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Contribution of IL-6/gp130 signaling in hepatocytes to the inflammatory response in mice infected with *Streptococcus pyogenes*

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Running title: "Contribution of IL-6/gp130 signalling in hepatocytes to S. pyogenes sepsis"

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FOOTNOTE:

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ABSTRACT

Background. Sepsis and septic shock due to gram-positive bacteria have become increasingly clinical problems. These conditions are accompanied by an overshooting inflammation in which the liver plays a central role as source and target of inflammatory mediators. Sepsis is still associated with high mortality rates and new intervention strategies directed to ameliorate the extent of the inflammatory reaction are strongly needed. Here, we investigated whether blockage of the transducer gp130, a receptor involved in the regulation of the inflammatory response, may be useful in the treatment of experimental gram-positive sepsis.

Methods. An experimental model of gram-positive sepsis was used where liver specific gp130-deficient mice (FVB/n alfpCre+ $gp130^{LoxP/LoxP}$) and wild-type control mice (FVB/n $gp130^{LoxP/LoxP}$) were intravenously infected with *Streptococcus pyogenes*. The following parameters were monitored: mortality, bacterial loads in systemic organs, serum inflammatory cytokines and organ damage.

Results. We show that infected gp130-deficient mice survived significantly longer, displayed lower bacterial loads, and developed slower pathology than infected control mice. Furthermore the levels of IFN-γ, IL-6 and the chemokine KC were significantly lower in gp130-deficient mice than control mice. Histopathological examination of the liver showed lower amounts of neutrophil infiltration, apoptosis, and tissue damage in infected gp130- deficient than in control mice.

Conclusion. Our results demonstrate that the gp130 receptor is involved in the regulation of inflammation during gram-positive sepsis and that blockage of gp130 signalling in hepatocytes could constitute a novel target for adjunctive therapy in septic patients.

Words: 237

INTRODUCTION

Sepsis remains the most common cause of death within intensive care units [1]. In particular, sepsis and septic shock caused by gram-positive bacteria such as streptococci and staphylococci have become an increasingly clinical problem in recent years [2-4]. Sepsis and septic shock are complex pathophysiological processes initiated by either the microbe itself or its products, and characterized by a massive systemic inflammatory response, circulatory insufficiency and organ damage. Despite aggressive antibiotic treatment and critical care support, the mortality rate of sepsis remains high, probably because the pathogenesis of this syndrome is poorly understood [5]. In addition, the treatment of septic patients has been further complicated by the emergence of antibiotic resistant gram-positive bacteria. Therefore, there is a clear need to develop more effective treatments for sepsis based on the identification of novel targets. The development of these new therapeutic approaches is not an easy task due to the complexity of the immunological defences and the feed-back mechanisms of the different components of the inflammatory cascade. Nevertheless, a number of adjuvant therapies, aimed at blunting/down regulating the host hyper inflammatory response, are currently under investigation.

Although strategies aimed at down-regulating pro-inflammatory cytokines by using antibodies directed against these molecules have proved to be uniformly disappointing [6], strategies targeting receptors involved in inflammatory signalling have shown promising results in experimental models of sepsis. Examples are provided by the attenuation of the hyper-inflammatory response and protective effects against polymicrobial septic peritonitis exhibited by mice deficient in the adaptor MyD88 molecule [7]. In addition, blocking TREM-1 (triggering receptor expressed on myeloid cells) signalling has also shown to be beneficial for the outcome of sepsis in a cecal ligation and puncture infection model [8-10]. In this study, we evaluated the gp130 transducing receptor as a potential therapeutic target to influence the hyper inflammatory

response in the course of gram-positive induced sepsis. The gp130 receptor is a membrane glycoprotein shared by interleukin-6 family of cytokines like IL-6, IL-11, ciliary neutrophic factor CNTF, cardiotrophin-1 (CT-1), cardiotrophin-like cytokine (CLC), leukaemia inhibitory factor (LIF), neuropoietin (NPN), oncostatin (OSM), IL-27, and IL-31, and is a critical component for the signal transduction of these cytokines [11-13]. Interfering with gp130 signalling by using recombinant soluble gp130 protein has proved very effective for therapy of inflammatory disorders in mouse models of human diseases such as Crohn's disease [14], rheumatoid arthritis [15], and colon cancer [16], suggesting that targeting of gp130 may be a promising therapeutic candidate for the treatment of sepsis. However, whole-body inactivation of molecules that are pivotal to innate immunity can also have harmful effects. These potential detrimental effects can be overcome by targeting these molecules in an organ-specific manner. As the liver plays a crucial role in the pathogenesis of bacterial sepsis as a source and target of inflammatory mediators, we examined the effect that liver-specific blockage of the gp130 signalling cascade has on the course of experimental gram-positive sepsis.

