resistance (*16*). Treatment of cultured cells with FFAs suppresses insulin action at the level of the insulin receptor (*16*). However, the specific mechanisms by which FFAs induce insulin resistance are not understood. Our results suggest that FFAs might induce insulin resistance by increasing the expression of *TNF-α* or other genes that interfere with insulin action. aP2 might play a role in regulation of gene expression by binding and shuttling these FFAs to the target cellular compartments.

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Liver Failure and Defective Hepatocyte Regeneration in Interleukin-6–Deficient Mice

Drew E. Cressman,* Linda E. Greenbaum, Robert A. DeAngelis, Gennaro Ciliberto, Emma E. Furth, Valeria Poli, Rebecca Taub†

Liver regeneration stimulated by a loss of liver mass leads to hepatocyte and nonparenchymal cell proliferation and rapid restoration of liver parenchyma. Mice with targeted disruption of the interleukin-6 (IL-6) gene had impaired liver regeneration characterized by liver necrosis and failure. There was a blunted DNA synthetic response in hepatocytes of these mice but not in nonparenchymal liver cells. Furthermore, there were discrete G_1 phase (prereplicative stage in the cell cycle) abnormalities including absence of STAT3 (signal transducer and activator of transcription protein 3) activation and depressed AP-1, Myc, and cyclin D1 expression. Treatment of IL-6–deficient mice with a single preoperative dose of IL-6 returned STAT3 binding, gene expression, and hepatocyte proliferation to near normal and prevented liver damage, establishing that IL-6 is a critical component of the regenerative response.

Because of the distinctive replication potential of adult hepatocytes, the liver has the ability to regenerate, allowing for rapid recovery after partial hepatectomy, liver transplant, or toxic injury (*1*, *2*). After a 70% partial hepatectomy, in which the two largest lobes of the liver are removed intact without injury to remnant liver cells, more than 95% of the mature, normally quiescent cells in the remnant liver rapidly proliferate, restoring liver mass in a few days. Answers to the central questions about liver regeneration remain elusive, including the identity of the initiating signals in regeneration and the mechanism by which liver cells continue to function while they are regenerating.

Two transcription factor complexes, nuclear factor kappa B (NF-κB) and STAT3, are rapidly activated by means of posttranslational modifications in the remnant liver within minutes to hours after hepatectomy and may provide clues to the initiating signals (*3*, *4*). Epidermal growth factor (EGF), IL-6, and related cytokines are among the factors capable of stimulating STAT3 DNA binding activity in mouse liver nuclei (*5*). Because it is a hepatocyte mitogen (*1*, *2*), initially it appeared likely that EGF was responsible for induction of STAT3 after hepatectomy. However, a role for gut and Kupffer cell (liver macrophage)–derived cytokines, IL-1, IL-6, and tumor necrosis factor–α (TNF-α), was suggested to us in part because the transcription complexes NF-κB and STAT3 are both linked to cytokine activation pathways (*2*–*5*), an intact portal circulation is required for normal regeneration, and germ-free and lipopolysaccharide (LPS)–deficient animals show a blunted regenerative response (*6*). An in vitro study suggests a supportive role for IL-6 in hepatocyte proliferation (*7*), but, although controversial, most of the data suggest that large quantities of TNF-α may actually increase liver injury (*8*).

With the availability of mice harboring a targeted disruption of the gene encoding IL-6, we were able to determine whether IL-6 is responsible for activating STAT3 after hepatectomy. IL-6–deficient ($IL\text{-}6^{-/-}$) mice are developmentally normal and display abnormalities in the hepatic acute-phase response, some immune mechanisms, and bone resorption in response to estrogen (*9*–*11*). Antibody supershift experiments demonstrated that a STAT3 complex was induced in hepatectomized mouse livers (*12*) (Fig. 1A). STAT5, also a 92-kD protein, is activated in mouse liver in response to EGF stimulation (*13*); however, antibodies to STAT5 did not supershift the complex after hepatectomy. STAT3 DNA binding was strongly induced in the IL-6–containing ($IL\text{-}6^{+/+}$) remnant liver nuclear extracts with appearance 0.5 hour, peak activity 2 hours, and continued elevation 8 hours after hepatectomy (Fig. 1B), correlating well with results in rats (*4*). In $IL\text{-}6^{-/-}$ mice, STAT3 DNA binding was virtually absent, suggesting that STAT3 induction during liver regeneration is strictly mediated by IL-6. There was little induction of STAT3 binding after sham surgery in $IL\text{-}6^{+/+}$ animals and none in the $IL\text{-}6^{-/-}$ mice (Fig. 1C), confirming that STAT3 activation is specific to the regenerative response of the liver and not due to an acute-phase response induced by the surgical procedure.

