After tissue loss the liver has the unique capacity to restore its mass by hepatocyte proliferation. Interleukin-6 (IL6)-deficient mice show a lack in DNA synthesis after partial hepatectomy (PH). To define better the role of IL6 and its family members for liver regeneration after PH, we used conditional knockout mice for glycoprotein 130 (gp130), the common signal transducer of all IL6 family members. We show that gp130-dependent pathways control Stat3 activation after PH. By using gene array analysis, we demonstrate that c-jun, NF-κB, c-myc, and tumor necrosis factor receptor expression is gp130-dependent. However, in gp130-deleted mice only minor effects on cell cycle and on the maximum of DNA synthesis after PH were found compared with controls. As in conditional gp130 animals, the acute phase response was completely abolished, we considered that other means are essential to define the role of gp130-dependent pathways for liver regeneration. LPS stimulation in gp130-deleted and also IL6−/− animals after PH leads to a significant reduction in survival and DNA synthesis, which was associated with decreased Bcl-xL expression and higher apoptosis in the liver. These results indicate that the phenotype concerning the reduction in DNA synthesis might be linked to the degree of infection after PH. Thus our results suggest that the role of gp130-dependent signaling is not a direct influence on cell cycle progression after partial hepatectomy but is to activate protective pathways important to enable hepatocyte proliferation.

Interleukin 6 (IL6) belongs to a family comprising of IL6, IL11, leukemia inhibitory factor, oncostatin M, ciliary neurotropic factor, and cardiotropin 1. They all need the gp130 molecule for signal transduction (1, 2). Knockout mice with deletion of individual cytokines of the IL6 family have a rather mild phenotype (3). Experiments elucidating the role of one specific cytokine of the IL6 family during different pathophysiological conditions are thus hampered by potential redundancy in activity by another family member. Because all known IL6 family members use gp130 for signal transduction, mice lacking functional gp130 are an attractive model to study the role of IL6-related pathways in vivo.

gp130 knockout mice die in utero or directly after birth due to myocardial hypoplasia and reduced hemopoiesis in the fetal liver (4, 5). In order to circumvent this problem, conditional gp130 knockout mice using the Cre/loxP system were generated (6). In these mice exon 16 coding for the gp130 transmembrane domain can be deleted as two loxP sites flank it. In animals with the Cre recombinase under the control of the Mx promoter, expression is induced by interferon-α or agents triggering internal interferon-α expression, e.g. pIpC (6, 7).

In the liver, IL6 is a major regulator of the acute phase response. It has also been shown that IL6 is essential for liver regeneration after partial hepatectomy (PH) (8, 9). First experiments indicated that there is an elevation of TNFα followed by IL6 in the serum of animals after PH (8). Further studies using IL6 and TNF receptor 1 (TNF-R1) knockout mice (9, 10) demonstrated that in both knockout animals S phase progression is impaired and some of the animals die after surgery. In both IL6 and TNF-R1 knockout mice, the defect in hepatocyte proliferation can be rescued by treating the animals with IL6. These results imply that the increase in TNFα levels is important to induce IL6, which is ultimately involved in triggering liver regeneration (9, 11).

However, other reports indicated the possibility that the role of IL6 on cell cycle progression during liver regeneration after PH is less essential as hypothesized previously (12). These results suggest the possibility that other factors, e.g. the expression of other IL6 family members, may modulate the outcome in IL6 family members knockout animals. We used conditional gp130 knockout mice to study liver regeneration after PH. By using this approach, we define more precisely the role of IL6/gp130-dependent signaling, and we show that these pathways may determine survival after PH but not the expression of genes directly involved in cell cycle control.

