p38 α MAPK inhibits JNK activation and collaborates with I κ B kinase 2 to prevent endotoxin-induced liver failure

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Activation of c-Jun amino-terminal kinase (JNK) facilitates tumour necrosis factor (TNF)-induced cell death. The p38 mitogen-activated protein kinase pathway is induced by TNF stimulation, but it has not been implicated in TNF-induced cell death. Here, we show that hepatocyte-specific ablation of p38 α in mice results in excessive activation of JNK in the liver after *in vivo* challenge with bacterial lipopolysaccharide (LPS). Despite increased JNK activity, p38 α -deficient hepatocytes were not sensitive to LPS/TNF toxicity showing that JNK activation was not sufficient to mediate TNF-induced liver damage. By contrast, LPS injection caused liver failure in mice lacking both p38 α and I κ B kinase 2 (IKK2) in hepatocytes. Therefore, when combined with partial nuclear factor- κ B inhibition, p38 α deficiency sensitizes the liver to cytokine-induced damage. Collectively, these results reveal a new function of p38 α in collaborating with IKK2 to protect the liver from LPS/TNF-induced failure by controlling JNK activation.

Keywords: cytokine signalling; liver failure; p38; JNK; NF- κ B

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INTRODUCTION

On binding to its receptors, tumour necrosis factor (TNF) regulates several intracellular signalling cascades that influence cell survival and death (Wajant *et al*, 2003). Activation of the nuclear factor- κ B (NF- κ B) pathway protects cells from TNF-induced apoptosis by inducing the expression of anti-apoptotic proteins (Hayden & Ghosh, 2004). Sustained c-Jun amino-terminal kinase (JNK) activation is important for TNF-induced killing of NF- κ B-deficient cells, by acting through the ubiquitin ligase ITCH to induce proteasome-dependent degradation of cellular FLICE inhibitory protein (c-FLIP; Chang *et al*, 2006). The mechanisms regulating TNF-induced death are particularly relevant for the liver, as TNF signalling regulates hepatocyte survival and proliferation, and is implicated in liver failure (Schwabe & Brenner, 2006). After *in vivo* administration of lipopolysaccharide (LPS), which acts as a potent inducer of endogenous TNF and other cytokines that can cause liver damage (Pfeffer *et al*, 1993; Pasparakis *et al*, 1996), NF- κ B signalling is essential to protect hepatocytes from TNF-induced death (Schwabe & Brenner, 2006; Luedde *et al*, 2007).

TNF stimulation also activates the p38 mitogen-activated protein kinase (MAPK) pathway, which regulates cellular responses to stress and is implicated in cell proliferation, differentiation and apoptosis (Nebreda & Porras, 2000; Schieven, 2005). Recently, p38 α has been suggested to influence chemical carcinogenesis in the liver (Hui *et al*, 2007); however, it remains unclear whether p38 signalling has a function in LPS/TNF-induced hepatocyte death. Mice lacking p38 α —the main isoform of the p38 MAPK family—die *in utero* (Adams *et al*, 2000; Allen *et al*, 2000; Mudgett *et al*, 2000; Tamura *et al*, 2000); therefore, we generated mice with liver parenchymal cell-specific ablation of p38 α by using Cre/loxP-mediated gene targeting to study the function of the p38 MAPK pathway in the liver. Here, we show that p38 α collaborates with the NF- κ B pathway to protect hepatocytes from TNF-induced death by controlling JNK activation.

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RESULTS AND DISCUSSION

Conditional ablation of p38 α in the liver

To study the cell-specific function of p38 α *in vivo*, we generated mice carrying conditional loxP-flanked p38 α (p38 α^{FL}) alleles (Fig 1A). p38 $\alpha^{FL/FL}$ mice expressed normal levels of p38 α and did not show any apparent abnormalities. To generate mice with liver parenchymal cell-specific p38 α deficiency (p38 α^{LPC-KO}), we crossed mice carrying p38 α^{FL} alleles with Alfp-Cre transgenic mice (Kellendonk *et al*, 2000). p38 α^{LPC-KO} mice were born at the expected Mendelian ratio, were viable and fertile, and did not show signs of hepatic alterations (data not shown).