D. E. Cressman, L. E. Greenbaum, R. A. DeAngelis, R. Taub, Department of Genetics and Medicine, University of Pennsylvania School of Medicine, 705a Stellar-Chance, 422 Curie Boulevard, Philadelphia, PA 19104–6145, USA.
G. Ciliberto and V. Poli, Instituto di Ricerche di Biologia Molecolare, IRBM Pietro Angeletti, Via Pontina Km 30.600, Pomezia (Roma), Italy.
E. E. Furth, Department of Pathology, University of Pennsylvania School of Medicine, 705a Stellar-Chance, 422 Curie Boulevard, Philadelphia, PA 19104–6145, USA.

*Present address: Duke University Medical Center, Research Drive, Durham, NC 27710, USA.
†To whom correspondence should be addressed.

Liver extracts from IL-$6^{-/-}$ mice killed in the absence of surgery 20 min after IL-6 injection (time 0) and IL-$6^{-/-}$ mice treated with IL-6 and subjected to partial hepatectomy showed high, prolonged STAT3 DNA binding greater than STAT3 binding in IL-$6^{+/+}$ animals at equivalent times, indicating that latent STAT3 exists in liver cells of IL-$6^{-/-}$ mice (Fig. 1D).

If IL-6 is important for normal liver regeneration, the regenerative response should be abnormal in IL-$6^{-/-}$ animals. By ~24 hours after surgery, 8 of 48 IL-$6^{-/-}$ animals had died, and between 24 and 96 hours, 11 of 40 others showed lethargy, anorexia, and tremulousness, some with gross jaundice. Mortality and morbidity were greatly increased in IL-$6^{-/-}$ (19 of 48, 40%) relative to IL-$6^{+/+}$ (3 of 29, 10%) (probability $P < 0.001$, χ^2) or IL-6–treated IL-$6^{-/-}$ mice (1 of 13, 8%) ($P < 0.001$) 24 hours and longer after hepatectomy. Normal liver architecture was seen in IL-$6^{-/-}$ animals before surgery (Fig. 2A), but the livers of most IL-$6^{-/-}$ animals, even those with no apparent morbidity, showed large areas of necrosis and ballooning degeneration of hepatocytes (without apoptosis) 36 to 72 hours after hepatectomy, an indication of hepatocyte injury and fragility (Fig. 2B) (*14*). The livers of jaundiced IL-$6^{-/-}$ animals were small and erythematous 4 to 5 days after hepatectomy, and massive hepatic necrosis was apparent (Fig. 2C). Little or no necrosis was seen in the livers of the ~50% of IL-$6^{-/-}$ animals that survived 96 and 120 hours after hepatectomy (*15*), indicating that either resolution or progression (Fig. 2C) can occur. Hepatic necrosis or signs of liver failure were not seen in any IL-$6^{+/+}$ or IL-6–treated IL-$6^{-/-}$ mice at any time after hepatectomy.

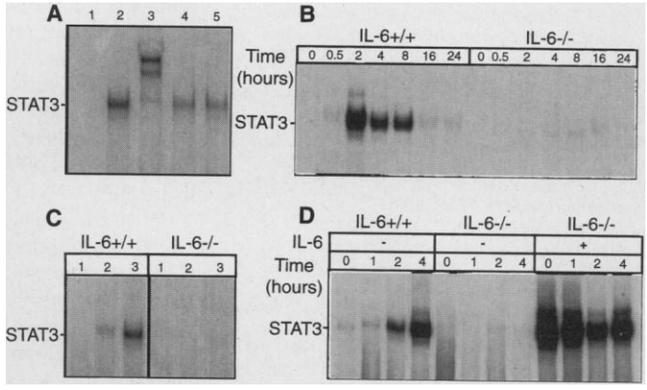

Fig. 1. STAT3 activation in IL-$6^{+/+}$ but not IL-$6^{-/-}$ livers after hepatectomy. (**A**) Gel mobility-supershift assays with nuclear extract made 1 hour after hepatectomy incubated alone or with STAT3, STAT5, or p50NF–κB1 (control) antibodies (lanes 2 to 5, respectively). Lane 1, probe alone. As a control for nonspecific antibody-DNA interactions, antibodies were incubated with oligonucleotide alone and no reaction occurred (*15*). The oligonucleotide probe contains the STAT binding site of the sis-inducible element (SIE) from the Fos promoter. (**B**) Gel mobility-shift assay of nuclear extracts from IL-$6^{+/+}$ or IL-$6^{-/-}$ mice made at the indicated times after hepatectomy. (**C**) Nuclear extracts were made 3 hours after sham surgery (lanes 2) or partial hepatectomy (lanes 3) in IL-$6^{+/+}$ or IL-$6^{-/-}$ livers and incubated with the SIE probe in gel mobility-shift assays. Lanes 1, normal liver extract. (**D**) Gel mobility-shift assay of liver nuclear extracts prepared at the indicated times after hepatectomy (0 hours, no surgery, normal liver) from IL-$6^{+/+}$ and IL-$6^{-/-}$ mice and IL-$6^{-/-}$ mice treated with recombinant IL-6 20 min before surgery [IL-$6^{-/-}$ (+IL-6)].

