Molecular Dissection of gp130-dependent Pathways in Hepatocytes during Liver Regeneration*

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Interleukin-6 (IL-6) via its signal transducer gp130 is an important mediator of liver regeneration involved in protecting from lipopolysaccharide (LPS)-induced liver injury after partial hepatectomy (PH). Here we generated mice either defective Δ in hepatocyte-specific gp130-dependent Ras or STAT activation to define their role during liver regeneration. Deletion of gp130-dependent signaling had major impact on acute phase gene (APG) regulation after PH. APG expression was blocked in gp130-ΔSTAT animals, whereas gp130-ΔRas mice showed enhanced APG response and stronger SOCS3 regulation correlating with delayed hepatocyte proliferation. To define the role of SOCS3 during hepatocyte proliferation, primary hepatocytes were co-stimulated with IL-6 and hepatocyte growth factor. Higher SOCS3 expression in gp130-ΔRas hepatocytes correlated with delayed hepatocyte proliferation. Next, we tested the impact of LPS, mimicking bacterial infection, on liver regeneration. LPS and PH induced SOCS3 and APG in all animal strains and delayed cell cycle progression. Additionally, IL-6/gp130-dependent STAT3 activation in hepatocytes was essential in mediating protection and thus required for maximal proliferation. Unexpectedly, oncostatin M was most strongly induced in gp130-ΔSTAT animals after PH/LPS-induced stress and was associated with hepatocyte proliferation in this strain. In summary, gp130-dependent STAT3 activation and concomitant SOCS3 during liver regeneration is involved in timing of DNA synthesis and protects hepatocyte proliferation during stress conditions.

The liver has the unique capacity to compensate tissue loss after injury through hyperplasia of remaining hepatocytes while keeping its organ-specific functions (1, 2). Liver mass is controlled in an intriguingly exact way, which becomes essential in situations where transplanted livers that are too small for matching the body weight of the recipient have to grow until they reach their optimal size, whereas transplants that are too big in size shrink to their optimal volume (3–5).

Partial (two-thirds) hepatectomy (PH)2 in rodents is a well-established model for studying liver regeneration and cell cycle regulation in vivo. In this model it has been a major challenge to evaluate the extra- and intracellular events that orchestrate this highly complex process. To undergo distinct stages including priming, cell cycle progression, and cessation of regeneration, fully differentiated hepatocytes are forced to leave their quiescent state (6, 7).

Growth factors and cytokines have been shown to play important roles in regulating this process. In the priming phase of hepatocytes, roles for cytokines like tumor necrosis factor α and IL-6 have been reported (8–12). First, it was suggested that both are directly involved in mediating cell cycle progression. However, in recent years, especially for IL-6, it has been demonstrated that this cytokine does not directly control hepatocyte proliferation after PH but activates signaling pathways that are protective for the progression of regeneration (13).

For signal transduction IL-6 first binds to the IL-6 receptor gp80. As a result these proteins build a complex with two gp130 signal transducers, which in turn results in the intracellular dimerization of these glycoproteins (14). This event triggers activation of Janus kinases in the cell, which phosphorylated distinct intracellular tyrosines of the gp130 receptor. They serve as docking sites and activate the downstream pathways. In the gp130 molecule six tyrosine residues have been defined (15). Phosphorylation of the four distal tyrosines is involved in mediating nuclear translocation of STAT3 and STAT1, whereas phosphorylation of the second most membrane-proximal tyrosine is linked to the Ras/Erk pathway as well as to negative regulation of gp130 signaling (16, 17).

In an earlier study we used conditional gp130 knock-out (LoxP/Cre system) mice to better characterize the role of IL-6-dependent signaling during liver regeneration. In these mice gp130 was deleted using a transgene for Cre, where its expression is controlled via the Mx promoter (MxCre gp130LoxP/LoxP) (13). Therefore, this approach had the advantage of deleting gp130 on demand but was active in all cells that are sensitive to interferon. The MxCre gp130LoxP/LoxP mice had a severe phenotype after PH, when they were co-stimulated...
with LPS. A high percentage, in contrast to controls, of the animals died and showed impaired hepatocyte proliferation. These results stressed two important questions: whether this phenotype is related to deletion of gp130 in hepatocytes and which of the two pathways, Ras/Erk and/or STAT, is involved in mediating protection during liver regeneration.

