as figure 8D in Naidu et al, 2009]

3.14 Genetic deficiency and pharmacological inhibition of p38 MAPK lead to increased accumulation of intracellular ROS

Dolado and co-workers previously reported that genetic deficiency of p38α MAPK leads to an increased accumulation of ROS (Dolado et al. 2007). Hence, we determined the ROS levels in the $p38^{+/+}$ and $p38^{-/-}$ MEF. In comparison to $p38^{+/+}$ MEF, $p38^{-/-}$ MEF exhibited significantly higher level of intracellular ROS as

measured by FACS (Fig. 28A, B). Furthermore, treatment with the p38 inhibitor SB 202190 markedly increased the ROS levels in $p38^{+/+}$ MEF in comparison to the control untreated group (Fig. 28A, B). To evaluate the role for ROS in this p38-mediated regulation of HO-1 gene expression, $p38^{+/+}$ and $p38^{-/-}$ MEF were treated with the antioxidant NAC.

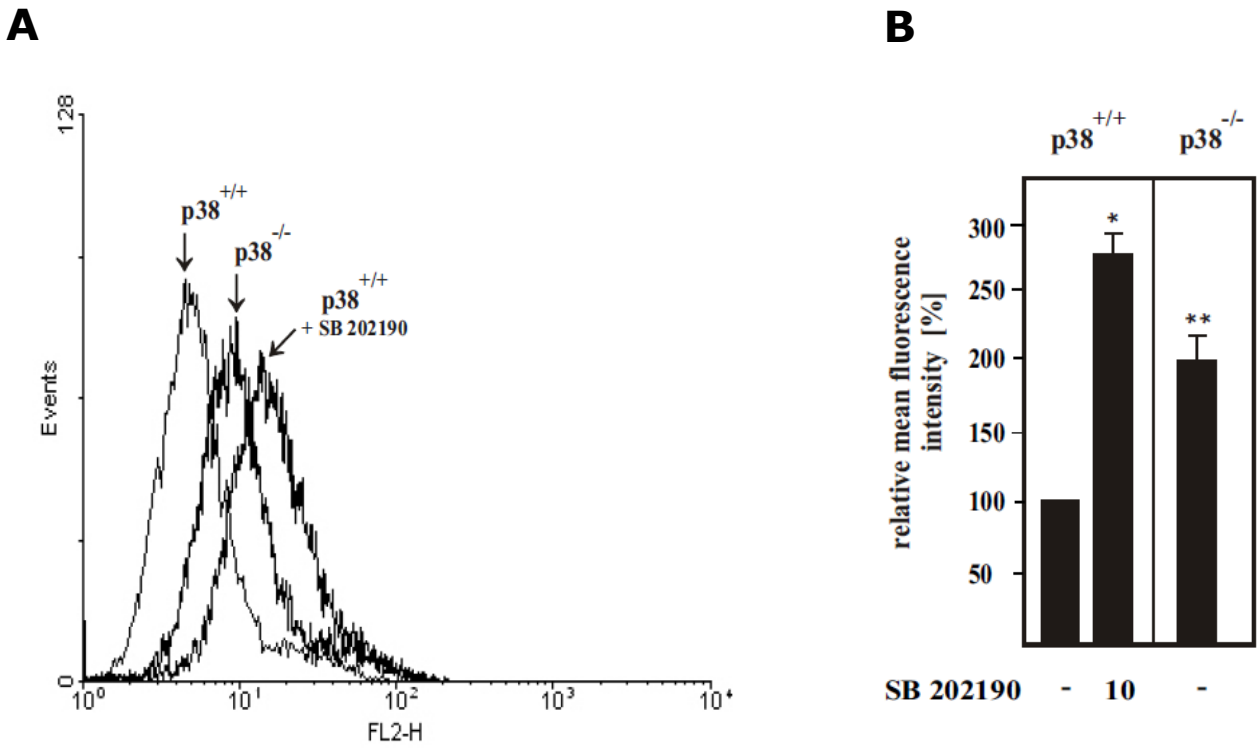


Figure 28: p38 MAPK deficiency leads to increase in intracellular ROS

A: $p38^{-/-}$ and $p38^{+/+}$ MEF were treated with SB 202190 (10 µM) for 1 h. Cells were washed and loaded with DHE stain (5 µM) for 20 min and after further washing steps analyzed by flow cytometry. B: Numbers indicate quantification of the mean fluorescence intensity relative to $p38^{+/+}$ MEF from at least three independent experiments ± SEM. Statistics: Student's *t-test* for paired values: *: significant differences treatment *versus* control; **: $p38^{+/+}$ control *versus* $p38^{-/-}$ control, $p \leq 0.05$. [Published as figure 9A-B in Naidu et al, 2009]

HO-1 gene expression has been shown to be induced by ROS in immunologically active cells (Maines 1997; Immenschuh and Ramadori 2000; Alvarez-Maqueda et al. 2004; Naidu et al. 2008). To evaluate the role for ROS in this p38-mediated regulation of HO-1 gene expression, $p38^{+/+}$ and $p38^{-/-}$ MEF were treated with the antioxidant NAC. The basal expression of HO-1 was significantly

reduced in p38$^{-/-}$ MEF when treated with NAC. Treatment with NAC had little effect in p38$^{+/+}$ MEF (Fig. 29A). In the following RAW264.7 cells were treated with NAC prior to the addition of SB 202190. Treatment with NAC blocked the SB 202190-mediated induction of the HO-1 gene expression. Similarly, upregulation of HO-1 by PMA which is a known inducer of ROS production in monocytes (Datta et al. 2000) was also attenuated by NAC (Fig. 29B). Taken together these results suggest that loss of p38 activity increases the intracellular generation of ROS and this might be involved in the induction of the HO-1 gene.

