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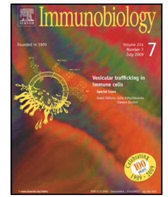

Hepatic interleukin-6 production is maintained during endotoxin tolerance and facilitates lipid accumulation

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ABSTRACT

Gut-derived bacterial endotoxins, such as lipopolysaccharide (LPS), contribute to the pathogenesis of steatosis and steatohepatitis by activating Kupffer cells, the resident liver macrophages. Exposure of macrophages to low doses of LPS causes hyporesponsiveness upon subsequent endotoxin challenge, a phenomenon termed endotoxin or LPS tolerance. In the present study, we aimed to examine whether LPS-induced lipid accumulation is affected by endotoxin tolerance.

LPS pretreatment reduced the expression of proinflammatory mediators upon subsequent high-dose LPS treatment in murine livers. Total lipid and lipid class analysis indicated that LPS-induced lipid accumulation was not affected by endotoxin tolerance, although it was dependent on the presence of Kupffer cells. Analysis of the expression of lipogenic genes revealed that sterol regulatory element binding transcription factor 1 (*Srebf1*) and its target ELOVL fatty acid elongase 6 (*Elovl6*) were upregulated upon LPS administration in livers from LPS-tolerant and non-tolerant mice, whereas the expression of peroxisome proliferator activated receptor-α (*Ppara*), a key inducer of lipid degradation, was decreased. Neither Interleukin (IL)-6 expression nor the activation of its downstream effector signal transducer and activator of transcription (STAT) 3 were suppressed in liver tissues of LPS-tolerized mice. In vitro experiments confirmed that recombinant or macrophage-derived IL-6 was a potent activator of the lipogenic factor STAT3 in hepatocytes. Accordingly, IL-6 treatment led to increased lipid levels in this cell type.

In summary, our data show that endotoxin tolerance does not influence LPS-induced hepatic lipid accumulation and suggest that IL-6 drives hepatic lipid storage.

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1. Introduction

Over the last decades, a lifestyle shift in Western societies has led to massively increased obesity rates (Swinburn et al., 2011). Obesity and diabetes mellitus are the key features of the metabolic syndrome, which strongly correlates with the development of non-alcoholic fatty liver disease (NAFLD) (Adams et al., 2005; Bellentani et al., 2010; Browning et al., 2004; de Alwis and Day, 2008).

With an estimated prevalence of 20–35% in the adult population in Western countries, NAFLD has been predicted to become the most common cause for liver transplantations by the year 2030 (Byrne and Targher, 2015; Sayiner et al., 2016). The pathogenesis of NAFLD is widely believed to start with simple steatosis, which is characterized by excessive hepatic lipid accumulation (Adams et al., 2005; Angulo, 2002). The progression from steatosis to advanced inflammatory states, such as alcoholic steatohepatitis (ASH) or non-alcoholic steatohepatitis (NASH), is mediated by the release of inflammatory cytokines (Day, 2010). Both ASH and NASH can further progress to hepatic cirrhosis and may finally result in

Abbreviations: ARG1, arginase 1; ASH, alcoholic steatohepatitis; CE, cholesteryl ester; CER, ceramides; CH, cholesterol; Clo, clodronate; CXCL10, C-X-C motif chemokine 10; DAMP, danger-associated molecular pattern; ELOVL6, ELOVL fatty acid elongase 6; GILZ, glucocorticoid-induced leucine zipper; HCC, hepatocellular carcinoma; HFD, high-fat diet; IGF2, insulin-like growth factor 2; IL, interleukin; iNOS, inducible NO synthase; LPS, lipopolysaccharide; MCD, methionine cholin-deficient diet; MCM, macrophage-conditioned medium; M-CSF, macrophage-colony stimulating factor; MDM, monocyte derived macrophage; MLXIPL, MLX interacting protein like; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NOS2, nitric oxide synthase 2; PAMP, pathogen-associated molecular pattern; PBMC, peripheral blood mononuclear cell; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PPAR, peroxisome proliferator activated receptor; R-LPS, rough LPS; S-LPS, smooth LPS; SPV, sulfophospho-vanillin; SRE, sterol regulatory element; SREBP, SRE binding protein; SREBF1, sterol regulatory element binding transcription factor 1; STAT3, signal transducer and activator of transcription 3; TG, triglycerides; TLC, thin layer chromatography; TLR, toll-like receptor; TNF, tumor necrosis factor.