Therefore, in the present study we used hepatocyte-specific gp130 knock-out (AlfpCre gp130LoxP/LoxP) mice and combined these animals with gp130 knock-in mice, deficient for either hepatocyte-specific gp130-dependent Ras/Erk (AlfpCre gp130Y757F/LoxP) or STAT (AlfpCre gp130ΔSTAT/LoxP) activation. This strategy allowed us to specifically address the role of IL-6/gp130-Ras- or IL-6/gp130-STAT-dependent signaling in hepatocytes and to investigate their relevance during different pathophysiological conditions. We thus used these animal strains to further characterize the relevance of gp130-dependent signaling during liver regeneration after partial hepatectomy.

**EXPERIMENTAL PROCEDURES**

**Generation and Genotyping of gp130-mutated Animals—**Hepatocyte-specific AlfpCre gp130LoxP/LoxP; mice were generated by breeding AlfpCre mice with mice expressing LoxP-flanked gp130 alleles. Gp130LoxP/LoxP mice without Cre expression were used as control (wild type) animals.

Hepatocyte-specific AlfpCre gp130ΔSTAT/LoxP; gp130ΔSTAT/LoxP animals were generated by breeding AlfpCre gp130LoxP/LoxP with gp130ΔSTAT/ΔSTAT knock-in mice. Gp130ΔSTAT/ΔSTAT mice express a truncated gp130 knock-in allele that lacks the essential regions encoding the carboxyl-terminal end of gp130 required for activation of STAT1 and -3 signaling (18).

**Hepatocyte-specific AlfpCre gp130Y757F/LoxP—**gp130Y757F/LoxP animals were generated by crossing AlfpCre gp130LoxP/LoxP with gp130Y757F/ΔSTAT knock-in mice. The gp130ΔSTAT/ΔSTAT mice express a truncated gp130 knock-in allele that lacks the essential regions encoding the carboxyl-terminal end of gp130 required for activation of STAT1 and -3 signaling (18).

**Two-thirds Hepatectomy and LPS Stimulation—**Pathogen-free 8–10-week-old male mice were used for PH and additional LPS injury experiments providing the different hepatocyte-specific defects in IL-6-gp130 signaling. PH and sham surgeries were performed under ketamin/xylazine anesthesia as described earlier (13). The mice were exposed to additional injury induced by intraperitoneal application of LPS (4 mg/kg) (Escherichia coli O55:B5; Sigma) injected 3 h after surgery. The mice subjected to LPS challenge in the absence of PH received 4 mg/kg of LPS. Control animals were treated with equal volumes of carrier solution (NaCl). Each time point provides at least five mice/genotype. The remaining livers were removed, rinsed in ice-cold phosphate-buffered saline, and individually frozen in liquid nitrogen and Tissue Tek (Sakura Europe, Zoeterwoude, The Netherlands).

**Isolation of Primary Hepatocytes—**Primary mouse hepatocytes were isolated and cultured as described earlier (20). Stimulation was performed with 50 ng/ml recombinant oncostatin M (OSM), 200 ng/ml recombinant IL-6, and 50 ng/ml recombinant HGF. For quantification of DNA synthesis [3H]thymidine was added 6 h before harvest at the indicated time points, and whole cell proteins and RNA were isolated.

**SDS-PAGE and Western—**Frozen liver tissues were lysed in cold lysis buffer as described earlier (21) and heat-denaturated in double-strength sodium dodecyl sulfate sample buffer containing dithiothreitol. After resolution in 10% SDS-PAGE, equal protein loading was confirmed by Ponceau Red staining. For primary antibody incubation, the membranes were probed with anti-P-STAT3 (Tyr705; 9131s; Cell Signaling, Danvers, MA), anti-4E-BP1 (clone C19; sc-596; Santa Cruz), anti-phospho-p44/42 (Erk1/2) mitogen-activated protein kinase (Thr202/Tyr204; #9106s; Cell Signaling), and anti-GAPDH (Biogenesis 4699-9555) antibodies. As a secondary antibody, horseradish peroxidase-linked anti-rabbit immunoglobulin G (#7074; GE Healthcare, Buckinghamshire, UK).

