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Neurokinin-1 receptor antagonists CP-96,345 and L-733,060 protect mice from cytokine-mediated liver injury

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a) Running Title: NK-1 receptor antagonists protect against liver inflammation

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d) ALT, alanine aminotransferase; AST, aspartate aminotransferase; IFNγ, interferon-γ; IL, interleukin; LPS, lipopolysaccharide; NK-1R, neurokinin-1 receptor; NPC, non-parenchymal liver cells; TNFα, tumor necrosis factor-α.

e) Inflammation & Immunopharmacology

Abstract

Previously, we have shown that primary afferent sensory neurons are necessary for disease activity in T cell-mediated immune hepatitis in mice. In the present study we analyzed the possible role of substance P (SP), an important pro-inflammatory neuropeptide of these nerve fibers, in an *in vivo* mouse model of liver inflammation. Liver injury was induced by bacterial lipopolysaccharide (LPS) in D-galactosamine (GalN)-sensitized mice. Depletion of primary afferent nerve fibers by neonatal capsaicin treatment down-regulated circulating levels of the pro-inflammatory cytokines TNFα and IFNγ and protected mice from GalN/LPS-induced liver injury. Likewise, pretreatment of mice with antagonists of the SP-specific neurokinin-1 receptor (NK-1R), i.e. CP-96,345 and L-733,060, dose-dependently protected mice from GalN/LPS-induced liver injury. The presence of the NK-1R in the murine liver was demonstrated by RT-PCR, sequence analysis as well as by immunocytochemistry. NK-1R blockade reduced inflammatory liver damage, i.e. edema formation, neutrophil infiltration, hepatocyte apoptosis and necrosis. To get further insight into the mechanism by which receptor blockade attenuated GalN/LPS-induced liver damage, we analyzed plasma levels and intrahepatic expression of TNFα, IFNγ, IL-6, and IL-10. NK-1R blockade clearly inhibited GalN/LPS-induced production of TNFα and IFNγ, whereas synthesis of the hepatoprotective cytokines IL-6 and IL-10 was increased. NK-1 receptor antagonists, most likely by inhibiting SP effects, might be potent drugs for treatment of inflammatory liver disease.

Capsaicin-sensitive primary afferent neurons, mainly equipped with unmyelinated C-fibers, are responsible for neurogenic inflammation in peripheral organs such as respiratory and gastrointestinal tract and skin (Holzer, 1988). The pro-inflammatory effects are most likely mediated by local effector functions of these neurons, i.e. by local release of several neuropeptides such as tachykinins and calcitonin gene-related peptide (CGRP) from their nerve terminals during peripheral inflammation (Holzer, 1988). However, these nerve fibers also transmit afferent signals to the spinal cord in response to pain and inflammation (Holzer, 1988). The tachykinin substance P (SP) is considered as a serious candidate mediator of neurogenic inflammation although it is probably not the only one (Harrison and Geppetti, 2001). Hallmarks of neurogenic inflammation are increase in vascular permeability, plasma extravasation, edema formation and leukocyte infiltration (Holzer, 1988; Harrison and Geppetti, 2001). *In vitro*, SP elicits activation of the pro-inflammatory transcription factor NF-κB (Marriott et al., 2000) and activates immune cells to produce cytokines (Lotz et al., 1988; Rameshwar et al., 1994). SP preferentially binds to the G protein-coupled neurokinin-1 receptor (NK-1R, Harrison and Geppetti, 2001). Studies using either NK-1R antagonists or mice genetically deficient in the NK-1R have proven a role for this receptor in asthma and chronic bronchitis, intestinal inflammation, pancreatitis, and resistance to infection (Harrison and Geppetti, 2001; Kincy-Cain and Bost, 1996).

Until now, only limited data are available regarding the role of primary afferent neurons in the liver under both, physiological and pathophysiological conditions. These nerve fibers are detectable by SP and CGRP immunoreactivity in the portal tract of human (Stoyanova and Gulubova, 1998) and rodent (Markus et al., 1998; Tiegs et al., 1999) livers. Immunoreactive nerve fibers were capsaicin-sensitive, i.e. they were completely absent in adult mice that have been treated with capsaicin three days after birth in order to permanently deplete the C-fibers (Tiegs et al., 1999). Evidence for a functional role of these capsaicin-sensitive nerve fibers in

liver injury has been demonstrated in experimental liver fibrosis induced by common bile duct ligation (Casini et al., 1990). We have shown recently that these nerve fibers are absolutely required for disease activity in T cell-mediated immune hepatitis in mice (Tiegs et al., 1999).

In the present study we analyzed the possible role of NK-1R, the principal SP binding receptor (Harrison and Geppetti, 2001), in an *in vivo* model of severe inflammatory liver injury inducible by bacterial lipopolysaccharaides (LPS) in D-galactosamine (GalN)-sensitized mice. In this model, LPS induces the release of a variety of cytokines including tumor necrosis factor-α (TNFα), interferon-γ (IFNγ) as well as interleukin (IL)-6 and IL-10. TNFα (reviewed in Schümann and Tiegs, 1999) and IFNγ (Car et al., 1994) are detrimental mediators of GalN/LPS-induced liver failure and lethality, whereas IL-6 and IL-10 are hepatoprotective (Mizuhara et al., 1994; Galun et al., 2000; Louis et al., 1997) and IL-6 is critical for liver regeneration (Streetz et al., 2000). We pretreated mice with antagonists of the NK-1R, i. e. CP-96,345 (Snider et al., 1991) and L-733,060 (Rupniak et al., 1996), and analyzed their hepatoprotective and anti-inflammatory potential in GalN/LPS-induced liver damage. Since high affinity binding sites have not yet been detected in the normal rodent liver (Hershey and Krause, 1990; Tsuchida et al., 1990), we analyzed NK-1R mRNA expression in livers of mice and compared it to NK-1R mRNA expression in lung and spinal cord. Moreover, since NK-1R expression has been detected on monocytes (Ho et al., 1997), macrophages and dendritic cells (Ho et al., 1997; Marriott and Bost, 2000 and 2001), and since macrophages are the prime inflammatory cells activated by LPS, we analyzed NK-1R mRNA expression in non-parenchymal liver cells which are enriched in Kupffer cells. We also investigated the cellular distribution of NK-1R specific immunoflourescence in mouse liver sections.

Methods

Animals. BALB/c mice (age, 6–8 weeks; weight range, 18–22 g) were obtained from the animal facilities of the Institute of Experimental and Clinical Pharmacology and Toxicology of the University of Erlangen-Nuremberg (Erlangen, Germany). All mice received humane care according to the guidelines of the NIH, as well as to the legal requirements in Germany. They were maintained under controlled conditions (22°C, 55% humidity, and 12-hour day/night rhythm) and fed a standard laboratory chow.

Dosages and application routes. Capsaicin pretreatment: Newborn BALB/c mice were injected with 100mg/kg capsaicin subcutaneously (s.c.) in a volume of 40 µL on the third day after birth. Capsaicin (RBI, Natick, MA) was initially dissolved in 100% ethanol and further diluted with sterile PBS and Tween 80 to a final concentration of 5 mg/mL in 10% Tween and 10% ETOH. Control animals received the solvent without capsaicin. Seven weeks after injection, the effectiveness of the capsaicin treatment was assessed by the eye-wiping test: a trop of a 0.1 mg/mL solution of capsaicin in saline was applied into one eye with a plastic Pasteur pipette. All control animals wiped the eye rigorously (>30 wipes in 30 s) with a latency of < 1 s. Capsaicin-treated animals showed a latency of > 5 s to the first wipe and did not carry out more than 5 wipes. Most treated animals did not respond at all.

All other reagents were injected in a total volume of 250 µL per 25 g mouse. LPS from *Salmonella abortus equi*, was purchased from Metalon (Ragow, Germany*)* and administered intraperitoneally at a concentration of 6.5 µg/kg together with 700 mg/kg GalN (Roth GmbH, Karlsruhe, Germany) in pyrogen-free saline in the same solution.

The NK-1R antagonists CP-96,345 (generous gift of Dr. H. Berghof, Pfizer GmbH, Karlsruhe, Germany) or L-733,060 (Tocris/Biotrend Chemikalien GmbH, Köln, Germany) were administered in doses of 1.25 to 20 mg/kg i.p. 30 minutes before GalN/LPS challenge.