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the development of hepatocellular carcinoma (HCC) (Adams et al., 2009; Ascha et al., 2010; Fabbrini et al., 2010).

Chronic alcohol consumption associated with ASH as well as high-caloric food intake resulting in NASH have been reported to increase the permeability of the intestinal barrier for bacteria and microbial products, such as lipopolysaccharides (LPS), an effect referred to as the leaky gut syndrome (Amar et al., 2008; Bode and Bode, 2003; Bode et al., 1987; Enomoto et al., 1998; Nanji et al., 1993). LPS exposure elicits strong immune responses by activation of Toll-like receptor 4 (TLR4). Interestingly, TLR4 knockout animals were protected from steatohepatitis in a methionin cholin-deficient (MCD) or high-fat diet (HFD) mouse model, two common models that exhibit signs of liver injury similar to human NASH (Csak et al., 2011; Li et al., 2011; Rivera et al., 2007). TLR4 has also been suggested to play a role in the pathogenesis of ASH (Uesugi et al., 2001), and a recently published report demonstrated that progression from steatohepatitis to HCC was linked to TLR4 expression in macrophages (Miura et al., 2016).

Kupffer cells are the resident liver macrophages, the main source of TLR4 in the liver (Fisher et al., 2013), and have been implicated in the regulation of the hepatic lipid content and composition in a NASH mouse model (Kessler et al., 2014). In chronic or acute liver diseases, Kupffer cells can be activated either via pathogen-associated or damage-associated molecular patterns (PAMPs/DAMPs). Upon stimulation, they secrete pro-inflammatory mediators, such as interleukin (IL)-6, IL-1β, and tumor necrosis factor (TNF)-α (Ganz and Szabo, 2013; Park et al., 2010). Permanent exposure of monocytes and macrophages to even low doses of endotoxins, such as LPS, leads to a state of hyporesponsiveness, a phenomenon termed LPS or endotoxin tolerance. LPS-tolerant macrophages are characterized by the lack of pro-inflammatory mediator production after re-stimulation with LPS. In contrast, the production of anti-inflammatory mediators, such as IL-10 or glucocorticoid-induced leucine zipper (GILZ), can even be enhanced (Biswas and Lopez-Collazo, 2009; Bohannon et al., 2013; Hoppstädter et al., 2015a; Hoppstädter and Kiemer, 2015; Pena et al., 2011).

Since macrophages are the central mediators of inflammation and have also been suggested to play a vital role in lipid homeostasis, we aimed to examine whether endotoxin tolerance influences hepatic lipid accumulation.

2. Materials and methods

2.1. Materials

Cell media, FCS, penicillin, streptomycin, and glutamine were from Sigma-Aldrich. Ultrapure LPS from *Escherichia coli* K12 was used for in vitro studies and LPS from *Salmonella minnesota* R595 for in vivo experiments. Both were purchased from Invivogen. Lymphocyte Separation Medium 1077 was obtained from PromoCell and Leucosep tubes were from Greiner. Primers were obtained from Eurofins MWG Operon. Human recombinant macrophage-colony stimulating factor (M-CSF), recombinant human IL-6, CD14 Microbeads, LS columns, and MACS accessories were purchased from Miltenyi Biotec. Recombinant human insulin-like growth factor 2 (IGF2) was obtained from R&D Systems. The neutralizing antibody against human IL-6 (clone 3H3) and the matching IgG1 control were from Invivogen, and pSTAT3 (Tyr705, clone D3A7) and STAT3 (clone 124H6) antibodies were from Cell Signaling. IRDye® 680 or IRDye® 800 conjugated secondary antibodies were from LI-COR Biosciences. Other chemicals were obtained from either Sigma-Aldrich or Carl Roth unless stated otherwise.

