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Host microbiota dictates the proinflammatory impact of LPS in the murine liver

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ABSTRACT

Gut microbiota can impact liver disease development *via* the gut-liver axis. Liver inflammation is a shared pathological event in various liver diseases and gut microbiota might influence this pathological process. In this study, we studied the influence of gut microbiota on the inflammatory response of the liver to lipopolysaccharide (LPS). The inflammatory response to LPS (1–10 μg/ml) of livers of specific-pathogen-free (SPF) or germ-free (GF) mice was evaluated *ex vivo*, using precision-cut liver slices (PCLS). LPS induced a more pronounced inflammatory response in GF PCLS than in SPF PCLS. Baseline TNF-α gene expression was significantly higher in GF slices as compared to SPF slices. LPS treatment induced TNF-α, IL-1β, IL-6 and iNOS expression in both SPF and GF PCLS, but the increase was more intense in GF slices. The anti-inflammatory markers *SOCS3 and IRAK-M gene expression was significantly higher in GF PCLS than SPF PCLS at 24h with 1 μg/ml LPS treatment, and IL-10 was not differently expressed in GF PCLS than SPF PCLS*. In addition, TLR-4 mRNA, but not protein, at basal level was higher in GF slices than in SPF slices. Taken together, this study shows that, in mice, the host microbiota attenuates the pro-inflammatory impact of LPS in the liver, indicating a positive role of the gut microbiota on the immune homeostasis of the liver.

1. Introduction

Inflammation of the liver, can be caused by alcohol abuse, viral infections and the metabolic syndrome, and the gut-liver axis is widely implicated in disease progression (Seki and Schwabe, 2015). Blood from the gut can reach the liver *via* the hepatic portal vein carrying with it microbiota-derived exogenous molecules (for example, lipopolysaccharide and lipoteichoic acid), also known as pathogen-associated molecular patterns (PAMPs) (Chassaing et al., 2014). PAMPs are recognized by pattern recognition receptors (PRRs), which are responsible for sensing invading pathogens and orchestrating the innate immune response (Arrese et al., 2016; Takeuchi and Akira, 2010).

Toll like receptors (TLRs) are members of the PRR family. Until now, 10 have been identified in human and 13 in mouse (West et al., 2006). Among these TLRs, TLR-4 has attracted particular interest in terms of hepatic inflammation and fibrogenesis due to its ligand, LPS, which is involved in the development of various liver diseases (Su, 2002). TLR-4 is expressed in almost every type of liver cell, including hepatocytes, Kupffer cells, hepatic stellate cells, biliary epithelial cells and sinusoidal endothelial cells (Kesar and Odin, 2014).

Both in healthy and pathological conditions, the liver is involved in the detoxification of LPS (Jirillo et al., 2002). In the healthy state, LPS from gut microbiota penetrates the intestinal wall only in trace amounts and is then cleared by Kupffer cells and hepatocytes, without inducing significant liver inflammation (Szabo and Bala, 2010). In both alcoholic and non-alcoholic liver disease patients, gut permeability is often increased (Miele et al., 2009; Parlesak et al., 2000), thus accelerating bacterial translocation to the liver (Parlesak et al., 2000). This facilitates an increased hepatic LPS translocation, which may act as a second hit promoting disease progression. Binding of LPS to TLR-4 stimulates the production of cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β). These cytokines have been suggested to

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drive the pathogenesis of alcoholic liver disease, nonalcoholic steatohepatitis and nonalcoholic fatty liver disease (Bugianesi et al., 2005; Miura et al., 2010; Tilg et al., 2016).

Germ free (GF) rodent is a popular model to study the gut-liver axis. In GF mice, the liver has limited history of LPS exposure, while in colonized (specific-pathogen-free, SPF) mice the liver is subjected to LPS (Caesar et al., 2012). The aim of this study is to investigate the influence of the gut microbiota on the inflammatory response of the liver to LPS. We hypothesize that the liver of SPF mice will respond less severe to LPS than the liver of GF mice. As a model, we used precision-cut liver slices (PCLS). Previously, we have shown that PCLS can be used as a multicellular model to study LPS-induced inflammation of the liver since hepatocytes, Kupffer cells and other (non)-parenchymal cells are still present in their original tissue environment (Olinga et al., 2001). This makes PCLS a unique model to study the innate immunity in the liver. To investigate the role of gut microbiota in facilitating tolerance of the liver to LPS, we compared the effect of LPS in PCLS from SPF and GF mice. These experiments will provide additional insight into the interaction between the liver and gut microbiota.

2. Materials and methods

2.1. Animals

Use of murine tissue for the preparation of PCLS was approved by the Animal Ethical Committee of University of Groningen (DEC 6416AA-001). Germ free C57BL/6 mice were housed in isolators at the Central Animal Facility of the University Medical Center Groningen and provided with sterile rodent chow diet and water *ad libitum*. Specific-pathogen-free C57BL/6 mice were purchased from Harlan (Zeist, The Netherlands) and were provided standard rodent chow diet and water. All mice were allowed to acclimatize at least 1 week prior to the experiments. Mice were sacrificed under 2% isofluorane/O_2 (Nicholas Piramal, London, UK) anesthesia, at the age of 8–10 weeks. Livers of the mice were resected immediately after sacrificing and stored in ice-cold University of Wisconsin (UW) organ preservation solution (DuPont Critical care, Waukegab, IL, USA.).

2.2. Preparation of mouse liver slices

Cylindrical cores of liver tissue were obtained using a 6 mm diameter biopsy punch and preserved in ice-cold UW solution. Precision-cut liver slices (PCLS) were prepared in Krebs-Henseleit buffer supplemented with 25 mM D-glucose (Merck, Darmstadt, Germany), 25 mM $NaHCO_3$ (Merck) and 10 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (MP Biomedicals, Ohio, USA), oxygenated with 95% O_2 and 5% CO_2 using a Krumdieck tissue slicer as previously described (de Graaf et al., 2010). Liver slices had a wet weight of 4–5 mg, with a thickness of approximately 250 μm.

2.3. Incubation of mouse liver slices

After slicing, PCLS were incubated individually in 12 well plates containing William's E medium with GlutaMAX (Life Technologies, Carlsbad, USA) supplemented with 2.75 g/ml D-glucose monohydrate (Merck, Darmstadt, Germany) and 50 μg/ml gentamicin (Invitrogen, Paislely, UK). PCLS were incubated for 1 h to restore viability and ATP content. To assess the full phenomena of LPS stimulation, slices were incubated for 48 h with 0, 1 or 10 μg/ml ultrapure LPS from *Escherichia coli* O111:B4 (InvivoGen, Toulouse, France) (Olinga et al., 2001). Medium was refreshed every 24 h. The plates were incubated in a shaking incubator (90 cycles/min) with continuous 5% CO_2 and 80% O_2 supply.