MATERIAL AND METHODS

Bacteria. *S. pyogenes* A20 strain is a human isolate obtained from the German Culture Collection (DSM 2071). Stocks were maintained at -70°C and were routinely cultured at 37°C in Todd-Hewitt broth (Oxoid), supplemented with 1% yeast extract for approximately 6 h. Bacteria were collected in mid-log-phase, washed twice with sterile PBS, diluted to the required inoculum and the number of viable bacteria determined by counting CFU after diluting and plating in blood agar plates (GIBCO) containing 5% sheep blood.

Mice. Female FVB/n alfpCre+ $gp130^{LoxP/LoxP}$ bearing an hepatocyte specific gp130 deficiency and wildtype FVB/n $gp130^{LoxP/LoxP}$ control mice, between 8-10 weeks of age, were obtained from the animal Research Institute of the Medical School Hanover [17]. They were housed in microisolator cages and given food and water ad libitum. All studies were approved by the appropriate authorities. For bio-burden experiments groups of 3-5 wilde type and hepatocyte – specific gp130-deficient mice were taken randomly from the infected cohort 24h and 48h of infection.

Infection model. The infection model used has been previously described [18, 19]. In brief, mice were inoculated with 10^5 CFU of *S. pyogenes* in 0.2 ml of PBS via a lateral tail vein. For kinetic studies, infected mice were sacrificed by CO_2 asphyxiation and bacteria from the liver and spleen were enumerated at indicated times, by preparing homogenates of these organs in PBS and plating 10-fold serial dilutions on blood agar. Colonies were counted following a 24 h incubation at 37°C. Viable bacterial counts of the blood were also determined by collecting blood samples from the tail vein at different times post-inoculation and plating serial dilutions on blood agar.

Determination of IL-10, IL-6, IFN-γ and KC concentrations. IL-6, IFN-γ and KC serum concentrations were determined by ELISA (IL-6 BD-Pharmingen), KC (R&D-Systems) as previously described [20]. IL-10 was measured with the IL-10 ELISA-Kit (BD Bioscience) according to the manufacturers instructions.

Determination of Serum transaminases. AST and ALT activity was determined by an automated enzyme assay as described [21, 22].

Quantification of polymorph nuclear neutrophils (PMN) infiltration and apoptotic bodies. Liver sections were prepared at 48 h post infection from 4 mice / group. The tissue was fixed with 10% buffered neutral formalin solution, embedded in paraffin, cut into 3-nm-thick sections and stained with haematoxylin-eosin. The amount of PMN´s and apoptotic bodies were determined by counting 10 representative high power fields after microscopic examination.

Myeloperoxidase (MPO) determination. Liver MPO was used as a quantitative indicator of neutrophils infiltration. The whole liver was homogenized in 5 mL of 50 mM HEPES and was centrifuged at 10,000 *g* for 30 min at 4°C. Pellets were resuspended in 2 mL of 0.5% cetyltrimethylammonium chloride (Sigma), extensively vortexed, and centrifuged at 12,000 x *g* for 15 min. The supernatant was then diluted 1:2 in 0.5% cetyltrimethylammonium and was mixed 1:2 with the MPO substrate solution 3,3-,5,5-tetramethyl-nezidineassay buffer. After 15 min, the reaction was stopped by addition of 4 *N* H_2SO_4. A standard curve was generated by use of purified human MPO (Sigma), and activity was expressed in units of MPO per mL of whole organ homogenate.

Statistical analysis. Statistical significance between paired samples was determined by Student's *t* test. Comparison of survival time curves was performed by use of the Wilcoxon rank sum test. $P < 0.05$ was considered to be statistically significant.

RESULTS

Hepatocyte-specific gp130-deficient mice were more resistant to S. pyogenes infection. Hepatocyte-specific gp130-deficient mice (n=19) and wild type mice (n=12) of the FVB/n background were intravenously infected with 10^5 CFU of *S. pyogenes* and the survival rate of individual mice was recorded daily for 10 days. The Kaplan-Mayer-Plots displayed in Fig. 1 show that hepatocyte-specific gp130-deficient mice survived significantly longer than wild type mice after *S. pyogenes* infection ($p < 0.001$). Thus while the mean survival time (MST) of wild type mice was 3 days with 100% mortality at day 4 of infection, the MST of hepatocyte-specific gp130-deficient mice was 4.5 days with a 100% mortality at day 8 of infection. In accordance with the longer survival, the hepatocyte-specific gp130-deficient mice displayed significant lower bacterial loads in blood and systemic organs than wild type mice with the greatest differences in bacterial loads in blood (Fig.2A), liver (Fig. 2B) and spleen (Fig. 2C) between the gp130-deficient and wild type mice observed at 48 h of infection.

