Experimental and Molecular Pathology 94 (2013) 216–227

Contents lists available at SciVerse ScienceDirect

Experimental and Molecular Pathology

journal homepage: www.elsevier.com/locate/yexmp

Distinct responses of lung and liver macrophages to acute endotoxemia Role of toll-like receptor 4

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ARTICLE INFO

Article history:
Received 25 July 2012
and in revised form 6 September 2012
Available online 19 September 2012

Keywords:
LPS
TLR4
Macrophages
Inflammation
Liver
Lung

ABSTRACT

Exposure to excessive quantities of bacterial-derived lipopolysaccharide (LPS) is associated with injury to the lung and the liver. Macrophages are thought to play a key role in the pathogenic response to LPS by releasing proinflammatory/cytotoxic mediators. Macrophage responses to LPS are mediated in large part by toll-like receptor 4 (TLR4). In the present studies we used C3H/HeJ mice, which possess a mutated nonfunctional TLR4, to examine its role in lung and liver macrophage responses to acute endotoxemia induced by LPS administration. Treatment of control C3H/HeOuJ mice with LPS (3 mg/ml, i.p.) was associated with a significant increase in the number of macrophages in both the lung and the liver. This was most prominent after 48 h, and was preceded by expression of proliferating cell nuclear antigen (PCNA), suggesting that macrophage proliferation contributes to the response. In liver, but not lung macrophages, LPS administration resulted in a rapid (within 3 h) increase in mRNA expression of Mn superoxide dismutase (SOD) and heme oxygenase-1 (HO-1), key enzymes in antioxidant defense. In contrast, HO-1 protein expression decreased 3 h after LPS administration in liver macrophages, while in lung macrophages it increased. mRNA expression of enzymes mediating the biosynthesis of eicosanoids, including cyclooxygenase-2 (COX-2) and microsomal prostaglandin E synthase-1 (mPGES-1), but not 12/15-lipoxygenase (LOX), was upregulated in liver macrophages 3–24 h after LPS, with no effect on lung macrophages. However, COX-2 protein expression increased in both cell types. Loss of functional TLR4 significantly blunted the effects of LPS. Thus, no major changes were observed after LPS administration in the number of lung and liver macrophages recovered from TLR4 mutant mice, or on expression of PCNA. Increases in HO-1, MnSOD, COX-2 and PGES-1 mRNA expression in liver macrophages were also reduced in these mice. Conversely, in lung macrophages, loss of functional TLR4 resulted in increased expression of COX-2 protein and 12/15-LOX mRNA. These results demonstrate distinct lung and liver macrophage responses to acute endotoxemia are mediated, in part, by functional TLR4.

Introduction

Lipopolysaccharide (LPS), typically found in the gastrointestinal tract, is a major component of the cell wall of Gram-negative bacteria. It is a glycolipid predominantly composed of oligo- and polysaccharides, and lipid A endotoxin (Raetz and Whitfield, 2002). LPS is primarily cleared from the body by Kupffer cells in the liver (Protzer et al., 2012; Vazquez-Torres et al., 2004). However, excessive levels of LPS can readily overwhelm this clearance mechanism resulting in acute endotoxemia. This is associated with systemic inflammation which can lead to septic shock, multiple organ failure and death (Annane et al., 2005). The lung is particularly sensitive to endotoxin and is usually the first organ to fail, followed closely by the liver (Ciesla et al., 2005; Fry, 2012). A characteristic feature of acute endotoxemia is an accumulation of macrophages in target tissues (Ahmad et al., 2002; Chen et al., 2007; McCloskey et al., 1992; Pilaro and Laskin, 1986; Wizemann and Laskin, 1994). These cells are activated by LPS to release reactive oxygen and nitrogen species, proinflammatory cytokines, proteases, and bioactive lipids, which are thought to contribute to tissue injury and the pathogenesis of organ failure (Laskin et al., 2011; Murray and Wynn, 2011).

