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Phagocytosis of gadolinium chloride or zymosan induces simultaneous upregulation of hepcidin- and downregulation of hemojuvelin- and Fpn-1-gene expression in murine liver

Federico Moriconi[1], Ghayyor Ahmad[1], Pierluigi Ramadori[1], Ihtzaz Malik[1], Nadeem Sheikh[1], Manuela Merli[2], Oliviero Riggio[2], Joszef Dudas[1] and Giuliano Ramadori[1]

The liver and the spleen are the organs in which cellular material and aged erythrocytes are eliminated from the blood. Within the liver, Kupffer cells (KCs) are mainly responsible for this task, as such KCs have a pivotal role in iron metabolism. The aim of this study is to investigate the changes of hepatic gene expression in two models of KC phagocytosis. Gadolinium chloride (GD) or zymosan was injected intraperitoneally into rats and to endotoxin-resistant mice (C3H/HeJ). The animals were killed at different time points and their livers were immediately frozen in liquid nitrogen for RNA isolation and immunohistological studies. RNA was analyzed by real-time PCR and northern blot. Sera were used to measure transaminases, hepcidin and iron levels. The expression of iron metabolism genes, hepcidin, hemojuvelin (Hjv), ferroportin-1 (Fpn-1) and of the inflammatory cytokines IL-6, IL-1β, TNF-α and IFN-γ was determined. Although phagocytosed material was detected in ED-1- and C1q-positive cells, no inflammatory cells were identified within the liver parenchyma. Serum levels of hepcidin, iron and transaminases did not differ from those of control animals. Both GD and zymosan induced an upregulation of hepcidin-gene expression in rat liver as early as 3 h, reaching a maximum 6 h after treatment. Hjv- and Fpn-1-gene expression was downregulated at the same time. IL-6 was by far the most induced acute-phase-cytokine in GD- and zymosan-treated livers, although IL-1β and TNF-α were also strongly upregulated by zymosan and to a lesser extent by GD. Similar results were obtained in the C3H/HeJ mouse strain excluding the possible role of contaminating endotoxin. This study shows that phagocytosis upregulates hepcidin-gene expression and downregulates Hjv- and Fpn-1-gene expression within the liver. These changes in iron-regulating-gene expression may be mediated by the locally produced acute-phase-cytokines.

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One of the most important functions of the liver is to clear microorganisms, bacterial products and other particulate material coming from the gut through the portal vein or from other organs through the arterial blood. Kupffer cells (KCs) within the liver are the first cells to be exposed to materials absorbed from the gastrointestinal tract.[1,2] Macrophages within the liver and spleen have a major role in iron metabolism by recycling 20–25 mg/day of iron from aged red blood cells.[3,4] As mammals lack a regulated pathway for iron excretion, excess free iron is toxic.

Hepatic injury induced by various toxic agents has been attributed in part to the production of pro-inflammatory mediators by resident KCs within the liver.[5] The precise role of KCs in this process may vary as a function of the specific model and pathophysiological conditions.

To study the effect of phagocytosis *in vivo*, two substances known to be taken up by hepatic macrophages, gadolinium chloride (GD) and zymosan were investigated. GD is a member of the trivalent cations of the lanthanides series and exhibits properties similar to that of calcium in biological

[1]Department of Internal Medicine, Section of Gastroenterology and Endocrinology, Georg August-University, Göttingen, Germany and [2]Department of Clinical Medicine, Section of Gastroenterology, University 'La Sapienza', Rome, Italy
Correspondence: Professor Dr med. G Ramadori, MD, Department of Internal Medicine, Section of Gastroenterology and Endocrinology, Georg-August-University Göttingen, Robert-Koch-Straße 40, 37075 Göttingen, Germany.
E-mail: gramado@med.uni-goettingen.de

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systems.[6] Suppression of KCs function by pretreatment with GD has been shown to ameliorate or even prevent chemically induced liver damage in rats and mice.[7,8]

Although it is known that GD selectively reduces the capacity of KCs to ingest cellular matter and reduces both cytokine and free radical production in response to hepatic injury,[9–11] the precise effects of GD administration on KCs are not well defined. Zymosan is a substance derived from the cell wall of the yeast *Saccharomyces cerevisiae.*[12,13] It has been shown to directly activate *in vitro* mouse macrophages as a result of phagocytosis,[14] to secrete IL-8[15] and TNF-α.[16] Saito *et al*[17] recently reported that intraperitoneal (IP) injection of zymosan after IP dexamethasone treatment was not able to induce an increase of the hepatic expression levels of the acute-phase-cytokines, IL-1β, IL-6 and TNF-α. On the other hand, Burdon *et al*[18] showed that an IP administration of zymosan to C57BL mice induced an upregulation of TNF-α-gene expression, but only at day 7 after treatment. IP or intravenous GD injection has been used to examine the role of macrophages in the regulation of hepcidin-gene expression. Hepcidin is a circulating antimicrobial peptide synthesized mainly by hepatocytes.[19] It inhibits iron efflux by binding to Fpn-1 and inducing Fpn-1 internalization, resulting in reduced iron release from cells.[20] Hemojuvelin (Hjv) is a membrane protein that appears to have an essential role in the regulation of hepcidin expression, although the specific function of Hjv is currently unknown.[21,22]

In this study, we show that IP administration of particulate material induces changes in the gene expression of the iron regulatory proteins, hepcidin, Hjv and Fpn-1, similar to those induced by acute-phase-cytokines[23] whose gene expression is upregulated at the same time.

In the two models of KC phagocytosis induced by GD or zymosan, Hjv-gene expression behaved in a way opposite to that of hepcidin. Specifically, hepcidin-gene expression was increased, whereas the gene expression of Hjv and Fpn-1 was decreased both in rats and mice. Neither serum pro-hepcidin or iron levels changed after GD or zymosan treatment.

To rule out the possibility that the observed effect could be due to endotoxin contamination, endotoxin-resistant (C3H/HeJ) mice were also studied. C3H/HeJ mice have a missense mutation in the third exon of TLR-4, yielding a nonfunctional TLR-4, and are hyporesponsive to the effects of LPS[24–26] and are resistant to lethal endotoxin-induced shock as compared with normal mice.[27]

The simultaneous induction of the principal acute-phase-cytokines suggests that a local paracrine mechanism may be responsible for these changes in the expression of genes involved in the regulation of hepatic iron metabolism.