Liver-specific gp130-deficient mice exhibited lower levels of inflammatory cytokines than wild type mice at 48h after bacterial inoculation. It was previously shown, that susceptibility of mice to *S. pyogenes* infection is associated with an overproduction of pro-inflammatory cytokines such as IFN-γ and IL-6 [19]. Therefore the level of these cytokines in the serum of hepacyte-specific gp130-deficient and wild type mice at 48 h after intravenous infection with *S. pyogenes* was determined. The results show that significant lower levels of IFN-γ (Fig. 3A) ($p < 0.05$) and IL-6 (Fig. 3B) ($p < 0{,}001$) were present in the serum of hepatocyte-specific gp130-deficient mice compared to wild type control mice after 48 h of bacterial inoculation. In contrast, the levels of IL-10, a gp130-independent cytokine, were undetectable in both wild type and hepatocyte-specific gp130-deficient mice after *S. pyogenes* infection.

Taken together, these results clearly indicate that blockage of gp130 signalling in hepatocytes can significantly attenuated the systemic inflammatory reaction taking place in septic mice after infection with *S. pyogenes*.

Disruption of gp130 signalling in hepatocytes results in reduced production of the cytokine - induced neutrophile chemoattractant (KC). We have previously shown that IL-6/gp130 engagement in hepatocytes induced expression and production of the chemo tactic factor cytokine-induced-neutrophile chemoattractant (KC) [22]. Therefore, we determined the levels of KC in wild type control and hepatocyte-specific gp130-deficient mice at 48 h of infection. The results in Fig. 4 show, that while significantly high levels of KC were detectable in wild type mice, levels of KC were almost undetectable in hepatocyte-specific gp130-deficient mice (Fig. 4A). This suggests, that the production of KC during *S. pyogenes* infection is primarily produced by hepatocytes in response to gp130 signalling and that KC may play an important role in the attraction of inflammatory neutrophils into infected organs.

Histopathological examination of liver tissue obtained from gp130-deficient and wild type control mice at 48h of infection with S. pyogenes. In previous studies, we have shown that organ damage occurs in *S. pyogenes* infected mice and that liver injury and dysfunction, caused by an accumulation of neutrophiles in this organ, contributed to the lethality in those animals. Histopathological evaluation of liver tissue obtained at 48 h of infection shows a significant higher numbers of neutrophils infiltrated into the liver of control animals than in hepatocyte-specific gp130-deficient animals (Fig. 4B). To substantiate the histochemical findings and provide more quantitative data, the amount of MPO, a heme protein abundantly expressed in PMNs, was determined in liver of infected wild type hepatocyte-specific gp130-deficient mice.

Higher levels of myeloperoxidase activity could be detected in the liver tissue of wild type (0.9 ± 0.03 U/ml) than in the liver tissue of hepatocyte-specific gp130-deficient mice (0.65 ± 0.08 U/ml).

The histological examination also shows a higher number of apoptotic bodies in the liver of control mice (Fig. 5A) than in the liver of hepatocyte-specific gp130-deficient mice (Fig. 5B) at 48 h of infection. Quantification of apoptotic bodies in the liver of infected control and gp130-deficient mice is shown in Fig. 5C.

The different degree of pathology observed in the liver of infected control and gp130-deifcient mice was correlated with the different levels of transaminases AST (Fig. 6A) and ALT (Fig. 6B) detected in the serum of these animals.

DISCUSSION

The current study shows that blockage of gp130 receptor signalling specifically in hepatocytes improved the survival of septic mice lethally infected with *S. pyogenes*. Improved survival in hepatocyte-specific gp130-deficient mice was associated with lower bacterial burdens in systemic organs, reduced levels of the inflammatory cytokines IFN-γ and IL-6 and lower levels of the neutrophil chemoattractant KC. Hepatocyte-specific gp130 blockage also resulted in reduced infiltration of PMNs and lower degree of pathology. Consistent with the histopathological findings, biochemical markers of liver damage were also reduced in hepatocyte-specific gp130-deficient mice. These data together with the fact that eventually hepatocyte-specific gp130-deficient mice develop similar pathology to wild-type control mice and also succumbed to *S. pyogenes* infection, further demonstrated that the survival advantage conferred by blockage of gp130 in hepatocytes resulted from delay in the development of the liver pathology in this experimental mouse model.

