Research Article

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Hepatocytes express functional NOD1 and NOD2 receptors: A role for NOD1 in hepatocyte CC and CXC chemokine production

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Background & Aims: NOD-like receptors are recently described cytosolic pattern recognition receptors. NOD1 and NOD2 are members of this family that recognize bacterial cell wall components, diaminopimelic acid and muramyl dipeptide, respectively. Both NOD1 and NOD2 have been associated with many inflammatory diseases, although their role in liver inflammation and infection has not been well studied.

Methods: We investigated the role of NOD receptors in mouse liver by assessing expression and activation of NOD1 and NOD2 in liver and primary isolated hepatocytes from C57BL/6 mice.

Results: Both NOD1 and NOD2 mRNA and protein were highly expressed in hepatocytes and liver. RIP2, the main signaling partner for NODs, was also expressed. Stimulation of hepatocytes with NOD1 ligand (C12-iEDAP) induced NFκB activation, activation of MAP kinases and expression of chemokines CCL5 (RANTES) and CXCL1 (KC). C12-iEDAP also synergized with interferon (IFN)γ to increase iNOS expression and production of nitric oxide. Despite activating NFκB, NOD1 ligand did not upregulate hepatocyte production of the acute phase proteins lipopolysaccharide binding protein, serum amyloid A, or soluble CD14 in cell culture supernatants, or upregulate mRNA expression of lipopolysaccharide binding protein, serum amyloid A, C-reactive protein, or serum amyloid P. NOD2 ligand (MDP) did not activate hepatocytes when given alone, but did synergize with Toll-like receptor ligands, lipopolysaccharide (LPS), and polyI:C to activate NFκB and MAPK.

Conclusions: All together these data suggest an important role for hepatocyte NOD1 in attracting leukocytes to the liver during infection and for hepatic NLRs to augment innate immune responses to pathogens.

Keywords: Infection; Inflammation; Pattern recognition receptors; Innate immunity; Liver.
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Abbreviations: NOD, nucleotide oligomerization domain; NLR, NOD-like receptor; TLR, Toll-like receptor; NALP, NACHT leucine-rich repeat and pyrin domain containing protein; CARD, caspase activation and recruitment domain; RIP2, receptor-interacting protein kinase; IL, interleukin; DAP, diaminopimelic acid; PGN, peptidoglycan; MDP, muramyl dipeptide; NO, nitric oxide; LPS, lipopolysaccharide; RANTES, regulated on activation normal T cell expressed and secreted; MIG, monokine induced by IFN-gamma; KC, keratinocyte-derived chemokine; MCP1, monocyte chemotactic protein 1; SAA, serum amyloid A; SAP, serum amyloid P; LBP, lipopolysaccharide binding protein; CRP, C-reactive protein.

Introduction

NOD-like receptors (NLRs) are a recently described family of pattern recognition receptors that have been associated with many inflammatory diseases in humans, highlighting their significant immunologic role [1]. NLRs are found in the cell cytosol, in contrast to membrane-associated pattern recognition receptors such as Toll-like receptors (TLRs), and contain a central nucleotide-binding and oligomerization (NOD) domain with a leucine-rich repeat domain responsible for pathogen sensing [2]. There are two major subgroups within NLRs: NODs and NACHT, leucine-rich repeat and pyrin domain containing proteins (NALP)s. NODs have amino terminal caspase activation and recruitment (CARD) domains [3], which allow them to associate with other CARD containing signaling adaptor molecules. NOD1 and NOD2 have been shown to associate with RIP2/RICK, via CARD–CARD interactions, which allow RIP2 to associate with TRAF6/TAK1 [4]. Subsequent signaling leads to activation of NFκB and upregulation of inflammatory mediators, such as interleukin (IL)-6 [4].

Specific ligands have been identified that stimulate NOD1 and NOD2. These ligands are all components of bacterial cell walls [5]. NOD1 responds to meso-diaminopimelic acid (DAP), a muropeptide found on most Gram-negative bacteria. NOD2 senses both Gram-positive and Gram-negative bacteria through peptidoglycans (PGN) and muramyl dipeptide (MDP) [5].

The liver is a sentinel organ in a unique position to monitor pathogen-associated molecules in the portal and systemic circulations. It is increasingly recognized that not only immune cells but also the parenchymal cells of the liver, including hepatocytes and liver endothelial cells, play important roles in the immune response to a wide range of liver problems, from alcoholic liver disease to acetaminophen toxicity to liver I/R injury. Hepatocytes represent the largest cell mass in the liver and we have shown these cells express TLRs. Our work [6–8] and the work of others [9,10] have shown that hepatocytes respond directly to TLR ligands and danger signals, and act together with non-parenchymal cells such as Kupffer cells (KC, resident liver macrophages) and dendritic cells (DC). The liver, and its multiple cell types, including hepatocytes, are therefore central components that initiate and regulate innate immune pathways. Little is known

about the expression or function of NLRs in specific liver cell populations such as hepatocytes.