2.4. Viability of mouse liver slices

For ATP analysis, slices were kept in 1 ml sonification solution (70% (vol/vol) ethanol (VWR, Paris, France), 2 mM EDTA (Merck), pH 10.9) (de Graaf et al., 2010), snap-frozen, and stored at −80 °C. The samples were homogenized using a Mini-Beadbeater (BioSpec Products, Bartlesville, USA) and centrifuged. Clear supernatant was used for ATP analysis and the remaining pellet for protein determination. ATP content of each slice was determined using the ATP bioluminescence assay kit class II (Roche Diagnostics GmbH, Mannheim, Germany) as previously described (de Graaf et al., 2010). Protein content of each slice was determined using the DC™ Protein Assay (Biorad, Veenendaal, the Netherlands) according to the manufacturer's instruction.

2.5. Quantitative real-time polymerase chain reaction

Total RNA, from pooled (n = 3) slices, was extracted using the FavorPrep tissue total RNA mini kit (FAVORGEN Biotech Corp, Vienna, Austria) according to the manufacturer's instructions and stored at −80 °C. RNA concentration was determined using the Synergy HT (Biotek, Swindon, UK) at a wavelength of 260/280. Total RNA (1 μg) was transcribed into cDNA using the Reverse Transcription Kit (Promega, Leiden, the Netherlands) following the manufacturer's instructions and stored at −20 °C. Gene expression was determined by either the SYBR Green or Taqman method (Roche Diagnostics GmbH, Mannheim, Germany) using gene specific primers (Supplementary Table I). Expression of each gene was normalized using the reference gene GAPDH (ΔCt) and expressed as percentage ($(2^{-\Delta Ct})$ *100).

2.6. ELISA and NOx colorimetric assay

Culture medium from 3 PCLS was pooled together after 0-24 h and 24-48 h incubation and stored at −20 °C. Concentrations of TNF-α and IL-1β were measured using the DuoSet® ELISA Development Systems (R &D Systems, Abingdon, UK) according to the manufacturer's protocol. Nitrate/nitrite (NO_x) was determined by a colorimetric assay according to Moshage et al. (Moshage et al., 1995).

2.7. Western blotting

TLR-4 protein expression was determined by immunoblotting. PCLS (n = 3) were lysed in RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific, Waltham, USA). Membranes were incubated with a TLR-4 antibody at 4 °C overnight, followed by incubation with a secondary antibody at room temperature for 1 h (Supplementary Table II for details for the antibodies). The protein signal was visualized with VisiGlo™ Prime HRP Chemiluminescent Substrate Kit (Amresco, Ohio, USA) and quantified with Image Lab software (Biorad, Veenendaal, the Netherlands).

2.8. Morphology

PCLS were incubated with or without LPS for 48 h. Slices, processed directly after slicing for morphological analysis, PCLS were fixed in 4% formaldehyde overnight and stored at 4 °C in 70% ethanol. Fixed slices were dehydrated, embedded in paraffin, sectioned (4 μm) and stained with hematoxylin and eosin as previously described (Hadi et al., 2013).

2.9. Statistics

Results are expressed as mean ± standard error of the mean (SEM). Student's *t*-test or ANOVA followed by Fisher's LSD multiple comparisons test were performed using Graphpad Prism 6.0 (La Jolla, CA, USA). A p-value of < 0.05 was considered significant when comparing differences between groups.

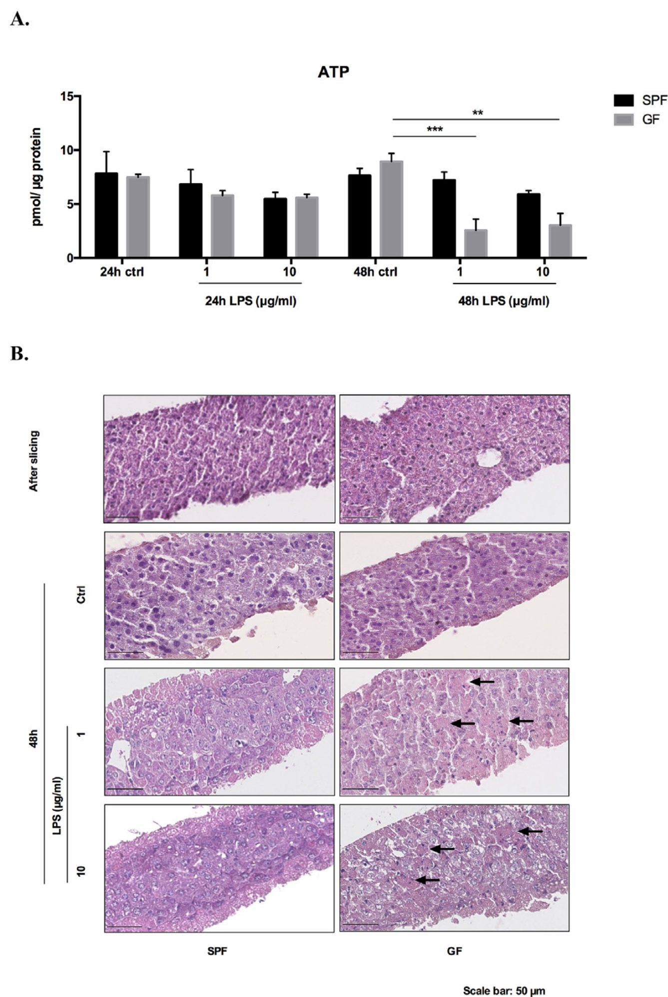

Fig. 1. Viability and morphology of PCLS. (A) Viability of PCLS was determined by ATP/protein (pmol/ µg) content. Data are shown as mean ± SEM; three PCLS were used for each group in both GF mice (n = 3–5) and SPF mice (n = 3–5). ** $p < 0.01$ and *** $p < 0.001$. (B) H&E staining of PCLS after 48 h incubation with or without LPS; arrows: necrotic area. GF mice (n = 3) and SPF mice (n = 3), scale bar = 50 µm.