MATERIALS AND METHODS

Animals

Male Wistar rats (8 weeks old) and C3H/HeJ mice were purchased from Charles River (Sulzfeld, Germany) and maintained with free access to food and water. The animals were cared for according to the University, the German Convention for the Protection of Animals and NIH guidelines.

Reagents

All the chemicals used were of analytical grade and purchased from commercial sources as indicated below: real-time PCR primers and primers for northern blot from Invitrogen (Carlsbad, CA, USA), Moloney murine leukemia virus reverse transcriptase (M-MLV RT), reverse transcription buffer and 0.1 M DTT; Platinum Sybr Green qPCR-UDG mix from Invitrogen; dNTPs, protector RNase inhibitor, primer oligo$(DT)_{15}$ for cDNA synthesis and salmon sperm DNA from Roche (Mannheim, Germany); α^{32}P-labelled deoxycytidine triphosphate (specific activity 3000 Ci/mmol), NICK TM columns and Hybond N nylon membranes from Amersham Pharmacia Biotech (Freiburg, Germany); hybridization solution QuickHyb from Stratagene (Heidelberg, Germany); iron ferrozine from Rolf Greiner BioChemica (Flacht, Germany); and Quantikine enzyme-linked immunosorbent assay (ELISA) kits for pro-hepcidin from DRG International (Marburg, Germany).

Antibodies

The monoclonal antibody directed against the ED-1 epitope was purchased from Biermann (Wiesbaden, Germany). The antibody against C1q was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

GD and Zymosan Treatment of Rats and C3H/HeJ Endotoxin-Resistant Mice

Rats received one IP injection of either pyrogen-free phosphate-buffered saline or 40 mg/kg body weight GD.[28] In a separate experiment, rats were injected intraperitoneally with 0.1 mg/g body weight of zymosan.[29] C3H/HeJ mice were treated with similar doses of GD and zymosan by an IP injection. *In vivo*, three independent experiments for rats or C3H/HeJ mice have been carried out leading to a final number of $n = 3$ for each time point analyzed. The animals were killed at 3, 6, 12, 24 and 48 h after GD or zymosan treatment and their livers were removed and snap frozen in liquid nitrogen.

Tissue Sections and Immunohistology

For routine histology and immunohistochemistry, tissue obtained from GD- and zymosan-treated liver was rinsed with 0.9% NaCl and snap frozen in liquid nitrogen. Sections of 5-μm thickness were obtained, air dried and fixed with methanol (–20°C, 10 min), acetone (−20°C, 10 s), and stored at −20°C until further use. After inhibition of endogenous peroxidase by incubating the slides with phosphate-buffered saline (PBS) containing glucose/glucose oxidase/sodium azide, the sections were treated with FCS for 30 min to minimize nonspecific staining. The serial sections were

Table 1 Sequence of gene-specific primers used for quantitative RT-PCR analysis

Primer	Forward 5′→3′	Reverse 5′→3′
Hepcidin	5′-GAAGGCAAGATGGCACTAAGCA-3′	5′-TCTCGTCTGTTGCCGGAGATAG-3′
Hjv	5′-ACTATGAAGCCCGGTTTTCCAG-3′	5′-TTGTGGAAGCTGCGCACAT-3′
Fpn-1	5′-TTCCGCACTTTTCGAGATGG-3′	5′-TACAGTCGAAGCCCAGGACTGT-3′
IL-6	5′-GTCAACTCCATCTGCCCTTCAG-3′	5′-GGCAGTGGCTGTCAACAACAT-3′
IL-1β	5′-TACCTATGTCTTGCCCGTGGAG-3′	5′-ATCATCCCACGAGTCACAGAGG-3′
TNF-α	5′-ACAAGGCTGCCCCGACTAT-3′	5′-CTCCTGGTATGAAGTGGCAAATC-3′
IFN-γ	5′-AGTCTGAAGAACTATTTTAACTCAAGTAGCAT-3′	5′-CTGGCTCTCAAGTATTTTCGTGTTAC-3′
β-actin	5′-TGTCACCAACTGGGACGATA-3′	5′-AACACAGCCTGGATGGCTAC-3′

incubated in a humidified chamber with the first antibody directed against ED-1 or C1q diluted in PBS at a ratio of 1:100 for 1 h at room temperature. Negative controls were incubated with isotype-specific mouse IgGs, instead of the specific primary antibody. After washing, the slides were covered with peroxidase-conjugated anti-rabbit/anti-mouse immunoglobulins pre-absorbed with normal rat serum to avoid cross-reactivity. Slides were washed and incubated with PBS containing 3,3′-diaminobenzidine (0.5 mg/ml) and H_2O_2 (0.01%) for 10 min to visualize immune complexes. Nuclei were counterstained with Meyer's hemalaun solution before the slides were mounted with coverslips.

Estimation of Total ED-1- and C1q-Positive Cell Numbers

To estimate the number of positive ED-1 and C1q cell numbers in liver samples, ten portal fields from three different sections were analyzed. Control, GD- and zymosan-treated liver 24 h after treatment were studied. Counting was performed using a $\times 40$ objective lens and $\times 10$ ocular lenses (Zeiss, Jena, Germany). Images of the portal area for ED-1- and C1q-positive cells were captured in an IBM personal computer (IBM, New York, USA) and projected onto the computer monitor. The mean value of liver macrophages calculated in the ten portal areas selected at the time of screening was recorded.

RNA Isolation

RNA was prepared using Trizol reagent according to the manufacturer's instructions (Invitrogen). The RNA obtained was quantified by measuring the absorbance at 260/280 nm.

Quantitative Real-Time PCR

The cDNA was generated by reverse transcription of 1 μg of total RNA with 100 nM of dNTPs, 50 pM of primer oligo$(dT)_{15}$, 200 U of M-MLV RT, 16 U of protector RNase inhibitor, 1 $\times$ RT buffer and 2.5 μl of 0.1 M DTT, for 1 h at 40°C. Gene expression was analyzed using Platinum Sybr Green qPCR mix UDG. β-actin was used as control housekeeping genes. The primer sequences used are given in Table 1.