**BrdUrd Labeling and Staining—**For in vivo pulse labeling of cells in S phase, 5-bromo-2′-deoxyuridine (BrdUrd) was injected intraperitoneally (300 μg/20 g mouse) 2 h before the animals were sacrificed. Cryosections were stained according to the cell proliferation kit manual (RPN20; GE Healthcare). BrdUrd-negative cells were counterstained with hematoxylin. For evaluation of the average percentage of BrdUrd-positive hepatocytes per liver sample, four fields (magnification, ×200) were analyzed by counting the total number of nuclei. At least five mice were analyzed per time point.

**Quantitative Real Time PCR—**Total RNA from frozen liver was isolated and treated with DNasel (Invitrogen) before cDNA synthesis as described earlier (20). Real time PCR was performed by using a Real Time PCR System 7300 (Applied Biosystems, Foster City, CA) and SYBRGreenER qPCR Super Mix (Invitrogen). The primers were as follows: SOCS3, forward, 5′-CCC GCG GCG ACC TTT CTT AT-3′, and reverse, 5′-CAC TGG ATG CGT AGG TTC TTG-3′; SAA primers, forward, 5′-TCT CTG GGG CAA CAT AGT ATA CCT CTC AT-3′, and reverse, 5′-TCTT ATT ACC CTC TCC TCC TCA AGC AGT TAC-3′; cyclin E1, forward, 5′-ACA GCA GGT CTG CTT GCA GAT C-3′, and reverse, 5′-CTG AAG CAG CCA CAT CCA G-3′; OSM, forward, 5′-CTG TGA GAG AGT GCG CAG TAT AGT-3′, and reverse, 5′-CGA AAC GCT CTT GCT GCA GAT C-3′; and GAPDH, forward, 5′-GAC ACC TGT GTC GGT CCA GGA C-3′, and reverse, 5′-GAC CTT CTG GAC TGG GAA GAA CCA CCA-3′. The expression levels were normalized with the levels of GAPDH. The specificity of PCR products was confirmed by melting curves and electrophoresis.

**H1 Histone Kinase Assay—**The activity of Cdk2 was measured by the histone H1 kinase assay. Liver protein extract (500 μg) was immunoprecipitated overnight at 4 °C with antibodies to Cdk2 (M2; sc-163; Santa Cruz). 40 μl of protein A-agarose beads (Roche Applied Science) were added to each immuno-
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FIGURE 1. Regulation of the acute phase response in gp130 hepatocyte-specific modified mice after PH. Analysis of the liver derived acute phase protein SAA, SOCS3, and P-STAT3 was performed by quantitative real time RT-PCR analysis, relative to a house keeping control gene (GAPDH) and Western blot analysis, respectively. Three to five individual livers were examined in each cohort at each time point following PH. A, quantitative analysis of SAA induction after PH (*, p = 0.04). The data are expressed as the means ± S.D. B, SOCS3 levels quantitatively evaluated by real time RT-PCR (**, p = 0.001). The data are expressed as the means ± S.D. C, for P-STAT3 analysis gels were run in parallel for direct comparability (more than four animals/time point). The membranes were reprobed with anti-GAPDH (loading control).

RESULTS

Alterations in Acute Phase Gene Regulation in Genetically Modified gp130 Mice after PH—IL-6 is an important mediator of liver regeneration (8). IL-6 via gp130 triggers pathways that are important in mediating protection after PH but has a minor role in directly mediating hepatocyte proliferation (13). In our present study we further dissected the role of gp130-dependent pathways specifically in hepatocytes to understand their relevance during liver regeneration after PH. We used hepatocyte-specific gp130 genetically modified mice that have a complete deletion of gp130 in hepatocytes (AlfpCre gp130loxP/loxP) or can selectively only activate the gp130-STAT (AlfpCre gp130Y757F/loxP) or gp130-Ras (AlfpCre gp130ΔSTAT/loxP) pathway in hepatocytes (19). Additionally, gp130 loxp/loxP mice were used. These animals express no Cre recombinase but only carry LoxP sites in the gp130 locus and thus were used as wild type (wt) control animals.

First, we studied the regulation of gp130-dependent pathways in these four animal strains following PH. Analysis of the major liver-derived acute phase protein, serum amyloid A (SAA) showed a more than 150-fold induction on the RNA level in wt mice 24 h after PH. This regulation was completely blocked in AlfpCre gp130loxP/loxP and AlfpCre gp130ΔSTAT/loxP strains. In contrast, compared with wt mice a significant 2.5-fold stronger induction (p = 0.043) of SAA levels was found in AlfpCre gp130Y757F/loxP animals after PH (Fig. 1A).