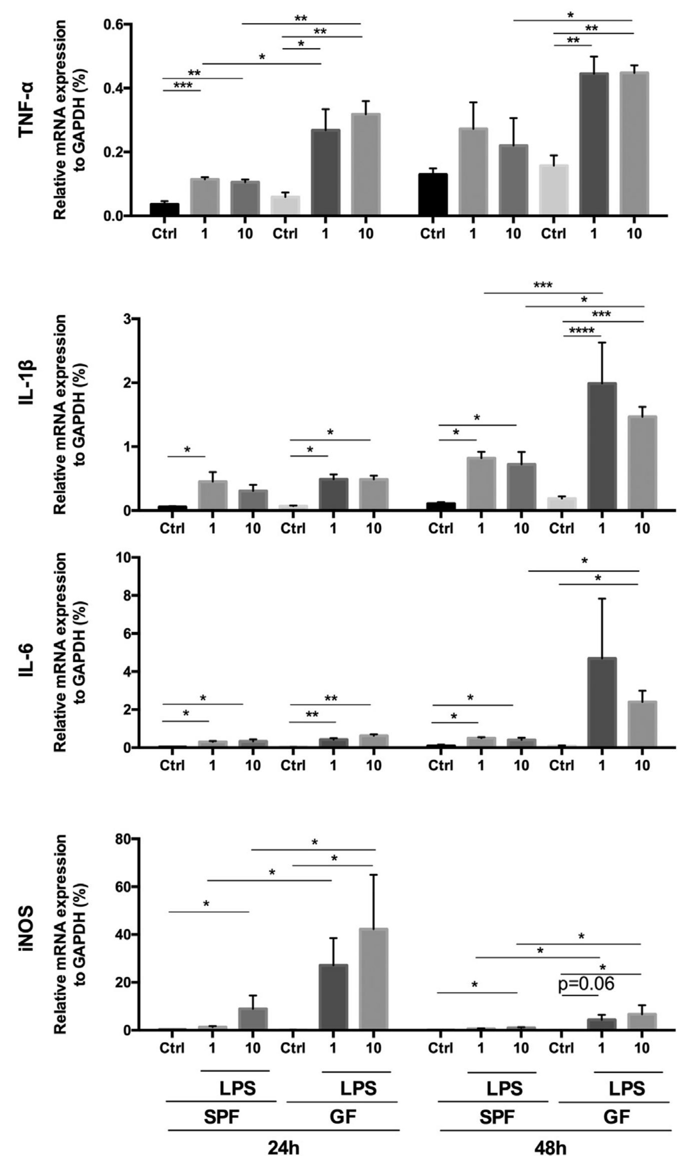

Fig. 2. The effect of LPS on mRNA levels of TNF-α, IL-1β, IL-6 and iNOS in PCLS from GF and SPF mice. mRNA levels of the above-mentioned genes were measured with qRT-PCR. Data are shown as mean ± SEM; three PCLS for each condition were pooled for RNA isolation. After slicing: GF (n = 6), SPF (n = 6); 24 h and 48 h: GF (n = 3), SPF (n = 3). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$.

3. Results

3.1. Viability of liver slices from GF and SPF mice

The ATP content of PCLS, as a measure of viability, was determined after culturing. As shown, slices remained viable for 48 h (Fig. 1A&B). Exposure of SPF liver to LPS for 48 h slightly lowered the ATP content (Fig. 1A) and caused the appearance of apoptotic cells (Fig. 1B). In contrast, treatment of GF liver slices with LPS for 48 h markedly reduced ATP levels (Fig. 1A) and resulted in the presence of necrotic areas (Fig. 1B). These data show that PCLS from SPF mice are more tolerant to LPS challenge than those from GF mice.

3.2. Expression of cytokines in GF and SPF mouse liver slices upon LPS challenge

To evaluate the inflammatory response of the liver after LPS challenge, gene expression of TNF-α, IL-1β, IL-6 and iNOS was determined (Fig. 2). Prior to culturing, TNF-α gene expression was 1.75-fold higher in GF compared to SPF PCLS; whereas the expression of IL-1β, IL-6 and iNOS did not differ (Supplementary Fig. II). During incubation without LPS for 48 h, an inflammatory response was induced in PCLS, as illustrated by an increase in the gene expression of TNF-α, IL-1β, IL-6 and iNOS in both GF and SPF PCLS (Supplementary Fig. II).

Upon LPS treatment, TNF-α gene expression increased in both GF and SPF mouse PCLS up to 24 h; this phenomenon was much stronger in GF PCLS than SPF PCLS. At 48 h, LPS did not significantly increase TNF-α gene expression in SPF PCLS, while in GF PCLS, LPS did induce TNF-α gene expression (Fig. 2). iNOS expression was higher in GF PCLS than SPF PCLS at 24 h and 48 h, while the expression of IL-1β and IL-6 was higher in GF PCLS than SPF PCLS at 48 h.

After 24 h of incubation of both SPF and GF PCLS, TNF-α and IL-1β cytokine release was markedly elevated in LPS challenged groups (Fig. 3). LPS evoked bigger extent of TNF-α secretion in the GF than SPF PCLS, but the IL-1β response was similar in both groups. Between 24 and 48 h, we did not detect any increase in cytokine release following LPS challenge in both GF and SPF slices (Fig. 4). LPS significantly increased NO_x production in GF PCLS both after 0–24 and 24-48 h incubation, while in SPF slices we observed a small but non-significant increase. Taken together, these data indicate that LPS treatment evokes

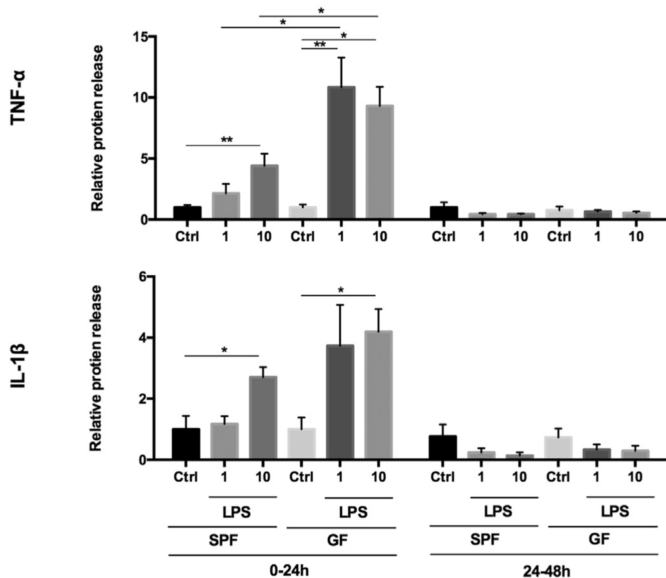

Fig. 3. The effect of LPS on protein release of TNF-α and IL-1β of PCLS from GF and SPF mice. Protein levels of TNF-α and IL-1β were measured in the culture medium from 0 to 24 h and from 24 to 48 h using ELISA. Cytokine release from LPS treated groups are expressed as relative value to the control group of GF or SPF mice after 24 h incubation. Data are shown as mean ± SEM; culture medium from three PCLS was pooled to represent each condition in GF mice (n = 3), SPF mice (n = 4–5). *p < 0 .05, **p < 0 .01.