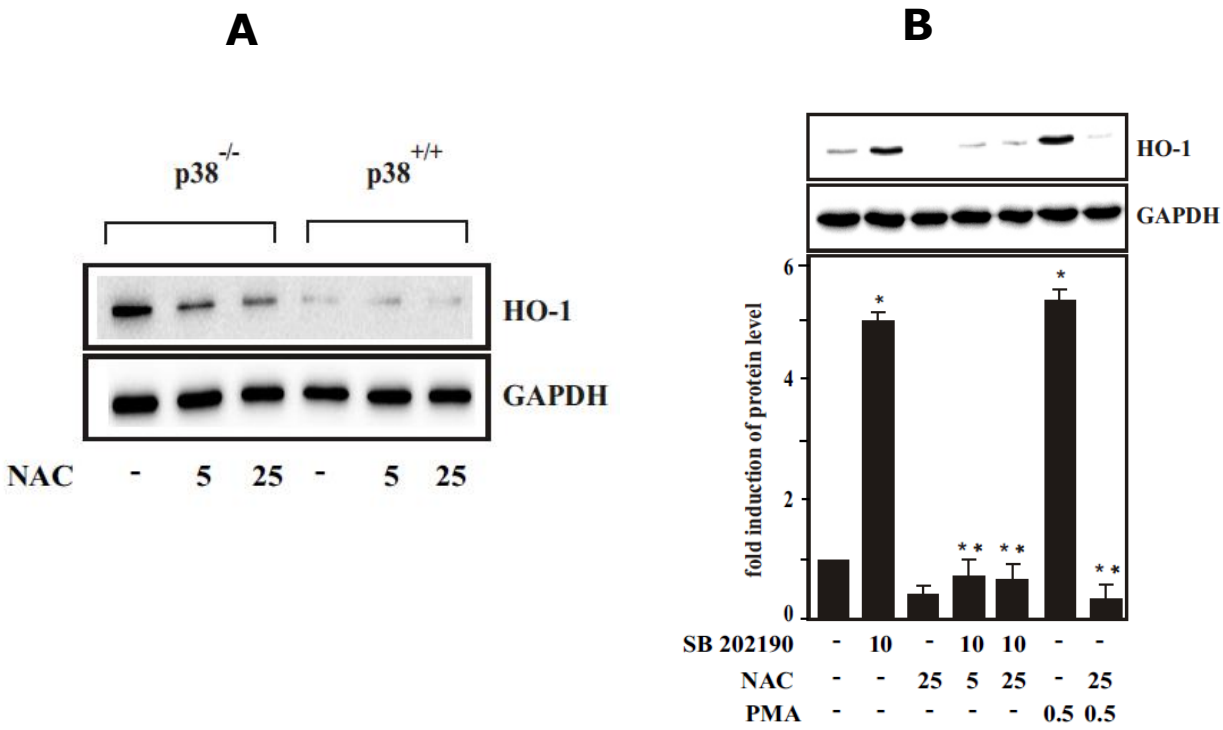


Figure 29: p38 MAPK deficiency leads to increase in intracellular ROS

A: p38$^{-/-}$ and p38$^{+/+}$ MEF were treated for 6 h with or without NAC (5 and 25 mM). B: RAW264.7 cells were pretreated for 30 min with NAC at the indicated concentrations (mM), after which treatment was continued for another 6 h with or without SB 202190 (10 μM) and PMA (0.5 μM). (B) Values ± SEM represent the fold-induction of the HO-1 normalized to the GAPDH from three independent experiments. Statistics: Student's *t-test* for paired values: *: significant differences treatment *versus* control; **: treatment *versus* NAC + treatment, $p \leq 0.05$. [Published as figure 9C-D in Naidu et al, 2009]

4. Discussion

Hemeoxygenase-1(HO-1) is the inducible isoform of the rate-limiting enzymatic step of heme catabolism and has major antioxidant and anti-inflammatory functions. The beneficial effects of this enzyme appear to be mainly mediated via degradation of proinflammatory heme and production of the anti-inflammatory compounds CO and bilirubin. More recently, myeloid/macrophage cell type specific effects of HO-1 have been implicated in central immunomodulatory functions of the innate and adaptive immune response. In this thesis experimental strategies were used to elucidate the signaling mechanisms regulating the HO-1 gene expression. Major results revealed an important role of the immunologically relevant kinase Bruton's tyrosine kinase (Btk) in the regulation of TLR-induced signaling of the HO-1 gene and a specific role of the p38 MAPK as a repressor of the HO-1 gene in mouse macrophages. TLR ligands, specifically the TLR4 ligand LPS induced the activation of the HO-1 gene expression via Btk. Moreover, inhibition of the p38 MAPK induced the HO-1 gene in cell cultures of macrophages. As revealed in this thesis, both mechanisms are mediated at the transcriptional level via the transcription factor Nrf2, a crucial regulator of the cellular antioxidant response, and involve ROS as signaling molecules.

4.1 Role of Btk in the induction of the HO-1 gene expression

The proinflammatory mediator LPS, a ligand of the central macrophage receptor TLR4, has previously been shown to induce HO-1 gene expression in mononuclear phagocytes (Camhi et al. 1995; Immenschuh et al. 1999; Wijayanti et al. 2004). The results of this thesis revealed that inhibition of the non-receptor tyrosine kinase Btk by the chemical inhibitor LFM-A13 or genetic Btk deficiency blocked the LPS-dependent HO-1 up-regulation in cell cultures of

the mouse macrophage cell line RAW264.7 and in murine alveolar macrophages (Figs. 7-10). The significance of Btk as an immunologically relevant kinase is clearly illustrated in X-linked agammaglobulinemia, a disorder in which a mutation of this kinase leads to impaired development of B-cells, making them incompetent to produce antibodies (Vetrie et al. 1993). Although earlier studies focussed on the role of this kinase in B-cell function, recent evidences indicated that Btk might play an important role in mediating inflammatory responses in other cell types, such as macrophages and mast cells (Hata et al. 1998; Kawakami et al. 1998; Kawakami et al. 1999; Jefferies and O'Neill 2004). Specifically, deficiency of Btk function leads to impaired cytokine response and poor nitric oxide induction in response to LPS (Horwood et al. 2003; Mangla et al. 2004; Horwood et al. 2006). Moreover, LPS treatment both *in vivo* and *in vitro* induces the HO-1 gene. Since various studies indicated that LPS-induced cytokine and NO production may influence the regulation of the HO-1 gene we hypothesized that Btk might also be an upstream candidate in LPS-dependent signaling of HO-1 gene up-regulation. The present findings are consistent with the notion that Btk might play a central role in mediating the HO-1 gene induction by LPS. As the Btk inhibitor LFM-A13 also inhibits the kinase JAK2, Btk knockout macrophages were used in this thesis to confirm that the specificity of Btk function in HO-1 regulation. Moreover, we have also analysed the effect of the JAK2 specific inhibitor AG490 on the regulation of HO-1. In contrast to LFM-A13, treatment with AG490 alone up-regulated the HO-1 gene expression (Fig. 7C), indicating that the blockage of the HO-1 gene induction with LFM-A13 is a specific effect caused by the inhibition of Btk and not JAK2. However, Btk has been shown to be a versatile protein that interacts with many other proteins.