SOCS3 is a negative regulator of STAT3-dependent signaling and has an important role during liver regeneration (23, 24). 6 h after PH SOCS3 was induced 13-fold in wt animals and 50-fold in AlfpCre gp130Y757F/loxP mice (p = 0.001 compared with wt), whereas this response was completely blunted in AlfpCre gp130loxP/loxP animals (Fig. 1B).

SAA and SOCS3 are STAT3 target genes and its phosphorylation (P) was studied. P-STAT3 was found in wt mice 6 h after PH, and this signal was significantly enhanced in AlfpCre gp130Y757F/loxP. Additionally, no P-STAT3 was found in AlfpCre gp130loxP/loxP and AlfpCre gp130ΔSTAT/loxP mice (Fig. 1C).

Impact of Hepatocyte-specific Modulation of gp130-dependent Pathways on Hepatocyte Proliferation—In further experiments the impact of these changes on cell cycle progression was quantified by BrdUrd pulse labeling experiments. A maximum in DNA synthesis was found in wt animals 40 h after PH and decreased at later time points. A similar kinetic was detected in AlfpCre gp130loxP/loxP and AlfpCre gp130ΔSTAT/loxP strains. In contrast to all other strains, AlfpCre gp130Y757F/loxP mice showed a different kinetic. Significantly less BrdUrd-positive hepatocytes were found 40 h after PH, and the maximum of DNA synthesis was evident 48 h after PH (Fig. 2, A and B).

To better define S phase progression, we studied activation of Erk1/2 triggered by growth factors like HGF and epidermal growth factor. Maximal Erk1/2 activity was found between 24 and 40 h after PH in wt, AlfpCre gp130loxP/loxP, and AlfpCre gp130ΔSTAT/loxP mice, whereas the maximum in Erk1/2 activation was evident 72 h after PH in AlfpCre gp130Y757F/loxP animals (Fig. 2C).

G/S Phase Progression Is Delayed in AlfpCre gp130Y757F/loxP Mice—DNA synthesis was delayed in AlfpCre gp130Y757F/loxP animals (Fig. 2B). We thus studied hepatocyte proliferation in more detail. All of the gp130-modified
mouse strains showed a peak in cyclin E1 expression 40 h after PH. Unexpectedly, cyclin E1 mRNA expression was significantly higher in AlfpCre gp130Y757F/LoxP mice compared with the other three mouse strains studied (Fig. 3A).

Western blot analysis evidenced cyclin A expression in wt mice between 40 and 72 h after PH. In AlfpCre gp130LoxP/LoxP and AlfpCre gp130ΔSTAT/LoxP mice a maximum of cyclin A expression was detected after 40 h. In contrast, AlfpCre gp130LoxP/LoxP animals showed a significantly lower cyclin A expression compared with AlfpCre gp130ΔSTAT/LoxP animals where low levels of cyclin A were detected up to 72 h. In contrast, AlfpCre gp130Y757F/LoxP animals showed delayed cyclin A expression with maximal levels at 48 h after PH (Fig. 3B).

BrdUrd analysis and cyclin A expression levels suggested delayed G1/S phase progression in AlfpCre gp130Y757F/LoxP animals. We now tested Cdk2 activity (25). Peak of Cdk2 activity after 40 h was found in all animal strains except in AlfpCre gp130Y757F/LoxP mice (Fig. 3C) where maximal Cdk2 activity was observed only 48 h after PH. AlfpCre gp130ΔSTAT/LoxP mice had the highest Cdk2 activity at the different time points investigated. Thus AlfpCre gp130Y757F/LoxP animals, compared with the other strains investigated, showed a stronger acute phase response and induction of SOCS3 associated with hyperactivation of STAT3 that correlated with a delay in G1/S phase progression after PH.
hepatocyte proliferation correlated with higher SOCS3 expression. To further test this hypothesis, we performed in vitro studies with primary hepatocytes from wt or AlfpCre gp130Y757F/LoxP mice. After isolation, the cells were stimulated with either HGF alone or in combination with IL-6. Proliferation was monitored by \[^{3}H\]thymidine incorporation.