Immunoblot analysis showed efficient ablation of p38 α in the liver of p38 α^{LPC-KO} mice (Fig 1B,E; supplementary Fig 1); however, some expression of p38 α was retained owing to the presence of non-parenchymal cells such as Kupffer and endothelial cells that are not targeted by the Alfp-Cre transgene. The expression levels of p38 δ were not changed in the liver of p38 α^{LPC-KO} mice compared with wild-type mice, whereas p38 β expression was not detected in these tissues (supplementary Fig 1 online), showing that there was no compensatory upregulation of these isoforms in the absence of p38 α . To examine the function of the p38 MAPK pathway in cytokine-induced signalling in the liver, we injected groups of p38 α^{LPC-KO} and littermate control mice with LPS. For all experiments described here, we used littermates carrying loxP-flanked p38 α alleles but lacking expression of Cre recombinase as wild-type controls. Measurement of serum liver aminotransferases, which are rapidly released in the circulation as a result of liver damage, did not show significant differences between LPS-injected p38 α^{LPC-KO} mice and control mice (Fig 1C). Moreover, TdT-mediated dUTP nick end labelling (TUNEL) of liver sections and biochemical assessment of caspase 3 activation in liver extracts did not show increased hepatocyte apoptosis in p38 α^{LPC-KO} mice compared with controls (Fig 1D,E). By contrast, as shown previously (Luedde *et al*, 2007), mice lacking the I κ B kinase (IKK) subunit NF- κ B essential modulator (NEMO, also known as IKK γ) in hepatocytes (NEMO $^{LPC-KO}$ mice), which show complete inhibition of canonical NF- κ B signalling in the liver, showed increased hepatocyte apoptosis and liver failure on administration of the same dosage of LPS (Fig 1D,E). Thus, in contrast to complete inhibition of the NF- κ B pathway, genetic disruption of p38 α in hepatocytes does not sensitize the liver to TNF-induced toxicity.

Increased activation of JNK in p38 α -deficient livers

Sustained activation of JNK has been shown to be essential for TNF-induced killing of NF- κ B-deficient liver cells (Kamata *et al*, 2005; Chang *et al*, 2006). Thus, we investigated the status of JNK activation in p38 α -deficient and control livers after LPS injection. Surprisingly, JNK activation was increased in livers from p38 α^{LPC-KO} mice compared with control mice after LPS administration (Fig 2A). The activation of JNK observed in p38 α -deficient livers was much stronger than that seen in NEMO-deficient livers (compare Fig 2A and B). Consistent with JNK activation, c-Jun phosphorylation was also induced in livers from p38 α^{LPC-KO} mice after LPS injection (Fig 2C). Although p38 α ablation leads to massively increased and prolonged JNK activation after LPS injection, it does not sensitize the liver to LPS-induced toxicity, showing that even high levels of sustained JNK activation downstream from TNF signalling are not sufficient to induce

death of hepatocytes. To assess whether increased activation of pro-survival pathways could compensate for the increased JNK activity in p38 α -deficient livers, we measured activation of NF- κ B, extracellular signal-regulated kinase (ERK) and AKT. Activation of NF- κ B, shown by immunoblot analysis of I κ B α phosphorylation and degradation, and by electrophoretic mobility shift assay measurement of nuclear DNA binding to NF- κ B dimers, was similar in the livers of p38 α^{LPC-KO} and control mice (supplementary Fig 2A,B online). Moreover, immunoblot analysis with phospho-specific antibodies showed that activation of ERK and AKT was slightly decreased in p38 α -deficient livers 60 min after LPS injection (supplementary Fig 2A,C online). Therefore, we could not detect substantially increased activation of pro-survival pathways correlating with the elevated JNK activity in the liver of LPS-challenged p38 α^{LPC-KO} mice.