Therefore, it is feasible that there might also exist protein-protein interactions between JAK2 and Btk, possibly affecting the HO-1 gene regulation.

4.2 Role of p38 MAPK in the induction of the HO-1 gene expression

Inhibition of p38 MAPK in monocytic cells by small molecule inhibitors and genetic deficiency of p38α in MEFs leads to an increased HO-1gene expression, in contrast to an unaltered Cox-2 gene (Fig. 20). Since chemical inhibitors of p38 MAPK block all four isoforms of this kinase, the specificity for p38α was analysed in MEFs deficient for p38α. Because, the induction of HO-1 was observed in both RAW264.7 cells and in p38-deficient MEFs in this thesis, it indicates that the observed mechanism may not be only restricted to macrophages. With regard to its physiological function p38α seems to be the most important isoform among the four p38 family, since the targeted deletion of the p38α gene in mice leads to embryonic lethality in mice (Adams et al. 2000). In this respect, it was interesting that HO-1 levels were constitutively high in $p38\alpha^{-/-}$ MEF, even though basal HO-1 expression is generally known to be barely detectable in most cells (Maines 1997; Ryter et al. 2006). Moreover, the upregulation of HO-1 by inhibition of p38 MAPKs was rather unexpected, since p38 MAPKs has previously been shown to mediate the activation of the HO-1 gene in response to various stimuli, such as LPS and TPA (Maines 1997; Immenschuh and Ramadori 2000; Ryter et al. 2006). Indeed, this has also been reproduced in our study, in which induction of HO-1 by TPA was partially blocked after inhibition of p38 MAPKs. The findings of this thesis are supported by a recent study demonstrating that the basal expression of many genes was increased without the presence of an external stress stimulus in immortalized $p38\alpha^{-/-}$ cardiomyocytes (Ambrosino et al. 2006). The present observations may therefore suggest that p38α has a dual function on the regulation of the HO-1 gene. On the one hand p38α may act as a

repressor of HO-1 in unstimulated cells and on the other hand it may promote also HO-1 activation in the presence of several external stress stimuli.

4.3 Transcriptional mode of the HO-1 gene induction

To investigate if the Btk- and p38α-mediated increase of the HO-1 expression occurs at the transcriptional level transient transfection experiments with reporter gene constructs of the mouse HO-1 gene promoter were performed in RAW264.7 cells. Additionally, MEFs were used that were genetically deficient for either p38α or Nrf2. Unfortunately, as noted by analysing the effect of LPS on MEFs, it was not possible to use appropriate MEFs for studies on Btk, since HO-1 is not induced by LPS in this cell type (data not shown in Results).

4.3.1 Transcriptional regulation of the HO-1 gene by Btk

This is the first report to demonstrate that Btk regulates the redox regulated transcription factor Nrf2. Btk had only been shown before to modulate the activity of other transcription factors, including TFII-I and NF-κB in B cells (Novina et al. 1999; Bajpai et al. 2000) or also NFAT in mast cells (Iwaki et al. 2005). The immunofluorescense studies showed a decreased nuclear translocation of Nrf2 in response to LPS and this indicate that Btk may play a role in the LPS mediated activation of Nrf2

In earlier reports it has been demonstrated that the LPS induced induction of the HO-1 gene is mediated by two upstream regions localized at -4kb and -12kb relative to the transcription initiation site of the mouse HO-1 gene promoter (Camhi et al. 1998). Initially, it was thought that transcriptional regulation via these sequences would be regulated by the transcription factor AP-1, because both regions contain potential AP-1 sites (Camhi et al. 1995). Later studies, however, revealed that these promoter regions are major nuclear targets for the transcription factor Nrf2 (Alam et al. 1999). Nrf2 binds to the ARE, also referred

to as antioxidant response element or electrophile response element (EpRE), several of which are located in the 5'-flanking sequence of the HO-1 gene promoter. AREs have been identified in a large number of phase II detoxifying enzymes and antioxidant stress proteins (Nguyen et al. 2003). The results of this thesis are consistent with this finding and revealed an Nrf2-mediated effect on the mouse HO-1 promoter by using reporter gene constructs with the proximal 4045 bp of the mouse HO-1 promoter and with ARE. Nrf2 controls the HO-1 gene induction in response to various oxidative/stress stimuli and by its interaction with keap1 is kept in an inactive state under normal conditions. Binding to keap1 in addition marks Nrf2 for proteasomal degradation via ubiquitination (Itoh et al. 1999; Kobayashi and Yamamoto 2005; Kensler et al. 2007). This Btk-mediated Nrf2 activation mechanism does not only regulate the HO-1 gene, but is also involved in the LPS-induced up-regulation of the NQO1 gene, a prototypical Nrf2-regulated gene. Thus, it would raise the possibility that also other Nrf2 target genes could also be altered in macrophages. In contrast to Nrf2, the transcription factor NF-κB, that has been shown earlier to regulate the HO-1 gene expression (Wijayanti et al. 2004; Naidu et al. 2008) and is one of the classical targets of LPS-dependent TLR4 signaling in mononuclear phagocytes, does not seem to play a major role in the HO-1 gene regulation via Btk. This assumption is supported by the observations that LPS-dependent induction of a NF-κB-regulated reporter gene construct (pTNF-585-luc) and phosphorylation as well as degradation of the NF-κB inhibitor IκB were not affected by pharmacological inhibition of Btk (Fig. 11). This notion is also in agreement with earlier reports, in which it was shown that the Btk-mediated induction of TNFα and IL-1ß by LPS was independent of the classical NF-κB pathway (Horwood et al. 2003; Doyle et al. 2005; Horwood et al. 2006).

However, it should be taken into consideration that other transcription factors such as AP-1 and USF or the transcription repressor Bach1 might in addition also be involved in LPS-mediated HO-1 regulation via Btk (Alam and Cook 2007).

