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Recommended Citation  
Gonzalez-Teran, Barbara; Cortes, Jose R.; Manieri, Elisa; Matesanz, Nuria; Verdugo, Angeles; Rodriguez, Maria E.; Gonzalez-Rodriguez, Agueda; Valverde, Angela M.; Martin, Pilar; Davis, Roger J.; and Sabio, Guadalupe, "Eukaryotic elongation factor 2 controls TNF-alpha translation in LPS-induced hepatitis" (2013). *Davis Lab Publications*. 3.  
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Eukaryotic elongation factor 2 controls TNF-alpha translation in LPS-induced hepatitis

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Citation: J Clin Invest. 2013 Jan;123(1):164-78. doi: 10.1172/JCI65124. Epub 2012 Dec 3. Link to article on publisher's site

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Bacterial LPS (endotoxin) has been implicated in the pathogenesis of acute liver disease through its induction of the proinflammatory cytokine TNF-α. TNF-α is a key determinant of the outcome in a well-established mouse model of acute liver failure during septic shock. One possible mechanism for regulating TNF-α expression is through the control of protein elongation during translation, which would allow rapid cell adaptation to physiological changes. However, the regulation of translational elongation is poorly understood. We found that expression of p38γ/δ MAPK proteins is required for the elongation of nascent TNF-α protein in macrophages. The MKK3/6-p38γ/δ pathway mediated an inhibitory phosphorylation of eukaryotic elongation factor 2 (eEF2) kinase, which in turn promoted eEF2 activation (dephosphorylation) and subsequent TNF-α elongation. These results identify a new signaling pathway that regulates TNF-α production in LPS-induced liver damage and suggest potential cell-specific therapeutic targets for liver diseases in which TNF-α production is involved.

Introduction
Infection and tissue injury trigger a protective inflammatory response that mobilizes a variety of effector mechanisms to contain and eliminate the injurious agent (1). Hepatic and circulating inflammatory cytokines initiate a positive feedback loop on the innate immune system (2); although cytokine production protects against pathogenic stimuli, it can cause more damage than the initiating event if its magnitude and duration are not strictly controlled by intrinsic negative regulators (1).

The cytokine TNF-α has been identified as a key regulator of the inflammatory response in animal models of fulminant hepatitis (3). Elevated concentrations of this proinflammatory cytokine are also found in acute and chronic inflammatory conditions, including trauma, sepsis, and rheumatoid arthritis (4). Upon binding to its receptors, TNF-α initiates several intracellular signaling cascades that influence cell survival, death, differentiation, proliferation, and migration (5, 6).

TNF-α signaling is particularly important in the liver. While it mediates hepatocyte survival and proliferation, it is also implicated in liver failure, since it triggers hepatocyte apoptosis and leads to upregulation of key adhesion molecules and chemokines involved in leukocyte migration and infiltration (6, 7). A well-established mouse model of acute liver failure and septic shock is the in vivo administration of bacterial LPS and D-galactosamine (D-gal), which strongly induce endogenous TNF-α and other cytokines that cause liver damage (8, 9).

TNF-α signaling can be mediated by protein kinases and phosphatases, in particular the MAPK family members ERK, JNK, and p38 MAPK. The p38 MAPK pathway transduces a variety of extracellular signals regulating cellular responses to stress, being implicated in cell proliferation, differentiation, and apoptosis (10, 11). Four p38 MAPK family members have been identified: p38α, p38β, p38γ, and p38δ, also known as stress-activated kinase 2 a (SAPK2a), SAPK2b, SAPK3, and SAPK4 (10, 12, 13). The p38 MAPK family can be further divided into 2 subsets, with p38α and p38β in one group and p38γ and p38δ in the other. All these kinases are activated by double phosphorylation mediated by the upstream kinases MKK3 and MKK6 (14, 15). To date, the best-characterized p38 isoform is p38α. This isoform is required for macrophage inflammatory responses (1), but in hepatocytes prevents endotoxin-induced liver damage (16, 17). Studies using inhibitors suggested that p38α and p38β cooperate in inflammatory processes; however, p38β-knockout mice show no differences in several in vivo and in vitro models of inflammation (18). Less is known about the role of the p38γ and p38δ MAPK. p38δ is known to phosphorylate eukaryotic elongation factor 2 (eEF2) kinase (eEF2K), inhibiting its activity (19). eEF2K blocks mRNA translation into protein through an inhibitory phosphorylation of eEF2.

The rate-limiting step in mRNA translation is thought to be the initiation step; however, far less is known about the regulation of the elongation step of protein biosynthesis (20). The process of peptide-chain elongation is at least partially regulated by eEF2 phosphorylation/dephosphorylation events induced by diverse stimuli (21). Moreover, the phosphorylation state of the upstream kinase eEF2K can be modulated by signaling molecules that regulate the activity of this factor, such as mTOR and MAPKs, including p38 kinases (19, 21–23). Because these kinases are activated by different stimuli, it is expected that the regulation of eEF2 will be important in cell adaptation to different physiological states. Here, we show that p38δ and p38γ expressed in myeloid cells promote eEF2 activity and, in consequence, TNF-α production and the inflammatory response. By blocking p38δ/γ activity in myeloid macrophages, we were able to suppress hepatitis caused by LPS-induced TNF-α expression.
Results
MKK3 and MKK6 collaborate in LPS-induced hepatitis. To study the role of p38 MAPK signaling in hepatitis, we examined the effect of D-gal+LPS treatment on mice deficient for the upstream kinases MKK3 and MKK6. No differences in mortality where detected in Mkk3–/– or Mkk6–/– mice compared with WT animals (Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI65124DS1). However, Mkk3–/– mice present less severe liver hemorrhage (Supplemental Figure 1B) and Mkk6–/– liver apoptosis was significantly reduced (Supplemental Figure 1C).
Figure 1C). Levels of alanine transaminase (ALT) and aspartate aminotransferase (AST), 2 well-established markers of hepatic necrosis, were not altered in Mkk3 Δ/− mice, but were significantly reduced in Mkk6 Δ/− mice, thus showing an effect on liver necrosis (Supplemental Figure 1D).