A number of receptors have been identified on macrophages that are involved in LPS responsiveness. These include CD14 and toll-like receptor 4 (TLR4) (Kawai and Akira, 2010; Schumann et al., 1990). Following its release from dividing or damaged bacteria, LPS is sequestered by an LPS-binding protein in serum, which transports it to CD14 on the surface of macrophages (Akira and Takeda, 2004; Wright et al., 1990). Subsequently LPS is transferred to MD2, a soluble protein which associates with the extracellular domain of TLR4. Activation of TLR4 initiates a cell signaling cascade leading to translocation of NF-κB into the nucleus and transcription of proinflammatory genes including inducible nitric oxide

Abbreviations: BAL, bronchoalveolar lavage; COX-2, cyclooxygenases-2; HBSS, Hank's balanced salt solution; HO-1, heme oxygenenase-1; iNOS, inducible nitric oxide synthase; LOX, lipoxygenase; LPS, lipopolysaccharide; mPGES-1, microsomal prostaglandin E synthase-1; NF-κB, nuclear factor-kappa B; PCNA, proliferating cell nuclear antigen; SOD, superoxide dismutase; TLR4, toll-like receptor 4; TNFα, tumor necrosis factor alpha.

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http://dx.doi.org/10.1016/j.yexmp.2012.09.006

synthase (iNOS), cyclooxygenase-2 (COX-2), and tumor necrosis factor alpha (TNFα), proteins implicated in lung and liver injury (Kawai and Akira, 2010; Laskin et al., 2011). Previous studies have shown protection against LPS-induced inflammation and tissue injury in animals with TLR4 deficiency (Guo et al., 2009; Roger et al., 2009; Wittebole et al., 2010). Similar protective effects of loss of TLR4 have been described in sterile inflammatory responses to tissue injury induced by various target-organ specific toxicants (Connor et al., 2012; Lin et al., 2011; Matzinger, 2002). The present studies demonstrate that macrophage accumulation and responsiveness in the lung and the liver following acute endotoxemia depend on functional TLR4. These data provide additional evidence for an essential contribution of TLR signaling to inflammatory pathologies (Jiang et al., 2005; Matzinger and Kamala, 2011; Ostuni and Natoli, 2011).

Materials and methods

Animals and treatments

Male TLR4 mutant C3H/HeJ and control C3H/HeOuJ mice (11–12 weeks) were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were housed in sterile microisolation cages and provided autoclaved food and water *ad libitum*. Animal care was in compliance with Rutgers University guidelines as outlined in the *Guide for the Care and Use of Laboratory Animals* prepared by the National Academy of Sciences. Acute endotoxemia was induced by i.p. injection of mice with 3 mg/kg *Escherichia coli* LPS (serotype 0128: B12 Sigma L4255, Sigma Chemical Co., St. Louis, MO), repurified as described previously (Chen et al., 2007). Phosphate buffered saline (PBS) was used as a control.

Macrophage isolation

Macrophages were isolated from the liver as described previously (Chen et al., 2007). Briefly, the liver was perfused though the portal vein with 10 ml of Ca^{+2}/Mg^{+2}-free Hank's balanced salt solution (HBSS) containing 25 mM HEPES, 0.5 mM EGTA and 4.4 mM $NaHCO_3$ (pH 7.3) at 37 °C for 2 min at a rate of 10 ml/min. This was followed by perfusion for 1 min with Leibovitz L-15 media containing 100 U/ml collagenase type IV. The liver was then removed, gently combed, and filtered through 220 μm nylon mesh. Hepatocytes were separated from nonparenchymal cells by centrifugation at 50×*g* for 5 min. Nonparenchymal cells were recovered by centrifugation of the supernatant at 330×*g* for 7 min. Macrophages were then purified according to their size and density on a Beckman J-6 elutriator (Beckman Instruments Inc., Fullerton, CA) equipped with centrifugal elutriation rotor speed set at 2500 rpm. The pump speed was set at 12 ml/min to load the cells and at 33 ml/min to collect macrophages, which were further purified using Nycodenz® (osmolality 265 mOsm, density 1.077 g/ml). Cells were identified morphologically by Giemsa staining and electron microscopy, and were >85% macrophages (Chen et al., 2007). The major contaminating cell population was endothelial cells. We have previously demonstrated that the inflammatory activity of hepatic endothelial cells is significantly reduced, relative to liver macrophages (Chen et al., 2007; McCloskey et al., 1992). Thus, their contribution to the observed responses in these studies is unlikely to be significant.