4.3.2 Transcriptional regulation of the HO-1 gene by p38 inhibitors

p38 MAPKs regulate gene expression by both transcriptional and post-transcriptional mechanisms (Ono and Han 2000). Similar to Btk-mediated induction of the HO-1 gene, inhibition or genetic deficiency of p38α activates the transcription factor Nrf2 for induction of HO-1(Fig 24-25). This conclusion is supported by the present finding that genetically deficient p38α MEFs possess higher Nrf2 levels in the nucleus. Moreover, Nrf2-deficient MEFs failed to upregulate the HO-1 gene after p38 inhibition. In this respect it is of interest that the expression of the peroxiredoxin 1 (Prdx1) gene, a gene regulated in a similar manner as HO-1 (Immenschuh et al. 1995) was also induced by the inhibition of p38. Previously Prdx1 1 has been shown to be a target of Nrf2 supporting the finding that p38 plays a negative role on Nrf2 activation (Ishii et al. 2000). Previous studies have reported that blockage of p38 augments Stress response element (StRE)-mediated induction of phase II detoxifying enzymes (Yu et al. 2000) and also sulforaphane-dependent activation of HO-1 via Nrf2 in human hepatoma HepG2 cells (Keum et al. 2006) and our findings corroborate these studies. Finally, it can, however, not be excluded that additional Nrf2-independent regulatory mechanisms may be involved in the up-regulation of the HO-1 gene expression via p38 MAPKs.

4.4 Signaling pathway

Btk- and p38α- dependent induction of HO-1 is mediated by distinct signaling cascades in macrophages. However, in both of these signaling pathways Nrf2 and ROS act as downstream regulators of the HO-1 gene expression.

4.4.1 Btk-dependent induction of HO-1 gene expression in macrophages

The results of this thesis indicate that Btk mediates the induction of HO-1 not only by stimulation of macrophages via TLR4/LPS, but also by ligands for TLR2, TLR7 and TLR9 (Fig. 16-17). These observations are in accordance with earlier reports which indicated that Btk might play a central role in macrophage stimulation by various TLRs (Koprulu and Ellmeier 2009). As an example, Btk has previously been demonstrated to be involved in TLR-dependent induction of pro- and anti-inflammatory cytokines (Horwood et al. 2006; Schmidt et al. 2006). Moreover, Btk has been shown to mediate TLR8- and TLR9-dependent signaling in the human macrophage cell line THP-1 (Doyle et al. 2007). The current observations of this thesis indicate that ROS may mediate the Btk-dependent activation of the HO-1 gene expression in TLR-stimulated macrophages. These results corroborate earlier findings that ROS generated upon TLR4 activation acts as a secondary signaling molecules and mediate cellular responses in mononuclear phagocytes (Asehnoune et al. 2004). Furthermore, direct interaction of TLR4 and NADPH oxidase 4 (NOX4), has previously been shown to mediate generation of ROS in phagocytes (Park et al. 2004). A similar mechanism has also been reported in an in vivo knockout mouse model (Kong et al. 2010). Previously, Mangla and colleagues have reported that in Btk-/- mononuclear phagocytes LPS failed to induce ROS production (Mangla et al. 2004). Our findings are consistent with this observation and further studies are required to characterize the exact mechanism by which Btk regulates ROS production in LPS-stimulated macrophages. Surprisingly, the present results indicate that Btk-dependent induction of the HO-1 gene via Nrf2 is mainly mediated by ROS but not RNS. Indeed, the activity of Nrf2 was not influenced by the specific iNOS inhibitor

1400W, but blocked by NAC (Fig. 15). However, Nrf2 has previously been shown to be activated by NO and reactive nitrogen species in Hela cells (Fourquet et al.), human umbilical vein endothelial cells (Heiss et al. 2009) or PC12 phaeochromocytoma cells (Li et al. 2007). Therefore, it might be feasible that cell type- or species-specific effects may play a role in the NO-dependent regulation of Nrf2. The Btk-mediated Nrf2 activation may involve direct modulation of the cytosolic inhibitor protein Keap1 as described in many scenarios (Kobayashi and Yamamoto 2005). Furthermore, ROS produced after LPS-induction could in addition regulate gene transcription by other mechanisms such as the inhibition of regulatory phosphatases as shown by others (Rhee et al. 2003).

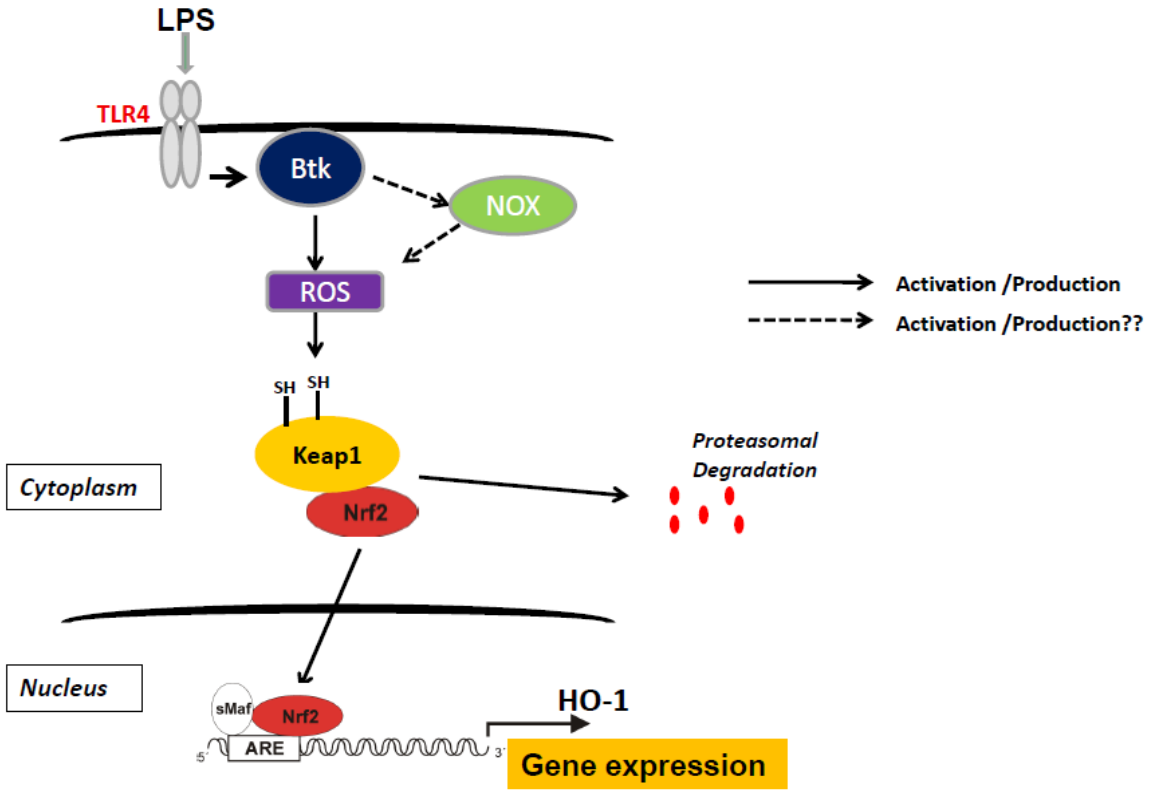


Figure 30: Model of TLR4 mediated activation of HO-1 gene expression in macrophages

Upon stimulation with LPS Btk mediates the generation of ROS and that leads to the activation of Nrf2. Nrf2 translocates to the nucleus binds to the ARE elements present on HO-1 gene promoter and induce the transcription of the HO-1 gene. Whether Btk directly influence NOX enzymes for the generation of ROS is not clear and should be explored in further studies. Nrf2: nuclear factor erythroid 2-related factor

E2; ARE: antioxidant response element; Keap: Kelch ECH associating protein; ROS: reactive oxygen species; sMaf: small Maf protein.