MATERIALS AND METHODS

Mice Strains, pIpC Stimulation, and Southern Blot Experiments—gp130loxP (+/−) Mx-Cre(−) mice were bred with gp130loxP (+/+) Mx-Cre(+) animals. By using this approach, littermates were always homozygote for gp130loxP and had a 50% chance for the Mx-Cre transgene. These animals with identical backgrounds were then used for further experiments. Cre+ and Cre− (controls) animals were always
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...treated with 80 μg of pIpC per mouse. At each time point indicated at least 4 animals were used in parallel. Blood was drawn from the animals at each time point. The remaining livers were reserved, pooled, and rinsed in ice-cold phosphate-buffered saline, and part of the livers were frozen immediately in liquid nitrogen or tissue-tek (Sakura Europe, Netherlands). The remaining liver was used for nuclear extracts (15).

SDS-PAGE and Western Blot Analysis—Nuclear extracts were separated on a 10% SDS-polyacrylamide gel and blotted onto a nitrocellulose membrane (Millipore Corp., Bedford, MA) as described previously (15), and Western blot analysis was performed. Membranes were probed with anti-cyclin E and anti-cyclin A as primary antibodies [cDNA (24)]. Liver nuclear extracts were incubated with 80 μg of pIpC per mouse. 10 days after stimulation and administration (data not shown). With 80 μg of pIpC injection each detectable band was performed from the digital light units generated by OptiQuant image analysis software (PerkinElmer Life Sciences). Quantification of each detectable band was performed from the digital light units generated by OptiQuant. The background was subtracted.

TUNEL Assay—The TUNEL Test (TdT-mediated DTU nick end labeling) was performed using the In Situ Cell Death Detection Kit, POD from Roche Diagnostics according to the instructions of the manufacturer. Sections were analyzed with a fluorescence microscope (Olympus, Hamburg, Germany).

RESULTS

gp130 Results in Exon 16 Deletion in gp130loxP/Mx-Cre Animals—gp130 induces interferon-α expression in vivo and activates the Mx promoter (7, 18). We injected increasing amounts of gp130 in gp130loxP (+/+)/Mx-Cre (+) animals and gp130loxP (+/+)/Mx-Cre (−) to analyze deletion of gp130 exon 16 in the liver. 72 h after pIpC injection, the liver of the animals was harvested, and DNA was prepared. As evidenced by Southern blot analysis, 20 μg of gp130 resulted in a more than 90% deletion of exon 16 in the gp130 locus in Mx-Cre (+) and not in Mx-Cre (−) animals (data not shown). With 80 μg (Fig. 1A) and higher amounts (160 and 320 μg, data not shown) of gp130 more than 95% of exon 16 was deleted in gp130loxP (+/+)/Mx-Cre (−) animals.

Next, we tested the functional consequences of a deletion higher than 95% of gp130 exon 16 after 80 μg of pIpC injection in the liver. gp130loxP (+/+)/Mx-Cre (+) animals were stimulated with 10 μg of IL6/mouse for 3 h at different times after gp130 treatment. Gel shift analysis with a 32P-labeled Stat3 consensus oligonucleotide showed that IL6-dependent Stat3 complex formation was completely blocked 10 days after gp130 administration (data not shown).

Stat3 Activation Is Blocked in gp130loxP/Mx-Cre Animals after PH—Earlier results using IL6 knockout mice implied that IL6 is involved in cell cycle progression after PH (9). We performed partial hepatectomies in conditional gp130 knockout mice. gp130loxP (+/+)/Mx-Cre (−) (also referred to as gp130-deleted) and gp130loxP (+/+)/Mx-Cre (−) (controls) animals were both treated with 80 μg of gp130. After 10 days PH was performed. For each time point indicated at least 4 animals...
were included, and the efficiency of gp130 exon 16 deletion was monitored by Southern blot analysis in the resected liver lobes. For further analysis only mice were included with a deletion efficiency >95%.

We first measured IL6 serum levels. In both groups IL6 serum levels increased 3 h after PH (Fig. 1B). In controls peak IL6 expression was found after 6 h and in the gp130-deleted group after 12 h. Maximal IL6 levels were more than 2-fold higher in the gp130-deleted group, and at later time points the decrease was slower compared with controls (Fig. 1B). Between the two groups there was no difference in mortality after PH.