The p38 MAPK pathway is a key regulator of the expression of inflammatory cytokines, including IL-6 and TNF-α, the main determinant of LPS-stimulated liver damage (24–27). Circulating levels of TNF-α and IL-6 after D-gal+LPS injection did not differ between WT mice and the Mkk3 Δ/− or Mkk6 Δ/− mice (Supplemental Figure 2A). In vitro stimulation of isolated BM macrophages from these mice with LPS also revealed no differences in proliferation rate or released TNF-α and IL-6 levels (Supplemental Figure 2, B and C). Moreover, supernatants from WT, Mkk3 Δ/−, and Mkk6 Δ/− macrophages induce apoptosis similarly in cultured hepatocytes (not shown), thus indicating that the partial protection against LPS-induced liver apoptosis was not due to circulating macrophages.

Neutrophil infiltration can cause mild to severe liver damage (28), and p38α was recently shown to control neutrophil cytokine production (29). We examined liver myeloid leukocytes based on CD11b, Gr-1, F4/80, and Ly6C markers (Supplemental Figure 2D and ref. 30). Analysis of liver cell populations revealed that D-gal+LPS injection increased liver infiltration by neutrophils (CD11b+Gr-1hi) in WT mice, but this effect was impaired in Mkk3 Δ/− or Mkk6 Δ/− mice (Supplemental Figure 2A). In vitro stimulation of isolated BM macrophages from these mice with LPS also revealed no differences in proliferation rate or released TNF-α and IL-6 levels (Supplemental Figure 2, B and C). Moreover, supernatants from WT, Mkk3 Δ/−, and Mkk6 Δ/− macrophages induce apoptosis similarly in cultured hepatocytes (not shown), thus indicating that the partial protection against LPS-induced liver apoptosis was not due to circulating macrophages.

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Mkk3 Δ/− Mkk6 Δ/− mice are protected against LPS-induced liver failure. Mkk3 and Mkk6Δ show functional redundancy in the activation of p38 MAPK (14), and while single mutants are viable, doubly deficient Mkk3 Δ/− Mkk6 Δ/− and Mkk3 Δ/− Mkk6 Δ/− mice die early in embryogenesis. To minimize interference from redundant actions, we used Mkk3 Δ/− Mkk6 Δ/− (ΔMkk3/6) mice, which are viable (14). As expected, ΔMkk3/6 mice were protected against LPS-induced hepatotoxicity, showing milder hemorrhage and better survival than WT mice (Figure 1, A and B). Immunoblot analysis demonstrated that D-gal+LPS injection activated caspase 3 and PARP cleavage in WT liver but not in ΔMkk3/6 livers (Figure 1D). Moreover, circulating transaminases were reduced in ΔMkk3/6 mice, indicating a reduction in liver necrosis (Figure 1C). These data demonstrate that Mkk3/6 deficiency severely limits LPS-stimulated hepatic apoptosis. Examination of serum TNF-α after D-gal+LPS injection revealed that the marked increase in TNF-α concentration observed in WT and singly deficient mice was not detected in ΔMkk3/6 mice (Figure 1E). Serum IL-6 was also lower in ΔMkk3/6 mice (Figure 1E). The loss of LPS-stimulated TNF-α expression in ΔMkk3/6 mice might account for the finding that LPS activation of JNK pathway in the livers of WT mice was not seen in ΔMkk3/6 mice (Figure 1D).

Mkk3 and Mkk6 are required for neutrophil migration after LPS injection. The lower TNF-α production in ΔMkk3/6 mice could be a consequence of the lower leukocyte infiltration in liver. To address this, we analyzed myeloid cell migration after D-gal+LPS injection. Quantification of liver chemokines showed that in WT mice, LPS treatment increased mRNA expression of molecules important for migration of macrophages (MCP1 and ICAM) and neutrophils (KC, MIP2), whereas induction of these chemokines and adhesion molecules was decreased in the livers of ΔMkk3/6 mice (Figure 2A). Phenotypic characterization of liver-infiltrating leukocytes from control and D-gal+LPS–treated livers revealed a significant increase in neutrophils in the inflammatory cell infiltrate of WT mice that was impaired in ΔMkk3/6 mice (Figure 2B), consistent with the reduced disease severity (28). However, the percentages of macrophages and monocytes did not differ between genotypes (Figure 2B). These findings correlated with lower RNA expression of neutrophil markers (Elastase 2, Ly6G) in the livers of LPS-injected ΔMkk3/6 mice (Supplemental Figure 3A).

The reduced liver neutrophil infiltration in ΔMkk3/6 mice could be due either to impaired neutrophil release from BM or to a defect in cell migration. The levels of circulating neutrophils after LPS injection showed a sharp increase in WT mice that was absent in ΔMkk3/6 mice (Figure 2C). These data show that the reduced neutrophil infiltration in liver is in part caused by lowered neutrophil mobilization from the BM. To investigate whether the low circulating levels of TNF-α in ΔMkk3/6 mice might be responsible for this alteration, we injected TNF-α+D-gal into WT and ΔMkk3/6 mice, resulting in increased circulating neutrophil levels in both genotypes (Supplemental Figure 3B). These data indicate that the defect in migration is secondary to the low production of TNF-α in ΔMkk3/6 mice.

ΔMkk3 and ΔMkk6 regulate TNF-α production by macrophages. To investigate the molecular mechanism underlying reduced circulating TNF-α in ΔMkk3/6 mice, we treated BM-derived macrophages from WT and ΔMkk3/6 mice with LPS. Whereas treatment of WT macrophages with LPS activated JNK, ERK, and p38 MAPK, in ΔMkk3/6 macrophages the activation of p38 MAPK was impaired (Supplemental Figure 3C). The ΔMkk3/6 macrophages also secreted markedly reduced amounts of TNF-α and IL-6 compared with WT macrophages, confirming that ΔMkk3/6 deficiency causes major defects in TNF-α production (Supplemental Figure 3D). Further analysis showed that neutrophils and Kupffer cells, 2 other myeloid cell types implicated in liver damage, also secreted reduced amounts of TNF-α and IL-6 (Supplemental Figure 3, E and F). However, in vivo analysis indicated that although the percentage of liver neutrophils expressing TNF-α after D-gal+LPS injection was the same in both genotypes, the percentage of monocytes and macrophages expressing TNF-α was higher in WT livers (Figure 2D).