4.4.2 Regulatory pathways of increased HO-1 gene expression by inhibition and genetic deficiency of p38 MAPK

The results of this thesis revealed that genetic deficiency of p38α causes an increase in ERK activity (Fig. 26-27) which is in agreement with an earlier report (Porras et al. 2004). Moreover, treatment with the ERK inhibitor PD 98059 in $p38\alpha^{-/-}$ MEF abolished the increase in expression of the HO-1 gene indicating that the MAPK p38α and ERK play opposing roles in the regulation of this gene. This assumption is corroborated by a previous study, in which pharmacological inhibition of p38 up-regulated the low density lipoprotein receptor gene expression through the activation of ERK in HepG2 cells (Singh et al. 1999). Moreover, another independent study has demonstrated that inhibition of p38 augmented LPS-mediated inducible NO synthase gene expression and NO production via JNK activation in macrophages (Lahti et al. 2006). The role of MAPK signaling in the regulation of the HO-1 gene is complex and varies among species. Elbirt and colleagues reported that in avian hepatocytes both activation of ERK and p38 MAPKs were required for the arsenite-dependent induction of the HO-1 gene expression (Elbirt et al. 1998). In contrast, both JNK and p38 MAPK were important for the HO-1 gene induction by this compound in rat hepatocytes (Kietzmann et al. 2003). Thus, one can assume that MAPK modulate HO-1 gene expression in a stimulus-, cell- and species-specific manner.

Since transformed p38α -deficient cells were reported to accumulate much larger amounts of ROS than wild type cells, p38α was suggested to play the role of a cellular oxidative stress sensor (Dolado et al. 2007). The current findings (Fig. 28) are in line with this suggestion. Moreover, ROS might be involved in the activation of ERK in the absence of p38 activity (Porras et al. 2004). This is also

corroborated by recent reports, which demonstrate that ROS causes a sustained activity of ERK in human hepatoma cells (Wu et al. 2006) as well as in a model of cerebral ischemia (Wu et al. 2008). Accordingly, this is in line with the results obtained in this thesis by treatment of p38$\alpha^{-/-}$ MEF with the antioxidant NAC, blocked the increase of the HO-1 gene expression (Fig. 29).

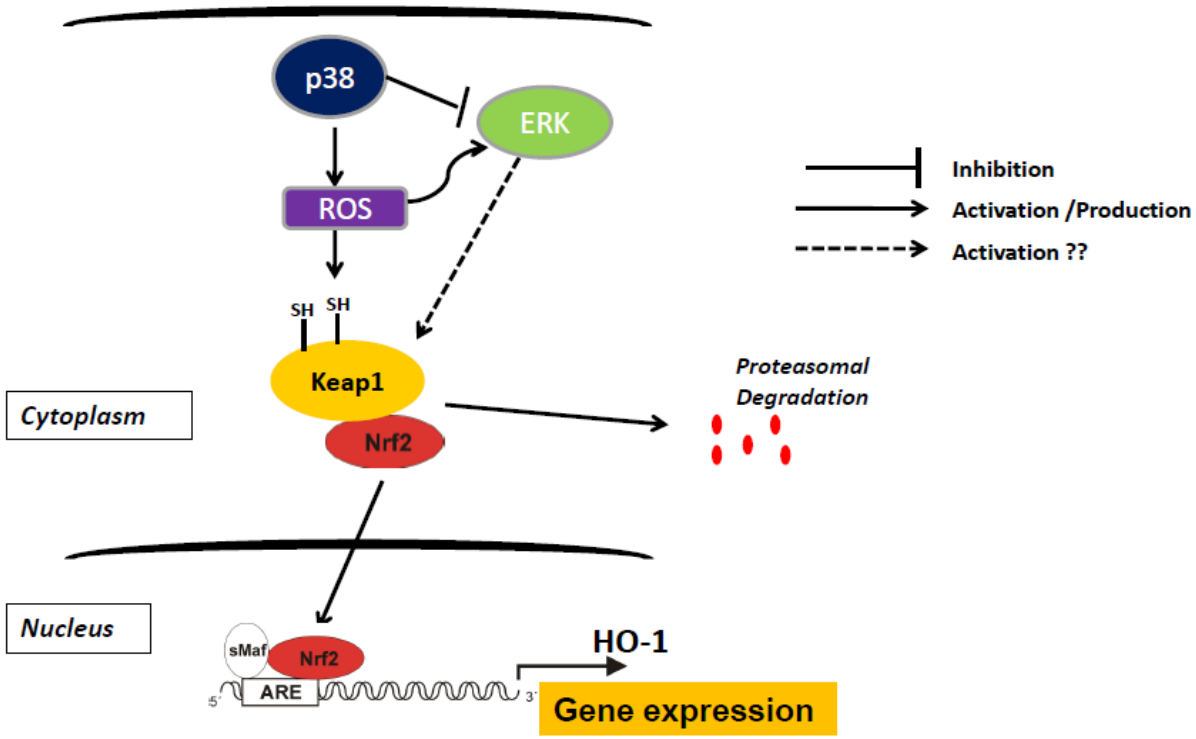


Figure 31: Inhibition of p38 activates HO-1 gene expression

Inhibition or genetic deficiency of p38α MAPK mediates the generation of ROS which leads to the activation of Nrf2. Nrf2 translocates to the nucleus binds to the ARE elements present on HO-1 gene promoter and induce the transcription of the HO-1 gene. p38α MAPK inhibition also leads to an increase in ERK activity which may play a role in this regulatory pathway. Nrf2: nuclear factor erythroid 2-related factor E2; ARE: antioxidant response element; Keap: Kelch ECH associating protein; ROS: reactive oxygen species; sMaf: small Maf protein.