In wt mice 6 h of HGF treatment resulted in a significant 25% increase in DNA synthesis (p < 0.02), and co-stimulation with IL-6 was also associated with higher proliferation (Fig. 4A). In contrast, in AlfpCre gp130Y757F/LoxP mice HGF had no major impact on hepatocyte proliferation, whereas IL-6/HGF co-stimulation inhibited DNA synthesis 6 h after treatment (p < 0.01). However, 12 h after IL-6/HGF stimulation, hepatocyte proliferation in AlfpCre gp130Y757F/LoxP-derived hepatocytes was higher compared with all of the other conditions (Fig. 4A).

HGF stimulation alone did not increase P-STAT3 expression in wt or AlfpCre gp130Y757F/LoxP hepatocytes. However, HGF/IL-6 treatment significantly stimulated STAT3 phosphorylation in AlfpCre gp130Y757F/LoxP but clearly less in wt cells (Fig. 4B). These differences in STAT3 activation directly correlated with a significantly higher SOCS3 expression in HGF/IL-6-stimulated AlfpCre gp130Y757F/LoxP hepatocytes (Fig. 4C).

LPS Stimulation Delays Cell Cycle Progression after Partial Hepatectomy—Our earlier results using inducible gp130 knock-out animals demonstrated that PH/LPS stimulation resulted in inhibition of cell cycle progression (13). We thus injected in all mouse strains LPS (4 mg/kg) 3 h after PH and quantified DNA synthesis via BrdUrd staining. In all PH/LPS-treated mouse strains the maximum in BrdUrd-positive cells could be detected 48 h after PH (Fig. 5A). In wt and AlfpCre gp130Y757F/LoxP animals, a maximum of 30% BrdUrd-positive hepatocytes was found. In contrast, in AlfpCre gp130LoxP/LoxP and AlfpCre gp130STAT/LoxP animals, quantification of BrdUrd-positive hepatocytes revealed 10 and 20%, respectively (Fig. 5A). These results demonstrated a reduction in DNA synthesis after LPS treatment (Fig. 2B versus Fig. 5B). No difference in mortality was found between the four groups during the first 72 h after PH (data not shown).

Reduced Cyclin A Expression and Cdk2 Activity after PH and LPS Stimulation in Animals with a Hepatocyte-specific Deleted gp130-STAT Pathway—We analyzed the progression of cell cycle markers in the PH/LPS-treated strains. Differences in cyclin E1 expression were evident. A maximum in cyclin E1 expression was found after 48 h in wt, AlfpCre gp130STAT/LoxP, and AlfpCre gp130Y757F/LoxP animals, whereas Alfp-
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Cdk2 assays revealed a strong activity 48 h after PH/LPS in wt mice, and in AlfpCre gp130Y757F/LoxP a similar activation was detected at the 48-h time point (Fig. 6C). However, in these animals an even stronger Cdk2 activity was still observed after 72 h. Dramatic differences were found in AlfpCre gp130ΔSTAT/LoxP and AlfpCre gp130LoxP/LoxP animals. Only weak activity was evident at 48 h after PH/LPS treatment in AlfpCre gp130LoxP/LoxP animals. In contrast to the other strains, AlfpCre gp130ΔSTAT/LoxP mice showed an earlier peak 40 h after PH. Therefore these results suggested that LPS treatment delays the start of DNA synthesis in all four mice strains. Additionally, lack of gp130-STAT-dependent signaling significantly reduced DNA synthesis.

Acute Phase Response Regulation in the Four Mouse Strains after Combined PH and LPS Treatment—LPS stimulation may trigger alternative pathways e.g. via Toll-like receptors that are involved in regulating the acute phase response. Therefore we first determined SAA mRNA expression in the four mouse strains. A strong increase with a maximum after 24 h was found after PH/LPS treatment in wt animals (Fig. 7A). Maximum SAA expression was 2-fold higher and more prolonged compared with wt animals treated with PH only (see Fig. 1A).

In AlfpCre gp130LoxP/LoxP animals no significant increase in SAA expression was observed during the first 72 h after PH despite additional LPS stimulation. However, in AlfpCre gp130ΔSTAT/LoxP mice after 24 h, a 250-fold induction of SAA was evident. This response was delayed as after 6 h, in contrast to wt animals, no significant up-regulation of SAA was found. SAA expression during the first 24 h showed a level comparable with that of wt mice. At a later time SAA expression continuously increased up to 72 h, when a more than 600-fold SAA elevation of RNA was evident compared with the pretreatment level.