The amplification was carried out at 95–60°C for 45 cycles in an ABI prism 7000 sequence detection system. All samples were assayed in duplicate. The results were normalized to the housekeeping gene and fold change expression was calculated using threshold cycle (C_t) values. During a real-time RT-PCR the number of cycles when gene expression of any specific gene under study reaches a predetermined threshold of detection is known as the 'C_t value'. The C_t value (threshold cycle) is defined as 'the fractional cycle number at which the fluorescence passes the fixed threshold'. The higher the C_t value is for the specific gene at a given time, the lower is the abundance of the specific mRNA.

Northern Blot Analysis

Total RNA (10 μg/lane) was size fractionated by electrophoresis in 1% agarose–formaldehyde gels, transferred to nylon membranes using the capillary transfer system and cross-linked by ultraviolet light. Rat hepcidin and Hjv cDNA were generated by PCR from rat hepatic RNA with the following primers: hepcidin, forward 5′-AGGACAGAAGG CAAGATGGCA-3′, reverse 5′-TGTTGAGAGGTCAGGACA AGGC-3′; Hjv, forward 5′-CCATGGCAGTCCTCCAACTC TA-3′, reverse 5′-AGACGCAGGATTGGAAGTAGGC-3′. Hybridization was carried out at 68°C for 2 h with random-primed ^{32}P-labeled cDNA probes for hepcidin and Hjv. GAPDH was used to confirm equal loading of the samples: forward 5′-TCCTGCACCACCACCAACTGCTTAG-3′, reverse 5′-TTCTGAGTGGCAGTGATGGCA-3′.

Detection of Serum Transaminases and Iron Levels

Serum transaminases (ALT, AST) levels were measured in sera from GD- and zymosan-treated rats as described elsewhere.[30] In serum samples from treated and control rats, iron levels were detected by colorimetric ferrozine-based assay.[31]

ELISA

For detection of pro-hepcidin in serum from GD- and zymosan-treated rats, a pro-Hepcidin ELISA kit was used.[32,33] Samples were processed according to the manufacturer's instructions.

Statistical Analysis

The data were analyzed using Prism Graph Pad 4 software (San Diego, CA, USA). Data are given as mean ± s.e.m. A Student's *t*-test was carried out to identify significant differences among treated (GD and zymosan) and control data groups were obtained in three (*in vivo*) independent experiments. Statistically significant differences as compared with the control groups were estimated by a one-way ANOVA. For calculation of relative changes, the gene expression before treatment was set as '1'. Differences with a *P*-value of ≤ 0.05 after adjusting for multiple comparisons were considered to be significant.

RESULTS

ED-1 and C1q Immunoreactivity in Livers from Normal and GD- or Zymosan-Treated Rats

Immunohistological analysis of the liver 24 h after GD administration with the ED-1 antibody showed a reduction in the number of positive cells compared with controls (Figure 1). Similarly, C1q staining showed a reduction in positively stained cells (Figure 1). The KCs of GD-treated animals, particularly those located around the portal field, could be shown to contain particulate material. Similar findings were observed in animals treated with zymosan. The only difference between zymosan- and GD-treated animals was that the ED-1 staining was not reduced 24 h after zymosan administration. Immunohistochemical analysis using antibodies against neutrophil elastase did not reveal an increase in the number of neutrophilic granulocytes in the liver of GD-treated rats compared with control rat livers (data not shown).

Estimation of Total ED-1- and C1q-Positive Cell Number from Livers of Untreated, GD- and Zymosan-Treated Rats

ED-1 cell density per ten portal fields from zymosan-treated livers was 55 ± 8 ($P < 0.05$), and was significantly higher than that of untreated livers (33 ± 5, $P < 0.05$) and of GD-treated livers (16 ± 2, $P < 0.05$). The mean value of C1q-positive cells of zymosan-treated livers was 25 ± 4 ($P < 0.05$), whereas 17 ± 3 and 14 ± 3 positive cells ($P < 0.05$) were counted in the liver of control and GD-treated rats, respectively.

Expressions of Hepcidin, Hjv and Fpn-1, of the Main Acute-Phase-Cytokines, and of ED-1 and ED-2 *In Vivo* IP GD-Treated Rats

Real-time PCR analysis of total RNA extracted from IP GD-treated rat livers showed a time-dependent upregulation of hepcidin-gene expression with a maximum at 6 h (7 ± 3-fold, Figure 2a). At the same time, the expression of Hjv-and Fpn-1-gene expressions was downregulated to a maximum of 0.5 ± 0.2 and 0.6 ± 0.1-fold, respectively, at 6 h after IP GD treatment (Figure 2a). In the GD-treated livers, a significant upregulation of IL-6 and IL-1β gene expression was seen 3 h after treatment (44 ± 8-fold and 4.5 ± 2-fold, respectively, $P < 0.05$). TNF-α gene expression was upregulated 3 and 6 h after GD injection (4.3 ± 2-fold and 3.5 ± 1-fold, Figure 2b). mRNA transcripts for IFN-γ were either very low or not detectable in the livers of IP GD-treated animals.

The immunohistochemical data relative to ED-1 and ED-2 were confirmed by a demonstration of a time-dependent downregulation of these genes at 24 h after IP GD treatment (0.4 ± 0.03-fold and 0.05 ± 0.03-fold, respectively, Figure 2c).

Expressions of Hepcidin, Hjv and Fpn-1, of the Main Acute-Phase-Cytokines, and of ED-1 and ED-2 *In Vivo* IP Zymosan-Treated Rats

Similar to the data obtained after GD administration, after a single IP injection of zymosan, hepcidin-gene expression was induced achieving a maximum level by 6 h (9 ± 2.3-fold, Figure 3a) and was accompanied by a parallel downregulation of Hjv and Fpn-1, reaching a nadir of respectively 0.04 ± 0.07 and 0.2 ± 0.04-fold at 3 h after treatment (Figure 3a). Furthermore, the genes of the acute-phase-cytokines IL-6, TNF-α and IL-1β were strongly upregulated at 3 h (IL-6, TNF-α) and 6 h (IL-1β) after injection (115 ± 38-fold at 3 h and 46 ± 10-fold at 3 h for IL-6 and TNF-α, and 70 ± 20-fold at 6 h for IL-1β, Figure 3b), whereas IFN-γ mRNA was not detectable in zymosan-treated livers.