Lung macrophages were isolated by bronchoalveolar lavage (BAL) as described previously with some modifications (Connor et al., 2012). After liver perfusion, the trachea was cannulated and the lung was removed from the chest cavity. BAL was collected by slowly instilling and withdrawing 1 ml of HBSS 7–10 times though the cannula. BAL fluid was centrifuged (300×*g* for 8 min). Cell pellets were washed 4 times with HBSS containing 2% FBS and then enumerated using a hemocytometer. Viability was 98% as determined by trypan blue dye exclusion, and cell purity>97% macrophages as assessed morphologically after Giemsa staining.

Immunohistochemistry

Tissues were fixed in 10% formalin buffer overnight at room temperature, followed by 50% ethanol. Sections (6 μm) were deparaffinized, then incubated overnight at 4 °C with rabbit antibody to heme oxygenase-1 (HO-1, 1:1000; Stressgen/Assay Designs, Ann Arbor, MI), proliferating cell nuclear antigen (PCNA, 1:250, Abcam, Cambridge, MA), COX-2, (1:400, Abcam) or normal rabbit serum followed by a 30 min incubation with biotinylated secondary antibody (Vector Labs, Burlington, CA). Binding was visualized using a VECTASTAIN® Elite ABC kit (Vector Labs).

cDNA synthesis and real time-PCR

Total RNA was extracted from cells using QIAshredder and RNeasy Mini kit with on-column DNase digestion (Qiagen, Valencia, CA) according to the manufacturer's protocol. RNA concentration was determined by absorbance at 260 nm. For cDNA synthesis, 200 ng of RNA was reverse transcribed using the High-capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Standard curves were generated using serial dilutions from pooled randomly selected cDNA samples. Real-time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) on a 7900HT Thermal cycler using 96-well optical reaction plates. Samples from three to four animals per treatment group were analyzed and gene expression presented relative to hypoxanthine phosphoribosyltransferase (HPRT) mRNA expression. All PCR primer sequences were generated using Primer Express 2.0 (Applied Biosystems) and primers were synthesized by Integrated DNA Technologies (Coralville, IA). Forward and reverse primer sequences were as follows: 12/15-lipoxygenase (LOX), TCGGAGGCAGAATTCAAGGT and CAGCAGTGGCCCAAGGTATT; COX-2, CATTCTTTGCCCAGCACTTCAC and GACCAGGCACCAGACCAAAGAC; microsomal prostaglandin E synthase (mPGES)-1, GGCCTTTCTGCTCTGCAGC and GCCACCGCGTACATCTTGAT;

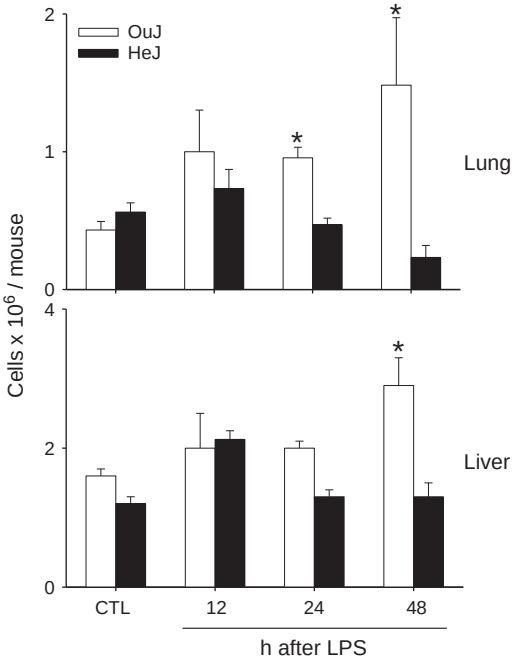

Fig. 1. Effects of the loss of TLR4 on the number of macrophages in the lung and liver following LPS administration. Macrophages were isolated from the lung and liver 12–48 h after treatment of C3H/HeOuJ (OuJ) and TLR4 mutant C3H/HeJ (HeJ) mice with control or LPS. Viable cells were counted on a hemocytometer using trypan blue exclusion. Upper panel: Each bar represents the mean±SE (n=3–21 mice). Lower panel: Each bar represents the mean±SE (n=11–72 mice). *Significantly different ($p \leq 0.05$) from control (ANOVA).