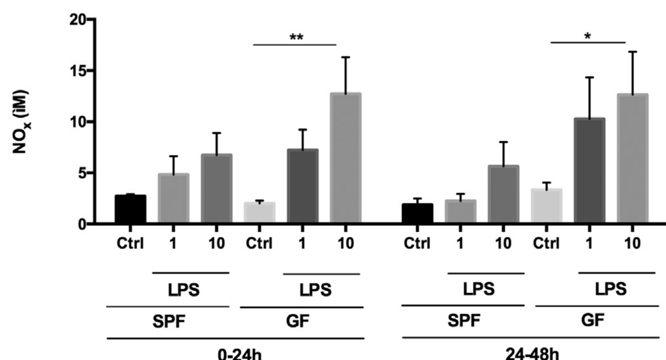

Fig. 4. The effect of LPS on nitrite/nitrate (NO_x) production of PCLS from GF and SPF mice. Nitrite/nitrate (NO_x) content in the culture medium from 0 to 24 h and from 24 to 48 h was determined using the NO_x colorimetric assay. Data are shown as mean ± SEM; culture medium from three PCLS was pooled to represent each condition in GF mice (n = 5), SPF mice (n = 9). *p < 0 .05, ** p < 0 .01.

a stronger inflammatory response in GF liver slices than in SPF liver slices.

3.3. LPS receptor TLR-4 mRNA but not protein expression was lower in SPF mice

To elucidate why SPF PCLS are more tolerant to LPS challenge, we examined the expression of the LPS receptor TLR-4. At baseline, TLR-4 gene expression in SPF PCLS was significantly lower as compared to GF PCLS (Fig. 5), but this difference disappeared after 24 h of culturing. In SPF mice, both 1 and 10 μg/ml LPS reduced the expression of TLR-4 at 24 h, but not in GF mice. At 48 h, LPS did not impact TLR-4 mRNA levels. The baseline expression of TLR-4 protein was not different between SPF *versus* GF slices, and also not different during incubation with or without LPS challenge (Fig. 6). Taken together, the divergent responses to LPS cannot be explained by differences in TLR-4 mRNA expression.

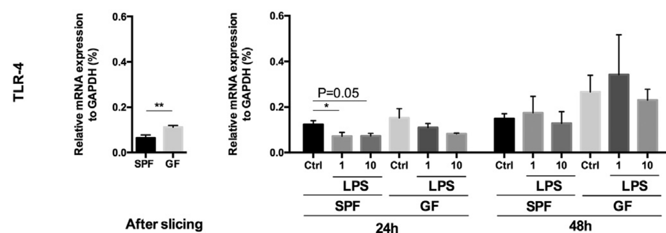

Fig. 5. The effect of LPS on mRNA levels of TLR-4 in PCLS from GF and SPF mice. mRNA level of TLR-4 was measured with qRT-PCR. Data are shown as mean ± SEM; three PCLS for each condition were pooled for RNA isolation. After slicing: GF (n = 6), SPF (n = 6); 24 h and 48 h: GF (n = 3), SPF (n = 3). *p < 0 .05, **p < 0 .01.

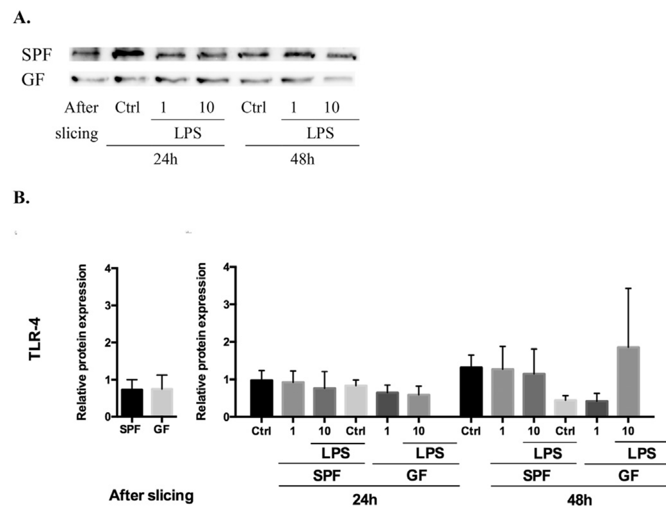

Fig. 6. The effect of LPS on protein level of TLR-4 in PCLS from GF and SPF mice. TLR-4 protein in PCLS from GF or SPF mice was measured by Western blotting. (A) Representative Western blots of TLR-4 expression at baseline and during incubation. (B) Average protein expression normalized to total protein loaded (Supplementary fig. I.). Data are shown as mean ± SEM; three PCLS for each condition were pooled for protein isolation. After slicing: GF (n = 6), SPF (n = 6); 24 h and 48 h: GF (n = 3), SPF (n = 3). *p < 0 .05.

3.4. Anti-inflammatory status in GF and SPF mouse liver slices upon LPS challenge

The expression of the anti-inflammatory mediators IL-10, IRAK-M and SOCS3 was determined by qRT-PCR to elucidate whether these factors cause the different responses to LPS observed in SPF and GF PCLS (Fig. 7). IL-10, IRAK-M and SOCS3 were not differently expressed at baseline in GF *versus* SPF PCLS. During incubation, there was upregulation of SOCS3 and IRAK-M in SPF PCLS, IRAK-M in GF mice, no significant change in the expression of IL-10 in both GF and SPF PCLS (Supplementary fig. III). LPS increased the expression of IL-10, IRAK-M and SOCS3 both in GF and SPF PCLS. For IL-10, this increase was much higher after 48 h than after 24 h, whereas for SOCS3 and IRAK-M, the opposite was true. Nevertheless, SOCS3 and IRAK-M gene expression was higher in GF PCLS than SPF PCLS at 24 h with 1 μg/ml LPS treatment, and IL-10 was not differently expressed in GF PCLS than SPF PCLS at. Surprisingly, SPF PCLS did not express more anti-inflammatory mediators than GF PCLS in response to LPS stimuli.