The fact that blockage of the gp130 signalling enhanced bacterial clearance, decreased the expression of inflammatory cytokines, and reduced the infiltration of inflammatory cells in the liver clearly indicates that the mechanisms leading to improved survival in hepatocyte-specific gp130-deficient mice may be multifactorial. This is not surprising since gp130 is the common receptor subunit for several cytokines including IL-6, IL-11, ciliary neutrophic factor CNTF), cardiotrophin-1 (CT-1), cardiotrophin-like cytokine (CLC), leukaemia inhibitory factor (LIF), neuropoietin (NPN) and oncostatin (OSM) [11] and has recently been enlarged by the addition of the newly characterized IL-27 and IL-31 [12, 13].

However, IL-6 is of particular importance since this cytokine has a pleiotropic effect as an inflammatory but also as an anti-inflammatory mediator during sepsis. IL-6 has been shown to be up-regulated in septic humans and is correlated with a poor outcome [23-26]. In addition,

multiple reports have demonstrated that high levels of IL-6 in the early phases of sepsis predict early mortality in experimental animal models [27-29]. However, mice completely deficient in IL-6 production do not show improved survival in CLP-induced sepsis [30]. Other studies have demonstrated a dose-dependent beneficial effect of IL-6 blockage in experimental sepsis models [31, 32]. These studies imply that although high IL-6 levels may be detrimental during the sepsis process, some low levels of IL-6 may be necessary for an appropriate acute-phase response, underlining how important an appropriate control of IL-6 levels is for the outcome of sepsis.

Because protection in hepatocyte-specific gp130-deficient mice was associated with inhibition of KC production by hepatocytes, a chemokine that has been shown to be a differentially IL-6-gp130 regulated gene [20], it can be suggested that the harmful effect of high levels of IL6 may be due to the up-regulation of KC production and the concomitant development of liver pathology. Thus, disruption of gp130 receptor specifically in hepatocytes might limit the harmful effect of high levels of IL-6 during sepsis but will not result in a global blockage of the whole IL-6 responses.

We have also observed that ablation of the gp130 signalling resulted in enhanced bacterial clearance and decreased the expression of inflammatory cytokines. It is reasonable to assume that inhibition of gp130 signalling in hepatocytes might also affect the biological activity of the other gp130 family cytokines. In this regard, it has recently been shown that IL-27 is a key negative regulator of innate immune cell function during experimental septic peritonitis [33]. Thus, *in vivo* blockage of IL-27 function has been shown to increase survival of mice subjected to CLP and enhance bacterial clearance [33]. To date, nothing is known about the potential contribution of IL-27 to gram-positive sepsis and shock. This issue is currently under investigation in our group.

In summary, our data demonstrated a major role of gp130 receptor signalling in

modulating the inflammatory response, bacterial clearance, and organ damage in an experimental mouse model of gram-positive sepsis. The blockage of gp130 signalling in hepatocytes could represent a novel strategy and adjunctive therapy for patients suffering sepsis.

Words: 2611

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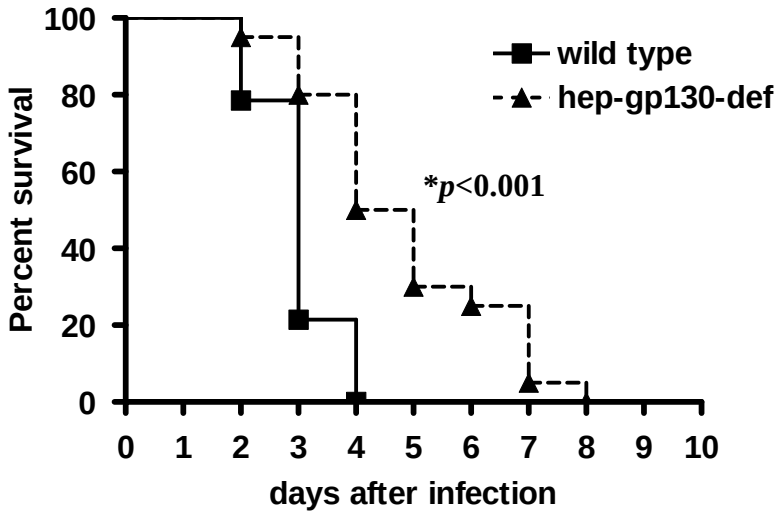
100
80
60
40
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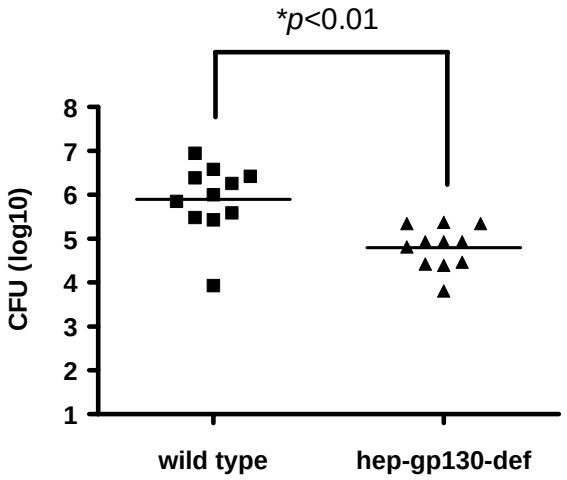
Percent survival
0 1 2 3 4 5 6 7 8 9 10
days after infection
wild type
hep-gp130-def
*p<0.001