To confirm that reduced cytokine production by liver macrophages is responsible for the protection against hepatocyte apoptosis in ΔMkk3/6 mice, we stimulated primary hepatocytes with conditioned medium from LPS-treated BM-derived macrophages. Conditioned medium from LPS-treated WT macrophages increased hepatocyte apoptosis, measured by cleavage of caspase 3 and PARP, whereas conditioned medium from LPS-treated ΔMkk3/6 macrophages had no effect (Supplemental Figure 3G).

ΔMkk3 and ΔMkk6 are not required for TNF-α–induced liver damage. Our results so far show that Mkk3/6 deficiency causes decreased TNF-α production in a TNF-α–dependent hepatitis model. This implies that ΔMkk3/6 mice are defective for TNF-α secretion, but not TNF-α responsiveness. To test this, we treated mice with TNF-α, finding that TNF-α caused similar mortality and liver damage in...
ΔMKK3/6 mice have lower liver inflammation after LPS injection. WT and ΔMKK3/6 mice were treated with D-gal+LPS or saline. (A) Total RNA was extracted from livers 6 hours after treatment and chemokine mRNA levels determined by qRT-PCR. mRNA expression was normalized to Gapdh (n = 5–8). (B) Liver myeloid subsets (CD11b+Gr-1<sup>+</sup>, CD11b+Gr-1<sup>intermediate</sup>, CD11b-Gr-1<sup>-</sup>) were assessed by flow cytometry of liver leukocytes isolated from WT and ΔMKK3/6 mice 4 and 6 hours after treatment. Representative dot plots are shown. Bar charts show each myeloid population as the percentage of total intrahepatic leukocyte population (n = 7). (C) Neutrophils as a percentage of circulating leukocytes, measured in total blood 4 hours after injection (n = 5–8). (D) TNF-α and IL-12 production by liver myeloid subsets were analyzed by intracellular staining in neutrophils (CD11b+Gr-1<sup>+</sup>), monocytes (CD11b+Gr-1<sup>intermediate</sup>) and CD11b-Gr-1<sup>-</sup> myeloid cells isolated from WT and ΔMKK3/6 mice 4 and 6 hours after injection. Representative dot plots are shown for all treatment groups, and bar charts show TNF-α-positive cells as the percentage of each myeloid population (n = 7). Data are means ± SD. *P < 0.05; **P < 0.01; ***P < 0.001 (2-way ANOVA coupled to Bonferroni’s post tests).
WT and ΔMKK3/6 mice (Figure 3, A and B). Immunoblot analysis demonstrated that TNF-α stimulated a similar degree of caspase 3 and PARP cleavage (Figure 3C). Moreover, TNF-α induced the same levels of neutrophil liver infiltration in both genotypes (Figure 3D). To confirm that the protection was due to defective cytokine production and not to a protective loss of responsiveness to TNF-α, we treated primary hepatocytes from WT or ΔMKK3/6 mice with TNF-α and the protein synthesis inhibitor cycloheximide (CHX). Both hepatocyte populations presented the same level of apoptosis (Figure 3E). These data demonstrate that ΔMKK3/6 mice do not exhibit resistance to TNF-α-induced hepatitis and indicate that the primary protection against LPS-induced hepatitis in these mice is due to an inability to produce TNF-α.

MKK3 and MKK6 are required in the hematopoietic compartment for LPS-induced liver damage. The source of TNF-α in mouse models of hepatitis is the hematopoietic compartment (31, 32). Therefore...
ΔMKK3/6 hematopoietic cells protect mice against hepatitis. Lethally irradiated WT mice were reconstituted with BM from WT or ΔMKK3/6 mice. Mice were treated with D-gal+LPS 2 months after transplantation. (A) 2 × 10^7 freshly prepared CD45.2 whole BM mononuclear cells were transplanted into lethally irradiated B6.SJL (CD45.1) mice, and engraftment by CD45.2 cells (%) was analyzed by antibody staining and FACS of peripheral blood after 4 months. Left panels: representative FACS dot plot of CD45.1 and CD45.2 expression in cells isolated from the blood of transplanted mice. Right panels: representative FACS dot plot of CD45.1 and CD45.2 expression in F4/80-positive cells isolated from the liver of transplanted mice (n = 3). (B) Survival curves after D-gal+LPS injection (n = 10). Survival curves were created with the Kaplan-Meier method and compared by log-rank (Mantel-Cox) test. (C) Representative H&E-stained sections of livers extracted 6 hours after injection. The chart presents hemorrhagic area as a percentage of the total area (n = 5–8). Scale bar: 50 μm. (D) Serum transaminase activity at 4 and 6 hours after injection (n = 5–8). (E) ELISA of serum TNF-α and IL-6 at different times after injection (n = 5–8). Data are means ± SD. *P < 0.05; **P < 0.01; ***P < 0.001 (2-way ANOVA coupled to Bonferroni's post tests).
Figure 5
p38γγ/δδ−/− mice are protected against LPS-induced liver damage. p38Δγ/δΔγ/δ, p38Δγδ−/−, p38δΔγ−/−, and control Lyz-cre transgenic mice were injected with D-gal+LPS or saline. (A) Mouse survival after D-gal+LPS injection (n = 14). Survival curves were created with the Kaplan-Meier method and compared by log-rank (Mantel-Cox) test. (B) Livers were removed at 6 hours after injection. Panels show representative H&E-stained liver sections and livers (n = 5–8). Scale bar: 50 μm. (C) Hemorrhagic area as a percentage of the total area on H&E-stained liver sections (n = 5–8). (D) Immunoblot analysis of liver extracts (n = 5–8). (E) Serum transaminase activity at 6 hours after injection (n = 8–10). (F) ELISA of serum TNF-α and IL-6 at different times after injection (n = 10). ΔP < 0.01 for Lyz-cre versus p38Δγδ−/− mice in TNF-α; ΔP < 0.001 for Lyz-cre versus p38γ/δ−/− mice for TNF-α and for Lyz-cres versus p38γ/δ−/−, p38Δγδ−/− or p38Δγ−/− mice in IL-6 (n = 6–8). (G) Liver myeloid subsets (CD11b+Gr-1−, CD11b+Gr-1+immature, CD11b+Gr-1+) were assessed by flow cytometry of liver leukocytes isolated from p38Δγδ−/−, p38Δγ−/− and control Lyz-cres transgenic mice 6 hours after injection. Representative dot plots are shown. Bar charts show each myeloid population as a percentage of the total intrahepatic leukocyte population (mean ± SD; n = 4–6). Circulating neutrophils in total blood were measured as a percentage of circulating leukocytes 4 hours after injection (n = 5–8). Data are means ± SD. **P < 0.01; ***P < 0.001 (2-way ANOVA coupled to Bonferroni's post tests).