4. Discussion

Liver inflammation is an underlying pathology in various liver diseases. Interaction between the liver and gut microbiota (the gut-liver axis) is an emerging but not fully understood topic. In this study, the relationship between liver inflammation and gut microbiota was investigated using an *ex vivo* model of liver inflammation in GF and SPF

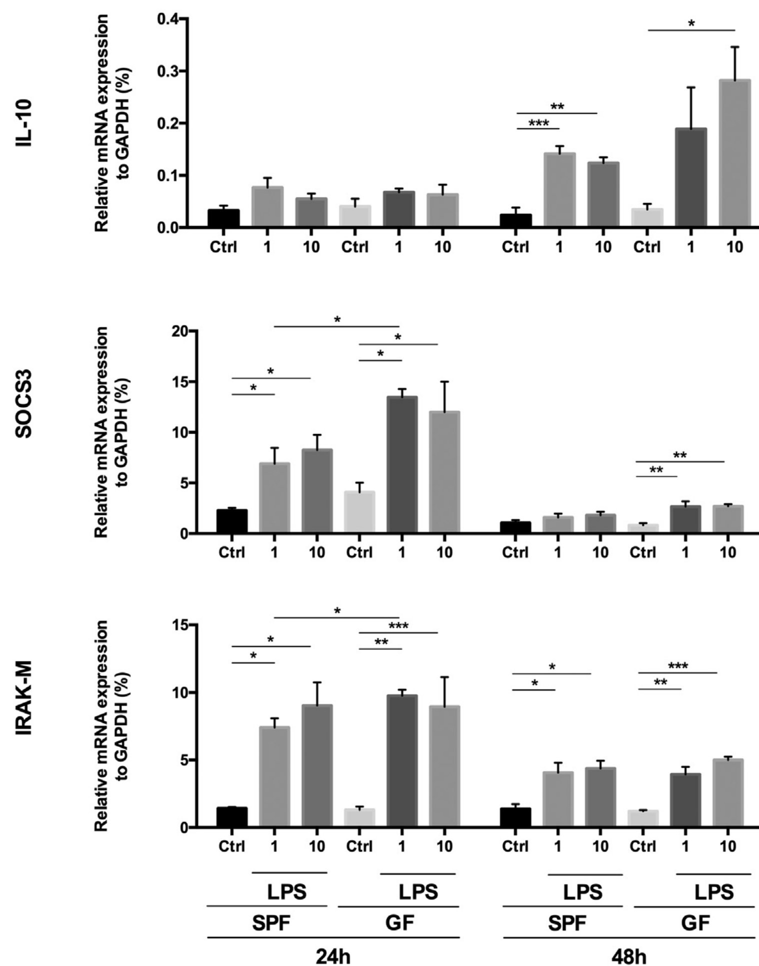

Fig. 7. The effect of LPS on mRNA levels of IL-10, SOCS3, IRAK-M in PCLS from GF and SPF mice. Expression of the above-mentioned genes was measured with qRT-PCR. Data are shown as mean ± SEM; three PCLS for each condition were pooled for RNA isolation. After slicing: GF (n = 6), SPF (n = 6); 24 h and 48 h: GF (n = 3), SPF (n = 3). *p < 0 .05.

mice. The data revealed that the presence of host microbiota attenuates the proinflammatory impact of LPS in the liver, by decreasing proinflammatory responses and improving cell survival even in the absence of circulating immune cells.

Our results demonstrated that PCLS from SPF mice are less vulnerable to an LPS challenge than PCLS from GF mice (Fig. 1). Previously, it has been shown that LPS leads to moderate tissue damage in SPF rat liver slices (Olinga et al., 2001), proposed to be mediated exclusively by TNF-α (Nowak et al., 2000). Since LPS evoked a more robust TNF-α in GF slices compared to SPF slices (Fig. 3), this might explain the worse survival of GF PCLS after LPS treatment.

The LPS-induced inflammatory response was more pronounced in GF PCLS as compared to SPF PCLS (Fig. 2-4). In SPF mice, LPS elevated TNF-α and IL-1β cytokine release during the first 24 h, however the slices did not produce any cytokines in the subsequent period, even though gene levels remained elevated. A similar observation was made in GF slices; however, it cannot be excluded that the reduction in cytokine production is due to changes in viability. The lack of TNF- α protein production between 24 and 48 h indicates a transient expression (Chanput et al., 2010), and this observation may be explained *via* the mechanism of endotoxin tolerance. Pena et al. found that restimulation of human mononuclear cells by LPS 24 h after the initial stimulation did not increase TNF-α gene expression and cytokine release (Pena et al., 2011). Similarly, Sun et al. showed in a human monocyte cell line (THP-1 cells) that restimulation with LPS did not enhance TNF-α and IL-1β cytokine production (Sun et al., 2014).

TLR-4 is tightly regulated to avoid uncontrolled inflammation and extensive tissue damage. In this study, LPS had the tendency to lower the gene expression of TLR-4 in both GF and SPF liver slices (Fig. 5). This is in agreement with Poltorak et al.'s finding that LPS strongly and transiently suppressed TLR-4 mRNA expression (Poltorak et al., 1998). Recent *in vivo* lung and *in vitro* macrophage studies argued that LPS shortens the half-life of TLR-4 mRNA (Fan et al., 2002). Decreased TLR-4 expression was reported to be associated with a tolerance towards LPS in neutrophils (Parker et al., 2005). Takahashi et al. showed that commensal microbiota are essential for epigenetic repression (*via* high methylation of the promoter) of TLR-4 mRNA expression in large intestinal epithelial cells, which was associated with a reduced inflammatory response of the intestine to LPS challenge (Takahashi et al., 2011). They also suggested that the responsiveness of intestinal epithelial cells to LPS is mainly regulated at the transcriptional level for TLR-4, although there might be additional post-transcriptional regulation present (Takahashi et al., 2009). The present study showed that TLR-4 mRNA expression was higher in GF than SPF mice, which might correlate with the observed responsiveness to LPS. However, protein expression of TLR-4 was not different between SPF and GF PCLS at baseline and during incubation, thus TLR-4 expression cannot fully explain the hyper-responsiveness of GF to LPS.