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Analysis of liver enzymes. Hepatocyte damage was assessed 8 hours after GalN/LPS administration by measuring plasma enzyme activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) using an automated procedure (Bergmeyer, 1984).

Isolation of non-parenchymal liver cells. Non-parenchymal liver cells (NPC) of untreated mice or mice that have been treated for either 3 h or 6 h with 6.5 μg/kg *Salmonella abortus equi* LPS were prepared as described previously (Gantner et al., 1996). Briefly, after collagenase digest, supernatants from two consecutive 100 x g centrifugations of liver cells were pooled and centrifuged for 8 min at 400 x g. The pellet containing the NPC fraction was washed twice and stored at –80°C until preparation of RNA. The NPC fraction contained about 50% Kupffer cells and less than 0.5% hepatocytes. The proportion of Kupffer cells in the NPC fraction was determined using cytospin slides stained with the rat anti-macrophage antibody (Ab) BM8 (Dianova, Hamburg, Germany) and a secondary rabbit anti-rat FITC labeled Ab (Dako, Hamburg, Germany). Hepatocytes were identified according to their morphology.

Cytokine determination by ELISA. Sandwich ELISAs for murine plasma TNFα, IFNγ, IL-6, and IL-10 were performed using flat-bottom high-binding polystyrene microtiter plates (Greiner GmbH, Frickenhausen, Germany). Antibodies were purchased from BD Biosciences (Heidelberg, Germany). Streptavidin-peroxidase (Jackson ImmunoResearch Laboratories Inc., West Grove, Pennsylvania, USA) and the peroxidase chromogen tetramethylbenzidine (Boehringer Mannheim, Mannheim, Germany) were used according to the manufacturers' instructions. Plasma cytokine levels were measured after the hepatotoxic challenge at the time points indicated, or at the time points of their maximal release (c.f. Fig. 3B), i.e. TNFα, IL-6, and IL-10 1 h and IFNγ 8 h after GalN/LPS administration.

Determination of cytokine and NK-1 receptor mRNA by RT-PCR and real-time RT-PCR. Total RNA was isolated from liver tissue, NPC, lung or spinal cord of untreated mice, or at

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the indicated time points after LPS administration, using the Nucleo Spin RNA Purification Kit (CLONTECH, Palo Alto, California, USA). To analyze altered gene expression, mRNA from 1 μg total RNA was transcribed into cDNA using SuperScript II RNase H reverse transcriptase (Life Technologies Inc., Grand Island, New York, USA). Oligonucleotides and Taq polymerase for subsequent PCR reactions were also obtained from Life Technologies Inc.

Primers were selected for murine TNFα, IL-6, IL-10, IFNγ, NK-1R and β-actin (GibcoBRL): TNFα: 5'-ATG AGC ACA GAA AGC ATG ATC (158-178) and 3'-GTC TGG GCC ATA GAA C (386-371 in GenBank X02611); IL-6: 5'-GCC TAT TGA AAA TTT CCT CTG (375-395) and 3'-GTT TGC CGA GTA GAT CTC (681-663 in GenBank J03783); IL-10: 5'-GTT ACT TGG GTT GCC AAG (76-91) and 3'-TTG ATC ATC ATG TAT GCT TC (294-276 in GenBank M37897); IFNγ: 5'-GAA CGC TAC ACA CTG CAT C (113-131) and 3'-GAG CTC ATT GAA TGC TTG G (513-494 in GenBank M28621); NK-1Rout: 5'-GCT TCA AGC ATG CCT TTC G (964-982) and 3'-GCC AGA ATG TTA GAG TAG AAG (1240-1220 in GenBank X62934); NK-1Rnest: 5'-GAT ACC TCC AGA CCC AGA G (1039-1055) and 3'-GCT GGA GCT TTC TGT CAT G (1220-1202 in GenBank X62934); β-actin: 5'-TGG AAT CCT GTG GCA TCC ATG AAA (729-752) and 3'-TAA AAC GCA GCT CAG TAA CAG TCC G (1076-1053 in GenBank X03765). For quantitative evaluation of cytokine and β-actin mRNA expression real time RT-PCRs of at least 6 livers of mice for each time point and treatment were used. For each liver β-actin expression, defined by its crossing point, was measured to verify equal amounts of cDNA. Using a Light Cycler system (Roche Molecular Biochemicals, Mannheim, Germany) a crossing point is defined as the third cycle in the exponential phase of amplification, which is specific for each sample. Crossing points of TNFα, IFNγ, IL-10 and IL-6 expression were measured in the same way using the specific primers. Quantification of expression levels was done by calculating the difference of β-actin

and the regulated PCRs crossing points giving distances of cycles, 1 cycle difference being equivalent to a 2^1-fold induction. Finally, expression of untreated samples was defined as 1-fold and the induction levels of all treated samples were related to that. The NK-1R PCR from liver and NPC cDNA was performed as nested-PCR with primary RT-PCR amplifications (15 cycles with the NK-1R out primer pair), which amplified a 276-bp fragment of the NK-1R sequence. For the second (nested) RT-PCR reaction the 276-bp fragment was used as template for amplification with the NK-1Rnest primer pair in the light cycler system. NK-1R mRNA expression in lung and spinal cord was measured in the light cycler system without previous amplification by RT-PCR. In order to demonstrate the length of the individual fragments, light cycler PCR products were analyzed by agarose gel electrophoresis. To confirm amplification specificity, PCR products were subjected to a melting curve analysis and, in the case of NK-1R, the PCR products were sequenced.

Cloning and DNA sequencing. PCR synthesized DNA was extracted by Concert Gel Extraction Systems (GIBCO/Invitrogen GmbH, Karlsruhe, Germany) and cloned into a plasmid vector by the TOPO TA Cloning method (Invitrogen GmbH, Karlsruhe, Germany). After transformation, Escherichia coli recombinants were selected for ampicillin resistance. The DNA sequence was subsequently determined using the M-13 forward and reverse primers. Resulting amplimers were subsequently analyzed by direct sequencing of both strands on an ABI 377 automated DNA sequencer.

Preparation of nuclear extracts. Nuclear extracts from frozen liver sections were prepared as described previously (Schreiber et al., 1989). Briefly, 200 mg of tissue were suspended in 3 of ml buffer A (10 mM HEPES pH 7.9; 10 mM KCl; 0.1 mM EDTA; 0.1 mM EGTA; 1 mM DTT; 0.5 mM PMSF) and homogenized using a dounce homogenizer. The homogenate was transferred to a polypropylene centrifuge tube, incubated on ice for 10 min and centrifuged in a pre-cooled Eppendorf 5810R centrifuge for 10 min. at 4,000 x g. After centrifugation the

pellet was resuspended in 1.4 ml of buffer A. 90 µL of Nonidet P-40 (10%) were added followed by 10 s of vigorous vortexing and incubation on ice for 10 min. After centrifugation (12,000 × g for 45 s) in an Eppendorf centrifuge 5417R the supernatant was removed and the nuclear pellet was extracted in 1.5 ml hypertonic buffer B (20 mM HEPES; (pH 7.9); 0.4 M NaCl; 1 mM EDTA; 1 mM EGTA; 1 mM DTT; 1 mM PMSF) by shaking at 4 °C for 60 min. The extract was centrifuged for 15 min at 12,000 × g, and the supernatant was frozen at –80 °C. The protein concentration was determined using the Bradford method (Bradford, 1976).

Electrophoretic Mobility Shift Assay (EMSA). The 22-mer double-stranded oligonucleotide probes containing a consensus binding sequence for NFκB (5'-AGT TGA GGG GAC TTT CCC AGG C-3') or AP-1 (5'-CGC TTG ATG AGT CAG CCG GAA-3') were 5'-end-labeled with [γ^{32}P]-ATP (10 µCi) using the Promega Labeling kit according to manufactors instructions. For DNA binding reaction 5 µg of nuclear protein were incubated for 20 min at RT in a 15 µl reaction volume containing 10 mM Tris-HCl, pH 7.5, 50,000 cpm radio-labelled oligonucleotide probe, 2 µg synthetic carrier copolymer (poly dI-dC), 4% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 50 mM NaCl and 0.5 mM DTT. Nucleoprotein-oligonucleotide complexes were resolved by electrophoresis (4.5 % non-denaturing polyacrylamide gel, 100 V). Signals were detected by phosphoimaging. The specificity of the DNA-protein complex was confirmed by competition with a 100-fold excess of unlabeled NF-κB, AP-1, and AP-2 (5'-GAT CGA ACT GAC CGC CCG CGG CCC GT-3') binding sequences, respectively.