2.1.1. Cell culture

Buffy coats were obtained from healthy adult blood donors (Blood Donation Center, Saarbrücken, Germany). The use of human material for the isolation of primary cells was approved by the local ethics committee (permission no. 130/08). For monocyte isolation, peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Lymphocyte Separation Medium and Leucosep tubes. After washing with PBS, monocytes were purified from PBMCs by magnetic cell sorting using anti-CD14 microbeads according to the manufacturer's instructions, except that 10% of the recommended bead amount was used (Stögbauer et al., 2008; Hoppstädter et al., 2015b; Seif et al., 2016). Monocyte purity was > 95% as assessed by CD14 expression (data not shown). Monocytes were differentiated into macrophages in RPMI-1640 supplemented with 10% FCS, 100 U/ml penicillin G, 100 mg/ml streptomycin, 2 mM glutamine, and 20 ng/ml M-CSF at 37 °C and 5% CO_2 for 7 d. To induce endotoxin tolerance, 0.5×10^6 cells/well were seeded into a 12-well plate and treated with 100 ng/ml LPS or medium only. After 24 h, the supernatant was removed, and fresh medium with or without LPS (1 μg/ml) was added for another 4 h. Subsequently, cells were harvested for qPCR analysis. Supernatants were pooled and stored at −80 °C until further use.

HepG2 cells were cultured in RPMI with 10% FCS, 100 U/ml penicillin G, 100 mg/ml streptomycin, and 2 mM glutamine as described previously (Laggai et al., 2014).

2.1.2. Mice

Mice were housed in a 12/12 h light/dark cycle with food and water ad libitum. For tolerance experiments, male C57BL/6J mice at the age of 10–12 weeks (22–25 g body weight) were tolerized to LPS as described previously (Hoppstädter et al., 2015a; Hoppstädter and Kiemer, 2015). Briefly, animals were treated with LPS by i.p. injections of low-dose LPS (10 μg per mouse dissolved in 0.9% NaCl) or vehicle alone on three consecutive days, followed by a final treatment with high-dose LPS (100 μg/mouse, 4 h) or vehicle on day 4. Mice were sacrificed, and liver tissues were collected and stored at −80 °C until further use. For Kupffer cell depletion, mice were repeatedly injected i.p. with clodronate liposomes (50 μg/10 μl suspension per g body weight) as described previously (Keller et al., 2005; Kessler et al., 2014), followed by injection of LPS (100 μg/mouse, 4 h) or vehicle as indicated. All animal procedures were performed in accordance with the local animal welfare committee (permission no. 34/2010 and 35/2013; Landesamt für Soziales, Gesundheit und Verbraucherschutz Saarland).

2.1.3. RNA isolation, reverse transcription, and real-time RT-PCR

Total RNA was extracted using Qiazol lysis reagent (Qiagen) according to the manufacturer's protocol. Residual genomic DNA was removed by treatment with DNase I (Ambion). Reverse transcription was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) as recommended by the supplier. Transcripts were detected using the 5 x HOT FIREPol® EvaGreen® qPCR Mix Plus (Solis BioDyne) according to the manufacturer's instructions using the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). Primer sequences are given in Table 1. For absolute quantification, standards of the respective PCR product cloned into pGEMTeasy (Promega) were run alongside the samples to generate a standard curve (Hoppstädter et al., 2012; Hoppstädter et al., 2015a; Hoppstädter et al., 2016; Kessler et al., 2016). All samples and standards were analyzed in triplicate. The relative gene expression was calculated by normalizing absolute gene expression to *Rn18s* values (Hoppstädter et al., 2015a; Kessler et al., 2016)

2.1.4. Lipid analysis

Freeze-dried liver tissues or HepG2 cells were dispersed in hexane/2-propanol [3:2 (v/v)]. After centrifugation for 10 min at

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