ED-1- and ED-2-gene expression was significantly downregulated 3 h after the zymosan treatment (0.6 ± 0.1-fold and 0.5 ± 0.1-fold, Figure 3c). Immunohistochemical analysis using antibodies against neutrophil elastase failed to show an increase in the number of neutrophilic granulocytes in the liver of zymosan-treated rats compared with that of control rats (data not shown).

Northern Blot Analysis of Total RNA from Livers of GD- and Zymosan-Treated Rats

Northern blot analysis was carried out to confirm the real-time PCR findings. An increase in hepcidin-gene expression with a maximum expression 6 h after IP GD injection was found. Hjv-gene expression was downregulated at the same time (Figure 4a). These results confirm the real-time PCR data. Hepcidin mRNA levels were increased achieving a maximum level at 6 h after an IP injection of zymosan. Hjv-gene expression was downregulated at the same time (Figure 4b). An oligonucleotide complementary to GAPDH was used to ensure equal loading of the RNA.

Expressions of Hepcidin, Hjv and Fpn-1, of the Main Acute-Phase-Cytokines, and of ED-1 and ED-2 *In Vivo* IP GD- or Zymosan-Treated C3H/HeJ Mice

To rule out a role for endotoxin contamination in the results observed in GD- and zymosan-treated rats, C3H/HeJ endotoxin-resistant mice were treated with GD or zymosan administered intraperitoneally. Hepcidin-1-gene expression was significantly induced by both GD and zymosan 3 h after administration (4 ± 0.4-fold and 5 ± 1-fold, respectively,

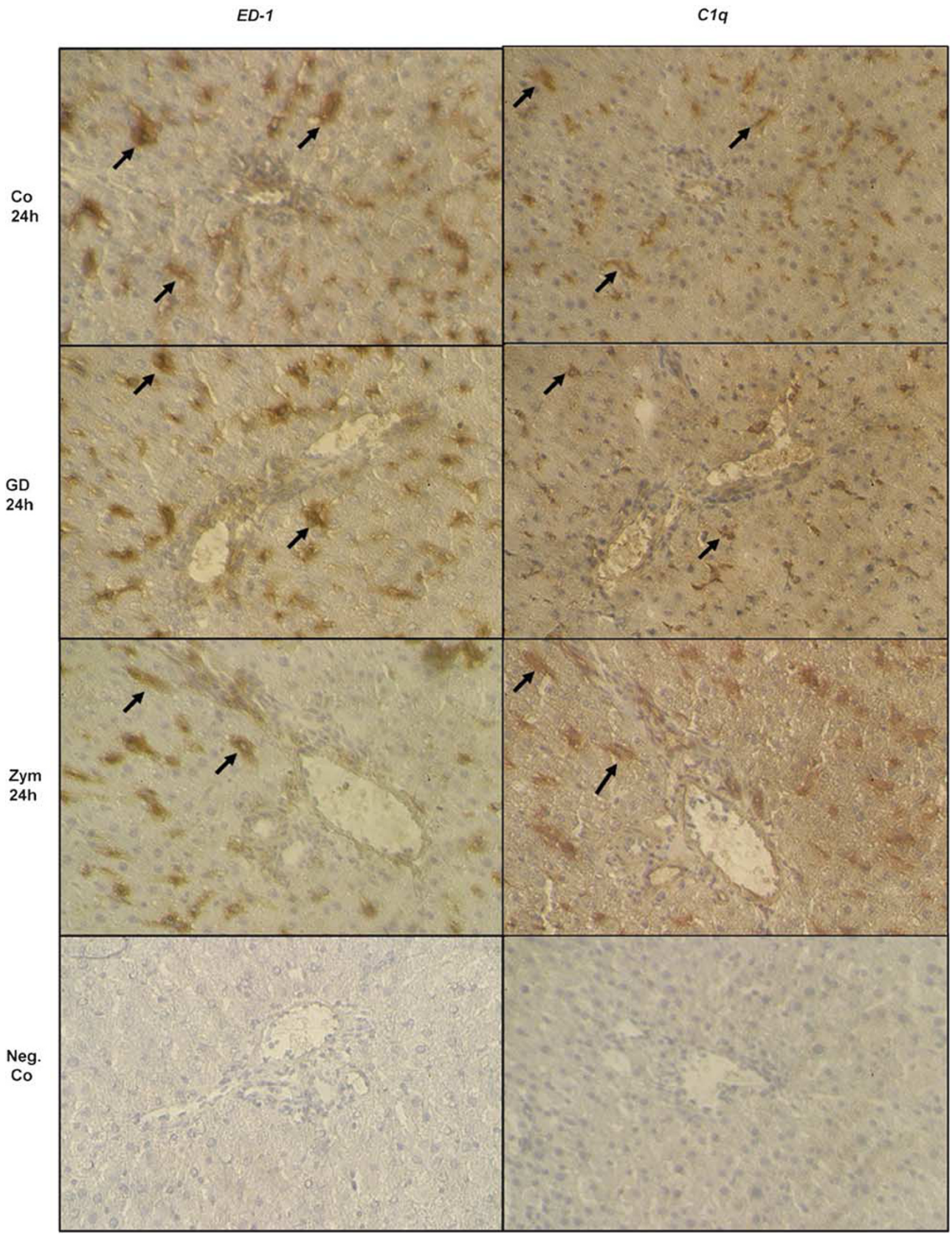

Figure 1 Immunohistochemical detection of ED-1 and C1q in normal, GD- and zymosan-treated rat liver (24 h). The arrows show ED-1- and C1q-positive cells in sequential liver sections.