To investigate further why TNF does not kill p38 α -deficient hepatocytes despite the strong activation of JNK, we examined the effects of p38 α ablation on downstream mediators of JNK function. JNK has previously been shown to facilitate TNF-induced apoptosis by promoting the proteasome-mediated degradation of the long c-FLIP isoform c-FLIP(L), an inhibitor of caspase 8 the expression of which is regulated by NF- κ B (Micheau *et al*, 2001; Chang *et al*, 2006). Immunoblot analysis showed a slight downregulation of the levels of c-FLIP(L) protein 4 h after LPS injection in livers from p38 α^{LPC-KO} mice compared with controls (Fig 2D). However, 10 h after LPS injection the levels of c-FLIP(L) were similar in p38 α^{LPC-KO} and control mice (Fig 2D). By contrast, the levels of c-FLIP(L) were diminished in NEMO-deficient livers 10 h after LPS injection (Fig 2D, lanes 7–9), which is consistent with the increased hepatocyte apoptosis detected at this point. These results show that even a massive activation of JNK is not sufficient to reduce intracellular levels of c-FLIP(L) to below the threshold required to sensitize hepatocytes to TNF-induced death. By contrast, even a moderate prolongation of JNK activity results in degradation of c-FLIP(L) and apoptosis of hepatocytes with defective activation of NF- κ B.

Activation of MKK4 and MKK3/6 in p38 α -deficient livers

In NF- κ B-deficient hepatocytes, the induction of sustained JNK activation by TNF is caused by the accumulation of reactive oxygen species that lead to the oxidation of JNK phosphatases (Kamata *et al*, 2005). However, analysis of the activity of JNK phosphatases in the livers of p38 α^{LPC-KO} mice did not show considerable differences when compared with control mice (data not shown). Moreover, administration of the antioxidant compound butylated hydroxyanisole did not lead to a substantial reduction of LPS-induced JNK activation in p38 α^{LPC-KO} mice (Fig 3A), suggesting that the mechanism leading to increased JNK activation in p38 α -deficient livers does not depend on oxidative stress. In addition, immunoblot analysis of the expression of a panel of phosphatases that have previously been reported to dephosphorylate JNK showed no differences between wild-type and p38 α -deficient livers (supplementary Fig 3 online).

Two upstream kinases control phosphorylation of JNK, of which MAP kinase kinase (MKK)7 specifically acts on JNK, and is activated by TNF and environmental stress, whereas MKK4 can phosphorylate both JNK and p38 MAPK, and is normally not activated by TNF (Derijard *et al*, 1995; Doza *et al*, 1995; Lin *et al*, 1995; Moriguchi *et al*, 1997; Tournier *et al*, 1999). It was recently

Fig 1 | p38 α ^{LPC-KO} mice are not sensitive to LPS-induced liver failure. (A) Schematic description of the targeting strategy for the generation of mice with loxP-flanked p38 α alleles. Filled boxes indicate the loxP-flanked exons 2 and 3 (E2, E3), which include the ATP-binding site of the kinase domain. B, *Bam*HI; H, *Hind*III. Black arrowheads indicate loxP sites; white arrows indicate FLP recombinase target (Frt) sites. (B) Immunoblot analysis of expression of p38 α in liver extracts from wild-type (WT) and p38 α ^{LPC-KO} mice. (C–E) Assessment of liver damage in p38 α ^{LPC-KO} and control mice after LPS injection. (C) Levels of free circulating alanine aminotransferases (ALTs) were measured in the serum of p38 α ^{LPC-KO} and control mice at the indicated time points after LPS injection. Error bars denote s.e.m. (n = 4). (D) Detection of apoptotic cells by TUNEL assay in livers from WT, p38 α ^{LPC-KO} and NEMO^{LPC-KO} (used as a positive control) mice 10 h after LPS injection. (E) Immunoblot analysis of caspase 3 activation using an antibody that specifically detects the cleaved form. DAPI, 4,6-diamidino-2-phenylindole; LPS, lipopolysaccharide; TUNEL, TdT-mediated dUTP nick end labelling.