EMSA experiments revealed strong Stat3 complex formation 1 h after PH in the control group. At later time points complex formation decreased and returned to background levels (Fig. 1C). In gp130-deleted animals Stat3 activation after PH was completely blocked. No increase in complex formation was found compared with the pretreatment level (Fig. 1C).

Suppressor of cytokine signaling 3 (SOCS-3) is a potent inhibitor of Stat3-dependent gene activation. A recent study by Campbell et al. (19) showed a strong regulation of SOCS-3 during liver regeneration. Therefore, we investigated SOCS-3 mRNA expression in both groups. SOCS-3 transcripts were strongly increased 3 and 6 h (more than 30-fold) after PH in the control group, whereas no regulation was found in gp130-deleted animals (Fig. 1D). No significant regulation was found for SOCS-1 and -2 in both groups after PH (data not shown).

DNA Synthesis after Partial Hepatectomy in gp130-deleted Mice—gp130-dependent signaling was blocked in gp130-deleted mice after PH. In further experiments we determined the effect on DNA synthesis after PH by BrdUrd pulse-labeling experiments in both groups.

In the control group a maximum of BrdUrd-positive cells was evident 48 h after PH. 39% ± 5 of all hepatocytes were BrdUrd-positive (Fig. 2, A and B). At later time points BrdUrd-positive cells decreased.

In gp130-deleted animals the maximum of BrdUrd-positive cells was observed 48 h after PH and was lower compared with controls. 31% ± 4 nuclei of all hepatocytes were BrdUrd-positive in gp130-deleted animals. At later time points the amount of BrdUrd-positive hepatocytes decreased (Fig. 2, A and B).

Delayed Induction of Cyclins in gp130-deleted Animals—In further experiments we studied the impact of gp130 deletion on cyclin expression after PH. Cyclin A and E expression was studied by Western blot experiments.

In control animals cyclin E expression was first positive 12 h after PH. There was a continuous increase in cyclin E expression for up to 48 h. At later time points cyclin E expression decreased (Fig. 3A). In gp130-deleted animals, cyclin E was only detected 24 h after PH, and a strong increase was observed up to 48 h after PH. At 72 h cyclin E expression declined (Fig. 3B).
Cyclin A expression in controls was detected 40 h after PH and decreased thereafter, whereas in gp130-deleted animals cyclin A expression was first positive at the 48-h time point (Fig. 3, C and D).

Gene Array Analysis in gp130-deleted Mice after IL6 Stimulation—Earlier experiments showed even more pronounced differences in cell cycle progression after hepatectomy in IL6 knockout animals compared with controls (9, 12). Therefore we searched for specific pathways to understand how IL6 may influence cell cycle progression in the liver. We stimulated gp130-deleted and control mice with IL6 for 6 h and prepared mRNA. The mRNAs of untreated and IL6-stimulated animals were used for gene array analysis. The focus of this analysis was on genes that are involved in cell cycle control. This analysis revealed two genes, c-jun and c-myc, that might have an impact on G0/G1 phase transition and are regulated in an IL6-dependent manner in the liver (Fig. 4A). No impact on genes directly involved in cell cycle control-like cyclins was found.

The gene array analysis showed also a strong effect on the expression pattern of the TNF-R1 and -R2 (Fig. 4B). The gp130-deleted animals are blocked in regulating the TNF-R expression.

Activation of Immediate Early Genes Is Inhibited in gp130-deleted Animals—In further experiments we tried to specify our gene array data by studying c-myc RNA expression by Northern blot analysis before and after PH. Basal expression and the induction after PH as found in controls were reduced in gp130-deleted animals. Thus gp130-dependent pathways contribute to the basal and inducible c-myc expression after PH (data not shown).

c-Jun is a member of the AP1 complex. Activation after PH was studied by gel shift experiments using a 32P-labeled AP1 consensus oligonucleotide. Strong complex formation was evident 1–36 h after PH in controls. At later time points complex formation decreased and was reduced to pretreatment levels (Fig. 4C). In the gp130-deleted animals specific complex formation was first evident only 36 h after hepatectomy and remained high 48 h after PH (Fig. 4C).