Figure 1

A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CFU (log10)
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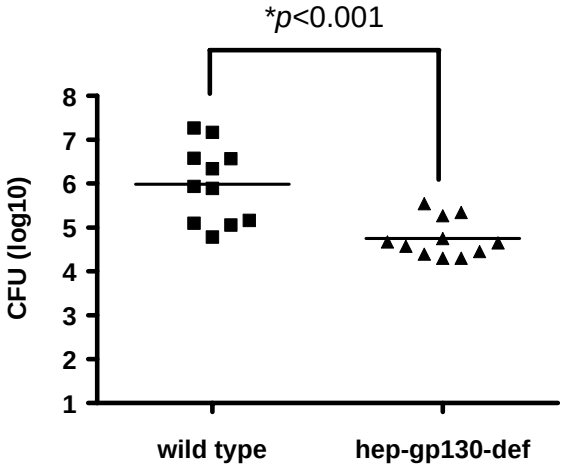
wild type
hep-gp130-def

B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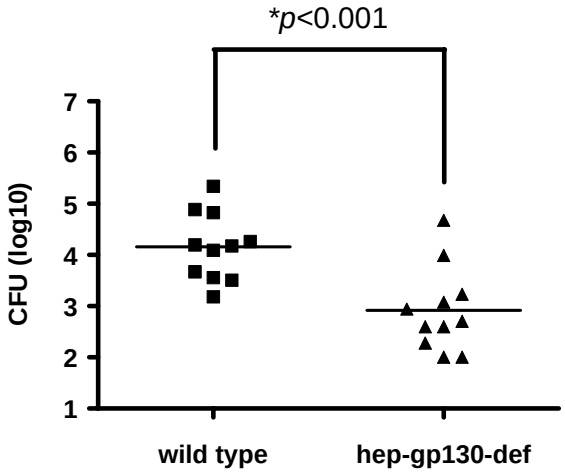
hep-gp130-def