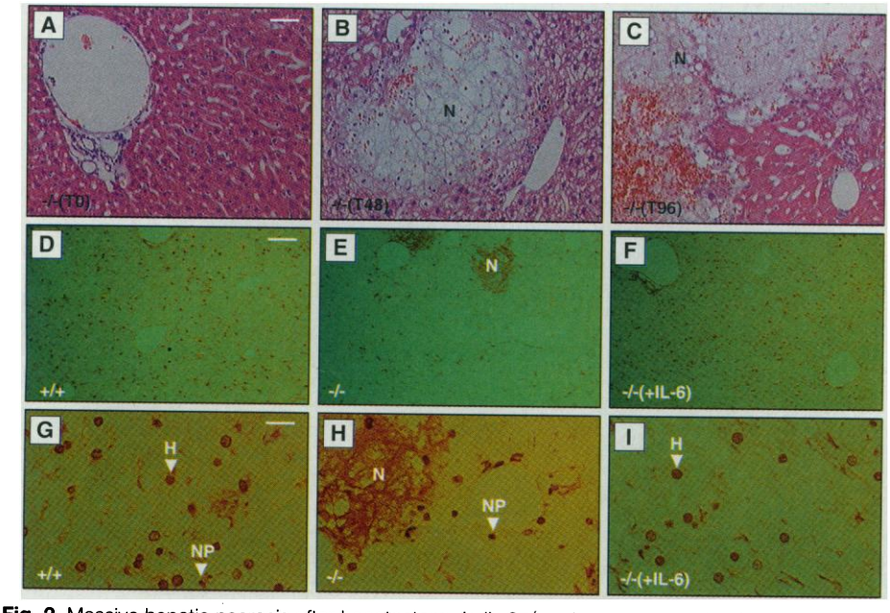

Fig. 2. Massive hepatic necrosis after hepatectomy in IL-$6^{-/-}$ mice was prevented by treatment with IL-6. Hematoxylin and eosin stain of IL-$6^{-/-}$ liver at (**A**) time 0 (T0), (**B**) 48 hours (T48), and (**C**) 96 hours (T96) after partial hepatectomy. Low-power photomicrographs of (**D**) IL-$6^{+/+}$, (**E**) IL-$6^{-/-}$, and (**F**) IL-$6^{-/-}$ (+IL-6) livers and high-power photomicrographs of (**G**) IL-$6^{+/+}$, (**H**) IL-$6^{-/-}$, and (**I**) IL-$6^{-/-}$ (+IL-6) livers 48 hours after hepatectomy and after BrdU labeling. Round, large nuclei indicate positively stained hepatocytes (H). NP, nonparenchymal cells; N, areas of necrosis. Scale bars, 40 μm for (A) through (F) and 10 μm for (G) through (I).

Bromodeoxyuridine (BrdU) incorporation detected by immunohistochemistry was used to measure the number of S phase cells at various times after partial hepatectomy in IL-$6^{+/+}$ and the cohort of IL-$6^{-/-}$ animals that appeared well after surgery (*16*). No BrdU staining occurred in IL-$6^{+/+}$ and IL-$6^{-/-}$ livers before surgery (0 hours after hepatectomy), consistent with the cells in a quiescent (G_0) stage. The level of induced hepatocyte DNA synthesis was abnormal in the IL-$6^{-/-}$ animals (Fig. 3A), with a reduction to between 20 and 25% of wild-type levels at the peak time 36 hours ($P < 0.001$) and later 48 hours ($P < 0.02$) after hepatectomy. Only a mild increase in periportal nonparenchymal cell DNA synthesis was seen in IL-$6^{-/-}$ livers compared with the dramatic activation of periportal progenitor cells observed in liver injury models (Fig. 2H) (*17*). On the other hand, DNA synthesis in nonparenchymal cells including endothelial, Kupffer, and other sinusoidal cells was unchanged in IL-$6^{+/+}$ and IL-$6^{-/-}$ livers (Fig. 3B). As expected, DNA synthesis peaked in nonparenchymal cells later than in hepatocytes (*1*, *2*).

If aberrant proliferation of hepatocytes after hepatectomy is the direct effect of IL-6 deficiency and not a secondary defect in IL-$6^{-/-}$ mice, then it should be possible to correct the regenerative defect by treating IL-$6^{-/-}$ mice with IL-6. Treatment with a single dose of IL-6 followed by partial hepatectomy restored hepatocyte BrdU incorporation 36 and 48 hours after hepatectomy in the IL-$6^{-/-}$ animals to 70% of the wild-type S phase hepatocytes at 36 hours (not statistically different from wild type, $P > 0.15$), and at 48 hours the number of S phase cells was slightly higher but not statistically different from wild type ($P > 0.4$) (Figs. 2 and 3). Like IL-$6^{+/+}$ mice, treated