In addition to c-Jun, it has been shown also for NF-κB that its activation correlates with liver regeneration after PH (20, 21). In controls, biphasic NF-κB activation was evident 1–6 and 24–36 h after PH (Fig. 4D). In gp130-deleted mice the first peak of NF-κB activation was strongly reduced, whereas the second peak could be detected equally to controls (Fig. 4D).

Activation of DNA Synthesis after Partial Hepatectomy in IL6 Knockout Animals—Our experiments in the gp130-deleted...

![Fig. 3. Cyclin E and A expression in gp130-deleted mice after partial hepatectomy.](image)

A–D, cyclin E (A and B) and A (C and D) expression was determined in gp130 loxP/Mx-Cre− (A and C) and + (B and D) animals at time points (n >4 animals per time point) before and after PH. An arrowhead shows the position of cyclin E and A. PK, pyruvate kinase.

![Fig. 4. Delayed activation of immediate early genes in gp130-deleted mice after partial hepatectomy.](image)

A, gp130 loxP/Mx-Cre− and + mice were included. Animals were stimulated for 6 h (+) with IL6. cDNA array analysis was performed. The impact on cell cycle-related genes is shown. B, basal and IL-6-inducible expression of TNF-R1 and TNF-R2 in control and gp130-deleted animals as evidenced by gene array analysis. C and D, gel shift experiments were performed using liver nuclear extracts derived from gp130 loxP/Mx-Cre− and + before and after PH. 32P-Labeled consensus oligonucleotides for AP1 (C) or NF-κB (D) were used in this analysis. Specific complex formation is indicated. EMSA experiments for both groups were performed in parallel.
animals indicated that gp130-dependent pathways are essential for liver cells in contributing to the activation of distinct immediate early genes as shown previously (9). Because in our experiments the impact on DNA synthesis was less prominent after PH as in IL6−/− mice (9), we performed partial hepatectomies in IL6 knockout animals (13) and monitored BrdUrd incorporation. DNA synthesis was increased in controls 24 h after PH. Maximal levels were found 48 h after PH. 72 h after PH the amount of BrdUrd-positive cells decreased (Fig. 5, A and B). Compared with controls in IL6−/− animals the increase and maximum in DNA synthesis was moderately reduced (Fig. 5, A and B).

As strain-specific differences may account for changes in hepatocyte proliferation, we additionally performed PHs in a second IL6−/− strain (14) and stained for BrdUrd-positive cells. These results were consistent with the data obtained in the first IL6−/− strain (data not shown).

**Impaired Acute Phase Gene Regulation in gp130-deleted Animals**—IL6 contributes to acute phase gene expression and represents a first line of defense after bacterial infections (22). Thus after abdominal surgery the outcome may depend upon the ability of the liver to control unspecific defense mechanisms. In further experiments the impact on acute phase gene expression was studied in gp130-deleted mice. As shown in Fig. 6A, serum amyloid A, haptoglobin and hemopexin gene expression were increased starting 3–6 h, and maximal levels were evident 24 h after PH in controls. In contrast, this increase in acute phase gene expression after PH was not found in gp130-deleted mice (Fig. 6A).

Albumin mRNA expression decreased after PH in the control group (Fig. 6A). In gp130-deleted animals only minor variations in albumin mRNA levels were detected in the first 12 h post-PH (Fig. 6A). These results demonstrate that after PH regulation of the acute phase response is blocked in gp130-deleted animals.

**gp130-deleted and IL6 Knockout Animals Are More Vulnerable to LPS after Partial Hepatectomy**—As regulation of the acute phase response was impaired in gp130-deleted animals, we investigated the impact of a bacterial infection, mimicked by LPS injection, on liver regeneration. LPS was injected 3 h after PH, and the impact on survival and DNA synthesis was studied.

LPS-treated, gp130-deleted animals had a significantly worse outcome compared with controls. In the gp130-deleted group 40% of the animals died during the first 48 h after PH. In contrast, the survival rate in controls was 85% after LPS injection (p < 0.01, Fig. 6B).