Divergent response to LPS was seen in GF and SPF slices. We hypothesized that SPF mice livers produce more anti-inflammatory mediators than GF upon LPS stimuli, so that the inflammation in the SPF mice is more negatively regulated than GF. IL-10 is an anti-inflammatory cytokine that regulates LPS tolerance (Iyer and Cheng, 2012). Interleukin-1 receptor-associated kinase-M (IRAK-M) is a serine/

threonine kinase that negatively regulates TLR signaling (Kobayashi et al., 2002). Suppressor of cytokine signaling-3 (SOCS3) negatively regulates cytokine signaling through blocking Janus kinases (JAK) activity (Kubo et al., 2003). Previously it has been shown that higher expression of IL-10, SOCS3 and IRAK-M is associated with LPS tolerance (Biswas and Lopez-Collazo, 2009). Thus, IL-10, IRAK-M and SOCS3 can be used as anti-inflammatory markers that regulate LPS responsiveness. *In vivo* IL-10 expression is upregulated following LPS administration, and functions to prevent excessive inflammation and protect against lethal amount of LPS stimulus (Berg et al., 1995; Berlato et al., 2002; Iyer et al., 2010). This process is potentially mediated by SOCS3 (Berlato et al., 2002; Biswas and Lopez-Collazo, 2009). IRAK- M is a negative regulator of the downstream signaling of TLR-4 after LPS stimuli. IRAK- M expression is increased by LPS and in LPS tolerant status (van 't Veer et al., 2007). Although these markers (IL-10, IRAK-M and SOCS3) are generally suggested to be negative regulators of the TLR pathway, the regulation of these genes and their induction kinetics are not completely understood. TNF-α upregulates SOCS3 and IRAK-M expression in macrophages in vitro (Bode et al., 1999; van 't Veer et al., 2007). The expression of SOCS3 is parallel to the proinflammatory cytokines (IL-1β, TNF-a and IL-6) in periodontal LPS model in vivo (Chaves de Souza et al., 2013). Kiyono et al. demonstrated *in vivo* that LPS administration in GF mice induces a higher and prolonged anti-LPS hemagglutinin titer than in conventional mice (Kiyono et al., 1980); and suggested that GF mice might lack a population of T lymphocytes that suppress the LPS response. SOCS3 and IRAK-M are expressed on T lymphocytes and regulates innate immunity (Yu et al., 2013; Zhang et al., 2017). In the PCLS-LPS model, upregulation of SOCS3 and IRAK-M did not show a clear parallel pattern with TNF-α, which might due to a lack of circulating T lymphocytes or other immune cells in the PCLS. Based on the presented data we found no indication that these anti-inflammatory mediators are responsible for the different LPS response between GF and SPF PCLS. Already in 1980, J.R. McGhee et al found that *in vitro* stimulation of splenocytes of GF mice gave 2- to 3-fold greater mitogenic stimulation than those observed in conventional spleen cells, in addition, lower responses to LPS were observed after conventionalization of GF mice with indigenous BALB/c flora, and suggested that these phenomena are associated with T cell population that regulates B cell responses to LPS (McGhee et al., 1980). Mitsuyama et al. suggested that the microbiota may play a role in regulating macrophage functionality (Mitsuyama et al., 1986); impaired or reduced phagocytosis of peritoneal macrophages was reported under stimulation of LPS *in vitro* or *in vivo* (Morland et al., 1979). Thus, macrophages in GF PCLS may not be fully developed and therefore cannot execute the complex tasks needed to control the response to LPS.

In a later study *in vivo*, injection of LPS induced less serum levels of TNF-α, IL-1β, IL-6 and greater corticosterone levels in GF compared to conventional mice. They speculated that high levels of serum corticosterone in GF might responsible for the lower responsiveness of inflammatory cytokines (Ikeda et al., 1999). A more recent study in periodontal tissue showed that local administration of LPS induced higher TNF-α expression in SPF mice and suggested that GF mice may be populated with decreased numbers of dendritic cells and macrophages that are the major cells producing cytokines and chemokines (Fukuhara et al., 2018). In contrast with these two *in vivo* observations, in the current study, the pro-inflammatory response of GF PCLS is greater than SPF, which might due to the lack of circulating immune cells or hormones which can influence immune reaction in PCLS compared to the *in vivo* situation. To our knowledge, no study previously compared the liver inflammation between SPF and GF mice upon LPS stimuli.

It is well-known that the gut microbiota influences host development and physiology, although it is unclear which signaling pathways are involved (Sommer and Backhed, 2013). The negative impact of gut microbiota on the development of different liver diseases is an emerging topic (Federico et al., 2016; Seki et al., 2007; Seki and Schnabl, 2012). However, it has also been described that the absence of gut microbiota contributes to liver pathology (Chen et al., 2015; Mazagova et al., 2015; Tabibian et al., 2016). The microbiota can be a double-edged sword. To illustrate, germ-free mice are resistant to diet-induced obesity (Backhed et al., 2007), but they are also more susceptible to chemical-induced liver fibrosis (Mazagova et al., 2015), alcohol-induced liver injury (Chen et al., 2015) and biliary injury (Tabibian et al., 2016), suggesting that the microbiota elicits hepatoprotective effects of microbiota. In accordance with these observations, we have shown in this study that LPS evokes a stronger pro-inflammatory response in GF PCLS than in SPF PCLS. The inflammatory response in GF PCLS was accompanied by a loss of viability, while SPF slices were less prone to LPS-induced damage, which may indicate that SPF PCLS develop tolerance against LPS when compared to GF PCLS. Since PCLS lack circulating immune cells the divergent response to LPS in GF and SPF slices is mediated by resident cells, this indicates that there is a close interaction between the gut and the hepatic innate immune system.

Whilst LPS is considered the main microbiota-derived PAMP, it would be of additional value to test other PAMPs to elucidate the organization and interaction of the liver's innate inflammatory response. Additionally, this study is based on an *ex vivo* model lacking circulating immune cells; adding immune cells to PCLS during incubation would aid in revealing the potential involvement of circulating immune cells in the inflammatory response of the liver. Lastly, *in vivo* studies could be designed to implant specific gram-negative or gram-positive bacteria in GF mice, to explore the contribution of these microbes on the inflammatory response in PCLS.

5. Conclusion

This study reveals that the presence of host microbiota mitigates the inflammatory response to LPS in the liver, by decreasing inflammatory processes and preventing cell death, even in the absence of circulating immune cells. Still, more research is needed to further unravel the relationship between the gut microbiota and the hepatic innate immune system.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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References

Arrese, M., Cabrera, D., Kalergis, A.M., Feldstein, A.E., 2016. Innate immunity and inflammation in NAFLD/NASH. Dig. Dis. Sci. 61, 1294–1303.
Backhed, F., Manchester, J.K., Semenkovich, C.F., Gordon, J.I., 2007. Mechanisms