C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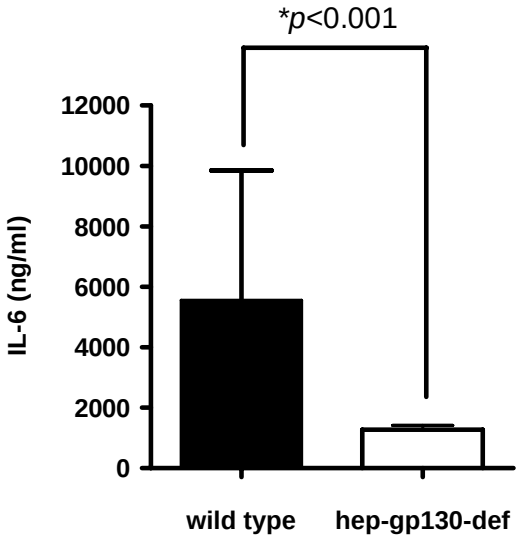
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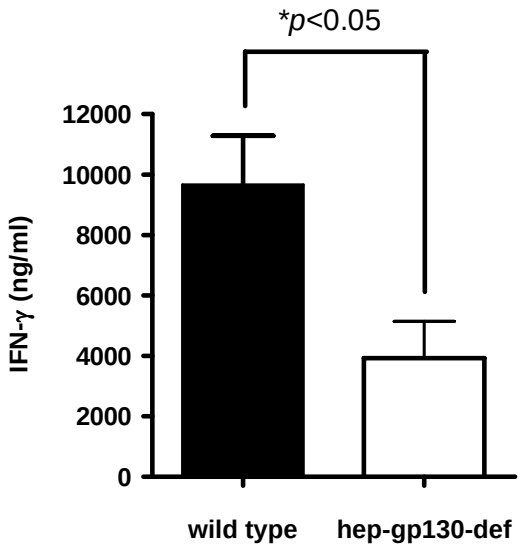
B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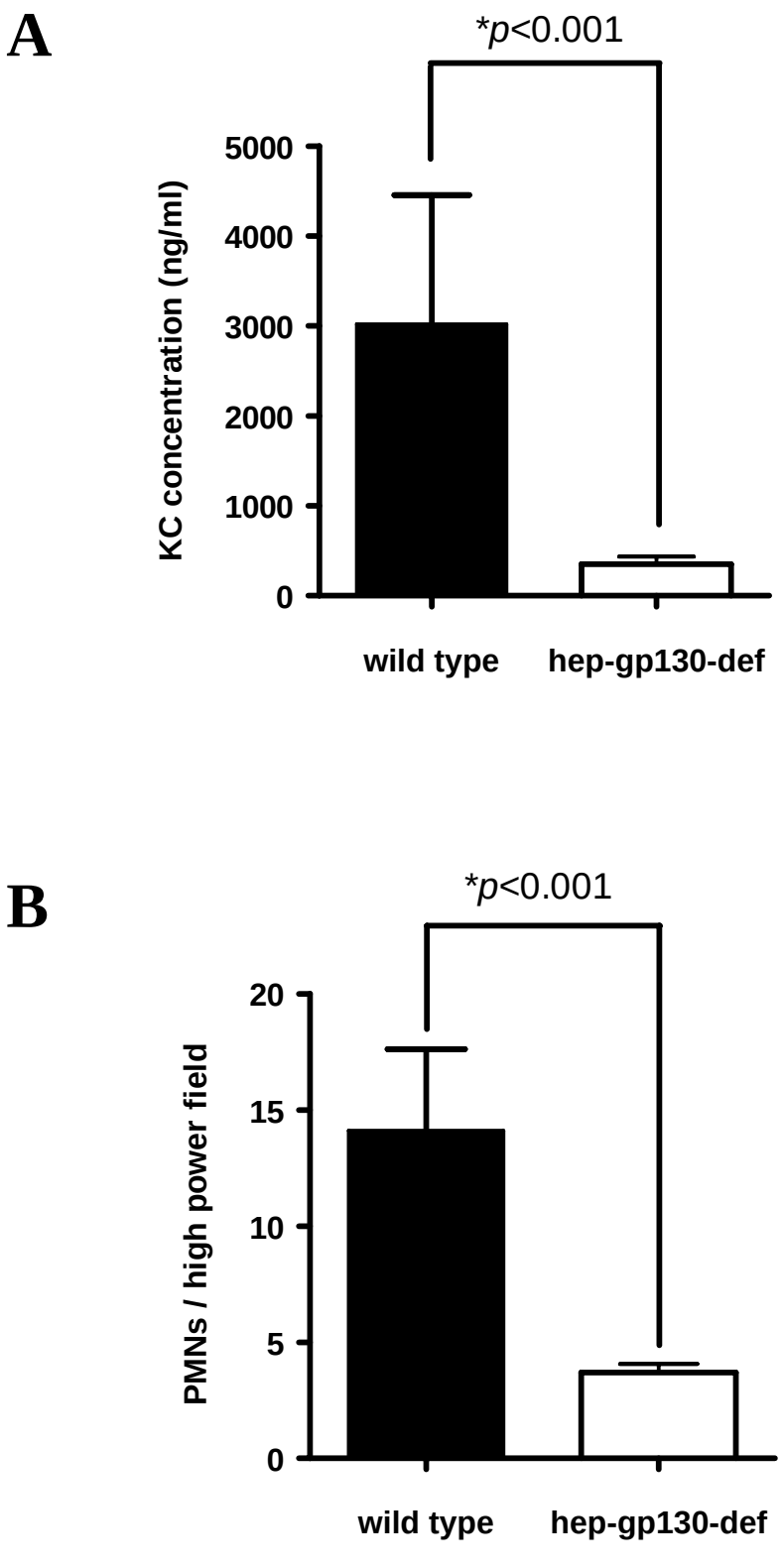
IFN-γ (ng/ml)
wild type
hep-gp130-def