P-STAT3 induction in the wt group was found 6 and 12 h after PH/LPS treatment, and this effect was stronger compared with the PH only group (Fig. 7C). In AlfpCre gp130LoxP/LoxP animals, no P-STAT3 expression was evident, whereas in AlfpCre gp130ΔSTAT/LoxP mice P-STAT3 induction was found 6 h after treatment. This regulation was comparable with the changes that were detected in the wt PH only group. In AlfpCre gp130Y757F/LoxP animals after PH/LPS treatment, strong STAT3 phosphorylation was obvious after 6 h and in contrast to the other strains P-STAT3 remained elevated at 12 h.

In wt animals the kinetic of SOCS3 expression showed a maximum 6 h after PH/LPS stimulation and after this time point continuously decreased and returned to pretreatment levels after 48 h (Fig. 7B). The SOCS3 kinetic was not significantly different compared with the results of wt mice in the PH only group.

SOCS3 expression in AlfpCre gp130LoxP/LoxP animals was induced at low levels 6 h after PH. Kinetic of SOCS3 expression in AlfpCre gp130ΔSTAT/LoxP and wt mice was similar during the first 6 h after PH, whereas SOCS3 mRNA further increased by 12 h in AlfpCre gp130ΔSTAT/LoxP mice. The strongest induction of SOCS3 RNA was found in AlfpCre gp130Y757F/LoxP animals. Peak expression was found at 6 h with a 3-fold higher induction compared with wt animals. Furthermore at

![Figure 6. Influence of LPS stimulation on cell cycle regulation after PH in hepatocyte-specific, gp130-modified strains. A, expression of cyclin E1 was quantified by real-time PCR at the indicated time following PH administration.**, p < 0.001. The data are expressed as the means ± S.D. B, Western blot analysis for cyclin A expression before and after PH/LPS stimulation. Equal protein loading was assessed by reprobing membranes for GAPDH. Gels of all genotypes were run in parallel (more than four animals/time point). C, Cdk2 activity was analyzed by H1 histone kinase assays. SDS-PAGEs of all genotypes were run in parallel (more than four animals/time point).]
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12 h after PH/LPS challenge, the expression levels remained high and then nearly returned to the pretreatment level.

**OSM Activation in AlfpCre gp130ΔSTAT/LoxP Animals**—Unexpectedly, we found STAT3 phosphorylation in AlfpCre gp130ΔSTAT/LoxP mice after PH/LPS treatment, which was not found only after PH alone. In these animals there is expression of the truncated gp130ΔSTAT receptor on hepatocytes. Some of the IL-6 family members signal by interacting with one gp130 receptor and a second gp130-related molecule (26). Therefore the truncated gp130ΔSTAT receptor could interact with another receptor and after ligand binding activate STAT3 in hepatocytes. Hence, we studied expression of other IL-6 family members.

Quantitative real time PCR analysis of RNA derived from total liver extracts was performed for the IL-6 family members OSM, leukemia inhibitor factor, and cardiotoxin 1. This analysis demonstrated specifically higher OSM expression in mice subjected to combined challenge of PH and LPS. Enhanced OSM expression was specifically evident in wt and AlfpCre gp130ΔSTAT/LoxP animals after PH/LPS treatment (Fig. 7D). 12 h after PH/LPS challenge OSM induction was 30-fold in wt animals and 130-fold in AlfpCre gp130ΔSTAT/LoxP animals. No regulation was found in the other two strains. Thus gp130-RAS-dependent signaling was associated with the induction of OSM after PH/LPS treatment, and this response was increased when gp130-dependent STAT signaling was impaired.

To further characterize this regulation we determined OSM expression in animals after PH or LPS treatment. After PH only minor OSM regulation compared with PH/LPS-treated animals was found 12 h after PH in AlfpCre gp130ΔSTAT/LoxP mice (Fig. 7E), whereas other IL-6 family members remained unaffected (data not shown).

After LPS injection phosphorylated STAT3 expression was observed in wt and AlfpCre gp130ΔSTAT/LoxP mice 6 and 12 h after injection (Fig. 8A). Only mild expression of OSM was found after LPS stimulation in wt and AlfpCre gp130ΔSTAT/LoxP animals. However, OSM expression in AlfpCre gp130ΔSTAT/LoxP animals was significantly higher compared with AlfpCre gp130LoxP/LoxP mice (Fig. 8B). STAT3 target genes, SAA (Fig. 8C) and SOCS3 (Fig. 8D) were significantly up-regulated in wt and AlfpCre gp130ΔSTAT/LoxP mice, in contrast to AlfpCre gp130LoxP/LoxP animals.