4.5 Physiological significance

4.5.1 Significance of Btk-dependent HO-1 regulation in innate and adaptive immune responses

Btk mutations in humans lead to X-linked agammaglobulinemia (XLA), which is a rare genetic immunological disorder (Vetrie et al. 1993) in which the B-cells are

not capable to produce antibodies. The phenotype observed in humans can be simulated successfully in mice by introducing a point mutation (R28C) in or by targeted disruption of the murine Btk gene (Vetrie et al. 1993; Khan et al. 1995) leading to X-linked immunodeficiency (XID). Thus, the blockage of the HO-1 gene induction in LPS-stimulated Btk-/- macrophages suggests that Btk-associated immunological defects may be partially caused by the immunomodulatory properties of HO-1. This assumption would correspond with a recent finding that a myeloid cell-specific knockout of the HO-1 gene impaired immunoregulatory functions (Tzima et al. 2009). The IFN-ß signaling pathways were severely impaired in these animals, leading to pathological inflammatory responses in experimental viral and bacterial infections and in an experimentally induced autoimmune encephalomyelitis. The assumption that decreased HO-1 inducibility in TLR ligand-stimulated macrophages may have profound effects in various immune responses also corresponds with one study revealing an impairment of TLR-dependent signaling in antigen-presenting cells in patients with XLA (Sochorova et al. 2007; Taneichi et al. 2008). Finally, Btk-mediated induction of the HO-1 gene may help in the resolution of inflammation by regulating the activation of TLR-stimulated macrophages.

This notion is supported by findings of Nakahira and colleagues who demonstrated that LPS-dependent production of CO, the by product of the HO-1 enzyme reaction forms a negative feedback loop that regulates the translocation of TLRs to lipid rafts in macrophages (Nakahira et al. 2006). Finally, an interconnection between Btk and HO-1 may also be important for the regulation of apoptosis. As mentioned earlier, HO-1 is known to have potent anti-apoptotic effects, and in earlier reports it was shown that Btk knockout macrophages exhibited an increased rate of apoptosis (Mangla et al. 2004) which might indeed

partly be caused by a decreased expression of HO-1. Because of the functional relevance of Btk and HO-1 in inflammation, targeted modulation of the related signaling pathways in myeloid/macrophage cells may help to develop novel therapeutic strategies in the treatment of inflammatory diseases. In conclusion, the current study reveals that the increase in HO-1 abundance in TLR-stimulated macrophages needs the activation of the Tec family kinase Btk. Due to the vital role played by myeloid/macrophage cells in inflammation, the present findings provide further insight into the complex signaling pathways regulating the anti-inflammatory HO-1 gene.

4.5.2 Physiological significance of HO-1 upregulation mediated by inhibition or genetic deficiency of p38 MAPK

HO-1 is known to have potent anti-apoptotic functions (Ryter et al. 2006), increased HO-1 expression levels in p38-/- MEF may partially be involved in the observation that p38α deficiency makes these cells more resistant to apoptosis via induction of anti-apoptotic genes (Porras et al. 2004). It is also noteworthy that degradation of p38α in endothelial cells have been connected with the anti-apoptotic role of HO-1 (Silva et al. 2006). In summary these findings suggest an important relation between HO-1 and p38 regulation. The present thesis revealed an activation of the HO-1 gene via pharmacological inhibition or genetic deficiency of p38 MAPK. These findings provide new insights into the regulatory mechanisms of the HO-1 gene expression in macrophages before and after they encounter an inflammatory signal.

Targeted over expression of HO-1 or induction by potential new drugs might open major potentials for the treatment of inflammatory disorders. The salutary effects of specific over expression of HO-1 either by pharmacological induction or by genetic approaches has been shown in experimental models of various

inflammatory disorders such as arthritis, encephalomyelitis and hepatitis (Ryter et al. 2006; Vijayan et al. 2010). Furthermore, targeted over expression of HO-1 in macrophages has also been shown to protect from ischemia-reperfusion injury in experimental mouse models of liver and kidney transplantation (Ferenbach et al. 2010; Ke et al. 2010).

4.6 Conclusion

Unfortunately, the signaling mechanisms leading to the induction of the HO-1 expression are highly complex, stimuli and cell-type specific. Although HO-1 is highly promising as a therapeutic target for intervening inflammation, our current knowledge on the complete properties and regulation of the gene HO-1 still too limited and critical questions remain to be answered before new clinical strategies and drug-induced activation of the HO-1 gene might be available for severe inflammatory diseases, such as sepsis (Vijayan et al. 2010).

5. Summary

Monocytes or macrophages are crucial regulators of inflammation. Intracellular signaling in macrophages is tightly regulated to ensure that they do not undergo excessive activation, which may lead to chronic inflammation accompanied with potential damage to the host tissue. Induction of HO-1 in macrophages has been shown to have potent immunomodulatory properties.

In this thesis two different signalling mechanisms are described which lead to the induction of HO-1 in macrophages. In the first part the signalling pathway leading to the induction of HO-1 via toll-like receptor (TLR)4 by the classical pro-inflammatory stimulus lipopolysaccharide (LPS) is described. The second part of this thesis deals with the induction of HO-1 caused by the inhibition of the mitogen activated protein kinase (MAPK) p38.

Induction of HO-1 by LPS in macrophages has been described earlier, but the exact regulatory mechanisms of this pathway are not well understood. In this thesis it is shown that Bruton's tyrosine kinase (Btk) mediates the LPS-induction of HO-1. LPS-dependent induction of HO-1 was blocked in macrophages treated with the Btk inhibitor, LFM-A13 or in $Btk^{-/-}$ alveolar macrophages. Promoter studies and quantitative real time PCR studies revealed a transcriptional regulatory mechanism. Btk was shown to mediate the production of ROS and activation of the transcription factor Nrf2.

p38 MAPK inhibition is shown to increase the expression of HO-1. This was rather surprising, because activation of p38 MAPK has earlier been shown to mediate HO-1 induction caused by various stimuli. This increase in HO-1 expression was also observed in $p38\alpha^{-/-}$ mouse embryonic fibroblasts. Further

analysis revealed that p38 inhibition leads to an increased production of reactive oxygen species (ROS) and activation of Nrf2. Furthermore, ERK MAPK was also shown to be involved in this pathway.

Taken together, this thesis demonstrates that signaling to HO-1 in macrophages is primarily mediated by the transcription factor Nrf2. Further studies to unravel the regulation of this gene may help to develop novel strategies for clinical intervention in inflammatory disorders.