Immunocytochemistry. Livers were excised from euthanized mice and immersion fixed in phosphate buffered formaldehyde for 4 to 6 hours. After cryoprotection in 15% buffered sucrose, 12 µm cryostat sections were mounted on poly-L-lysine coated slides, pre-incubated in TBS containing 1% bovine serum albumin (BSA), 0.5% Triton X 100, 0.05% Thimerosal and 5% goat normal serum for 1 hour at room temperature and incubated with rabbit anti-NK-1R (Chemicon, Hofheim, Germany) diluted 1:500 in TBS containing 1% BSA, 0.5% Triton X

100 and 0.05% Thimerosal for 48 hours at 4°C. After three rinses in TBS, binding sites of antibodies were revealed using goat anti rabbit IgG tagged with Alexa 488 (Molecular Probes, MoBiTec GmbH, Göttingen, Germany) diluted 1:1000 in TBS with the same additives as for the primary antibody. After another 3 rinses in TBS sections were coverslipped in TBS-glycerol 1:1, pH 8.6. As a positive control, sections from mouse brain and spinal cord were processed in parallel. Negative controls included replacement of the primary antibody by rabbit normal serum or TBS.

Confocal laser scanning microscopy. Confocal single optical sections at a resolution of about 500 nm were taken on a Biorad MRC 1000 scanning system (Biorad, Hemel Hempstead, UK) equipped with an argon-krypton laser (American Laser Corporation, Salt Lake City, USA) attached to a Nikon diaphot 300. A 60x oil immersion lens was used. The Alexa 488 signal was recorded in one channel while a second channel was used to detect background fluorescence of the tissue elicited by the 568 nm line of the laser. Merged images were formatted as TIFF and adjusted for contrast and brightness using Adobe Photoshop 6.0.

Histology. Formalin-fixed liver tissue was embedded in paraffin and 4 μm thick sections were randomly taken throughout the whole organ and stained with hematoxylin and eosin (H&E) using a standard protocol. In each section, three randomly selected areas measuring 86,400 μm^2 each, were screened for edema, granulocytes, and hepatocyte apoptosis and necrosis.

Statistical analysis. The results were analyzed using Student's t test if two groups were compared or by ANOVA followed by the Dunnett test if more groups were tested against a control group. If variances were inhomogeneous in the Student's t test, the results were analyzed using the Welsh test. All data in this study are expressed as a mean ± SEM. P values less than or equal to 0.05 were considered significant.

Results

Role of capsaicin-sensitive primary afferent neurons for liver injury and cytokine production. We have described previously that permanent depletion of primary sensory neurons by neonatal capsaicin treatment prevented immune-mediated liver injury in mice elicited by activation of T cells (Tiegs et al.; 1999). In order to prove whether these nerve fibers are also critical for liver damage and cytokine production in the macrophage-dependent GalN/LPS model (Freudenberg et al., 1986) we treated 7 to 8 weeks old mice, which had received either capsaicin or solvent (c.f. Material and Methods) 3 days after birth, with GalN and LPS. As shown in Figure 1A, capsaicin-pretreated mice were protected from liver damage as assessed by significantly reduced levels of the plasma transaminases ALT and AST 8 h after the concomitant administration of GalN and LPS. The protective capsaicin effect was accompanied by dramatically reduced circulating maximal levels of the pro-inflammatory mediators TNFα and IFNγ at 1 h and 8 h, respectively (Fig. 1B, for cytokine maximum levels see Fig. 4B).

Dose-dependence of the protective effect of NK-1 receptor antagonists against GalN/LPS-induced liver injury. Since SP is the most prominent pro-inflammatory neuropeptide of primary afferent neurons, we investigated the pharmacological effects of antagonists directed against the prime receptor of SP, i.e. NK-1R. Pretreatment of mice with the specific NK-1R antagonists CP-96,345 and L-733,060, respectively, 30 min. before administration of GalN/LPS, dose-dependently reduced plasma transaminase activities as well as maximum levels of TNFα and IFNγ (Fig. 2). Both NK-1R antagonists led to significantly reduced plasma transaminase activities at doses ≥ 5 mg/kg. CP-96,345 pretreatment resulted in a significant reduction of plasma levels of both cytokines at 10 mg/kg, whereas a higher dose of L-733,060, i.e. 20 mg/kg, was necessary for same effect. Histological examination of livers

from treated and control mice revealed signs of severe inflammatory liver damage in the GalN/LPS-treated group and virtual absence of the inflammatory infiltrate, mainly consisting of granulocytes, as well as reduction of hepatic edema and almost complete absence of hepatocyte apoptosis and necrosis in the L-733,060 pretreated group (Fig 3). Taken together, NK-1R antagonists exerted effects in the liver comparable to those of permanent depletion of C-fibers by capsaicin.

Time course of the protective effect of L-733,060 against GalN/LPS-induced liver injury. In order to monitor the whole time-course of the protective effect of NK-1R blockade in inflammatory liver injury, we pretreated mice with the NK-1R antagonist L-733,060 30 min. before GalN and LPS and measured activities of plasma transaminases as well as plasma levels and intrahepatic mRNA expression of the cytokines TNFα, IFNγ, IL-6 and IL-10 at several time points following the hepatotoxic challenge. GalN/LPS induced a dramatic increase of plasma ALT and AST activities within 8 h after intervention which was significantly reduced by the NK-1R antagonist (Fig. 4A). LPS induced the release of cytokines into plasma of GalN-sensitized mice showing different kinetics and peak concentrations (Fig. 4B). L-733,060 significantly reduced high plasma levels of TNFα observed at 0.5 and 1 h after GalN/LPS administration. IFNγ levels were reduced only at 8 h after intervention with GalN/LPS. In contrast, the NK-1R antagonist significantly increased the release of the anti-inflammatory and hepatoprotective cytokines IL-10 and IL-6 at the time points of their maximal release. The GalN/LPS-induced time course of cytokine production as well as the protective effect of L-733,060 were also evident at the intrahepatic mRNA level (Fig. 4C and D).

L-7330,060 affects transcription factor activation in vivo. Since SP has been described to activate the transcription factor NF-κB *in vitro* (Marriott et al., 2000) and since the NK-1R antagonist L-7330,060 differentially affected LPS-induced expression of TNFα and IL-6 *in*

vivo (this study), i.e. two cytokines carrying a NF-κB binding site in their promoter sequence, we examined the activation of the transcription factors NF-κB and AP-1 *in vivo*. As shown in Fig. 5, L-7330,060 pre-treatment attenuated DNA-binding of NF-κB in GalN/LPS challenged mice compared to controls receiving saline before GalN/LPS treatment, whereas the antagonist increased DNA-binding of AP-1. DNA binding activity of both transcription factors was determined 1 h after GalN/LPS administration, i.e. close to maximal TNFα and IL-6 production (c.f. Fig. 4). Specificity of the DNA-protein complexes was confirmed by incubation of nuclear extracts from GalN/LPS-treated mice with a 100-fold excess of unlabeled ("cold") NF-κB, AP-1, or AP-2 binding sequences. Excess of unlabeled specific oligonucleotides (i.e. NF-κB and AP-1, respectively) abolished binding reactions, whereas the AP-2 binding sequence showed no significant influence on binding reactions (data not shown). Hence, LPS-inducible transcription factor activation is affected by blockade of the NK-1R *in vivo*.