Application of Recombinant OSM Results in Phosphorylation of STAT3 in wt and AlfpCre gp130ΔSTAT/LoxP Primary Hepatocytes—Next, primary hepatocytes derived from livers of wt, AlfpCre gp130LoxP/LoxP, and AlfpCre gp130ΔSTAT/LoxP mice were stimulated with OSM. Strong P-STAT3 expression was found in wt hepatocytes 30 min and 1 h after OSM stimulation (Fig. 8E). In contrast, this response was completely blocked in AlfpCre gp130LoxP/LoxP animals but was still detected in AlfpCre gp130ΔSTAT/LoxP mice.
IL-6 is involved in controlling hepatocyte proliferation and cell protection after PH (10, 11, 13). IL-6 activates intracellular pathways via the signal transducer gp130, and its dimerization results in phosphorylation of tyrosine residues at the intracellular part of the receptor (27). The aim of the present study was to assess the role of distinct gp130-dependent pathways in hepatocytes during liver regeneration; two major pathways leading to Ras/Erk or STAT1/3 activation are essential. To differentiate between these two pathways in hepatocytes they were dissected by combining gp130 knock-in mutant mice with tissue-specific gp130 knock-out animals. Through this approach we are able to identify gp130-dependent mechanisms in hepatocytes and specifically link the effect to one pathway.

Between the strains major differences in acute phase gene (APG) regulation were found after PH. In wt controls transient APG induction was triggered after PH. This regulation was completely blocked in animals that lack gp130-dependent STAT3 activation in hepatocytes. In contrast, APG expression was enhanced in AlfpCre gp130Y757F/LoxP animals. Additionally, SOCS3 levels were strongly up-regulated. SOCS3 is a STAT3 target gene and via the second phosphorylated gp130 tyrosine limits activation of gp130-dependent signaling pathways, including activation of STAT3 (28). Because this feedback loop is blocked in AlfpCre gp130Y757F/LoxP animals, prolonged STAT3 phosphorylation and significantly higher SOCS3 mRNA levels were evident. Unexpectedly, this response was associated with a later start of hepatocyte proliferation 48 h after liver resection. A more detailed analysis of cell cycle parameters demonstrated that G1/S phase transition was delayed in these animals.

To further identify the potential molecular mechanisms, we tested Erk1/2 activation. Erk1/2 is activated through growth factors like HGF and epidermal growth factor that are involved in triggering cell cycle progression of hepatocytes (24, 29–32). This analysis revealed that specifically in the AlfpCre gp130Y757F/LoxP animals, Erk1/2 activation was delayed compared with the other mouse strains. SOCS3 regulation is not only linked to gp130, and earlier results identified SOCS3 as a negative regulator of HGF and epidermal growth factor signaling (24, 29–31, 33). These results suggested that prolonged SOCS3 activation in AlfpCre gp130 Y757F/LoxP animals may interfere with growth factor-dependent signaling and as a consequence results in a delay of G1/S phase transition (29, 34–37).

To further test this hypothesis, primary hepatocytes were stimulated with HGF or HGF/IL-6. HGF resulted in an increase in DNA synthesis of primary wt hepatocytes, which was slightly reduced with IL-6 co-treatment. In contrast, in AlfpCre gp130Y757F/LoxP hepatocytes the impact of HGF on DNA synthesis was significantly reduced.
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...synthesis was diminished, and IL-6 co-stimulation significantly reduced DNA synthesis after 6 h. Reduced DNA synthesis in these animals correlated with higher SOCS3 expression. Therefore our in vivo and in vitro experiments suggest that the strength of SOCS3 expression could be involved in controlling DNA synthesis.