IL-6$^{-/-}$ mice had four to five times more S phase hepatocytes than untreated IL-6$^{-/-}$ mice 36 ($P < 0.04$) and 48 ($P < 0.02$) hours after hepatectomy (Figs. 2 and 3). There was some increase in DNA synthesis in IL-6–treated IL-6$^{-/-}$ compared with IL-6$^{+/+}$ hepatocytes 24 and 96 hours after hepatectomy, but the difference was not statistically significant. In the absence of partial hepatectomy, treatment with IL-6 (three IL-6$^{-/-}$ animals) did not result in DNA synthesis 36 hours later. In addition, the mitotic index of IL-6$^{+/+}$ and IL-6$^{-/-}$ (+IL-6) hepatocytes after hepatectomy was significantly increased ($P < 0.02$) relative to IL-6$^{-/-}$ hepatocytes (Fig. 3D), and they were not different from each other. In IL-6$^{+/+}$ animals, liver mass was virtually restored after 4 days, and mass restoration was not significantly different between IL-6$^{+/+}$ and IL-6–treated IL-6$^{-/-}$ animals. The recovery of liver mass in IL-6$^{-/-}$ animals that survived was significantly delayed at all times relative to IL-6$^{+/+}$ and treated IL-6$^{-/-}$ animals until 5 days after hepatectomy when mass was not different from normal (animals with morbidity were excluded). In IL-6$^{-/-}$ animals with restored liver mass, there was no evidence of a delayed increase in S phase or M phase hepatocytes during the 5-day time course examined (Fig. 3, A and D), but significant hypertrophy of liver cells can occur even in the absence of DNA synthesis (*18*).

A subset of genes that are activated early in regeneration (*2*, *19*), including those encoding c-Fos, c-Myc, LRF-1 (rat ATF-3), STAT3, and JunB, showed a 3- to >10-fold reduction in expression in hepatectomized IL-6$^{-/-}$ livers from early to mid-G_1 phase (Fig. 4, A and B) (*20*). Two of the genes most strongly affected, those encoding JunB and c-Fos, are positively transactivated by STAT promoter elements; in particular, full expression of the c-Fos gene in vivo requires the STAT DNA binding element (*21*, *22*). In addition to transcription factors, β-actin, an integral component of the cytoskeletal structure that is encoded by a growth-associated gene, was expressed at a reduced level in IL-6$^{-/-}$ livers. Induction of hepatic acute-phase genes encoding SAP and HPX was low in hepatectomized IL-6$^{+/+}$ livers as expected (*10*, *23*), and SAP mRNA induction was less in IL-6$^{-/-}$ livers. In IL-6$^{-/-}$ livers, expression was normal for genes encoding PRL-1, HGF, and c-Jun, all of which are proteins involved in cell growth (*24*); two hepatocyte-specific genes, encoding glucose 6-phosphatase and insulin-like growth factor binding protein–1 (*15*, *25*, *26*); ODC; and C/EBPβ. The gene for C/EBPβ is regulated by IL-6 in some cell models (*27*) but apparently not in regenerating liver. IL-6$^{-/-}$ (+IL-6) livers had 70 to 100% of IL-6$^{+/+}$ gene expression for β-actin, JunB, LRF-1, c-Fos, c-Myc, STAT3, and SAP (Fig. 4B), an increase of >10-fold relative to IL-6$^{-/-}$ for some genes. Genes that were normally regulated in IL-6$^{-/-}$ livers (for example, gene for C/EBPβ) showed no change in their expression after IL-6 treatment. Thus, IL-6, by means of its activation of STAT3 or other factors, controls expression of a limited number of immediate-early genes during liver regeneration, implying that it has a role in specific intracellular pathways. Some mitogenic signals are reaching the hepatectomized IL-6$^{-/-}$ liver, because nonparenchymal DNA synthesis is normal, and some genes are induced normally in hepatocytes.

In IL-6$^{+/+}$ livers, c-Fos protein expression was biphasic with peaks 2 and 8 hours after hepatectomy (Fig. 4C). In IL-6$^{-/-}$ livers, c-Fos was slightly reduced and absent 2 and 8 hours, respectively, after hepatectomy. In IL-6$^{-/-}$ livers, total AP-1 activity was reduced by at least a factor of 2 at all time points. In particular, the JunB component of the 8-hour complex was reduced by a factor of 10, and the AP-1–related factor LRF-1 was also reduced (*15*). However, the activation of NF-κB was no different in IL-6$^{+/+}$ and IL-6$^{-/-}$ livers, which suggests that the signal, possibly TNF-α (*3*, *4*), that results in NF-κB induction is present in a normal amount in IL-6$^{-/-}$ mice.

The gene encoding cyclin D1 is transcriptionally regulated by AP-1 and c-Myc (*28*), both of which were expressed at a reduced level in IL-6$^{-/-}$ mice and, of the cyclins, is the best marker for the G_1-to-S transition in regenerating liver (*29*). Levels of cyclin D1 protein were greatly decreased in the IL-6$^{-/-}$ livers 36, 48, and 60 hours after hepatectomy (Fig. 4C). This result suggests that the amount of cyclin D1 that is attained in most IL-6$^{-/-}$ hepatocytes is insufficient to drive the cells into S phase.