Higher mortality correlated with a decrease in DNA synthesis in gp130-deleted animals who survived. BrdUrd staining after 48 h revealed an 80% lower rate of BrdUrd-positive hepatocytes in gp130-deleted animals compared with controls (Fig. 6C).

As LPS had a significant effect on survival and DNA synthesis of gp130-deleted animals, we also tested the impact of LPS on survival in IL6 knockout animals compared with controls (Fig. 7A). IL6−/− animals and controls were treated with LPS 3 h after PH. Survival was evaluated in the first 48 h after PH. In the IL6−/− animals significantly more animals died after PH (p < 0.05) when they were treated with LPS compared with controls and untreated animals. Thus as found in the gp130-deleted animals, IL6−/− mice were more susceptible to LPS after PH (Fig. 7A).

These differences were also reflected when BrdUrd staining was performed in the liver of these animals. DNA synthesis 48 h after PH in LPS-treated IL6−/− mice was significantly reduced compared with controls (Fig. 7B).

**Increased Apoptosis in gp130-deleted Animals after Partial Hepatectomy and LPS Stimulation**—The LPS/PH experiments...
indicated that protective mechanism might be impaired in gp130-deleted animals. Additionally, earlier experiments showed that IL6 might stimulate anti-apoptotic pathways by inducing higher Bcl-xL expression (23). Therefore we studied Bcl-xL expression through an RT-PCR approach early after PH. In control animals we found an increase in Bcl-xL expression 3 h after PH which was not found in gp130-deleted animals (Fig. 8, A and B).

As differences in Bcl-xL regulation might be one of the gp130-dependent mechanisms protecting from apoptosis after LPS stimulation, we now studied if there is a difference in the degree of apoptosis between controls and gp130-deleted animals. At the 3-h LPS/6-h PH time point we found a significant increase in apoptosis in the gp130-deleted animals compared with controls (Fig. 8C). In gp130-deleted mice 15-fold more liver cells were TUNEL-positive compared with controls (Fig. 8D).

DISCUSSION

The molecular mechanisms essential to restore liver mass after injury are complex, and the intra- and extracellular pathways that orchestrate liver regeneration have recently become clearer on a molecular level (11, 24, 25). Earlier experiments showed that after partial hepatectomy there is activation of Stat3 in the first hours after surgery (8, 26). Further experiments using IL6 knockout mice demonstrated that this cytokine is involved in regulating the regenerative response after PH (9). Additionally, TNF-R1 knockout mice showed a lack in liver regeneration that could be rescued by injecting IL6 (10). Based on these data a model has been deducted where first TNFα and then IL6 serum levels are increased, and the intra-cellular pathways that are activated through these two cytokines are essential to trigger cell cycle progression after PH.

In the present study we were interested to examine further the role of IL6 for liver regeneration after partial hepatectomy using conditional gp130 knockout mice. After birth Cre expression in gp130loxP/Mx-Cre animals was induced by pIpC injection. Earlier experiments demonstrated that in these animals efficient deletion of gp130 exon 16 was achieved in the liver and other tissue contributing to the immune response (spleen, bone marrow, thymus) (9). In the liver more than 95% of exon 16 in the gp130 locus was deleted, and IL6-dependent Stat3 activation was inhibited, in contrast to controls. All of the IL6 family members need gp130 for signal transduction, and thus the use of gp130loxP/Mx-Cre animals circumvents the possibility that other cytokines of this family may compensate for the loss of each other (1, 2).

After PH in hepatocytes of the control group strong Stat3 activation was restricted to the first hours after PH, whereas maximal IL6 serum levels were found after 6 h. Recent experiments published by Fausto and co-workers (19) have revealed a molecular explanation for this observation. They demonstrated that only 1 h after partial hepatectomy SOCS-3 is induced on the mRNA level and reached maximum expression after 2 h. In the present analysis we further characterized this mechanism and showed that SOCS-3 is controlled via gp130-dependent pathways in hepatocytes. Thus, through increasing SOCS-3 expression via gp130, Stat3 activation is strongly restricted after PH.