Several studies demonstrated no direct effect of IL-6 on cell cycle progression but identified a role during recovery, survival, and infection after PH (13, 38, 39). In our earlier study we used MxCre gp130 LoxP/LoxP animals and performed the LPS/PH challenge. These mice have a more general gp130 knock-out. In contrast, in the present study we specifically addressed the relevance of gp130-dependent signaling in hepatocytes. In all mouse strains LPS injection delayed DNA synthesis to 48 h after PH, and major differences in APG regulation were evident. In contrast to the PH only animals, SAA expression was significantly increased in wt mice, whereas no SAA induction was found in AlfpCre gp130 LoxP/LoxP mice, despite PH and LPS stimulation. These results show that the acute phase protein SAA in mice is primarily regulated via gp130 in hepatocytes, whereas other factors like LPS or tumor necrosis factor have minor impact.

In AlfpCre gp130Y757F/LoxP mice SAA induction further increased 24 h after PH/LPS. After 72 h SAA levels were 700-fold higher compared with the pretreatment level. In contrast, the induction of SOCS3 lasted only until 24 h after PH/LPS. In these animals, despite prolonged SAA expression, the start of DNA synthesis was not further delayed and was also found 48 h after PH/LPS. Together with the PH only experiments and the studies in primary hepatocytes, these results suggest that the timing of SOCS3 expression is involved in determining the start of the G1/S phase transition after PH/LPS treatment, whereas SAA expression shows no correlation with the start of DNA synthesis. These results are consistent with the hypothesis that SOCS3 has an impact on the timing of DNA synthesis most likely by interfering with growth factor-dependent signaling (24, 29–32).

In our earlier study using MxCre gp130LoxP/LoxP mice, with efficient gp130 deletion in several tissues, most of the animals died after PH/LPS challenge (39). In contrast, AlfpCre gp130LoxP/LoxP animals survived the PH/LPS stimulus. These results suggest that other cells besides hepatocytes have a major impact on survival after PH/LPS challenge. However, these observations together with our present study where LPS-stimulated AlfpCre gp130LoxP/LoxP mice showed a dramatic 65% reduction in hepatocyte proliferation further confirm the protective role of IL-6 during liver regeneration. To further dissect the contribution of gp130-dependent mechanisms in other tissues, we are currently generating additional tissue-specific knock-out mice to address their relevance during liver regeneration.

AlfpCre gp130ΔSTAT/LoxP mice express a truncated gp130 receptor in hepatocytes that lacks the docking sites required for STAT activation. IL-6 induction after PH was unable to trigger APG regulation. However, the addition of LPS stimulated SAA and SOCS3 expression in these mice. APG levels were not significantly different compared with wt animals. This unexpected observation led to the hypothesis that LPS triggers expression of IL-6 family members that use the truncated gp130 receptor in a heterodimeric receptor complex, thereby retaining some capacity for STAT3 stimulation.

The implication of OSM-mediated signaling during liver regeneration has been previously described (40). Our analysis showed OSM expression in AlfpCre gp130ΔSTAT/LoxP mice specifically after PH/LPS treatment. OSM is a potent activator of the Ras/Erk and STAT pathway, and in particular, OSM is induced by bacterial products (41). This fact may explain why AlfpCre gp130ΔSTAT/LoxP mice showed stronger DNA synthesis compared with AlfpCre gp130LoxP/LoxP animals after PH/LPS challenge. Thus it is likely that ligands that trigger gp130 heterodimerization (including OSM) overcome the detrimental effect of IL-6-dependent lack of STAT1/3 activation in these animals.

To characterize the role of OSM in AlfpCre gp130ΔSTAT/LoxP mice for STAT3 and APG expression, we stimulated these animals with LPS. As found after PH/LPS treatment, LPS stimulation alone also induced OSM expression significantly stronger in these animals compared with the two control strains. Additionally, OSM induction in AlfpCre gp130ΔSTAT/LoxP animals, in contrast to AlfpCre gp130LoxP/LoxP, was associated with P-STAT3 and APG expression. The relevance of a heterodimeric gp130 ΔSTAT/OSM-R receptor complex for inducing OSM-dependent signaling was further confirmed in primary AlfpCre gp130 ΔSTAT/LoxP-derived hepatocytes. Thus these results suggested that OSM was able to rescue a lack of IL-6 signaling during liver regeneration in these mice. In summary, our results indicate that gp130-dependent STAT signaling during liver regeneration in hepatocytes via SOCS3 controls the timing of G1/S phase transition, thereby providing protective signals that are important to allow hepatocyte proliferation during stress conditions. Additionally, OSM potentially substitutes the lack of IL-6-dependent signaling during liver regeneration.

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