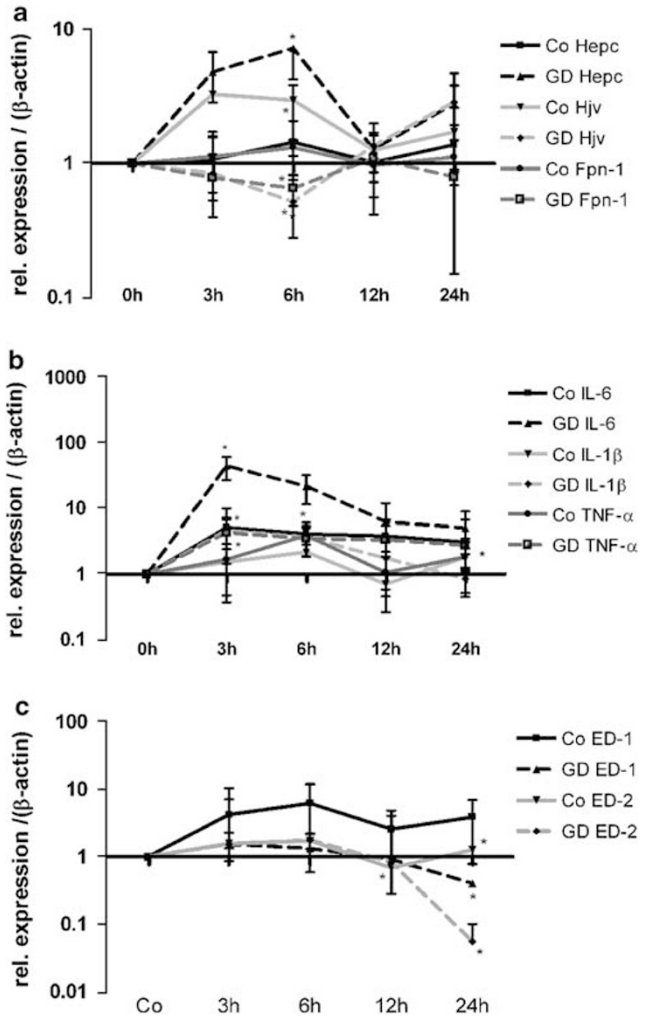

Figure 2 Measurement of mRNA expressions of hepcidin, Hjv and Fpn-1, of acute-phase-cytokines (IL-1β, TNF-α and IL-6), and of ED-1, ED-2 in the liver of rats after intraperitoneal (IP) administration of GD (**a**–**c**) measured by quantitative RT-PCR, showing a time-dependent GD-inducing hepcidin upregulation and a downregulation of Hjv-gene expression (**a**). Data are shown relative to the expression of β-actin (control levels of pyrogen-free, phosphate-buffered saline (PBS)-treated rats have been normalized to one and are not being directly represented). Values represented are means ± s.e.m. of analyzed RNA extracted from four different animals. *P*-values were calculated compared with pyrogen-free PBS control levels. $^*P \leq 0.05$.

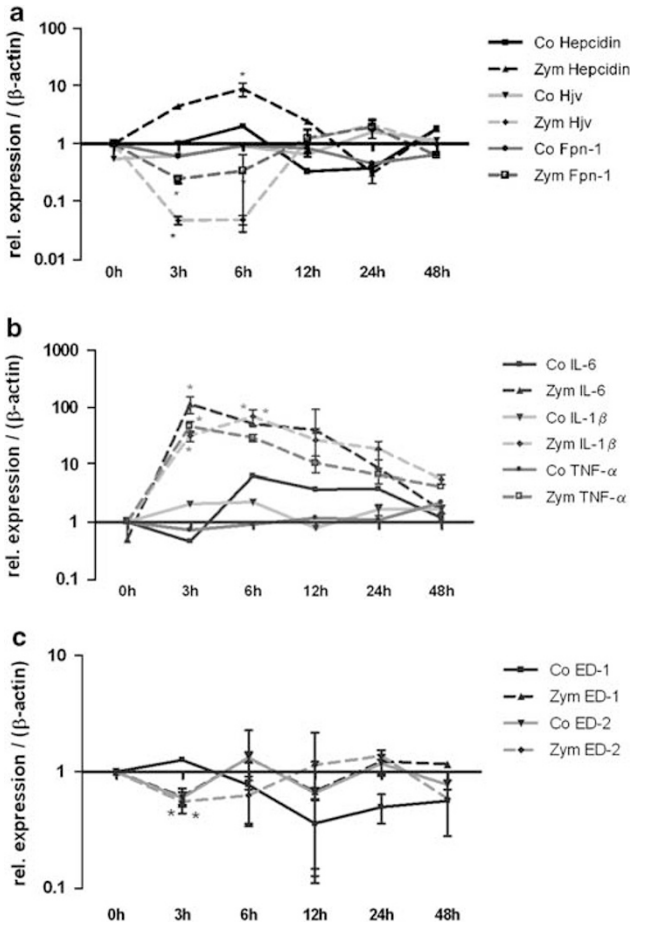

Figure 3 mRNA expressions of hepcidin, Hjv and Fpn-1, of the acute-phase cytokines (IL-6, IL-1β, TNF-α), and of ED-1 and ED-2 in the liver of rats after IP administration of zymosan (**a**–**c**) measured by quantitative real time RT-PCR. The results here reported show a time-dependent zymosan-inducing hepcidin upregulation and a downregulation of Hjv-gene expression (**a**). Data are shown relative to the expression of β-actin (control levels of pyrogen-free, phosphate-buffered saline (PBS)-treated rats have been normalized to one and are not directly represented). Values represented are means ± s.e.m. of analyzed RNA extracted from four different animals. *P*-values were calculated compared with pyrogen-free PBS control levels. $^*P \leq 0.05$.

Figure 5a). Hjv-gene expression was significantly down-regulated by both GD and zymosan, reaching a nadir at 3 h after beginning the experiment (0.2 ± 0.1-fold and 0.1 ± 0.01-fold, respectively, Figure 5a). The mRNA for IFN-γ was not detectable, whereas mRNA for IL-1β was sharply upregulated at 3 h by GD and zymosan (25 ± 2-fold and 25 ± 6-fold, respectively, Figure 5b). TNF-α was induced by zymosan at 3 h (10 ± 2-fold) and to a lesser extent by GD after 3 h (4.5 ± 0.8-fold). IL-6 mRNA expression was induced by zymosan at 3 h (27 ± 3-fold) and to a lesser extent by GD (4 ± 2.8-fold). After GD IP injection, a reduction in both ED-1- and ED-2-gene expression reaching a nadir at 12 h (0.7 ± 0.1-fold, 0.5 ± 0.05-fold, respectively, Figure 5c) was seen. No such changes were observed in C3H/HeJ mice treated with zymosan.