The liver is the first line of defense against ingested toxins, which, like hepatectomy, consistently result in increased LPS and cytokine concentrations in the portal circulation (*30*). Gut-derived cytokines stimulate hepatic Kupffer cells and endothelial cells to produce IL-1, TNF-α, and, subsequently, IL-6, which may be induced in part by TNF-α and IL-1 by means of activation of NF-κB (*31*). The ability of hepatocytes to proliferate in response to IL-6 (and other cytokines or growth factors) provides a protective mechanism by which this vital organ is able to recover from toxin-mediated damage and other forms of injury. In liver cirrhosis and alcoholic liver disease, continuous elevation of IL-6 and other cytokines may lead to conversion of hepatic fat-storing cells to collagen-producing

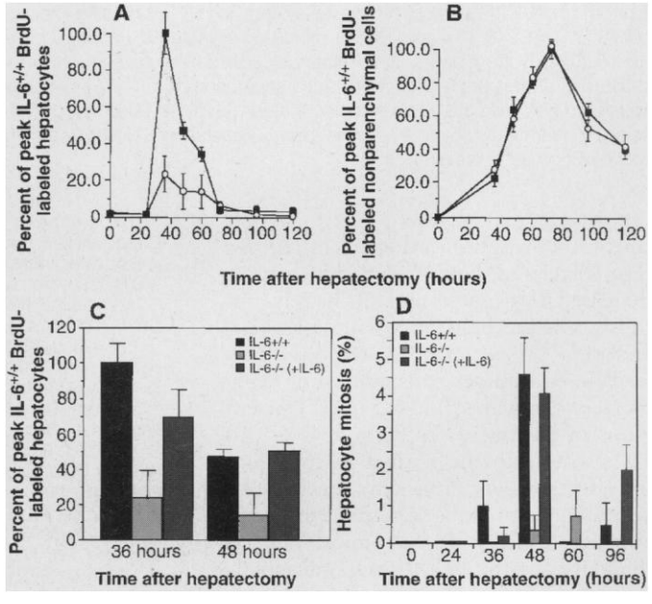

Fig. 3. Reduction of S phase and M phase hepatocytes in IL-6$^{-/-}$ livers after hepatectomy restored by treatment with IL-6. IL-6$^{+/+}$ and IL-6$^{-/-}$ mice were subjected to partial hepatectomy; 1 hour before harvest, animals were injected with BrdU. The remnant liver was harvested at the indicated times, fixed, sectioned, and stained with antibodies to BrdU. Positive, dark-staining hepatocyte (**A**) and nonparenchymal cell (**B**) nuclei were quantitated in each sample by counting the number of cells per low-power field for three fields by two investigators. Slides from IL-6$^{+/+}$ and IL-6$^{-/-}$ animals were processed simultaneously. The mean for each time point was plotted as a percentage of the number of labeled cells at the peak time of IL-6$^{+/+}$ animals. Standard deviations are indicated, and significance was determined on the basis of at least three animals. (**C**) Positively stained hepatocyte nuclei were quantitated and plotted from IL-6$^{+/+}$, IL-6$^{-/-}$, and IL-6$^{-/-}$ (+IL-6) animals as a percentage of the number of positive hepatocytes from IL-6$^{+/+}$ mice 36 or 48 hours after hepatectomy. Standard deviations are shown. (**D**) Mitotic figures in hepatocytes were counted and quantitated as a percentage of the total number of hepatocytes in four or five high-power fields at the indicated times after hepatectomy. No IL-6$^{-/-}$ (+IL-6) livers were evaluated 60 hours after hepatectomy.