NK-1 receptor expression in the liver. In order to prove the occurrence of the NK-1R in the liver, total RNA was isolated from liver tissue, non-parenchymal liver cells (NPC), lung and spinal cord and reverse transcribed into cDNA. The NK-1R PCR from liver and NPC cDNA was performed as nested-PCR with primary RT-PCR amplification and a second (nested) PCR reaction in the light cycler system. NK-1R mRNA expression in lung and spinal cord was measured in the light cycler system without previous amplification by a RT-PCR. The light cycler PCR products were analyzed on an agarose gel and demonstrated an equal length of the individual fragments in liver, lung and spinal cord (Fig. 6B). In order to confirm amplification specificity, PCR products were subjected to a melting curve analysis. The melting points of the PCR products of NK-1R mRNA were identical in all three tissues tested, i.e. spinal cord, lung, and liver (Fig. 6A). The PCR products of spinal cord and liver were sequenced and found to be identical to accession number GenBank X62934 (Fig. 6C). NK-1R

mRNA was also detectable in NPC and was inducible by pretreatment of mice with LPS 3 h or 6 h before isolation of the non-parenchymal liver cell fraction (expression levels, given in x-fold induction: saline treated group 1.0 ± 0.3; LPS 3 h 23.1 ± 7.9*; LPS 6 h 19.4 ± 9.4*; n = 5; *$p \leq 0.05$ *vs* saline control).

NK-1R immunoreactivity was detected in numerous rounded to elongated cells scattered throughout the liver, representing most likely monocytes/macrophages, lymphocytes and granulocytes (Fig. 7A). Many cells were found in the sinusoids while others were seen in small veins. Immunoreactivity was concentrated on the cell membrane. Granular NK-1R immunoreactivity was also detected in scattered hepatocytes (Fig. 7B). In brain and spinal cord, the pattern of NK-1R immunostaining found in our material was compatible with published data (Fig. 7C). In the liver, no nerve fibres were immunoreactive (c.f. Fig. 7A and B). Negative controls were devoid of immunostaining (Fig. 7D). Taken together, these results clearly demonstrate the presence of the NK-1R in mouse liver where it appeared predominantly in non-parenchymal mononuclear cells but also in hepatocytes.

Discussion

In our present study we have demonstrated that permanent depletion of C-fibers by neonatal treatment of mice with capsaicin as well as antagonists to the NK-1R, which is the high affinity receptor for SP, i.e. the prime pro-inflammatory neuropeptide of C-fibers (Harrison and Geppetti, 2001), protected mice against LPS-induced inflammatory liver injury. Although these nerve fibers are well known to affect peripheral inflammation in skin and joints as well as in gastrointestinal and respiratory tract (Holzer, 1988; Harrison and Geppetti, 2001), only limited information is available on their role in liver inflammation and fibrosis (Casini et al., 1990, Tiegs et al., 1999) and a hepatoprotective effect of NK-1R antagonists has never been demonstrated. The effects of SP and other tachykinins released from the peripheral endings of C-fibers are collectively referred to as 'neurogenic inflammation', which has been characterized so far by flare, plasma extravasation, edema formation and leukocyte infiltration (Holzer, 1988; Harrison and Geppetti, 2001; Severini et al., 2002). Indeed, most of these hallmarks of neurogenic inflammation were histologically also observable in our liver injury model (c.f. Fig. 3). Neurogenic inflammation can be elicited by electrical, mechanical, or chemical stimulation of C-fibers (Severini et al., 2002). Chemically, C-fibers can be selectively stimulated by low doses of capsaicin, which has recently been shown to specifically activate vallinoid receptors expressed by these neurons (Caterina et al., 1997). In human skin, the pro-inflammatory effect of low capsaicin concentrations has been reported to be partially inhibited by glucocorticoids (Tafler et al., 1993). Hence, it seems that the local pro-inflammatory effector function elicited by capsaicin is mediated by arachidonic acid metabolites and/or cytokines produced by inflammatory cells. Accordingly, the cytokines TNFα and IL-1β have been shown to potentiate capsaicin-induced tracheal neuropeptide release (Hua et al., 1996). Taking into account that neuropeptides such as SP activate monocytes and other immune cells to produce cytokines (Lotz et al., 1988; Rameshwar et al.,

1994), this scenario implicates that bi-directional communication between neurons and immune cells elicits a 'circulus vitiosus' that finally results in neurogenic inflammation. On the other hand, several immunocytes have been described to synthesize and release tachykinins (extra-neuronal source) which may in turn activate immune cells in an autocrine and/or paracrine fashion (Ho et al., 1997; Maggi, 1997). However, since both, NK-1R antagonization as well as chemical depletion of C-fibers prevented LPS-induced liver inflammation (this study) and since capsaicin sensitive C-fibers are present in the mouse liver (Tiegs et al., 1999), it seems that the communication between local inflammatory cells, e.g. Kupffer cells which are the main target cells of LPS in the liver and which seem to express the NK-1R (this study), and nerve terminals of C-fibers is critical for the pathogenic process.

The recent development of selective, nonpeptide NK-1R antagonists has enabeled investigation of the physiological and pathophysiological role of SP. Although SP is best known as a pain neurotransmitter, clinical trials reveal that NK-1R antagonists failed to alleviate pain (Rupniak and Kramer, 1999). However, these antagonists, lacking important side effects (Severini et al., 2002), exhibit potent anti-depressant and anti-emetic efficacy in patients (Rupniak and Kramer, 1999). Evidence from experimental animal studies suggest that NK-1R antagonists are powerful drugs for treatment of inflammatory disease in skin, respiratory and gastrointestinal tract (Harrison and Geppetti, 2001). In these studies, suppression of neurogenic inflammation was measured as attenuation of vasodilation, plasma extravasation and edema formation, but only limited data are available that correlate these effects with the cytokine response *in vivo*. In a Salmonella infection model it has been shown that the SP antagonist spantide II reduced IL-12 and IFNγ mRNA expression (Kincy-Cain and Bost, 1996), and LPS-induced TNFα production in mice was attenuated by the NK-1R antagonist SR 140333 (Dickerson C, 1998). However, the latter study did not correlate alterations in TNFα production to LPS-induced pathology.

The first high-affinity nonpeptide NK-1 receptor antagonist, CP-96,345, was published by Snider et al. in 1991. The *in vivo* pharmacological activity of CP-96,345 was investigated in the classical bioassay for SP, i.e. stimulation of salivation in the anesthetized rat, that was inhibited by the antagonist at an i.v. dose of 3.4 mg/kg (Snider et al., 1991). In mice (Perretti et al., 1993; Veronesi et al., 1995) or guinea-pigs (Costello et al., 1998, Lin et al., 2001) neurogenic inflammation was attenuated by CP-96,345 at doses of 5-15 mg/kg given either i.p., i.v., or s.c.. More recently, L-733,060 has been described to inhibit the formalin paw late phase at doses of 0.1 to 10 mg/kg when given intravenously (Rupniak et al., 1996). In our study, both receptor antagonists significantly inhibited liver damage, i.e. release of transmaninases, at the same doses range (Fig. 2). Although CP-96,345 significantly inhibited the production of TNFα, the major mediator of GalN/LPS-induced liver injury (Schümann and Tiegs, 1999), at doses of 2.5 to 10 mg/kg i.p., L-733,060 was less effective in suppression of the cytokine response (Fig.2). This suggests on the one hand, that L-733,060 has a reduced anti-inflammatory potency compared to CP-96,354, and, on the other hand, that NK-1R antagonists may affect additional pathophysiological pathways in the liver. Indeed, NK-1R antagonists inhibited apoptosis of hepatocytes when liver injury was induced by direct administration of TNFα to GalN-sensitized mice (Bang & Tiegs, unpublished). However, also L-733,060 significantly suppressed expression and release of TNFα and IFNγ at 20 mg/kg i.p. as shown in the time course experiments (Fig. 4). Accordingly, gene targeted disruption of the NK-1R was associated with a reduction of the TNFα and IFNγ response in intestinal (Castagliuolo et al., 1998) and chronic liver inflammation (Blum et al., 1999), respectively, and chemical depletion of C-fibers resulted in dramatically reduced release of both cytokines (this study). Since the cytokine inhibitory efficacy of C-fiber depletion was more pronounced compared to that of NK-1R antagonists, it seems that other neuropeptides besides SP are also responsible for this effect. Last but not least, this study shows for the first time that NK-1R

antagonists not only suppress the production of LPS-inducible pro-inflammatory cytokines, TNFα and IFNγ, but also augment expression and synthesis of the anti-inflammatory and hepatoprotective cytokines IL-10 (Louis et al., 1997) and IL-6 (Mizuhara et al., 1994; Streetz et al., 2000).