6. Zussamenfassung

Monozyten und Makrophagen spielen eine bedeutsame Rolle für die Regulation von Entzündungsreaktionen. Die intrazelluläre signaltransduktion in Makrophagen wird streng reguliert, um eine überschießende Aktivierung dieser Zellen zu vermeiden, die zu einer chronischen Entzündung mit möglicher Gewebeschädigung führen könnte. Für die Induktion der Hämoxygenase (HO)-1 in Makrophagen konnte eine wirksame immunmodulatorische Wirkung gezeigt werden.

In der vorliegenden Arbeit werden zwei verschiedene Signalübertragungwege beschrieben, die zu einer Induktion der HO-1 in Makrophagen führen. Im ersten Teil der Arbeit ist ein Signalweg beschrieben, der zu einer Induktion der HO-1 über eine Aktivierung von Toll-like Rezeptor (TLR)-4 durch den klassischen pro-inflammatorischen Stimulus Lipopolysaccharid (LPS) führt. Der zweite Teil der Arbeit beschäftigt sich mit der Induktion der HO-1 durch die Inhibition der Mitogen-aktivierten Proteinkinase (MAPK) p38.

Die Induktion der HO-1 durch LPS wurde bereits früher erstmals beschrieben, aber die genauen regulatorischen Mechanismen sind nicht genau bekannt. In der vorliegenden Arbeit wurde gezeigt, dass die Bruton-Tyrosinkinase (Btk) die LPS-abhängige Induktion der HO-1 vermittelt. Die HO-1 Induktion durch LPS wurde durch Behandlung von Makrophagen mit dem Btk Inhibitor LFM-A13 oder in $Btk^{-/-}$ Alveolarmakrophagen blockiert. Promoterstudien und quantitative *real time* PCR Studien zeigten einen zugrundeliegenden transkriptionalen Mechanismus.

Weiterhin konnte gezeigt werden, dass die Inhibition der p38 MAPK die HO-1 Expression heraufreguliert. Dieser Befund war unerwartet, weil in früheren Untersuchungen gezeigt worden war, dass p38 Aktivierung die Induktion der

HO-1 vermittelt hatte. Diese HO-1 Induktion konnte auch in $p38^{-/-}$ murinen embryonalen Fibroblasten beobachtet werden. Weitere Untersuchungen zeigten, dass die Inhibition von p38 zu einer gesteigerten Produktion von reaktiven Sauerstoffspezies (ROS) und einer Aktivierung von Nrf2 führte. Außerdem konnte gezeigt werden, dass die MAPK ERK an dieser Regulation beteiligt war.

Zusammenfassend zeigt diese Arbeit, dass die signaltransduktion zum HO-1 Gen in Makrophagen in erster Linie durch den Transkriptionsfaktor Nrf2 vermittelt wird. Weitere Studien zur Aufklärung der genauen Regulation dieses Genes könnten dabei helfen, neue Behandlungsstrategien bei der entzündlichen Erkrankungen zu entwickeln.

7. Appendix

Plasmids

BtkDNK430R and BtkDNR28C:

These Btk dominant negative expression vectors express Btk proteins containing mutations which make them non-functional. These plasmid vectors were a generous gift from Dr. Sarah Doyle (Trinity College, Dublin, Ireland). The point mutations, resulting in either the dominant negative (K430R) or the Xid (R28C) version of the Btk protein, were introduced and the sequence was cloned into a pCDNA3 expression vector (Jefferies et al. 2003).

pAP-1 luc:

pAP-1 luciferase construct (AP-1-Luc) was a generous gift from Dr. Craig A. Hauser (The Burnham Institute, La Jolla, CA). This plasmid was generated by cloning three AP-1 repeats into a luciferase reporter gene containing a minimal Fos promoter (Galang et al. 1994).

pARE luc:

pARE-luc contains a copy of the antioxidant response element and was a generous gift from Dr. William E. Fahl (University of Wisconsin, Madison, WI, USA). It is a TI-luciferase based plasmid which was constructed with either double-stranded oligonucleotide or PCR products inserted between the MluI and BglII sites of the TI luciferase vector (Wasserman and Fahl 1997).

pE2 luc:

pE2-luc was a generous gift from Dr. Jawed Alam (Alton Ochsner Medical Center, New Orleans, LS). This plasmid was generated by transferring the HO-1 enhancer region containing the stress response element and minimal promoter

sequences from the corresponding chloramphenicol acetyltransferase reporter gene construct pMHO1catD-44 1 AB1 (used in an earlier study), into the luciferase reporter plasmid pSKluc (Alam et al. 2000).

pHO-4045 luc:

HO-1 luciferase reporter plasmid contains the proximal 4045bp of the mouse HO-1 promoter and was a generous gift from Dr. Mark A. Perrella (Harvard Medical School, Boston, MS, USA). It was constructed by amplification of HO-1-(–4045/+74) from the mouse genomic DNA by PCR and then subcloned into the pGL2 luciferase reporter vector (Chung et al. 2005).

pTNF-585-luc:

This plasmid contains the proximal region of the TNF-alpha promoter which consists of two NF-kB elements (Wilson et al. 1997). This plasmid was a generous gift from Dr. Gordon Duff (University of Sheffield, Sheffield, UK) which was subcloned into a luciferase reporter construct in our lab.

The other plasmids used in this thesis were purchased from companies as mentioned in the Materials and Methods. The details regarding the plasmids can be found on the respective company website.

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9. Index of abbreviations

AP-1	activator protein-1
ARE	antioxidant response element
bZIP	basic leucine zipper
Btk	Bruton's tyrosine kinase
Cox-2	cyclooxygenase-2
DCFDA	dichlorofluorescein diacetate
DHE	dihydroethidium
DMEM	Dulbecco's modified Eagle's medium
ERK	extracellular signal-regulated kinase
FCS	fetal calf serum
GAPDH	glyceraldehydes-3-phosphate dehydrogenase
HO	heme oxygenase
IκB	inhibitor of NF-κB
JNK	c-jun N-terminal kinase
LFM-A13	α-Cyano-β-hydroxy-β-methyl-N-(2,5-dibromophenyl)propenamide
LPS	lipopolysaccharide
MAPKs	mitogen activated protein kinases
MEF	mouse embryonic fibroblast(s)
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF-κB	nuclear factor-κB
NO	nitric oxide
Nrf2	nuclear factor-erythroid-2 related factor 2
PBS	phosphate buffered saline
PMA	phorbol myristate acetate
Prx	peroxiredoxin
ROS	reactive oxygen species
RT	room temperature
SDS	sodium dodecyl sulfate
StRE	stress response element
TLR	toll-like receptor
TNF-α	tumor necrosis factor-α

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PUBLICATIONS:

1.Bruton's tyrosine kinase is required for TLR-dependent heme oxygenase-1 gene activation via Nrf2 in macrophages. Vijayan V, Baumgart-Vogt E, Naidu S, Qian G, Immenschuh S. J Immunol. 2011 Jul 15;187(2):817-27.