In our study there is a discrepancy between the relatively early activation of Stat3 after partial hepatectomy and the late peak of IL6 serum expression. As in the gp130-deleted animals
no Stat3 DNA binding was found, and activation is likely to be mediated through a gp130-dependent cytokine. Additionally in IL6+/− animals no Stat3 activation was found after PH (9). These two observations clearly indicate that IL6 induces Stat3 after PH. Therefore it is likely that increased IL6 levels are first present in the liver after PH as the cytokine at this stage may mainly bind to membrane-bound gp80. At later time points, gp80 receptors in the liver are saturated, and increased IL6 levels are present in the serum of the animals. In the serum IL6 is bound to soluble gp80 and soluble gp130 receptors that limit the activation of gp130-dependent pathways also in liver cells. As the pool of soluble gp130 is increased in the gp130-deleted animals, IL6 has a longer half-life in the serum of these animals.

Liver regeneration in gp130-deleted animals showed marked differences compared with controls. Stat3 activation was completely blocked, and gene array analysis defined additional gp130-dependent downstream targets, c-myc and c-jun, after PH. Additionally, our experiments suggest that NF-κB activation at the early time points during liver regeneration is gp130-dependent.

The explanation for diminished NF-κB and AP1 activation after partial hepatectomy might be caused on different levels. However, our gene array analysis also included TNF-R1 and TNF-R2. Interestingly, the expression of both receptors is controlled via IL6 as in gp130-deleted animals the basal and the inducible expression of TNF-R1 and the inducible expression of TNF-R2 are significantly reduced (Fig. 4F). As NF-κB and AP1 are controlled via TNF after PH, these results would explain in part a reduction in the activation of both transcription factors in the gp130-deleted animals after PH.

Our work is in agreement and expands earlier reports of Taub and co-workers (9) and Fausto and co-workers (10) indicating that activation of distinct immediate early genes is dependent on IL6 after PH (9, 10). However, DNA synthesis was reduced in conditional gp130 knockout animals but less severely impaired as described previously in IL6−/− animals. The increase in cyclin A and E expression was delayed in the gp130-deleted animals after PH. Therefore it is likely that the role of IL6 in the liver after PH is not mainly to trigger cell cycle progression through immediate early genes but instead is to protect the liver from injury during this complex step. We show in our study that in gp130-deleted animals acute phase gene regulation and thus early defense mechanisms are blocked after PH.

Therefore, to mimic a bacterial infection, we injected LPS in gp130-deleted and control animals after PH. These experiments revealed striking differences in the outcome of these animals similar to the results obtained in the initial PH experiments reported in the IL6−/− animals (9). After PH DNA synthesis was reduced in the surviving LPS-injected gp130-deleted animals, and the same phenotype was found when LPS was injected into IL6−/− mice after PH. Consistent with this observation, hepatocyte-specific gp130 knockout animals are also hypersensitive to LPS indicating that gp130-dependent pathways in hepatocytes are essential for this observation (30).

In further experiments we investigated the possible mechanism that might account for higher LPS sensitivity. As in earlier experiments Kovalovich et al. (23) showed that IL6 induces Bcl-xL expression, and we investigated its expression by RT-PCR and found lower expression in the gp130-deleted animals. After PH the finding correlated with a higher rate of apoptosis in the gp130-deleted animals after LPS stimulation indicating that this is most likely an important mechanism that contributes to the worse outcome in these animals after partial hepatectomy and LPS stimulation. Based on these results we define more specifically the role of the IL6/gp130-dependent pathway during liver regeneration. Higher IL6 expression after PH is important to activate protective pathways that render hepatocytes more resistant toward stress-inducing signals, e.g. bacterial infections after PH. Our results suggest that the phenotype of gp130-deleted and IL6 knockout animals after PH is related to the degree of additional injury. These results would nicely explain why there are differences in the literature upon the strength of the phenotype in IL6−/− mice after partial hepatectomy.

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