In this context, if defective TNF-α expression accounts for the protection of ΔMKK3/6 mice against TNF-α-dependent hepatitis, transplantation of the hematopoietic compartment of ΔMKK3/6 mice to immunodeficient donors should confer this protection. To test the role of hematopoietic cells, we conducted radiation chimera experiments by transplanting BM from WT and ΔMKK3/6 donor mice into lethally irradiated congenic WT and ΔMKK3/6 recipients. Efficient reconstitution of B6.SJL (CD45.1) mice with BM from WT C57BL/6j (CD45.2) or ΔMKK3/6 (CD45.2) mice was confirmed by flow cytometry analysis of peripheral blood leukocytes stained with antibodies to CD45.1/CD45.2. Moreover, analysis of Kupffer cells (F4/80+) in the liver of recipients after transplantation showed that more than 90% of Kupffer cells originated from the donor mice (Figure 4A).

We found that lethally irradiated mice reconstituted with ΔMKK3/6 BM were indeed protected against LPS-induced mortality and exhibited lower levels of LPS-stimulated serum TNF-α and hepatic damage than mice reconstituted with WT BM (Figure 4, B–E). These data confirm that MKK3 and MKK6 expression in hematopoietic cells play a critical role in the expression of TNF-α that leads to the development of hepatitis.

Inhibition of p38γδ and p38δ protects against LPS-induced hepatitis. MKK3 and MKK6 are the main regulators of the α, β, γ, and δ p38 MAPK isoforms. To determine which isoforms mediate MKK3/MKKδ-induced TNF-α expression, we analyzed the effect on D-gal+LPS-induced liver damage of 2 inhibitors: SB203580, which inhibits p38α and p38β, and BIRB796, which inhibits all isoforms (33). We treated mice with BIRB796, SB203580, or DMSO prior to LPS injection. We first examined the specific action of these inhibitors on MAPK pathway activity. Both compounds efficiently inhibited p38α/β, as shown by the loss of the phosphorylation of the known p38α substrate HSP27 (Supplemental Figure 4A and ref. 34). In contrast, only BIRB796 was able to inhibit p38δ activation, as indicated by suppression of the phosphorylation of its substrate, eEF2K (19). No changes in ERK or JNK phosphorylation were observed (Supplemental Figure 4A).

Injection of BIRB796 into WT mice protected against D-gal+LPS-induced liver damage, whereas SB203580 exacerated liver damage (hemorrhage, apoptosis, and necrosis) (Supplemental Figure 4, B–D). Injection of BIRB796 also reduced serum levels of IL-6 and TNF-α in D-gal+LPS-treated mice (Supplemental Figure 4E). These results suggest that LPS-induced liver injury might be mediated by p38γδ and p38δ.

Myeloid expression of p38γδ and p38δ is required for LPS-induced hepatitis. To investigate the role of p38γδ and p38δ in LPS-induced hepatitis, we generated mice with myeloid cell–specific ablation of these p38 isoforms, separately and in combination. Mice carrying conditionalloxP-flanked alleles for p38γδ, p38δ, and p38β/δ (p38γδ−, p38δ−, and p38β/δ−/− mice) were crossed with Lyz-cres transgenic mice (35), yielding p38γ−/−, p38δ−/−, and p38β−/− mice. Immunoblot analysis confirmed efficient ablation of the appropriate kinases in macrophages, neutrophils, and Kupffer cells (Supplemental Figure 5, A and B).

Compared with control Lyz-cres transgenic mice, p38γ−/− and p38δ−/− mice showed a delay in mortality in response to injection of D-gal+LPS (Figure 5A). These mice were also protected against liver damage and apoptosis, showing milder liver hemorrhaging, reduced serum levels of ALT and AST, and reduced caspase 3 cleavage (Figure 5, B–E). Analysis of cytokines revealed significantly lower circulating TNF-α and IL-6 in LPS-injected p38γ−/− and p38δ−/− mice than in similarly treated Lyz-cres controls (Figure 5F). Other chemokines involved in leukocyte migration (MIP1α and MIP1β) were also reduced (Supplemental Figure 6). In contrast, p38γ−/− mice presented only a modest effect on circulating TNF-α levels and no differences in the levels of other cytokines (Figure 5F and Supplemental Figure 6).