Since antagonization of the NK-1R *in vivo* differentially regulated LPS-induced expression of two NF-κB and AP-1 dependent cytokines, i.e. TNFα and IL-6 (Baud and Karin, 2001; Dendorfer et al., 1994), respectively, we examined the activation of the transcription factors NF-κB and AP-1. Our results revealed an attenuation of NF-κB and a concomitant increase of AP-1 binding to DNA in livers from L-7330,060 pre-treated, GalN/LPS challenged mice, indicating that the antagonist might have interfered with LPS-inducible transcription factor activation. Provided that these events occurred within the main target cells of LPS in the liver, i.e. the Kupffer cells, our results suggest that the attenuation of NF-κB activation was sufficient to suppress TNFα expression but still allowed transcription of the IL-6 gene, and that the increase in AP-1 activation augmented IL-6 expression. This may be explained by different usage of certain regulatory elements in dependence of intracellular signaling which has been described for regulation of IL-6 gene expression (Dendorfer et al., 1994). An alternative explanation is that NF-κB was down-modulated in Kupffer cells thereby suppressing TNFα production, while AP-1 might have been up-regulated in a different cell population, e.g. in T-helper-2 cells, which are a source of IL-6.

The expression of the NK-1R by human and murine monocytes/macrophages and dendritic cells is well documented (Ho et al., 1997; Marriott and Bost, 2000 and 2001), however, NK-1R expression in the liver is only mentioned in one report. Mice chronically infected with *Schistosoma mansoni* express the receptor on $CD4^+$ granuloma T lymphocytes (Cook et al., 1994). Using classical RT-PCR technique, the authors failed to identify the receptor in healthy liver tissue. With the help of nested PCR, we were able to identify the receptor also in

the normal liver. The identity of the RT-PCR product from liver mRNA with the NK-1R was verified by two means, i) by showing identical melting points of the RT-PCR products from liver, spinal cord and lung, and ii) by cDNA cloning and sequence analysis proving that the sequence of the receptor in the liver was identical to that in spinal cord (Fig. 5). Moreover, using the same PCR technique, we could identify the NK-1R in NPC enriched in Kupffer cells, which are the cell population primarily activated by LPS in the liver. Differences in receptor levels of NPC isolated 3 h and 6 h after LPS treatment, respectively, suggest induction by cytokines (Marriott and Bost, 2000). Our PCR data were supported by the detection of NK-1R-specific immunofluorescence on mononuclear non-parenchymal cells and hepatocytes in liver sections.

In conclusion, the present and our previous studies (Tiegs et al., 1999) indicate that inflammatory cytokine-mediated liver injury is affected by neuropeptides released from peripheral endings of capsaicin-sensitive nerves. SP seems to be a major player in this scenario up-regulating the pro-inflammatory cytokine response by activation of NK-1 receptors, which are also present in the liver. Thus, cytokine-mediated liver diseases might be successfully treated with antagonists to the NK-1R.

Acknowledgments

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Footnotes

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Figure legends

Fig. 1: Protection against GalN/LPS-induced liver injury in mice by capsaicin-induced depletion of primary afferent sensory neurons. Newborn BALB/c mice were pretreated with 100 mg/kg capsaicin s.c. at the third day after birth. Seven weeks later they were challenged with 700 mg/kg GalN i.p. together with 6.5 µg/kg LPS i.p. TNFα was detected 1 h and IFNγ, ALT and AST 8 h after GalN/LPS administration. Data are expressed as mean values ± SEM; n = 5; *p ≤ 0.05 *vs* capsaicin non-pretreated control.

Fig. 2: Protection by the NK-1R antagonists CP-96,345 and L-733,060 against GalN/LPS-induced liver injury in mice. NK-1R antagonists were administered i.p. at the doses indicated 30 min prior to injection of 700 mg/kg GalN together with 6.5 µg/kg LPS i.p. TNFα was detected 1 h, IFNγ and ALT 8 h after GalN/LPS administration. Data are expressed as mean values ± SEM; n = 4; *p ≤ 0.05 *vs* saline treated control, n.d. not determined (open bars: CP-96,345 pretreated mice; black bars: L-773,060 pretreated mice).

Fig. 3: The NK-1R antagonist L-733,060 reduced edema formation, granulocyte infiltration and hepatocellular apoptosis in livers of GalN/LPS treated mice Representative liver sections of untreated mice (A) compared to saline pretreated (B) or L-733,060 pretreated (C) mice, challenged with GalN/LPS for 8 hrs, were subjected to H&E staining and light microscopy. In section (B) numerous granulocytes, apoptotic bodies and hyperchromatic nuclear membranes of hepatocytes [c.f. also insert (a)], indicating apoptotically dying hepatocytes typical for liver damage in the GalN/LPS model, are visible (Leist et al., 1995). E, edema; arrow heads, granulocytes; scale bar 100µm.

Fig. 4: Time-course of transaminase and cytokine release into plasma of GalN/LPS treated and L-733,060 pretreated mice. The NK-1R antagonist L-733,060 (20 mg/kg) was administered i.p. 30 min prior to injection of 700 mg/kg GalN together with 6.5 µg/kg LPS

i.p. At the time points indicated, plasma samples were taken for determination of transaminases (A) and plasma cytokines (B), cytokine mRNA expression in liver tissue was measured by light cycler RT-PCR (C). Data are expressed as mean values ± SEM, n = 8, $p \leq 0.05$ *vs* saline-treated control (gray bars: untreated control; open bars: saline pretreated, GalN/LPS treated mice; black bars: L-733,060 pretreated, GalN/LPS treated mice).

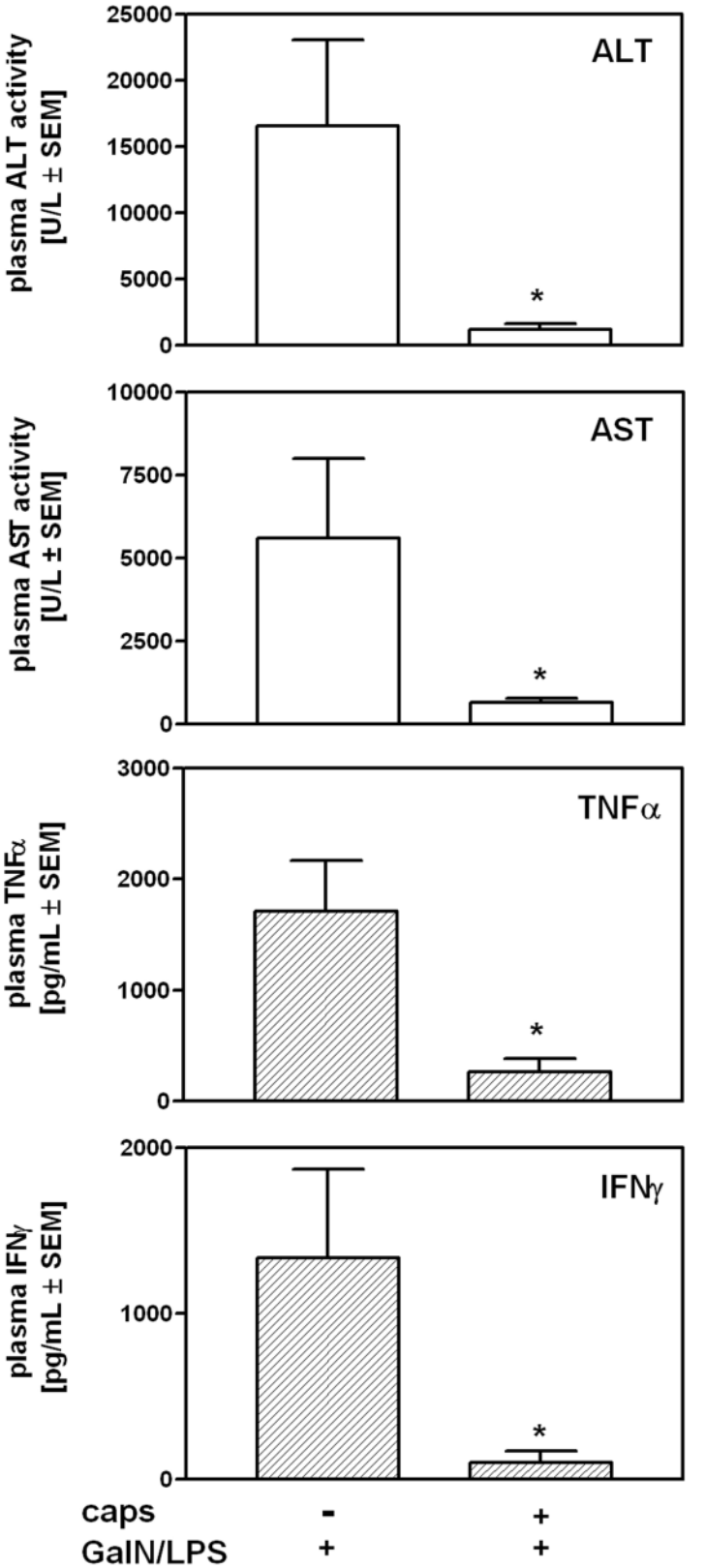
Fig. 5: Activation of NF-κB and AP-1 in livers of GalN/LPS treated and L-733,060 pretreated mice. The NK-1R antagonist L-733,060 (20 mg/kg) was administered i.p. 30 min prior to i.p. injection of 700 mg/kg GalN together with 6.5 µg/kg LPS. 1 h after GalN/LPS treatment, nuclear extracts were prepared from liver tissue as described in Materials and Methods. DNA binding capacity of NF-κB and AP-1 was determined by electrophoretic mobility shift assay. Excess of unlabeled specific oligonucleotides (i.e. NF-κB and AP-1, respectively) abolished binding reactions, whereas the AP-2 binding sequence showed no significant influence on binding reactions (data not shown).