Changes in the Serum Levels of Iron, Pro-Hepcidin and Transaminases (AST, ALT)

Changes in the level of iron and pro-hepcidin by ELISA, measured at different time points in serum and tissue lysates after IP GD and zymosan administration, were compared with values obtained in control rats. No significant changes were observed in the sera of any group.

Similarly, no significant changes in serum ALT and AST levels between groups could be identified (data not shown).

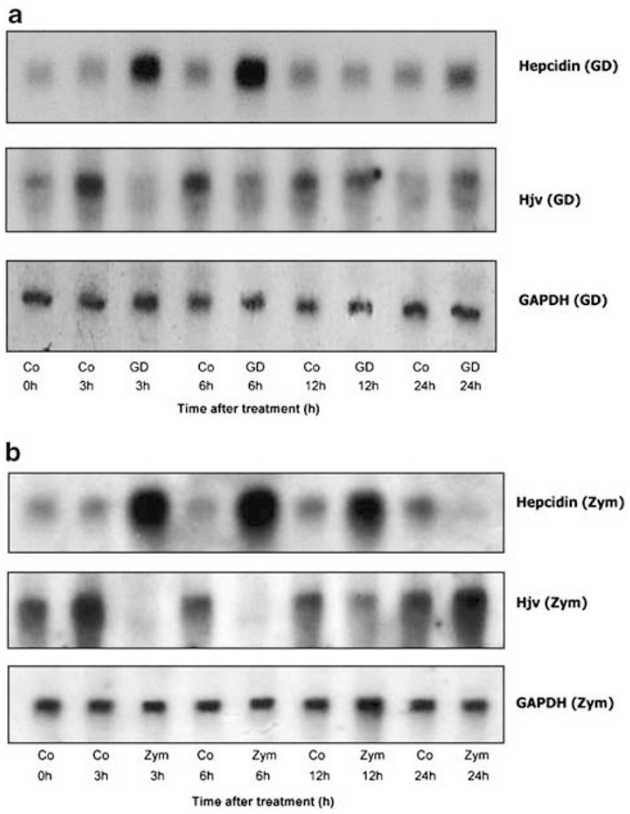

Figure 4 Northern blot analysis of hepcidin- and Hjv-specific transcripts (black = amount of specific transcript) in rat livers at 3, 6, 12 and 24 h after IP administration of GD (**a**), zymosan (**b**) and in control livers. The results reported here confirm the RT-PCR data showing a time-dependent GD- and zymosan-inducing hepcidin upregulation and a downregulation of Hjv-gene expression. Total RNA was electrophoresed and blotted onto nylon membranes, and hybridized with a cDNA specific for rat hepcidin and Hjv. GAPDH was used as the housekeeping gene. This figure shows results representative for three experiments.

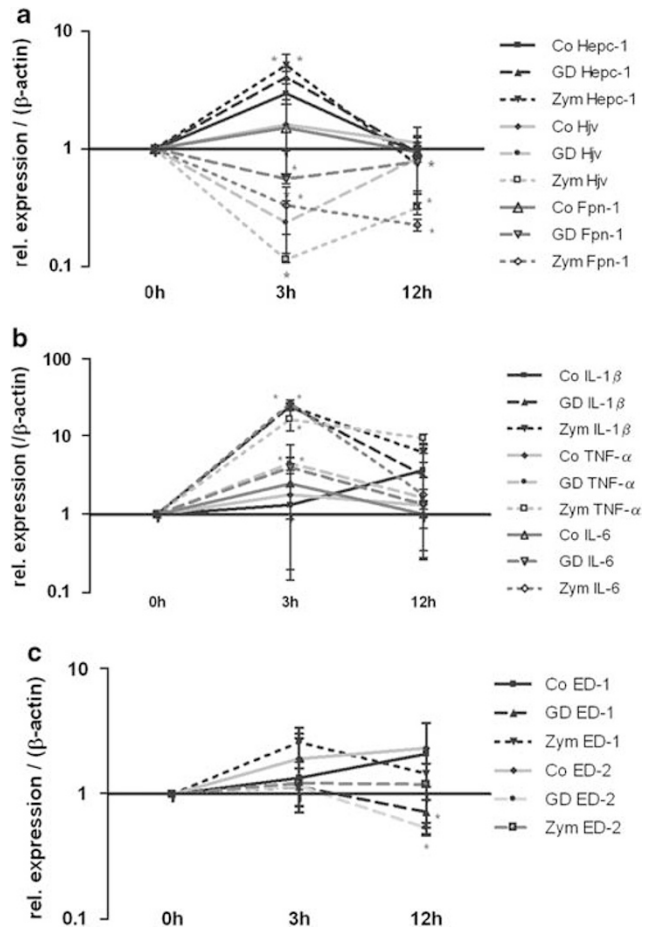

Figure 5 mRNA expressions of hepcidin-1, Hjv and Fpn-1, of acute-phase cytokines (IL-6, IL-1β, TNF-α), and of ED-1 and ED-2 in the liver of C3H/HeJ mice after IP administration of GD and of zymosan measured by quantitative RT-PCR (**a**–**c**). IFN-γ mRNA expression was not detected by using RT-PCR. Data are shown relative to the expression of β-actin (control levels of pyrogen-free, phosphate-buffered saline (PBS)-treated rats have been normalized to one and are not being directly represented). Values represented are means ± s.e.m. of analyzed RNA extracted from four different animals. *P*-values were calculated compared with pyrogen-free PBS control levels. $^*P \leq 0.05$.

DISCUSSION

To our knowledge, this is the first report of the changes in gene expression induced in liver tissue by the administration of particulate material, which is supposed to be taken up by liver macrophages, such as GD or zymosan, in rats and CH3/HeJ endotoxin-resistant mice.

Phagocytosis is one of the most important innate effector functions of tissue macrophages, serving to clear particulate matter, apoptotic cells and microorganisms.[34] Little is known about the changes in the expression of hepatic secretory proteins such as the proteins related to iron metabolism induced by phagocytosis by hepatic macrophages.