suggested that mouse embryonic fibroblast (MEF) cells lacking p38 α expression show increased phosphorylation of MKK7 (Hui *et al*, 2007). Assessment of the activation of MKK4 and MKK7 using immunoblot analysis with antibodies specifically recognizing their phosphorylated forms showed increased phosphorylation of MKK4, but not of MKK7, in the liver of p38 α ^{LPC-KO} mice compared with wild-type mice after LPS injection (Fig 3B). Further analysis showed increased phosphorylation of the p38 α -activating kinases MKK3/6 in livers from LPS-injected p38 α ^{LPC-KO} mice (Fig 3C). Taken together, these findings suggest that, in hepatocytes—in contrast to MEFs—p38 α controls LPS/TNF-induced JNK activation by inhibiting MKK4 activation, indicating that the role of p38 α in controlling MAPK activation might be cell and/or stimulus specific. At this stage, the mechanisms by which p38 α controls MKK4 and MKK3/6 activity in the liver after LPS

stimulation remain unclear. However, the fact that MKK4, MKK3 and MKK6 are all kinases able to activate p38 (Dong *et al*, 2002) suggests that p38 α activity might be required to negatively regulate its upstream kinases in response to LPS signalling. In this scheme, the increased activation of JNK in p38 α -deficient cells might be a ‘side effect’ caused by the double function of MKK4 in activating both p38 and JNK.

p38 α and IKK2 collaborate to prevent liver failure

We considered that p38 α -deficient livers were not sensitive to LPS/TNF challenge despite massive JNK activation because the NF- κ B-dependent transcriptional induction of anti-apoptotic genes such as c-FLIP(L) was sufficient to prevent TNF-induced apoptosis of hepatocytes lacking p38 α . To test this hypothesis, we used mice with liver parenchymal cell-specific ablation of IKK2

Fig 2 | Increased activation of JNK in the liver of p38 α ^{LPC-KO} mice by LPS injection. (A) Phosphorylation of JNK was assessed in liver extracts from wild-type (WT) and p38 α ^{LPC-KO} mice by immunoblot analysis using phospho-JNK-specific antibodies (upper panel). JNK immunoblot acts as a loading control. (B) Immunoblot analysis of phosphorylation of JNK in NEMO^{LPC-KO} and control (WT) mice. (C) Immunoblot analysis with antibodies recognizing phosphorylated c-Jun (upper panel), total c-Jun (middle panel) and tubulin (lower panel) as a loading control. (D) The levels of c-FLIP(L) in WT, p38 α ^{LPC-KO} and NEMO^{LPC-KO} mice were analysed by immunoblot at the indicated time points after LPS injection. JNK, c-Jun amino-terminal kinase; LPS, lipopolysaccharide.

Fig 3 | Hyperphosphorylation of JNK in the liver of LPS-injected p38 α ^{LPC-KO} correlates with increased activation of MKK4. (A) Wild-type (WT; lanes 1–3), p38 α ^{LPC-KO} (lanes 4–6) and p38 α ^{LPC-KO} mice that had been pretreated with the antioxidant compound BHA (lanes 7 and 8) were injected with LPS and killed at the indicated time points. Immunoblot analysis was carried out on extracts of liver protein using antibodies against phosphorylated JNK or total JNK proteins. (B) Immunoblot analysis of the phosphorylation status of the JNK-activating kinases MKK4 and MKK7 in the liver extracts from WT and p38 α ^{LPC-KO} mice at the indicated time points after LPS injection. Antibodies recognizing phosphorylated or total MKK7 or MKK4 were used. Tubulin acts as a loading control. n.s. indicates a nonspecific band. (C) Immunoblot analysis using an antibody detecting the phosphorylated forms of MKK3 and MKK6 (upper panel). Immunoblots with antibodies against total MKK3 and tubulin (lower panels) act as a loading control. BHA, butylated hydroxyanisole; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; MKK, mitogen-activated protein kinase.