Fig. 6: Detection of an identical NK-1R mRNA fragment in liver, lung and spinal cord. Hepatic mRNA was analysed in the light cycler system for the expression of NK-1R mRNA in comparison to lung and spinal cord (s.c.). In all tissues analyzed identical fragments of NK-1R mRNA were expressed. A: the PCR products of s.c., lung and liver had the same melting point. B: the PCR products of the light cycler run (A) were transferred to an agarose gel to analyze the fragment size. All PCR products had an identical length of 181 bp (dotted box: liver RT-PCR was performed as nested PCR, to enrich the low NK-1R mRNA amount). C: Sequencing of the liver PCR product (lane 2) and the s.c. PCR product (bold, lane 1) showed mRNA fragments identical to the NK-1R, GenBank X62934.

Fig. 7: NK-1R immunoreactivity in murine liver and striatum. NK-1 immunoreactive leukocytes (green) in a small branch of the portal vein. Immunostaining is concentrated on the cell membrane (A). Granular NK-1R immunoreactivity in hepatocytes bordering a small

branch of the portal vein. Both, immunopositive (green) and – negative (red background autofluorescence) hepatocytes are distinguishable (B). NK-1R immunoreactivity in the striatum. Perikaryal contours and dendrites are intensely stained (C). Green immunostaining is complete absent in a negative control section (replacement of the primary antibody by rabbit normal serum) through the liver. Hepatocytes stain red due to their background autofluorescence (D). Confocal single sections. Bar = 25μm.

Figure 1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15000
10000
5000
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ALT
*
plasma AST activity [U/L ± SEM]
10000
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5000
2500
0
AST
*
plasma TNFα [pg/mL ± SEM]
3000
2000
1000
0
TNFα
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plasma IFNγ [pg/mL ± SEM]
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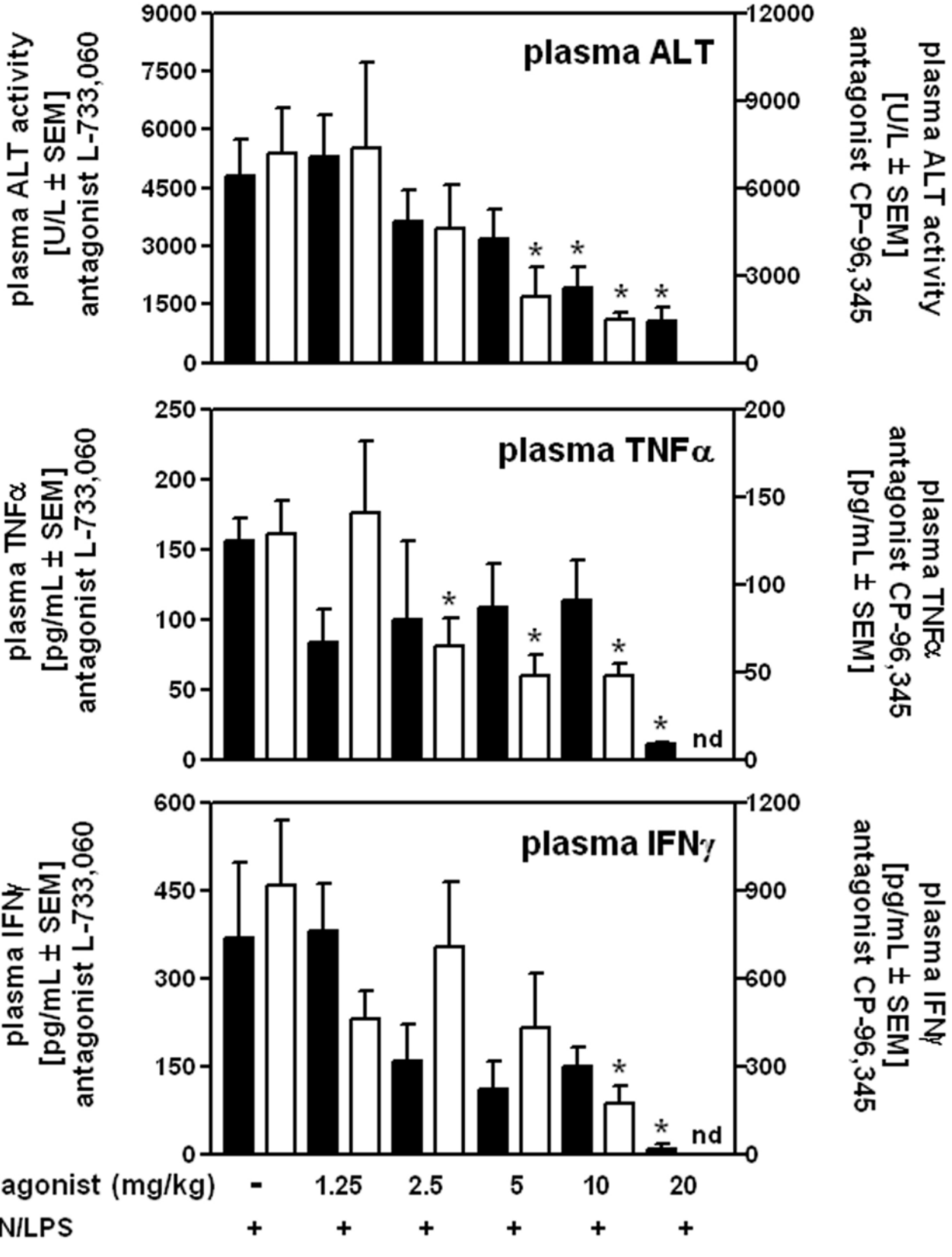
IFNγ
*
caps - +
GalN/LPS + +

Figure 2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plasma ALT activity [U/L ± SEM] antagonist CP-96,345
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6000
4500
3000
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0
12000
9000
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3000
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plasma TNFα
plasma TNFα [pg/mL ± SEM] antagonist L-733,060
plasma TNFα antagonist CP-96,345 [pg/mL ± SEM]
250
200
150
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50
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200
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100
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plasma IFNγ
plasma IFNγ [pg/mL ± SEM] antagonist L-733,060
plasma IFNγ antagonist CP-96,345 [pg/mL ± SEM]
600
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300
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Figure 3