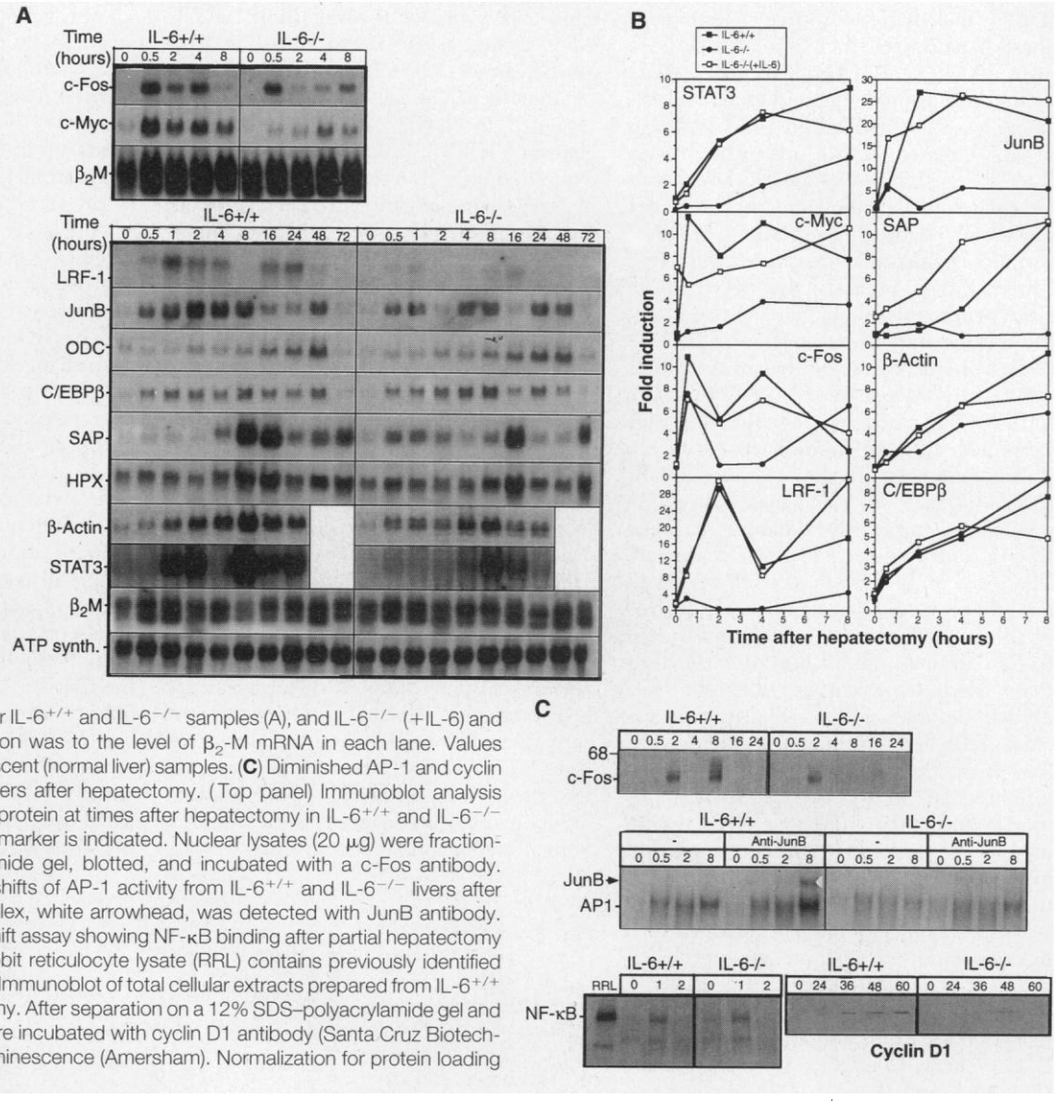

Fig. 4. Altered patterns of gene expression in IL-6$^{-/-}$ livers after hepatectomy, corrected by IL-6 treatment. (**A**) RNA was prepared from IL-6$^{+/+}$ and IL-6$^{-/-}$ livers as described (*20*) at the indicated times after hepatectomy. RNA (10 μg) was gel electrophoresed and probed with nick-translated cDNA probes for the indicated genes. β_2-microglobulin (β_2M) and ATP-synthetase (ATP synth.) were used as normalization controls. SAP, serum amyloid protein; HPX, hemopexin; ODC, ornithine decarboxylase. (**B**) Correction of gene expression in IL-6$^{-/-}$ mice treated with IL-6 after hepatectomy. Three IL-6$^{-/-}$ and IL-6$^{+/+}$ animals at each time point (0 to 4 hours) and two animals at 8 hours were used to demonstrate consistency in the Northern blots for some genes, and one animal was used for each of five different time points for the IL-6$^{-/-}$ animals treated with IL-6. After densitometric scanning of representative Northern blots for IL-6$^{+/+}$ and IL-6$^{-/-}$ samples (A), and IL-6$^{-/-}$ (+IL-6) and IL-6$^{+/+}$ samples (*15*), normalization was to the level of β_2-M mRNA in each lane. Values represent fold induction over quiescent (normal liver) samples. (**C**) Diminished AP-1 and cyclin D1, but not NF-κB, in IL-6$^{-/-}$ livers after hepatectomy. (Top panel) Immunoblot analysis showing expression of the c-Fos protein at times after hepatectomy in IL-6$^{+/+}$ and IL-6$^{-/-}$ liver. Position of 68-kD molecular marker is indicated. Nuclear lysates (20 μg) were fractionated on a 10% SDS–polyacrylamide gel, blotted, and incubated with a c-Fos antibody. (Middle panel) Gel mobility supershifts of AP-1 activity from IL-6$^{+/+}$ and IL-6$^{-/-}$ livers after hepatectomy. Supershifted complex, white arrowhead, was detected with JunB antibody. (Bottom panel, left) Gel mobility-shift assay showing NF-κB binding after partial hepatectomy in IL-6$^{+/+}$ and IL-6$^{-/-}$ livers. Rabbit reticulocyte lysate (RRL) contains previously identified NF-κB (*3, 4*). (Bottom panel, right) Immunoblot of total cellular extracts prepared from IL-6$^{+/+}$ and IL-6$^{-/-}$ livers after hepatectomy. After separation on a 12% SDS–polyacrylamide gel and transfer to nitrocellulose, blots were incubated with cyclin D1 antibody (Santa Cruz Biotechnology) and detected by chemiluminescence (Amersham). Normalization for protein loading was by Coomassie staining.