Figure 3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*p<0.001
5000
4000
3000
2000
1000
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KC concentration (ng/ml)
wild type
hep-gp130-def
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*p<0.001
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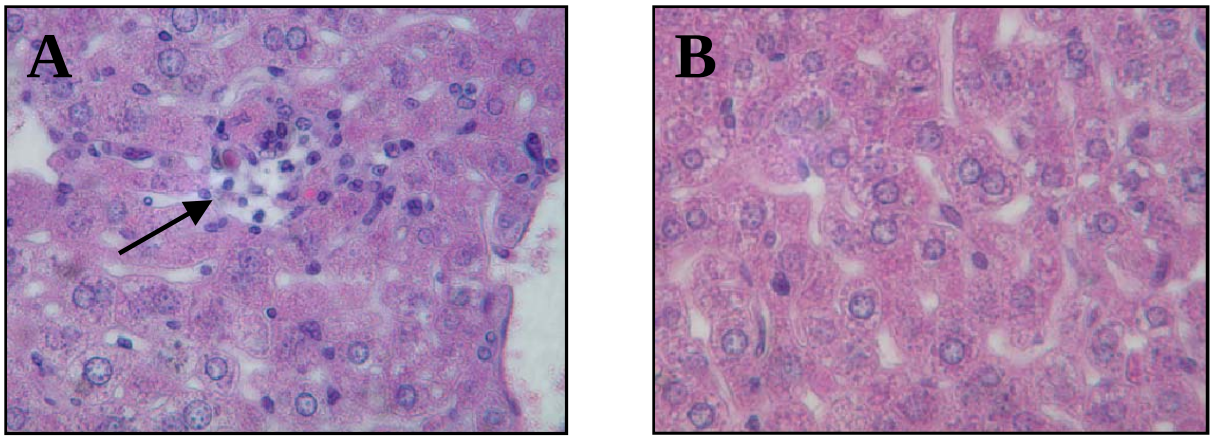
10
5
0
PMNs / high power field
wild type
hep-gp130-def

Figure 4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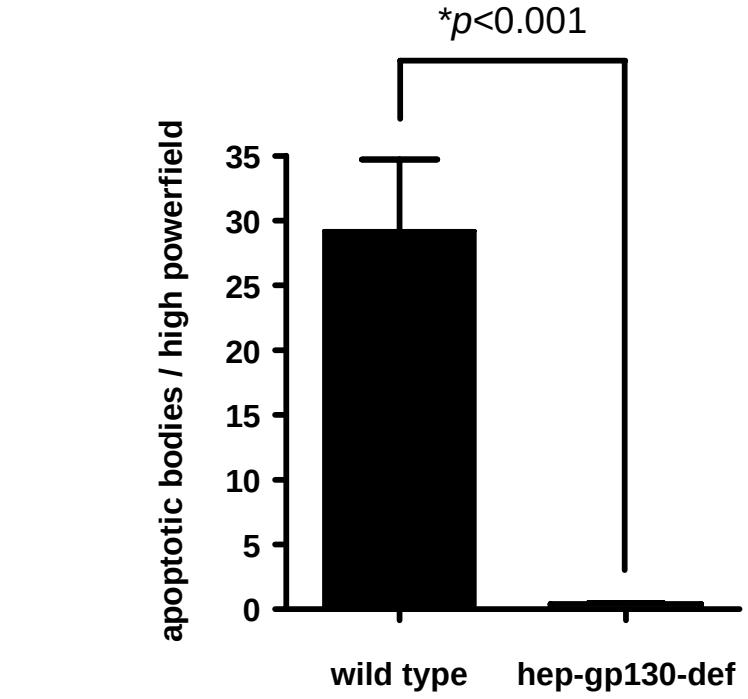
C
*p<0.001
apoptotic bodies / high powerfield
35
30
25
20
15
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5
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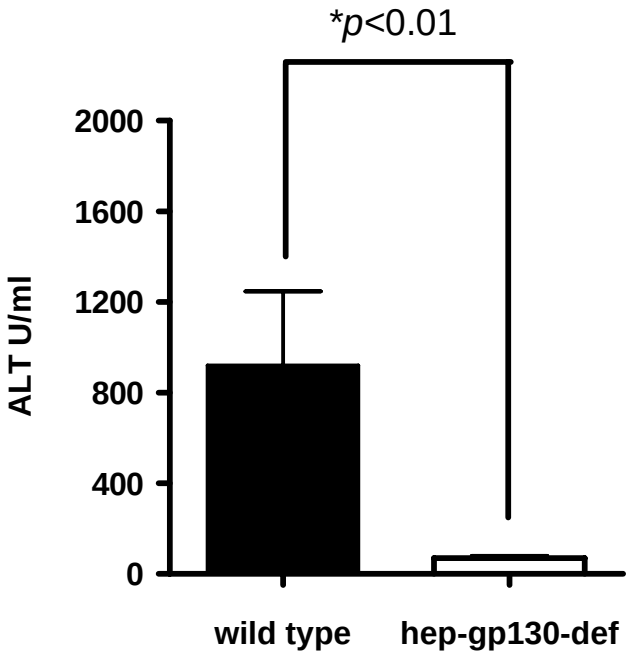
wild type
hep-gp130-def