(IKK2^{LPC-KO}), which show only partial inhibition of NF- κ B activation and are not sensitive to LPS/TNF-induced liver failure, in contrast to NEMO^{LPC-KO} mice that cannot activate NF- κ B in the

liver and show massive apoptosis of hepatocytes after similar challenge (Luedde *et al*, 2005, 2007). We reasoned that although p38 α and IKK2 single-mutant mice were protected

Fig 4 | p38 α collaborates with IKK2 to protect the liver from LPS-induced toxicity. (A) Immunoblot analysis for the expression of IKK2 and p38 α in the extracts of liver protein from wild-type (WT), p38 α ^{LPC-KO} and p38 α /IKK2^{LPC-KO} mice. (B) Levels of free circulating ALT were measured in IKK2^{LPC-KO}, p38 α /IKK2^{LPC-KO} and control (WT) mice before and 10 h after LPS injection. Error bars denote s.e.m. *Statistical significance by Student's *t*-test with *P* < 0.05 relative to control (*n* = 4). Mean values are depicted above each column. (C) Detection of apoptotic cells by TUNEL assay in liver sections from WT, IKK2^{LPC-KO} and p38 α /IKK2^{LPC-KO} mice 10 h after LPS injection. (D) Immunoblot analysis of caspase 3 activation using antibodies that specifically detect total caspase 3 (top panel) or the cleaved form of caspase 3 (middle panel) in liver extracts from mice with the indicated genotypes 10 h after LPS injection. NEMO^{LPC-KO} mice were used as a positive control. Tubulin acts as a loading control. (E) The levels of c-FLIP(L) were measured by immunoblot analysis in livers from WT, p38 α ^{LPC-KO} and p38 α /IKK2^{LPC-KO} mice at the indicated time points after LPS injection. Each lane represents an individual mouse. ALT, alanine aminotransferase; DAPI, 4,6-diamidino-2-phenylindole; IKK, I κ B kinase; LPS, lipopolysaccharide; TUNEL, TdT-mediated dUTP nick end labelling.

from LPS-induced liver failure, the combined liver parenchymal cell-specific ablation of both p38 α and IKK2 could sensitize hepatocytes to LPS/TNF challenge owing to increased JNK activation in the presence of partial NF- κ B inhibition. We thus generated mice with combined ablation of both p38 α and IKK2 in liver parenchymal cells (p38 α /IKK2^{LPC-KO} mice; Fig 4A) to test this hypothesis. *In vivo* challenge with LPS led to liver damage in p38 α /IKK2^{LPC-KO} mice but not in single-mutant mice, as shown by the analysis of serum alanine aminotransferase levels (Fig 4B). Measurement of apoptosis in the liver by TUNEL assay and by immunoblot analysis of caspase 3 cleavage showed increased cell death in p38 α /IKK2^{LPC-KO} mice compared with wild-type or p38 α and IKK2 single-mutant mice at 10 h after LPS injection (Fig 4C,D). These results show that p38 α and IKK2 collaborate to protect the liver from LPS/TNF-induced toxicity. Furthermore, immunoblot analysis showed reduced levels of c-FLIP(L) in p38 α /IKK2^{LPC-KO} double-mutant mice at 10 h after LPS injection compared with single-mutant animals (Fig 4E), suggesting that control of the levels of anti-apoptotic proteins such as c-FLIP(L) is one of the mechanisms by which p38 α and IKK2 collaborate to protect the liver from LPS/TNF-induced hepatocyte apoptosis.

Collectively, our findings uncover a new function of p38 α MAPK in the liver, where it cooperates with NF- κ B to protect

hepatocytes from LPS/TNF-induced death. p38 α mediates its protective function in the liver by controlling JNK activation through a mechanism involving the regulation of MKK4 activity. These results have important implications for our understanding of the mechanisms regulating TNF responses in the liver. Although JNK activation facilitates TNF-induced apoptosis in NF- κ B-deficient liver cells, our results show that even excessive JNK activity is not sufficient to sensitize hepatocytes with intact NF- κ B signalling to TNF toxicity. However, when p38 α deficiency is combined with the ablation of IKK2, the strong JNK activation synergizes with the partial impairment of NF- κ B activity to sensitize the liver to endotoxin-induced failure (Fig 5).