cells (*30*). An untoward effect of chronic IL-6, IL-1, and TNF-α elevation may be increased fibrogenesis and, ultimately, cirrhosis. On the basis of our findings, IL-6 elevation is an adaptive response that leads to continuous regeneration of hepatocytes within the diseased liver. Current forms of treatment for chronic liver diseases with cytokine antibodies to reduce the effects of overactive cytokines must be carefully designed, because too little cytokine activity, as in IL-6$^{-/-}$ mice, hampers the ability of the liver to respond rapidly and completely to tissue damage.

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12. IL-6$^{-/-}$ mice (*10*) were generated on C57BL/6 backgrounds and tested for homozygosity for the knockout as described. For regenerating liver, 12- to 16-week-old animals were anesthetized with metafane and subjected to midventral laparotomy with ~70% liver resection (left lateral and median lobes) [G. M. Higgins and R. M. Anderson, *Arch. Pathol.* **12**, 186 (1931)]. Animals were killed by cervical dislocation. For IL-6–treated mice, animals were injected subcutaneously with human recombinant IL-6 (1 mg per kilogram of body weight) [0.5% solution in phosphate-buffered saline (PBS)] 20 min before surgery. Human recombinant IL-6 was produced in *Escherichia coli* and purified to homogeneity as described [R. Arcone *et al.*, *Eur. J. Biochem.* **198**, 541 (1991)] with minor modifications. Protein concentration was determined by analysis of the purified product, and the specific activity (1×10^9 U/mg) was assessed by the 7TD1 cell-growth assay [J. Van Snick *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 9679 (1986)]. Human IL-6 is effective in rodents (*7*). Sham-operated animals were subjected to midventral laparotomy with minimal trauma to the liver. Nuclear extracts from livers were prepared from the remaining liver lobes as described (*4*) and stored in 20 mM Hepes (pH 7.6), 0.2 mM EDTA, 100 mM KCl, 20% glycerol (v/v), 1.0 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride with protease inhibitors, 1 mM NaF, 1 mM Na_2MoO_4. All extracts were prepared in the presence of antipain, aprotinin, bestatin, and leupeptin (each at 2 μg/ml) (Boehringer Mannheim). Protease inhibitors were added immediately at the time of

remnant liver removal. Binding reactions were performed essentially as described (*3, 4*) with nuclear extracts from mouse liver cells after hepatectomy. For STAT binding, the probe used was a previously annealed high-performance liquid chromatography–purified double-stranded oligonucleotide from the sis-inducible factor binding element in the c-*fos* promoter (5′-GATCCTCCAGCATTTCCCGTAAATCCTCCAG-3′) (*22*) and end-labeled with [γ-^{32}P]adenosine triphosphate (ATP). Supershift experiments were performed by incubating 1 μl of primary antibody with the nuclear extracts in binding buffer for 1 to 2 hours at 4°C before addition of the labeled oligonucleotide. Antibody to STAT3 (anti-STAT3) and anti-STAT5 were from Santa Cruz Biotechnology.