The phenotype found in p38γδ−/− mice might reflect the function of these kinases in several myeloid cell populations implicated in D-gal+LPS damage (such as neutrophils, Kupffer cells, and macrophages). Analysis of these cell types in Lyz-cres mice revealed expression of p38γδ and p38δ in macrophages, predominant expression of p38δ in neutrophils, and exclusive expression of p38γδ in Kupffer cells (Supplemental Figure 5A). Moreover, p38γδ is strongly activated in Kupffer cells by LPS or TNF-α treatment (Supplemental Figure 5B), as judged by the impaired p38 phosphorylation observed by FACS in Kupffer cells from p38β/δ−/− mice. Furthermore, analysis of phospho-p38 by Western blot confirmed that p38δ was the isoform activated in these cells after LPS treatment (Supplemental Figure 5C). LPS and TNF-α also activated p38δ in macrophages (Figure 6), while in neutrophils, p38δ was the only isoform activated after either stimulus (Supplemental Figure 5, B and D). Since neutrophils, Kupffer cells, and macrophages express p38γδ, p38δ, or both isoforms, any of these cell populations could have a role in the protection observed in p38γδ−/− mice. We therefore analyzed the different leukocyte populations that infiltrated the liver after D-gal+LPS injection. Analysis of liver-infiltrating leukocytes showed lower numbers of neutrophils in LPS-injected p38γδ−/− mice than in similarly treated Lyz-cres mice. However, this reduction was not significant in LPS-injected p38δ−/− mice (Figure 5G). None of the myeloid-specific p38-deficient mice showed alterations in the percentages of infiltrated macrophages or monocytes. Collectively, these data show that p38γδ−/− mice are more strongly affected than either of the single conditional knockouts; however, p38δ−/− animals have a stronger phenotype than p38γδ−/− mice, indicating partial...
p38γ/δγ/δ Knockout Macrophages Promote TNF-α Translation through Phosphorylation of eEF2 kinase. (A–D) BM-derived macrophages from p38γ/δγ/δ KO and Lyz-cre mice were treated with LPS (10 μg/ml) or TNF-α (20 ng/ml). Data were analyzed by 2-way ANOVA coupled to Bonferroni’s post tests. (A) ELISA of TNF-α and IL-6 in supernatants. (B) qRT-PCR of Tnfα and Il6 mRNA normalized to Gapdh. (C) Immunoblot of BM-derived macrophage (BMM) lysates. (D) Flow cytometry of phospho-p38 in BM-derived macrophages stimulated with LPS and TNF-α for 30 minutes. Intracellular phospho-p38 is depicted in black; gray corresponds to the isotype control. (E–I) Cells were treated with LPS. Tnfα mRNA was detected by qRT-PCR in immunoprecipitates (1 mg protein) obtained with anti-eEF2. mRNA amounts are expressed relative to the amount detected in control IgG immunoprecipitates. Data were analyzed by Student’s t test unless otherwise indicated. (E) Tnfα mRNA in RAW 264.7 cell immunoprecipitates. (F) Tnfα, Hif1α, and Il6 mRNA in RAW 264.7 cell immunoprecipitates. The presence of eEF2 was determined by immunoblot (upper panel) and quantified with ImageJ (t-way ANOVA coupled to Bonferroni’s post tests). (G) RAW 264.7 cells were pretreated with DMSO or BIRB796 (10 μM) for 30 minutes before stimulation with LPS (60 minutes). Tnfα mRNA was measured in immunoprecipitates. (H and I) Tnfα mRNA in immunoprecipitates of BM-derived macrophages from Lyz-cre and p38γ/δγ/δ mice (H) or WT or ΔMKK3/6 mice (I) after stimulation (60 minutes). eEF2 in immunoprecipitates was detected by immunoblot (upper panel). Data are means ± SD (n = 4). *P < 0.05; **P < 0.01; ***P < 0.001.

Redundancy between p38γ and p38δ, with a predominant role of p38δ. This overlapping action has been shown before and might account for the limited protection observed in single conditional knockouts (36, 37). We therefore used p38γ/δγ/δ mice for further experiments. Further studies showed that the reduced neutrophil infiltration in liver is, in part, caused by lowered neutrophil mobilization from the BM (Figure 5G), probably due to the defective production of TNF-α. Furthermore, measurement of TNF-α-stimulated chemotaxis in a chamber assay revealed a slightly lower-than-control mobility in p38γ/δγ/δ neutrophils (Supplemental Figure 8A), and this effect was not increased by stimulation with a cytokine cocktail (Supplemental Figure 8B). Neutrophils produce several cytokines, and this function can be controlled by p388x (29). Examination of cytokine production in our system showed that p38γ/δγ/δ neutrophils express slightly reduced levels of TNF-α and IL-6 after LPS treatment, but not sufficient to explain the phenotype observed (Supplemental Figure 8C).

To investigate whether the lower neutrophil infiltration in the livers of p38γ/δγ/δ mice could account for the protection observed, we investigated whether neutrophil-specific depletion in Lyz-cre mice results in milder liver damage upon D-gal+LPS treatment, similar to the phenotype of p38γ/δγ/δ mice. GR1 is highly expressed on neutrophils, and anti-GR1 antibody treatment has been shown to specifically deplete neutrophils (38). Treatment of mice with anti-GR1 one day before D-gal+LPS injection completely depleted Gr1+ neutrophils (Supplemental Figure 8D). Neutrophil depletion did not affect liver damage in Lyz-cre mice, and neither did it abolish the protection observed in p38γ/δγ/δ, as assessed by the levels of ALT and AST, cleaved caspase 3, and PARP, or liver damage observed in H&E-stained sections (Supplemental Figure 8, E–G).

These data indicate that neutrophils do not play a dominant role in the protection against D-gal+LPS-stimulated hepatic damage observed in p38γ/δγ/δ mice and therefore indicate that a different myeloid cell type underlies this phenotype. Available evidence suggests an involvement of cytokine and chemokine production by activated Kupffer cells and infiltrated macrophages in the pathogenesis of liver damage (39). We therefore examined cytokine production by macrophages and Kupffer cells upon LPS treatment. ELISA analysis demonstrated that LPS-induced production of TNF-α and IL-6 was lower in p38γ/δγ/δ macrophages and Kupffer cells than in Lyz-cre control cells (Figure 6A and Supplemental Figure 7C). Moreover, only the supernatant from macrophages of p38γ/δγ/δ animals was unable to induce apoptosis in primary hepatocytes (Supplemental Figure 7A), and analysis of this supernatant indicated a defect in cytokine production (Figure 6A and Supplemental Figure 7B). Similarly, induction of apoptosis by supernatant from control Lyz-cre macrophages was blocked by addition of anti-TNF-α antibody, resulting in a protection similar to that obtained with p38γ/δγ/δ supernatant (Supplemental Figure 7C). This finding thus indicates that the low TNF-α levels in p38γ/δγ/δ macrophages and Kupffer cells could account for the protection against D-gal+LPS-induced liver damage.