METHODS

Generation of conditional knockout mice. Mice with loxP-flanked p38 α alleles were generated by homologous recombination in C57Bl/6-derived ES cells (Bruce-4) using the targeting strategy shown in Fig 1A. p38 α ^{FL}, NEMO^{FL} (Schmidt-Suppran *et al*, 2000) and IKK2^{FL} mice (Pasparakis *et al*, 2002) were crossed with Alfp-Cre transgenic mice (Kellendonk *et al*, 2000) to generate liver parenchymal cell-specific knockout of the respective gene (p38 α ^{LPC-KO}, NEMO^{LPC-KO} and IKK2^{LPC-KO} mice). p38 α /IKK2 double-mutant mice were generated by crossing p38 α ^{LPC-KO} mice

Fig 5 | p38 α collaborates with IKK2 to prevent liver failure in response to *in vivo* LPS/TNF challenge. TNF binding to TNFR1 induces the activation of NF- κ B and MAPK pathways, but it can also induce cell death through the activation of caspase 8. Activation of NF- κ B protects cells from TNF-induced cell death by inducing the expression of anti-apoptotic proteins such as c-FLIP. Activation of JNK induces the E3 ubiquitin ligase ITCH to ubiquitinate c-FLIP leading to its degradation. Lack of p38 α in hepatocytes leads to hyperactivation of MKK3/6, MKK4 and JNK on *in vivo* LPS challenge. The increased sustained activation of JNK is not sufficient to induce cell death in the p38 α -deficient liver. When p38 α ablation is combined with moderate inhibition of NF- κ B, achieved by hepatocyte-restricted IKK2 ablation, *in vivo* LPS challenge results in increased degradation of c-FLIP and liver damage through caspase 8-mediated hepatocyte apoptosis. IKK, I κ B kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; NF- κ B, nuclear factor- κ B; TNF, tumour necrosis factor.

with IKK2^{FL/FL} mice (p38 α /IKK2^{LPC-KO}). All mice were kept in individual ventilated cages in the animal facilities of EMBL Monterotondo and the Institute for Genetics in Cologne. All experiments were performed according to the European Union, national and institutional guidelines.

Liver injury models. Experiments were performed on male mice between 8 and 10 weeks of age. LPS (Sigma-Aldrich Chemie GmbH, Munich, Germany) was administered i.p. at 25 μ g/10 g of body weight. For antioxidant treatment, mice were fed on a diet containing 0.7% butylated hydroxyanisole (Sigma-Aldrich) for 3 days before LPS injection.

Immunoblot analysis. Protein lysates were prepared from liver samples, separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose and analysed by immunoblotting. Membranes were probed with antibodies specific for: tubulin- α (Sigma-Aldrich), p38 α , IKK2, c-Jun, phospho-c-Jun, JNK, phospho-JNK, cleaved caspase 3, total caspase 3, phospho-MKK7, MKK7, phospho-MKK4, MKK4, MKK3, phospho-MKK3/6, p38 δ , phospho-I κ B α , phospho-AKT, AKT, phospho-ERK1/2, ERK1/2 (Cell Signaling, Danvers, MA, USA), c-FLIP (Alexis, San Diego,

CA, USA), p38 β , I κ B α , MKP1, MKP2, MKP3 (Santa Cruz Biotechnology) pp5 (BD Biosciences, Franklin Lakes, NJ, USA), pp1me (Abgent, San Diego, CA, USA), pp1a (Biozol, Eching, Germany) and pp2a (Upstate/Millipore, Schwabach, Germany). Horseradish peroxidase-conjugated anti-rabbit, anti-mouse and anti-rat secondary antibodies were used (Amersham/GE, Freiburg, Germany).

Electrophoretic mobility shift assay. Gel retardation assays were performed on nuclear extracts as described previously (Luedde et al, 2005, 2007). DNA-protein complexes were resolved on a 6% polyacrylamide gel. A ³²P-labelled oligonucleotide representing an NF- κ B consensus site (5'-CGGGCTGGGGATTCCCCA TCTCGGTAC-3') was used as a probe. For supershifts, high-concentrated antibodies against p50, p65, c-Rel and RelB (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used.

TUNEL assay. The TUNEL test was performed using the 'In situ cell death detection Kit, POD' (Roche Diagnostics, Basel, Switzerland) according to the instructions of the manufacturer.

Statistics. Results are expressed as the mean $\pm$ standard error of the mean (s.e.m.). Statistical significance between experimental groups was assessed using an unpaired two-sample Student's *t*-test.

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>)

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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