underlying the resistance to diet-induced obesity in germ-free mice. Proc. Natl. Acad. Sci. U. S. A. 104, 979–984.
Berg, D.J., Kuhn, R., Rajewsky, K., Muller, W., Menon, S., Davidson, N., Grunig, G., Rennick, D., 1995. Interleukin-10 is a central regulator of the response to LPS in murine models of endotoxic shock and the Shwartzman reaction but not endotoxin tolerance. J. Clin. Invest. 96, 2339–2347.
Berlato, C., Cassatella, M.A., Kinjyo, I., Gatto, L., Yoshimura, A., Bazzoni, F., 2002. Involvement of suppressor of cytokine signaling-3 as a mediator of the inhibitory effects of IL-10 on lipopolysaccharide-induced macrophage activation. J. Immunol. 168, 6404–6411.
Biswas, S.K., Lopez-Collazo, E., 2009. Endotoxin tolerance: new mechanisms, molecules and clinical significance. Trends Immunol. 30, 475–487.
Bode, J.G., Nimmesgern, A., Schmitz, J., Schaper, F., Schmitt, M., Frisch, W., Haussinger, D., Heinrich, P.C., Graeve, L., 1999. LPS and TNFalpha induce SOCS3 mRNA and inhibit IL-6-induced activation of STAT3 in macrophages. FEBS Lett. 463, 365–370.
Bugianesi, E., McCullough, A.J., Marchesini, G., 2005. Insulin resistance: a metabolic pathway to chronic liver disease. Hepatology 42, 987–1000.
Caesar, R., Reigstad, C.S., Backhed, H.K., Reinhardt, C., Ketonen, M., Lunden, G.O., Cani, P.D., Backhed, F., 2012. Gut-derived lipopolysaccharide augments adipose macrophage accumulation but is not essential for impaired glucose or insulin tolerance in mice. Gut 61, 1701–1707.
Chanput, W., Mes, J., Vreeburg, R.A., Savelkoul, H.F., Wichers, H.J., 2010. Transcription profiles of LPS-stimulated THP-1 monocytes and macrophages: a tool to study inflammation modulating effects of food-derived compounds. Food Funct. 1, 254–261.
Chassaing, B., Etienne-Mesmin, L., Gewirtz, A.T., 2014. Microbiota-liver axis in hepatic disease. Hepatology 59, 328–339.
Chaves de Souza, J.A., Nogueira, A.V., Chaves de Souza, P.P., Kim, Y.J., Silva Lobo, C., Pimentel Lopes de Oliveira, G.J., Cirelli, J.A., Garlet, G.P., Rossa Jr., C., 2013. SOCS3 expression correlates with severity of inflammation, expression of proinflammatory cytokines, and activation of STAT3 and p38 MAPK in LPS-induced inflammation in vivo. Mediators Inflamm. 2013, 650812.
Chen, P., Miyamoto, Y., Mazagova, M., Lee, K.C., Eckmann, L., Schnabl, B., 2015. Microbiota protects mice against acute alcohol-induced liver injury. Alcohol. Clin. Exp. Res. 39, 2313–2323.
Fan, J., Kapus, A., Marsden, P.A., Li, Y.H., Oreopoulos, G., Marshall, J.C., Frantz, S., Kelly, R.A., Medzhitov, R., Rotstein, O.D., 2002. Regulation of toll-like receptor 4 expression in the lung following hemorrhagic shock and lipopolysaccharide. J. Immunol. 168, 5252–5259.
Federico, A., Dallio, M., Godos, J., Loguercio, C., Salomone, F., 2016. Targeting gut-liver axis for the treatment of nonalcoholic steatohepatitis: translational and clinical evidence. Transl. Res. 167, 116–124.
Fukuhara, D., Irie, K., Uchida, Y., Kataoka, K., Akiyama, K., Ekuni, D., Tomofuji, T., Morita, M., 2018. Impact of commensal flora on periodontal immune response to lipopolysaccharide. J. Periodontol. 89, 1213–1220.
de Graaf, I.A., Olinga, P., de Jager, M.H., Merema, M.T., de Kanter, R., van de Kerkhof, E.G., Groothuis, G.M., 2010. Preparation and incubation of precision-cut liver and intestinal slices for application in drug metabolism and toxicity studies. Nat. Protoc. 5, 1540–1551.
Hadi, M., Westra, I.M., Starokozhko, V., Dragovic, S., Merema, M.T., Groothuis, G.M., 2013. Human precision-cut liver slices as an ex vivo model to study idiosyncratic drug-induced liver injury. Chem. Res. Toxicol. 26, 710–720.
Ikeda, M., Hamada, K., Sumitomo, N., Okamoto, H., Sakakibara, B., 1999. Serum amyloid A, cytokines, and corticosterone responses in germfree and conventional mice after lipopolysaccharide injection. Biosci. Biotechnol. Biochem. 63, 1006–1010.
Iyer, S.S., Cheng, G., 2012. Role of interleukin 10 transcriptional regulation in inflammation and autoimmune disease. Crit. Rev. Immunol. 32, 23–63.
Iyer, S.S., Ghaffari, A.A., Cheng, G., 2010. Lipopolysaccharide-mediated IL-10 transcriptional regulation requires sequential induction of type I IFNs and IL-27 in macrophages. J. Immunol. 185, 6599–6607.
Jirillo, E., Caccavo, D., Magrone, T., Piccigallo, E., Amati, L., Lembo, A., Kalis, C., Gumenscheimer, M., 2002. The role of the liver in the response to LPS: experimental and clinical findings. J. Endotoxin. Res. 8, 319–327.
Kesar, V., Odin, J.A., 2014. Toll-like receptors and liver disease. Liver Int. 34, 184–196.
Kiyono, H., McGhee, J.R., Michalek, S.M., 1980. Lipopolysaccharide regulation of the immune response: comparison of responses to LPS in germfree, Escherichia coli-monoassociated and conventional mice. J. Immunol. 124, 36–41.
Kobayashi, K., Hernandez, L.D., Galan, J.E., Janeway Jr., C.A., Medzhitov, R., Flavell, R.A., 2002. IRAK-M is a negative regulator of toll-like receptor signaling. Cell 110, 191–202.
Kubo, M., Hanada, T., Yoshimura, A., 2003. Suppressors of cytokine signaling and immunity. Nat. Immunol. 4, 1169–1176.
Mazagova, M., Wang, L., Anfora, A.T., Wissmueller, M., Lesley, S.A., Miyamoto, Y., Eckmann, L., Dhungana, S., Pathmasiri, W., Sumner, S., Westwater, C., Brenner, D.A., Schnabl, B., 2015. Commensal microbiota is hepatoprotective and prevents liver fibrosis in mice. FASEB J. 29, 1043–1055.
McGhee, J.R., Kiyono, H., Michalek, S.M., Babb, J.L., Rosenstreich, D.L., Mergenhagen, S.E., 1980. Lipopolysaccharide (LPS) regulation of the immune response: T lymphocytes from normal mice suppress mitogenic and immunogenic responses to LPS. J. Immunol. 124, 1603–1611.