p38γ and p38δ Mediate TNF-α Translation in Myeloid Cells by Phosphorylating eEF2K. p38 MAPK can promote cytokine expression at the level of transcription, after transcription (by promoting mRNA stability), and translation (27, 40). Il6 mRNA expression was reduced in p38γ/δγ/δ macrophages, but levels of Tnfα mRNA were comparable to those in Lyz-cre macrophages (Figure 6B). The stability of Tnfα mRNA also showed no difference between the 2 genotypes (Supplemental Figure 7F). The translational action of p38δ has been linked to eEF2 kinase, the enzyme that inactivates eEF2. This kinase is inhibited by p38δ-mediated phosphorylation on Ser359 and also by ribosomal protein S6 kinase-mediated phosphorylation on Ser366 (19, 23). Since little is known about the regulation of eEF2K in macrophages, we investigated the effect of p38δ deletion on the phosphorylation of eEF2K and its downstream substrate eEF2. LPS stimulation of Lyz-cre macrophages induced eEF2K phosphorylation on Ser359, but this was markedly impaired in p38γ/δγ/δ macrophages (Figure 6C). This observation was also corroborated in Kupffer cells (Supplemental Figure 7D). eEF2K phosphorylation in Lyz-cre macrophages correlated with dephosphorylation of eEF2, implying activation of elongation. In contrast, p38γ/δγ/δ macrophages sustained high levels of eEF2 phosphorylation after the stimulus, suggesting that elongation is abolished.

To assess whether eEF2 controls TNF-α elongation, we examined the binding of eEF2 to Tnfα mRNA in immunoprecipitates of mRNA-eEF2 complexes in RAW cells. RAW extracts were incubated with anti-eEF2 or an isotype control antibody, and the presence of Tnfα mRNA in the immunoprecipitates was examined by quantitative (q) real-time PCR. High relative levels of Tnfα mRNA were detected in the anti-eEF2 immunoprecipitate, and this association was increased by LPS treatment (Figure 6, E and F). High mRNA levels for Hif1α, a known eEF2 target, were also detected in the anti-eEF2 immunoprecipitate (Figure 6F). In contrast, Hif6 mRNA levels did not differ between the anti-eEF2 and isotype control immunoprecipitates (Figure 6F). Pretreatment of RAW cells with BIRB796 abolished the association of eEF2 with Tnfα mRNA (Figure 6G). Moreover, Tnfα mRNA was detected in anti-eEF2 immunoprecipitates from LPS-treated Lyz-cre or WT BM–derived macrophages but not in immunoprecipitates from similarly treated p38γ/δγ/δ or ΔMKK3/6 macrophages (Figure 6, H and I). Together, our data indicate that Tnfα mRNA is a selective target of eEF2 and that
Figure 7
eEF2 controls TNF-α elongation, and its gene silencing protects against LPS-induced liver damage. (A) BM-derived macrophages from Lyz-cre and p38γδ−/− mice were transduced with GFP+ lentivirus expressing eEF2 shRNA (shEF2) or with empty vector (shΔ). ELISA of TNF-α and IL-6 in culture supernatants was performed 16 hours after LPS (10 μg/ml) stimulation. One-way ANOVA coupled to Bonferroni’s post tests (n = 6). (B–F) WT mice were i.v. injected with shEF2 or shΔ GFP+ lentiviral vectors. After 7 days, mice were injected with D-gal+LPS or saline. Data are means ± SD. *P < 0.05; **P < 0.01; ***P < 0.001 (2-way ANOVA coupled to Bonferroni’s post tests). (B) ELISA of TNF-α and IL-6 in mouse serum 6 hours after treatment (n = 6). (C) Representative livers and H&E-stained sections after removal at 6 hours after injection The chart presents hemorrhagic area as a percentage of the total area (n = 6). Scale bar: 50 μm. (D) Serum transaminase activity at 6 hours after injection (n = 6). (E) Immunoblot analysis of liver extracts (n = 6). (F) Mouse survival after D-gal+LPS injection (n = 10). Survival curves were created with the Kaplan-Meier method and compared by log-rank (Mantel-Cox) test. (G) The MKK3/6-p38δ/eEF2K pathway is necessary for LPS-induced hepatitis. LPS induces leukocyte egress from BM and cytokine/chemokine production in peripheral blood and by Kupffer cells. This leads to massive leukocyte infiltration in the liver and hepatocellular injury. TNF-α plays a dominant role in LPS-induced liver injury. Activation of the MKK3/6-p38δ/eEF2K pathway in macrophages/Kupffer cells controls TNF-α production at the mRNA elongation level in acute inflammatory responses to LPS.

The MKK3/6-p38δ/eEF2K pathway, by releasing eEF2K-mediated inhibition of eEF2 activity, promotes the translational elongation of TNF-α in macrophages.

eEF2 controls TNF-α translation elongation. To confirm eEF2 control of TNF-α translation elongation, we used a recombinant lentivirus expressing an eEF2 shRNA (shEF2), which causes robust eEF2 knockdown in RAW cells (Supplemental Figure 9A). In macrophages, shEF2-mediated loss of eEF2 caused a decrease in TNF-α production of a magnitude similar to that caused by loss of p38δ (Figure 7A). In contrast, p38γδ-dependent IL-6 production was eEF2 independent (Figure 7A). These data indicate that the expression of TNF-α is controlled by eEF2, while the reduced IL-6 production in p38γδ−/− mice is caused by a different mechanism, most likely the loss of p38δ-regulated Il6 gene transcription.