Abstract

Heme oxygenase (HO)-1 is the inducible isoform of the rate-limiting enzyme of heme degradation and provides cytoprotection against oxidative stress by its products carbon monoxide and biliverdin. More recently, HO-1 has also been shown to exert immunomodulatory functions via cell type-specific anti-inflammatory effects in myeloid/macrophage cells. In the current study, it is demonstrated that Bruton's tyrosine

kinase (Btk), the gene of which is mutated in the human immunodeficiency X-linked agammaglobulinemia, is involved in the upregulation of HO-1 gene expression via TLR signaling in macrophages. The specific Btk inhibitor LFM-A13 blocked HO-1 induction by the classical TLR4 ligand LPS in cell cultures of RAW264.7 monocytic cells and primary mouse alveolar macrophages. Moreover, upregulation of HO-1 gene expression was abrogated in LPS-stimulated alveolar macrophages from Btk(-/-) mice. Transfection studies with luciferase reporter gene constructs demonstrated that LPS-dependent induction of HO-1 promoter activity was attenuated by pharmacological Btk inhibition and by an overexpressed dominant-negative mutant of Btk. This induction was mediated by the transcription factor Nrf2, which is a master regulator of the antioxidant cellular defense. Accordingly, nuclear translocation of Nrf2 in LPS-treated macrophages was reduced by Btk inhibition. The generation of reactive oxygen species, but not that of NO, was involved in this regulatory pathway. Btk-dependent induction of HO-1 gene expression was also observed upon macrophage stimulation with ligands of TLR2, TLR6, TLR7, and TLR9, suggesting that Btk is required for HO-1 gene activation by major TLR pathways.

2.Heme oxygenase-1 as a therapeutic target in inflammatory disorders of the gastrointestinal tract. Vijayan V, Mueller S, Baumgart-Vogt E, Immenschuh S. World J Gastroenterol. 2010 Jul 7;16(25):3112-9. Review

Abstract

Heme oxygenase (HO)-1 is the inducible isoform of the first and rate-limiting enzyme of heme degradation. HO-1 not only protects against oxidative stress and apoptosis, but has received a great deal of attention in recent years because of its potent anti-inflammatory functions. Studies with HO-1 knockout animal models have led to major advances in the understanding of how HO-1 might regulate inflammatory immune responses, although little is known on the underlying mechanisms. Due to its beneficial effects the targeted induction of this enzyme is considered to have major therapeutic potential for the treatment of inflammatory disorders. This review discusses current knowledge on the mechanisms that mediate anti-inflammatory protection by HO-1. More specifically, the article deals with the role of HO-1 in the pathophysiology of viral hepatitis, inflammatory bowel disease, and pancreatitis. The effects of specific HO-1 modulation as a potential therapeutic strategy in experimental cell culture and animal models of these gastrointestinal disorders are summarized. In conclusion, targeted regulation of HO-1 holds major promise for future clinical interventions in inflammatory diseases of the gastrointestinal tract.

3.Inhibition and genetic deficiency of p38 MAPK up-regulates heme oxygenase-1 gene expression via Nrf2. Naidu S, Vijayan V, Santoso S, Kietzmann T, Immenschuh S.J Immunol. 2009 Jun 1;182(11):7048-57.

Abstract

Heme oxygenase (HO)-1 is the inducible isoform of the first and rate-limiting enzyme of heme degradation. The HO products carbon monoxide and bilirubin not only provide antioxidant cytoprotection, but also have potent anti-inflammatory and immunomodulatory functions. Although HO-1 has previously been shown to be induced by various stimuli via activation of the p38 MAPK signaling pathway, the role of this protein kinase for HO-1 gene regulation is largely unknown. In the present study, it is demonstrated that pharmacological inhibitors of p38 induced HO-1 expression in monocytic cells. Moreover, basal HO-1 gene expression levels were markedly higher in untreated murine embryonic fibroblasts (MEF) from p38alpha(-/-) mice compared with those from wild-type mice. Transfection studies with

luciferase reporter gene constructs indicate that increased HO-1 gene expression via inhibition of p38 was mediated by the transcription factor Nrf2, which is a central regulator of the cellular oxidative stress response. Accordingly, inhibitors of p38 induced binding of nuclear proteins to a Nrf2 target sequence of the HO-1 promoter, but did not affect HO-1 protein expression and promoter activity in Nrf2(-/-) MEF. Genetic deficiency of p38 led to enhanced phosphorylation of ERK and increased cellular accumulation of reactive oxygen species. In addition, pharmacological blockage of ERK and scavenging of reactive oxygen species with N-acetylcysteine reduced HO-1 gene expression in p38(-/-) MEF, respectively. Taken together, it is demonstrated that pharmacological inhibition and genetic deficiency of p38 induce HO-1 gene expression via a Nrf2-dependent mechanism in monocytic cells and MEF.

SKILL PROFILE;

- Knowledge of biological techniques such as cell culture, isolation of alveolar, peritoneal and bone marrow derived macrophages, immunocytochemistry, Western blot, molecular biology (mainly PCR), Transient transfection and luciferase reporter assays, confocal microscopy (basic), fluorescent microscopy, flowcytometry.

Scientific Presentations:

-Abstract selected for a talk and poster presentation at the *Annual meeting of American Association of Immunologist*, 2011, San Fransisco, U.S.A.
-Abstract selected for poster at the *Annual meeting of Signal Transduction Society*, 2011, Weimar, Germany.
-Abstract selected for poster presentation Arbeitstagung der *Anatomischen Geselleschaft*, 2011, Wurzburg, Germany.
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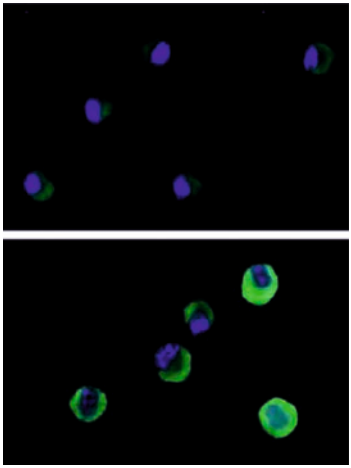

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