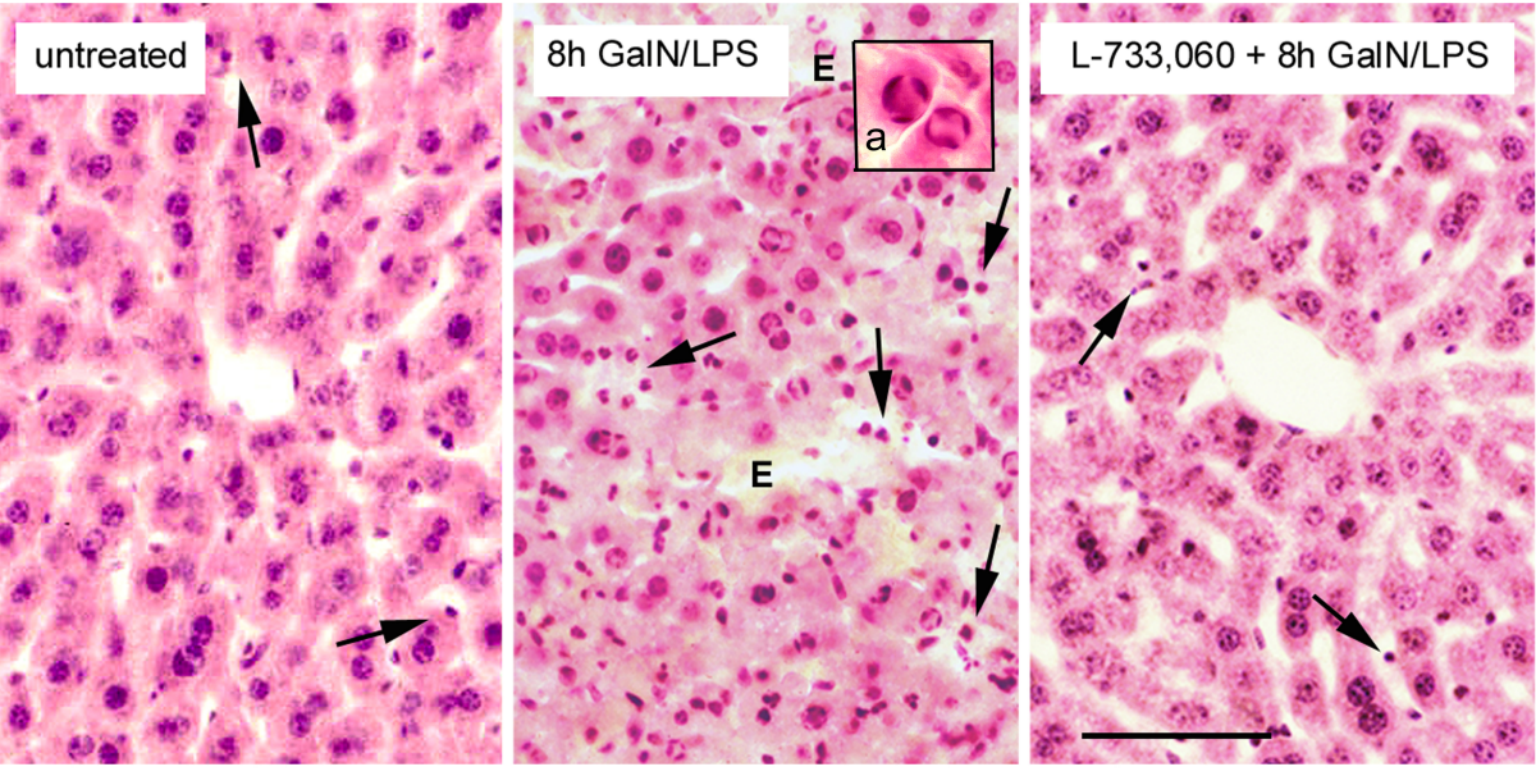
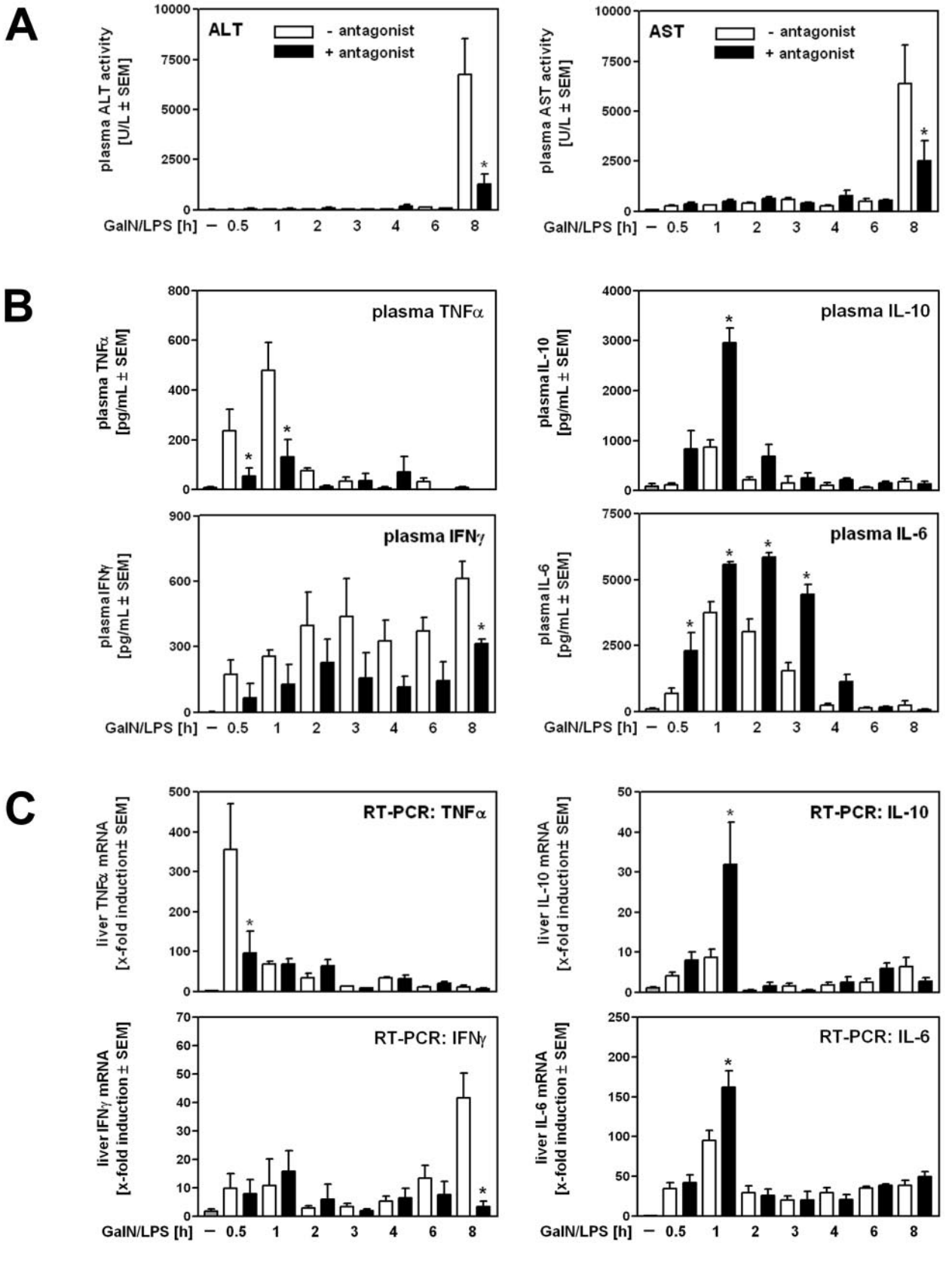


Figure 4


A
ALT
- antagonist
+ antagonist
plasma ALT activity [U/L ± SEM]
AST
plasma AST activity [U/L ± SEM]
GalN/LPS [h] – 0.5 1 2 3 4 6 8
B
plasma TNFα
plasma TNFα [pg/mL ± SEM]
plasma IL-10
plasma IL-10 [pg/mL ± SEM]
plasma IFNγ
plasma IFNγ [pg/mL ± SEM]
plasma IL-6
plasma IL-6 [pg/mL ± SEM]
C
RT-PCR: TNFα
liver TNFα mRNA [x-fold induction± SEM]
RT-PCR: IL-10
liver IL-10 mRNA [x-fold induction± SEM]
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liver IL-6 mRNA [x-fold induction ± SEM]