To test the role of eEF2 in vivo, we injected mice with the shEF2 lentivirus or control shRNA lentivirus 1 week before exposure to D-gal+LPS. Blood levels of TNF-α were strongly reduced by the shEF2-injected mice compared with controls (Figure 7B). Moreover, this reduction in TNF-α was associated with protection against hepatic cell death, measured by cleavage of caspase 3 and PARP and protection against hepatic necrosis, and measured by liver hemorrhage and blood levels of ALT and AST (Figure 7, C–E). Collectively, these data indicate that p38γδ MAPK proteins are required for the translation elongation of TNF-α in macrophages mediated by phosphorylation of eEF2K and the activation of eEF2.

Discussion

In this study, we show that protein kinases p38γδ control cytokine production in myeloid cells. Our findings raise 3 key areas of interest: the specific role of myeloid cells, the involvement of p38γδ compared with that of other kinases of the same family, and how acute inflammatory responses can be controlled through tight regulation of translational elongation.

Myeloid cells such as Kupffer cells and other macrophages are well known to be a critical source of TNF-α in LPS-induced hepatitis (14, 32). This specific role of hematopoietic cells is consistent with our finding that the protective effects of MKK3/6 deficiency are also present in irradiated WT mice reconstituted with ΔMKK3/6 BM (Figure 5). Furthermore, this protection was also observed in mice specifically lacking p38γδ in the myeloid compartment (Figure 6). Two cell types of special relevance in acute hepatitis are neutrophils and monocytes/macrophages (including Kupffer cells), and both of these BM-derived cell populations are essential for the innate immune response (42). Monocytes/macrophages bind to microbial constituents (such as LPS and cell wall constituents of Gram-positive bacteria), producing large amounts of pro- and antiinflammatory cytokines (3). Neutrophil recruitment and activation occur through TNF-α-mediated chemokine production (3). This is evidenced by our finding that low expression of TNF-α in ΔMKK3/6 or p38γδ−/− mice results in decreased chemokine production and neutrophil migration, an effect reversed by injection with TNF-α. We did not find, however, any evidence for a role of p38γδ in neutrophil migration, and we found only a minor effect on cytokine expression. This contrasts with the recent finding that p38α can control cytokine production by neutrophils (29), illustrating the different roles and clinical potential of p38 kinase isoforms as targets for preventing LPS-induced damage.

Previous reports have identified p38α as a key kinase involved in TNF-α production (43). p38α MAPK deficiency in macrophages results in decreased TNF-α production, with a modest effect on IL-6 expression (44). However, specific inhibition of this kinase has been shown to be hepatotoxic, hindering its clinical use. For example, the p38α inhibitor AMG 548 showed more than 85% inhibition of ex vivo LPS-induced TNF-α in healthy males, but its production and clinical use were suspended due to random liver enzyme elevations that were not dose or exposure dependent (45). Our results show that specific inhibition of p38α with SB203580 intensifies D-gal+LPS–induced liver damage, whereas BIRB796, which inhibits all 4 p38 isoforms (α/β/γ/δ) improves liver condition, including a reduction in apoptosis and necrosis. The toxicity associated with SB203580 might be caused by inhibition of p38α in hepatocytes, since mice with specific p38α deficiency in hepatocytes have increased JNK activity and increased susceptibility to liver damage (17, 43). In clinical use, BIRB796 also produces some liver enzyme elevations (46), most likely because of inhibition of p38. Our findings indicate that the generation of inhibitors that specifically target p38γδ might avoid the adverse effects found with p38α inhibitors.

Our results further show that p38γδ control macrophage production of TNF-α by promoting protein elongation during translation by eEF2. Protein synthesis is tightly regulated at transcriptional and posttranscriptional levels. Owing to the relatively long time of mRNA transcripts, transcriptional regulation is commonly involved in slow, long-term cell responses, whereas immediate cell responses require posttranscriptional regulation of mRNA stability or translation. Two physiological situations requiring rapid regulation of protein synthesis are starvation and inflam-
inflammatory conditions. In starvation conditions, mTOR stops protein synthesis at the elongation step — the step that consumes most metabolic energy — by inhibiting eEF2, thereby preventing cell energy depletion. Less is known about the mechanisms involved in the termination of the inflammatory response once it has achieved its goal. This process needs to be rapidly regulated in order to avoid more tissue damage. Our results suggest that control of protein elongation might be an important mechanism by which cells rapidly shut down production of proinflammatory proteins that could extend tissue damage in the body, such as TNF-α during an inflammatory response. This might be an important mechanism by which cells tightly regulate cytokine production induced by stress kinases activated during inflammation.

In summary, we conclude that p38/δ in myeloid cells promote TNF-α production by activating its translation without changes in mRNA levels. This is achieved by phosphorylation of eEF2K, releasing the inhibitory action of this kinase on eEF2. This post-transcriptional regulation might be an important mechanism regulating cytokine secretion during the innate immune response and provides potential targets for the treatment of liver diseases.

**Methods**

*Mice. Mkk3<sup>−/−</sup> (B6.129-Mp2k<sup>5tri</sup>Δ8.9) (47, 48) and Mkk6<sup>−/−</sup> (B6.129-Mp2k<sup>1tri</sup>Δ8.9) mice (49) were as previously described (14). p38δ (B6.129-Mp2k<sup>1tri</sup>Δ8.9) and p38β (B6.129-Mp2k<sup>1tri</sup>Δ8.9) mice were crossed with B6.129P2-Lys2tm1(cre)f/J and backcrossed for 10 generations to the C57BL/6j background (Jackson Laboratory) and genotyped by PCR analysis of genomic DNA. Radiation Mice. Mice were injected in the tail vein with 200 μl lentiviral particles suspension of the liver (56). Primary monolayer cultures were established by plating 1.5 x 10⁶ viable cells/cm² in 6-well collagen IV-coated plates. After 16 hours, primary hepatocytes were stimulated for 24 hours with TNF-α (20 ng/ml) plus CHX (100 μg/ml) or with 500 μl of macrophase-conditioned medium for 12 hours. Neutrophil migration assays were performed using BD Falcon FluoroBlok 96 Multi-Well insert systems. Neutrophils were stained for 1 hour with 1.5 μM calcein AM. Then, 1 x 10⁵ cells were plated per well in the upper chamber in 50 μl of culture medium without FBS. Lower chambers contained 200 μl of culture medium alone or supplemented with the chemoattractant (20 ng/ml TNF-α or 1/100 cytokine cocktail). Fluorescence emission was measured at different time points with a fluorescent plate reader with bottom-reading capability (Fluoroskan Ascent; Thermo Scientific).