The current data are consistent with the concept that administration of GD or zymosan leads to an induction of hepatic gene expression of proteins involved in iron metabolism and further that KCs are involved in this process.

These data confirm previous studies[35–37] in different rat models of liver injury, such as partial hepatectomy, CCl_4 administration, liver irradiation and turpentine oil-induced acute-phase response, that hepcidin- and Hjv-gene expression changes in the liver occur simultaneously and in an opposite direction. Specifically, when rats or mice are treated with GD intraperitoneally, hepcidin-gene expression is upregulated, whereas Hjv- and Fpn-1-gene expression are downregulated. The same results occurred in rats after an IP injection of zymosan; an upregulation of hepcidin-gene expression as early as 3 h reaching a maximum at 6 h associated with a time-dependent downregulation of Hjv- and Fpn-1-gene expression was observed. Northern blot analysis confirmed the real-time PCR findings for hepatic hepcidin- and Hjv-gene expression (Figure 3). Moreover, upregulation of genes for the acute-phase-cytokines, IL-6, TNF-α, IL-1β, but not of IFN-γ, was identified. These findings were not a consequence of an endotoxin contamination of the injected

substances, as the same results were obtained in a mouse strain (CH3/HeJ) resistant to the effects of endotoxin.

Serum levels of iron and pro-hepcidin were determined and no difference in the concentration of these markers between control and GD- or zymosan-treated animals was rated (data not shown). A possible explanation for the lack of change in the serum levels of iron and pro-hepcidin could be that upregulation of hepcidin-gene expression within the liver, although significant, was not sufficient enough to induce a measurable change of the serum levels within the time frames of the study as we have previously found.[35] This conclusion, however, must be tempered by the fact that the ELISA measurement of pro-hepcidin may not detect a change in functional hepcidin.[38] The changes in gene expression of these iron metabolism-related proteins occurred in the absence of hepatotoxicity, as serum AST and ALT levels did not increase in these experiments.

These findings indicate that uptake of corpusculate matter induces changes of hepatic gene expression which are qualitatively similar to those observed after LPS injection.

This leads to the question what are the putative mechanisms responsible for KC-mediated upregulation of hepcidin-gene expression. Acute-phase-cytokines are stimulators of early hepcidin-gene expression in inflammatory processes[39,40] and KCs produce several acute-phase-cytokines either constitutively or in response to inflammatory stimuli.[41] Cytokine regulation of iron metabolism in hepatocytes has been reported previously.[42–43] It remains a matter of debate whether IL-6 is more potent than IL-1β, TNF-α or IFN-γ in inducing hepcidin-gene expression in hepatocytes.[23,44]

Regardless of this debate that is suggested by this data, the induction of hepcidin-gene expression in hepatocytes occurs as a consequence of the synthesis and secretion of acute-phase-cytokines by KCs. This hypothesis is supported by the immunohistochemical analysis performed showing ED-1- and C1q-positive cells containing zymosan or GD up to 24 h after the administration of either compound (Figure 1), indicating that enough KCs are detectable in the liver and that they are still functionally active.

In GD- and zymosan-treated rats, as well as C3H/HeJ mice, an upregulation of IL-6, IL-1β and TNF-α gene expression can be detected using RT-PCR, suggesting that the changes of hepcidin-, Hjv- and Fpn-1-gene expression may be mediated by locally produced KC-derived acute-phase-cytokines and that the effect is not endotoxin dependent. Further studies are underway to elucidate the mechanisms involved in the upregulation of acute-phase-mediators gene expression induced by phagocytosis in liver macrophages *in vivo*.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

1. Smedsrod B, De Bleser PJ, Braet F, *et al.* Cell biology of liver endothelial and Kupffer cells. Gut 1994;35:1509–1516.
2. Laskin DL, Pendino KJ. Macrophages and inflammatory mediators in tissue injury. Ann Rev Pharmacol Toxicol 1995;35:655–677.
3. Chaston T, Chung B, Mascarenhas M, *et al.* Evidents for differential effects of hepcidin in macrophages and intestinal epithelial cells. Gut 2008;57:374–382.
4. Knutson M, Wessling-Resnick M. Iron metabolism in the reticuloendothelial system. Crit Rev Biochem Mol Biol 2003;38:61–88.
5. Laskin DL. Non parenchymal cells and hepatotoxicity. Semin Liv Dis 1990;10:293–304.
6. Palasz A, Czekaj P. Toxicological and cytophysiological aspects of lanthanides action. Acta Biochim Pol 2000;47:1107–1114.
7. Michael SL, Pumford NR, Mayeux PR, *et al.* Pretreatment of mice with macrophage inactivators decreases acetaminophen hepatotoxicity and the formation of reactive oxygen and nitrogen species. Hepatology 1999;30:186–195.
8. Sauer JM, Waalkes MP, Hooser SB, *et al.* Suppression of Kupffer cell function prevents cadmium induced hepatocellular necrosis in male Sprague-Dawley rats. Toxicology 1997;121:155–164).
9. Hardonk MJ, Dijkhuis FW, Hulstaert CE, *et al.* Heterogeneity of rat liver and spleen macrophages in gadolinium chloride-induced elimination and repopulation. J Leukoc Biol 1992;52:296–302.
10. Lazar Jr G, Farkas G, Csanadi J, *et al.* Gadolinium chloride-induced macrophage blockade prevents rejection of human insulinoma cell xenograft in rats. Transplantation 1997;63:729–732.
11. Olynyk JK, Yeoh GC, Ramm GA, *et al.* Gadolinium chloride suppresses hepatic oval cell proliferation in rats with biliary obstruction. Am J Pathol 1998;152:347–352.
12. Sanguedolce MV, Capo C, Bouhamadan M, *et al.* Zymosan-induced tyrosine phosphorylations in human monocytes. Role of protein kinase C. J Immunol 1993;151:405–414.
13. Zaffran Y, Escallier JC, Ruta S, *et al.* Zymosan-triggered association of tyrosine phosphoproteins and Lyn kinase with cytoskeleton in human monocytes. J Immunol 1995;154:3488–3497.
14. Underhill DM. Macrophage recognition of zymosan particles. J Endotoxin Res 2003;9:176–180.
15. Friedland JS, Remick DG, Shattock R, *et al.* Secretion of interleukin-8 following phagocytosis of Mycobacterium tuberculosis by human monocyte cell lines. Eur J Immunol 1992;22:1373–1378.
16. Sanguedolce MV, Capo C, Bongrand P, *et al.* Zymosan-stimulated tumor necrosis factor-α production by human monocytes. Down-modulation by phorbol ester. J Immunol 1992;148:2229–2236.
17. Saito M, Watanabe S. Differential modulation of lipopolysaccharide- and zymosan-induced hypophagia by dexamethasone treatment. Pharmacol Biochem Behav 2008;90:428–433.
18. Burdon D, Tiedje T, Pfeffer K, *et al.* The role of tumor necrosis factor in the development of multiple organ failure in a murine model. Crit Care Med 2000;28:1962–1967.
19. Ganz T, Nemeth E. Iron imports. IV. Hepcidin and regulation of body iron metabolism. Am J Physiol Gastrointest Liver Physiol 2006;290:G199–G203.
20. Nemeth E, Tuttle MS, Powelson J, *et al.* Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. Science 2004;306:2090–2093.
21. Pietrangelo A, Caleffi A, Henrion J, *et al.* Juvenile hemochromatosis associated with pathogenic mutations of adult hemochromatosis genes. Gastroenterology 2005;128:470–479.
22. Niederkofler V, Salie R, Arber S. Hemojuvelin is essential for dietary iron sensing, and its mutation leads to severe iron overload. J Clin Invest 2005;115:2180–2186.
23. Lee P, Peng H, Gelbart T, *et al.* Regulation of hepcidin transcription by interleukin-1 and interleukin-6. Proc Natl Acad Sci USA 2005;102:1906–1910.
24. Poltorak A, He X, Smirnova I, *et al.* Defective LPS signalling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science 1998;282:2085–2088.
25. Hoshino K, Takeuchi O, Kawai T, *et al.* Cuttinge edge: Toll-like receptor 4 (TLR-4)-deficient mice are hyporesponsive to lipopolysaccharide:

evidence for TLR4 as the Lps gene product. J Immunol 1999;162: 3749–3752.
26. Morrison DC, Ryan JL. Bacterial endotoxins and host immune responses. Adv Immunol 1979;28:293–450.
27. Wong PM, Kang A, Chen H, *et al.* Lps(d)/Ran of endotoxin-resistant C3H/HeJ mice is defective in mediating lipopolysaccharide endotoxin responses. Proc Natl Acad Sci USA 1999;96:11543–11548.
28. Montosi G, Corradini E, Garuti C, *et al.* Kupffer cells and macrophages are not required for hepatic hepcidin activation during iron overload. Hepatology 2005;41:545–552.
29. Nieuwenhuijzen GA, Haskel Y, Lu Q, *et al.* Macrophage elimination increases bacterial translocation and gut-origin septicemia but attenuates symptoms and mortality rate in a model of systemic inflammation. Ann Surgery 1993;218:791–799.
30. Schumann G, Bonora R, Ceriotti F, *et al.* IFCC primary reference procedures for the measurement of catalytic activity concentrations of enzymes at 37°C. International Federation of Clinical Chemistry and Laboratory Medicine Part 4 Reference procedure for the measurement of catalytic concentration of alanine aminotransferase. Clin Chem Lab Med 2002;40:718–724.
31. Riemer J, Hoepken HH, Czerwinska H, *et al.* Colorimetric ferrozine-based assay for the quantitation of iron in cultured cells. Anal Biochem 2004;331:370–375.
32. Kulaksiz H, Gehrke SG, Janetzko A, *et al.* Pro-hepcidin: expression and cell specific localisation in the liver and its regulation in hereditary haemochromatosis, chronic renal insufficiency, and renal anaemia. Gut 2004;53:735–743.
33. Kulaksiz H, Theilig F, Bachmann S, *et al.* The iron-regulatory peptide hormone hepcidin: expression and cellular localization in the mammalian kidney. J Endocrinol 2005;184:361–370.
34. Underhill DM, Ozinsky A. Phagocytosis of microbes: complexity in action. Ann Rev Immunol 2002;20:825–852.
35. Sheikh N, Batusic DS, Dudas J, *et al.* Hepcidin and hemojuvelin gene expression in rat liver damage: *in vivo* and *in vitro* studies. Am J Physiol Gastrointest Liver Physiol 2006;291:G482–G490.
36. Christiansen H, Sheikh N, Saile B, *et al.* x-Irradiation in rat liver: consequent upregulation of hepcidin and downregulation of hemojuvelin and ferroportin-1 gene expression. Radiology 2007;242:189–197.
37. Sheikh N, Dudas J, Ramadori G. Changes of gene expression of iron regulatory proteins during turpentine oil-induced acute-phase response in the rat. Lab Invest 2007;87: 713–725.
38. Koliaraki V, Marinou M, Vassilakopoulos TP, *et al.* A novel immunological assay for hepcidin quantification in human serum. PLoS ONE 2009;4:e4581.
39. Sharma N, Butterworth J, Cooper BT, *et al.* The emerging role of the liver in iron metabolism. Am J Gastroenterol 2005;100: 201–206.
40. Decker K. Biologically active products of stimulated liver macrophages (Kupffer cells). Eur J Biochem 1990;192:245–261.
41. Nicolas G, Chauvet C, Viatte L, *et al.* The gene encoding the iron regulatory peptide hepcidin is regulated by anemia, hypoxia, and inflammation. J Clin Invest 2002;110:1037–1044.
42. Nemeth E, Rivera S, Gbayan V, *et al.* IL-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin. J Clin Invest 2004;113:1271–1276.
43. Moriconi F, Christiansen H, Raddatz D, *et al.* Effect of radiation on gene expression of rat liver chemokines: *in vivo* and *in vitro* studies. Radiat Res 2008;169:162–169.
44. Lee P, Peng H, Gelbart T, *et al.* The IL-6- and lipopolysaccharide-induced transcription of hepcidin in HFE, transferrin receptor 2-, and beta 2-microgobulin-deficient hepatocytes. Proc Natl Acad Sci USA 2004;101:9263–9265.