Figure 5

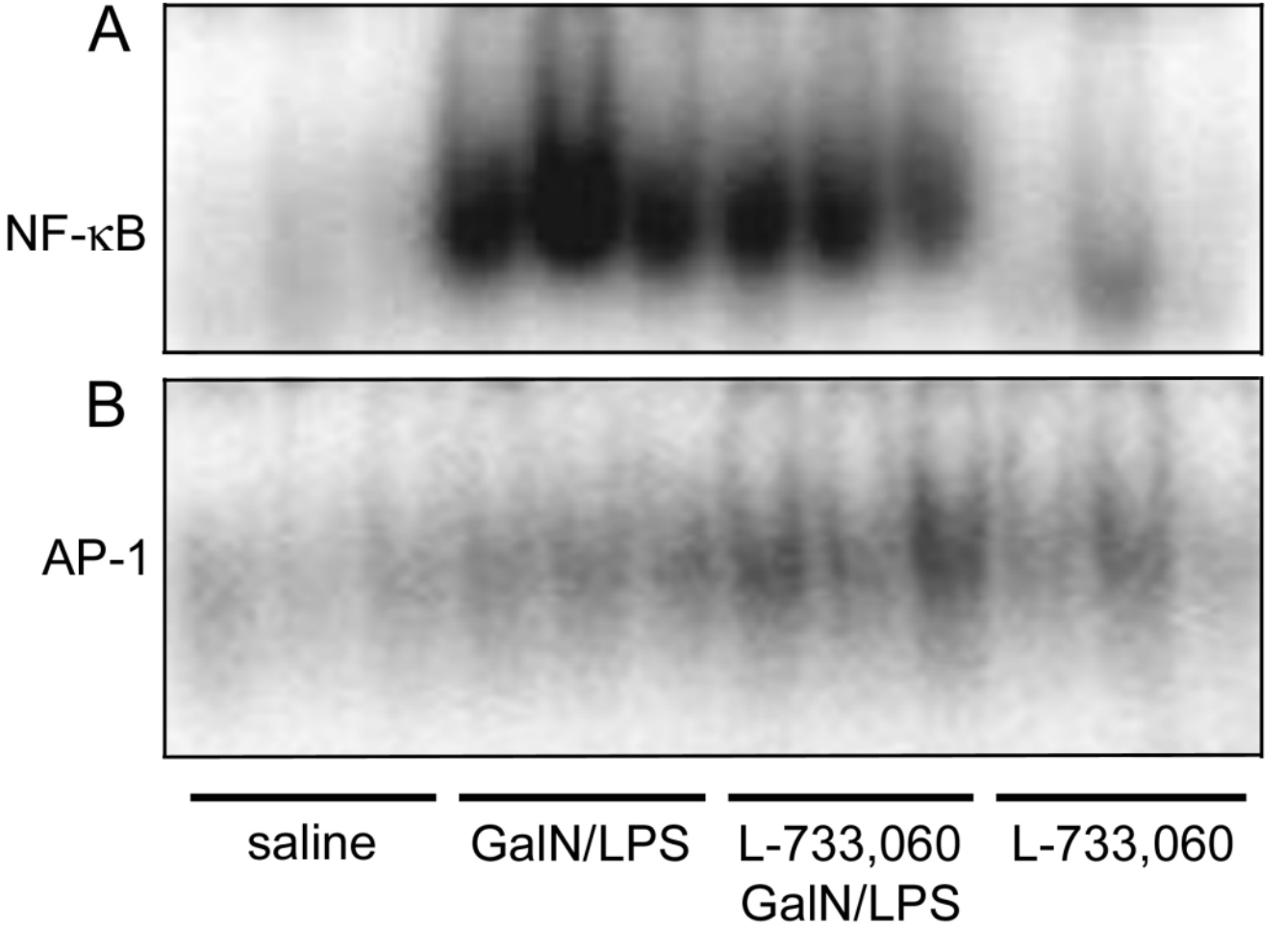
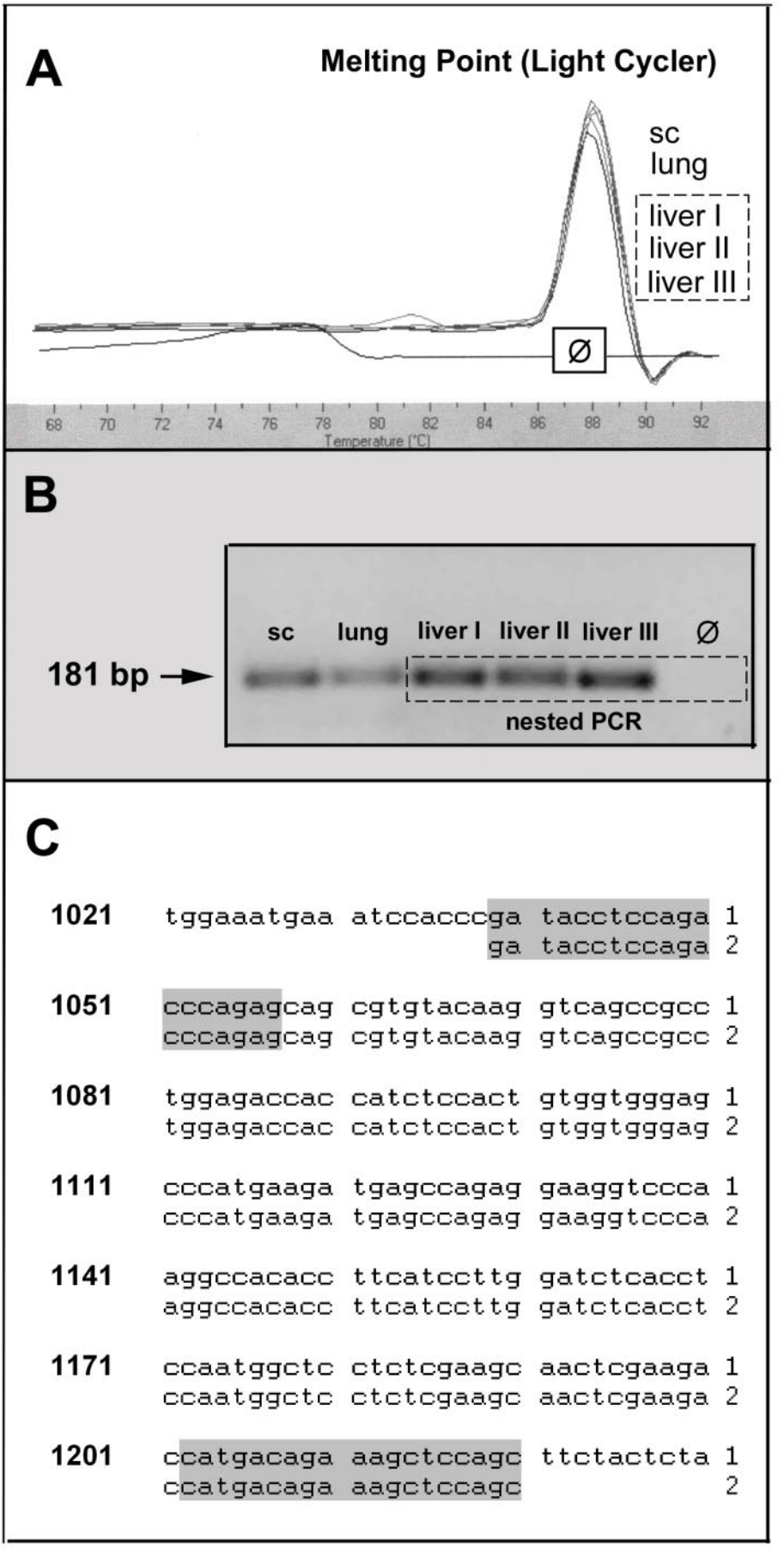


Figure 6


A
Melting Point (Light Cycler)
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lung
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liver II
liver III
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Temperature (°C)
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sc
lung
liver I
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liver III
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181 bp
nested PCR
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Figure 7