Miele, L., Valenza, V., La Torre, G., Montalto, M., Cammarota, G., Ricci, R., Masciana, R., Forgione, A., Gabrieli, M.L., Perotti, G., Vecchio, F.M., Rapaccini, G., Gasbarrini, G., Day, C.P., Grieco, A., 2009. Increased intestinal permeability and tight junction alterations in nonalcoholic fatty liver disease. Hepatology 49, 1877–1887.
Mitsuyama, M., Ohara, R., Amako, K., Nomoto, K., Yokokura, T., Nomoto, K., 1986. Ontogeny of macrophage function to release superoxide anion in conventional and germfree mice. Infect. Immun. 52, 236–239.
Miura, K., Kodama, Y., Inokuchi, S., Schnabl, B., Aoyama, T., Ohnishi, H., Olefsky, J.M., Brenner, D.A., Seki, E., 2010. Toll-like receptor 9 promotes steatohepatitis by induction of interleukin-1beta in mice. Gastroenterology 139, 323–334 e327.
Morland, B., Smievoll, A.I., Midtvedt, T., 1979. Comparison of peritoneal macrophages from germfree and conventional mice. Infect. Immun. 26, 1129–1136.
Moshage, H., Kok, B., Huizenga, J.R., Jansen, P.L., 1995. Nitrite and nitrate determinations in plasma: a critical evaluation. Clin. Chem. 41, 892–896.
Nowak, M., Gaines, G.C., Rosenberg, J., Minter, R., Bahjat, F.R., Rectenwald, J., MacKay, S.L., Edwards 3rd, C.K., Moldawer, L.L., 2000. LPS-induced liver injury in D-galactosamine-sensitized mice requires secreted TNF-alpha and the TNF-p55 receptor. Am. J. Physiol. Regul. Integr. Comp. Physiol. 278, R1202–R1209.
Olinga, P., Merema, M.T., de Jager, M.H., Derks, F., Melgert, B.N., Moshage, H., Slooff, M.J., Meijer, D.K., Poelstra, K., Groothuis, G.M., 2001. Rat liver slices as a tool to study LPS-induced inflammatory response in the liver. J. Hepatol. 35, 187–194.
Parker, L.C., Jones, E.C., Prince, L.R., Dower, S.K., Whyte, M.K., Sabroe, I., 2005. Endotoxin tolerance induces selective alterations in neutrophil function. J. Leukoc. Biol. 78, 1301–1305.
Parlesak, A., Schafer, C., Schutz, T., Bode, J.C., Bode, C., 2000. Increased intestinal permeability to macromolecules and endotoxemia in patients with chronic alcohol abuse in different stages of alcohol-induced liver disease. J. Hepatol. 32, 742–747.
Pena, O.M., Pistolic, J., Raj, D., Fjell, C.D., Hancock, R.E., 2011. Endotoxin tolerance represents a distinctive state of alternative polarization (M2) in human mononuclear cells. J. Immunol. 186, 7243–7254.
Poltorak, A., He, X., Smirnova, I., Liu, M.Y., Van Huffel, C., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freudenberg, M., Ricciardi-Castagnoli, P., Layton, B., Beutler, B., 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science 282, 2085–2088.
Seki, E., Schnabl, B., 2012. Role of innate immunity and the microbiota in liver fibrosis: crosstalk between the liver and gut. J. Physiol. 590, 447–458.
Seki, E., Schwabe, R.F., 2015. Hepatic inflammation and fibrosis: functional links and key pathways. Hepatology 61, 1066–1079.
Seki, E., De Minicis, S., Osterreicher, C.H., Kluwe, J., Osawa, Y., Brenner, D.A., Schwabe, R.F., 2007. TLR4 enhances TGF-beta signaling and hepatic fibrosis. Nat. Med. 13, 1324–1332.
Sommer, F., Backhed, F., 2013. The gut microbiota–masters of host development and physiology. Nat. Rev. Microbiol. 11, 227–238.
Su, G.L., 2002. Lipopolysaccharides in liver injury: molecular mechanisms of Kupffer cell activation. Am. J. Physiol. Gastrointest. Liver Physiol. 283, G256–G265.
Sun, Y., Li, H., Sun, M.J., Zheng, Y.Y., Gong, D.J., Xu, Y., 2014. Endotoxin tolerance induced by lipopolysaccharides derived from Porphyromonas gingivalis and Escherichia coli: alternations in toll-like receptor 2 and 4 signaling pathway. Inflammation 37, 268–276.
Szabo, G., Bala, S., 2010. Alcoholic liver disease and the gut-liver axis. World J. Gastroenterol. 16, 1321–1329.
van 't Veer, C., van den Pangaart, P.S., van Zoelen, M.A., de Kruif, M., Birjmohun, R.S., Stroes, E.S., de Vos, A.F., van der Poll, T., 2007. Induction of IRAK-M is associated with lipopolysaccharide tolerance in a human endotoxemia model. J. Immunol. 179, 7110–7120.
Tabibian, J.H., O'Hara, S.P., Trussoni, C.E., Tietz, P.S., Splinter, P.L., Mounajjed, T., Hagey, L.R., LaRusso, N.F., 2016. Absence of the intestinal microbiota exacerbates hepatobiliary disease in a murine model of primary sclerosing cholangitis. Hepatology 63, 185–196.
Takahashi, K., Sugi, Y., Hosono, A., Kaminogawa, S., 2009. Epigenetic regulation of TLR4 gene expression in intestinal epithelial cells for the maintenance of intestinal homeostasis. J. Immunol. 183, 6522–6529.
Takahashi, K., Sugi, Y., Nakano, K., Tsuda, M., Kurihara, K., Hosono, A., Kaminogawa, S., 2011. Epigenetic control of the host gene by commensal bacteria in large intestinal epithelial cells. J. Biol. Chem. 286, 35755–35762.
Takeuchi, O., Akira, S., 2010. Pattern recognition receptors and inflammation. Cell 140, 805–820.
Tilg, H., Moschen, A.R., Szabo, G., 2016. Interleukin-1 and inflammasomes in alcoholic liver disease/acute alcoholic hepatitis and nonalcoholic fatty liver disease/nonalcoholic steatohepatitis. Hepatology 64, 955–965.
West, A.P., Koblansky, A.A., Ghosh, S., 2006. Recognition and signaling by toll-like receptors. Annu. Rev. Cell Dev. Biol. 22, 409–437.
Yu, C.R., Kim, S.H., Mahdi, R.M., Egwuagu, C.E., 2013. SOCS3 deletion in T lymphocytes suppresses development of chronic ocular inflammation via upregulation of CTLA-4 and expansion of regulatory T cells. J. Immunol. 191, 5036–5043.
Zhang, M., Chen, W., Zhou, W., Bai, Y., Gao, J., 2017. Critical role of IRAK-M in regulating antigen-induced airway inflammation. Am. J. Respir. Cell Mol. Biol. 57, 547–559.