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16. Hepatectomized animals were anesthetized and ventral laparotomy was performed. Normal liver was prepared by subjecting animals to laparotomy followed by perfusion as described (*26*). One hour before the remnant liver was harvested and fixed, animals were injected intraperitoneally with BrdU (50 mg/kg) (0.2% solution in PBS) [B. Schutte, M. M. J. Reynders, F. T. Bosman, G. H. Blijham, *J. Histochem. Cytochem.* **35**, 1343 (1987)]. The portal vein was cannulated with a 22-gauge angiocatheter, the liver was flushed with PBS, and 4% paraformaldehyde (pH 7.2) (4°C) was then perfused for 10 min at a rate of 6 ml/min. The fixed liver was removed and cut into 5-mm slices with a razor blade and then fixed for 1 hour in 4% paraformaldehyde at 4°C. An automated tissue processor was used to embed liver slices with paraffin. Tissue sections (5 μm) were cut on a microtome and adhered to poly-L-lysine–coated glass slides. Staining of fixed tissue samples with an antibody to BrdU (Boehringer Mannheim) allows one to discern proliferating cells (brown, stained nuclei) from quiescent ones (clear, unstained nuclei). The immunohistochemical study was performed essentially as described [S. M. Hsu, L. Raine, H. Fanger, *Am. J. Clin. Pathol.* **75**, 734 (1981); L. E. Greenbaum, D. E. Cressman, B. A. Haber, R. Taub, *J. Clin. Invest.* **96** (1995)]. StatWorks and Student's *t* test, respectively, were used for statistical analyses on animal liver weights and DNA synthesis.
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20. For RNA preparation, animals were killed at the indicated times, and total liver RNA preparation, Northern blots, and hybridizations were performed as described [K. L. Mohn *et al.*, *Mol. Cell. Biol.* **11**, 381 (1991)]. For immunoblots, 20 μg of nuclear or whole-cell extract was electrophoresed on 10 to 15% SDS–polyacrylamide gels, transferred to nitrocellulose, and detected by chemiluminescence (Amersham) according to the instructions of the manufacturer as described (*3, 4*). Primary antibodies used in protein immunoblots, electrophoretic gel-mobility supershift, and immunohistochemistry studies were anti-Fos and anti-cyclin D1 (Santa Cruz Biotechnology), anti-p50–NF-κB1 (*2*), anti-JunB, and anti-LRF [J.-C. Hsu, R. Bravo, R. Taub, *Mol. Cell. Biol.* **12**, 4654 (1992)]. The AP-1 probe was a double-stranded oligonucleotide containing the consensus AP-1 site (3′-CGCTTGATGAGTCAGCCGGAA-5′) (Promega). The NF-κB probe was a previously annealed high-performance liquid chromatography–purified double-stranded oligonucleotide from the class I major histocompatibility complex enhancer element H2-κB (5′-TCGAGGGCTGGGGATTCCCCATCTC-3′) (*2*).
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32. We thank J. Darnell for the STAT3 cDNA, U. Muller Eberhard and S. Maeda for the hemopexin (HPX) and serum amyloid P-component (SAP) probes, and C. Steer for the gift of the cyclin D1 cDNA and helpful discussions. We also thank C. Deutschmann, R. Diamond, D. Tewari, P. Traber, M. Lazar, and F. Rauscher for helpful discussions; S. Hwang and V. Miles for technical assistance; and J. Matthews for assistance with manuscript preparation. This work was in part supported by NIH grants DK44237, DK49210, and DK49629 (to R.T.); NIH grant K08 (to L.E.G.); the University of Pennsylvania Genetics Training Grant (to R.A.D.); and the Howard Hughes Medical Institute.

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Neuroprotection by Aspirin and Sodium Salicylate Through Blockade of NF-κB Activation

Mariagrazia Grilli,* Marina Pizzi, Maurizio Memo, PierFranco Spano

Aspirin (acetylsalicylic acid) is a commonly prescribed drug with a wide pharmacological spectrum. At concentrations compatible with amounts in plasma during chronic anti-inflammatory therapy, acetylsalicylic acid and its metabolite sodium salicylate were found to be protective against neurotoxicity elicited by the excitatory amino acid glutamate in rat primary neuronal cultures and hippocampal slices. The site of action of the drugs appeared to be downstream of glutamate receptors and to involve specific inhibition of glutamate-mediated induction of nuclear factor kappa B. These results may contribute to the emerging theme of anti-inflammatory drugs and neurodegeneration.

Glutamate is the most abundant excitatory neurotransmitter in the brain; however, under certain conditions, it may become a potent excitotoxin and contribute to neurodegeneration (*1*). On the other hand, an accumulation of clinical and experimental evidence suggests that neurodegeneration is often associated with inflammation (*2*). We tested the possibility that the anti-inflammatory drugs aspirin [acetylsalicylic acid (ASA)] and sodium salicylate (NaSal), because of their wide spectrum of pharmacological activities and multiple sites of action (*3*), may confer neuroprotective properties.

Several models of neurons in culture have been used to unravel the molecular events triggered by glutamate that lead to cell death as well as to develop pharmacological compounds able to counteract excitotoxicity. Here we used primary cultures of rat cerebellar granule cells, where a brief pulse of glutamate, through activation of the *N*-methyl-D-aspartate (NMDA) type of glutamate receptor, induces cell death (*4*). ASA and NaSal were added to the culture medium 5 min before and during a 15-min application of 50 μM glutamate (*5*), a concentration that reduced cell survival by 70 to 80%. The range of concentrations for both drugs was correlated with the amounts in plasma (1 to 3 mM) for optimal anti-inflammatory effects in patients with rheumatic diseases (*3*). A concentration-dependent protection against glutamate-induced neurotoxicity was observed in the presence of both drugs (Fig. 1A). For ASA, the calculated median effective concentration (EC_{50}) was 1.7 mM, with maximal effect (83% protection) at 3 mM. The concentration of NaSal giving 50% protection was 5 mM, and maximal response (87% protection) was observed at 10 mM. Unlike salicylates, at concentrations compatible with the plasma levels during chronic drug treatment (1 to 20 μM) (*3*), the anti-inflammatory drug indomethacin was unable to prevent glutamate-induced cell death (*6*).

Neuroprotection was also evaluated in hippocampal slices of 8-day-old rat brain

Division of Pharmacology, Department of Biomedical Sciences and Biotechnologies, University of Brescia Medical School, Brescia, I-25123 Italy.

*To whom correspondence should be addressed. E-mail: mpharm@master.cci.unibs.it