**Serum analysis.** Serum activities of ALT and AST were measured using the ALT and AST Reagent Kit (BioSystems Reagents) with a Benchmark Plus Microplate Spectrophotometer. Serum concentrations of cytokines were measured by multiplexed ELISA with a Luminex 200 analyzer (Millipore).

**Biochemical analysis.** Tissue extracts were prepared using Triton xylol buffer (20 mM Tris [pH 7.4], 1% Triton X-100, 10% glycerol, 137 mM NaCl, 2 mM EDTA, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml aprotenin and leupeptin). Extracts (30 μg protein) and immunoprecipitates (prepared from 2 mg protein) were examined by protein immunoblot analysis with antibodies to ERK1/2, phospho-ERK1/2, phospho-MKK3/6, p38α MAPK, phospho-p38, phospho-hsp27, phospho-JNK1/2, JNK1/2, caspase 3, cleaved caspase 3, cleaved PARP, and phospho-eEF2 kinase (Ser366), all from Cell Signaling and MKK3 (BD Biosciences — Pharmingen), MKK6 (Stressgen), and GAPDH and eEF2 (Santa Cruz Biotechnology Inc.). The anti-p38δ and p38β antibodies were raised against the peptides PPRQL-GARVPKETAL and PIARKDSRRRSGLMKL, respectively, which correspond to the C terminus of the proteins.

**Immunohistochemistry.** Livers were fixed in 4% paraformaldehyde, processed, and embedded in paraffin. Sections (5 μm) were stained with H&E. *Isolation of liver-infiltrating monoclonal leukocytes.** Mouse livers were collected, and a single-cell suspension was obtained and passed through a 70-μm strainer. Leukocytes were collected from the interphase of centrifuged Ficoll gradients. Cells were counted with a CASY Cell Counter (57). Then 10⁶ Cells were labeled by surface staining (PE-conjugated anti-CD11b and PerCP-conjugated anti-Gr-1; Invitrogen), and another 10⁶ cells were collected and processed, and embedded in paraffin. Sections (5 μm) were stained with H&E.

**Flow cytometry.** Flow cytometry was performed with a FACScan cytofluorometer (FACS Canto BD), and data were examined using FlowJo software.

**Phospho-p38 FACS analysis.** Primary myeloid cells were isolated (neutrophils, BM macrophages, and Kupffer cells) and stimulated for 30 minutes with LPS (10 μg/ml) or with 500 μl of macrophase-conditioned medium. Cells were stained with anti–phospho-p38 antibody (Cell Signaling) or control rabbit IgG followed by FITC–anti-rabbit IgG. Signal was detected by flow cytometry.

**RNA-immunoprecipitation assay.** RAW 264.7 cells (mouse leukemic monocyte macrophage cell line) were stimulated with LPS (10 μg/ml) and RNA-immunoprecipitation assays (RIPs) were performed using antibodies against eEF2 (Santa Cruz Biotechnology Inc.). The efficiency of immunoprecipitation was checked by immunoblot analysis. RNA was isolated from the input lysate, isotype control immunoprecipitate, and the anti-eEF2 immunoprecipitate. After reverse transcription, the levels of
TNF-α mRNA were determined by qRT-PCR. Relative mRNA levels were calculated by subtracting background from the level of the target mRNA and then using the standard ΔΔCt formula.

qRT-PCR. The expression of mRNA was examined by qRT-PCR using a 7900 Fast Real Time thermocycler and FAST SYBR GREEN assays (Applied Biosystems). Relative mRNA expression was measured by Gapdh mRNA measured in each sample. Hif-1α, Gr-1, KC (Cxcl1), Mip-2 (Cxcl2), Mcp-1, and Icam-1 were amplified using the primers shown in Supplemental Table 1.

Statistics. Differences between groups were examined for statistical significance using 2-tailed Student’s t test or ANOVA coupled to Bonferroni’s post-test. Kaplan-Meier analysis of survival was performed using the log-rank test.

Study approval. Animal studies were approved by the local ethics committee and by the IACUC of the University of Massachusetts Medical School. All animal procedures conformed to EU Directive 86/609/EEC and Recommendation 2007/526/EC regarding the protection of animals used for experimental and other scientific purposes, enacted under Spanish law 12/2005.

Acknowledgments

We thank J.M. Vazquez and E. Calvo for the proteomics analysis; T. Barrett and L.G. Leiva for technical assistance; S. Bartlett for English editing; and M. Rincon and F. Sanchez-Madrid for kindly revising and critically reading the article. Phospho-eEF2K (Ser359) antibody was provided by the Division of Signal Transduction Therapy (DSTT), University of Dundee, Dundee, United Kingdom. p8A.9 and pSV-G were kindly provided by Katia Urso and Juan Miguel Redondo (Centro Nacional de Investigaciones Cardiovasculares, Spain). FACS cytometry was conducted at the CNIC-Cellomics Unit. G. Sabio is an investigator of the Ramón y Cajal Program and a recipient of a Príncipe de Girona Impulse award. R.J. Davis is an investigator of the Howard Hughes Medical Institute. Á. Verdugo is a recipient of a Madrid Regional Government fellowship. E. Manieri is a fellow of the La Caixa Foundation. Á. González-Rodríguez was supported by CIBERDEM (ISCIII, Spain). This work was funded by the following grants: ERC 260464, EFSF 2030, MICINN SAF2010-19347, and Comunidad de Madrid S2010/BMD-2326 (to G. Sabio); MICINN SAF2011-27330 (to P. Martin); and MICINN SAF2012-3328 (to Á. Valverde). The CNIC is supported by the Ministry of Economy and Competitiveness and the Pro-CNIC Foundation.

Received for publication May 31, 2012, and accepted in revised form October 4, 2012.

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