Figure 5

A


*p<0.01
2000
1600
1200
800
400
0
ALT U/ml
wild type
hep-gp130-def

B

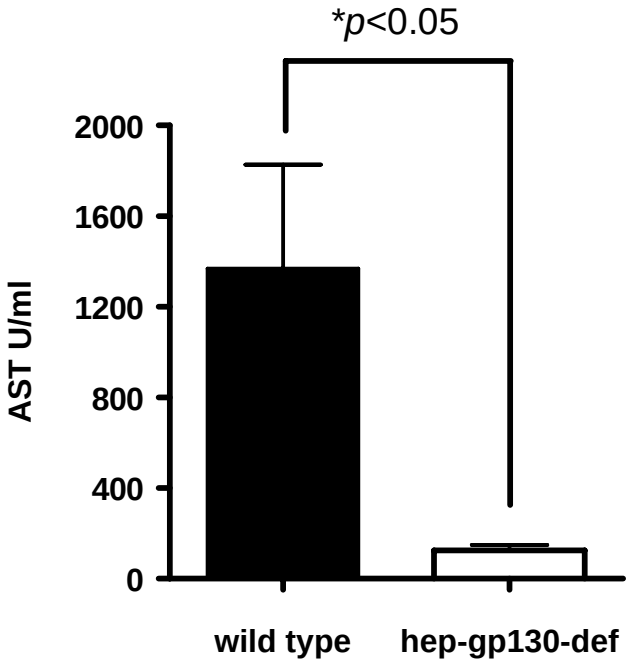

*p<0.05
2000
1600
1200
800
400
0
AST U/ml
wild type
hep-gp130-def

FIGURE LEGENDS

Figure 6

Fig. 1. Survival of hepatocyte-specific gp130-deficient mice (FVB/n alfpCre+ $gp130^{LoxP/LoxP}$, n=19) and wild type mice (FVB/n $gp130^{LoxP/LoxP}$, n=12) to *S. pyogenes*-induced sepsis. Mice were intravenously infected with 10^5 CFU of *S. pyogenes* in 0.2 ml of PBS via a lateral tail vein and survival was monitored over a period of 8 days. Survival curves represent the compilation data from 3 independent experiments.

Fig. 2. Bacterial loads in blood (A), livers (B), and spleen (C) of hepatocyte-specific gp130-deficient mice (■, n=11) and wild type mice (▲, n=11) at 48 h after intravenous inoculation with 10^5 CFU of *S. pyogenes*. The results were obtained in 3 independend experiments.

Fig. 3. Serum levels of IL-6 (A) and IFN-γ (B) in hepatocyte-specific gp130-deficient mice (n=22) and wild type mice (n=12) at 48 h after intravenous inoculation with 10^5 CFU of *S. pyogenes*. The levels of cytokines in serum were determined by ELISA. Each bars represent the mean value ± SD of infected samples over uninfected background levels.

Fig. 4. (A) Serum levels of cytokine-induced-neutrophile chemoattractant (KC) in hepatocyte-specific gp130-deficient (white bars) and wild type (black bars) mice at 48 h after intravenous inoculation with 10^5 CFU of *S. pyogenes*. The levels of KC in serum was determined by ELISA. Each bars represent the mean value ± SD of infected samples over uninfected background levels. (B) Quantification of PMN infiltrate into the livers of gp130-deficient (white bars) and control (black bars) mice at at 48 h after intravenous inoculation with 10^5 CFU of *S. pyogenes*. Liver

tissue samples obtained from infected were fixed, embedded in paraffin and microscopically analysed. Bars represent the number of PMN per high power field ± SD.

Fig. 5. Histopathological examination of hematoxylin/eosin-stained liver sections of wild type (A) and hepatocyte-specific gp130-deficient (B) mice at 48 h after intravenous inoculation with 10^5 CFU of *S. pyogenes*. Apoptotic bodies are indicated by the arrow. (C) Quantification of apoptotic bodies in liver tissue of control (black bars) and gp130-deficient (white bars) mice. Each bar represent the mean ± SD of apoptotic bodies per high powerfield.

Fig. 6. Levels of ALT (A) and AST (B) in hepatocyte-specific gp130-deficient (white bars) and wild type (black bars) mice at 48 h after intravenous inoculation with 10^5 CFU of *S. pyogenes*. Each bar represent the mean ± SD of 10 mice per group.