In the present study, we sought to determine whether hepatocytes express functional NOD receptors and whether NOD expression is altered in response to specific NOD or TLR ligands. We also examined the response of the liver *in vivo* to NOD ligand exposure. Our data show high expression of both NOD1 and NOD2 and that hepatocytes and liver respond to NOD1 ligands to activate NFκB, which results in increased CC and CXC chemokine release and increased nitric oxide (NO) production. Stimulation with NOD1 ligand did not, however, upregulate the production or release of acute phase proteins in hepatocytes. NOD2 ligand, MDP, did not by itself activate hepatocytes but did synergize with TLR ligands and lead to NFκB translocation to the nucleus.

Materials and methods

Reagents

Ultrapure LPS (*Escherichia coli* 0111:B4) from List Biological Laboratories, Inc. (Vandell Way, CA). Endotoxin-free C12-iEDAP and MDP from Invivogen (San Diego, CA). Cytokines: IFNγ (Cell Sciences Inc., Canton, MA), TNF (R&D systems, Minneapolis, MN), IL1β (Leinco Technologies, St. Louis, MO). Rabbit anti-mouse phospho-ERK, ERK, phospho-p38, p38, JNK, phospho-JNK from Cell Signaling Technologies (Beverly, MA). Anti-NOD1 and NOD2 from Imgenex (San Diego, CA). Anti-RIP2 from ProSci Inc., Poway, CA. NFκB consensus-oligonucleotides from Promega (Madison, WI). ELISAs: RANTES, MIG, KC, MCP1 from R&D Systems (Minneapolis, MN); LBP from Hycult Biotechnologies (Netherlands); SAA from Biosource (Camarillo, CA).

Animals

Experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. C57BL/6 mice were from Charles River Laboratories (Wilmington, MA). C57BL/10 and TLR4−/− (C57BL/10ScN) mice were from Jackson Laboratories. MyD88−/− mice were on a C57BL/6 background (a kind gift from R. Medzhitov [HHMI, New Haven]). LPS2 (TRIF−/−) mice were a kind gift from B. Beutler (Scripps Institute, CA). All mice used were specific pathogen-free, between 8- and 10-weeks old, and allowed rodent chow and water *ad libitum*. For *in vivo* studies mice were injected intraperitoneally with LPS (5 mg/kg), MDP (10 mg/kg), or iEDAP (5 mg/kg).

Hepatocyte isolation and cell culture

Hepatocytes were isolated from mice by an *in situ* collagenase (type VI; Sigma) perfusion technique, modified as described previously [11]. Hepatocyte purity exceeded 99% by flow cytometric assay, and viability was typically over 95% by trypan blue exclusion. Hepatocytes (150,000 cells/ml) were plated on gelatin-coated culture plates in Williams medium E with 10% calf serum, 15 mM HEPES, 10^{-6} M insulin, 2 mM L-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin. Hepatocytes were allowed to attach to plates overnight and prior to treatments the cell culture media was changed to serum-free media.

Analysis of chemokine, acute phase protein, and nitrite levels in cell culture supernatants

Chemokine and acute phase protein levels were detected in cell culture supernatants and plasma by ELISA according to the manufacturers' instructions. Nitrite levels in cell supernatants were detected by Greiss reaction.

Immunoblotting and EMSA

Treated hepatocytes were washed twice in PBS. Cells were lysed, and Western blots performed as previously described [7]. Nuclei were also extracted from some cells as previously described, and NFκB was detected by EMSA as previously described [7].

Comparative PCR

Total RNA was extracted from hepatocytes or liver using RNeasy mini extraction kits from Qiagen (Valencia, CA) according to the manufacturer's protocol. cDNA was synthesized using 1 μg RNA and oligo dT primers (Qiagen) and Omniscript™ reverse transcriptase (Qiagen). PCR reaction mixtures were prepared using SYBR Green PCR master mix (PE Applied Biosystems, Foster City, CA). SYBR Green two-step real-time RT-PCR was performed using forward and reverse primer pairs pre-validated and specific for NOD1, NOD2, RIP2, LBP, SAA, SAP, and CRP (Qiagen). All samples were run in triplicate. The level of gene expression for each sample was normalized to β-actin mRNA expression using the comparative C_t method.

Statistical analysis

Data are presented as mean ± standard deviation (SD). Experimental results are analyzed for their significance by the Student's *t*-test. Significance was established at the 95% confidence level ($p < 0.05$).

Results

Hepatocytes highly express NOD1, NOD2, and RIP2

We first wanted to determine whether primary isolated mouse hepatocytes expressed NOD1, NOD2, and RIP2 at baseline. We isolated RNA or collected whole-cell lysates from C57BL/6 mouse hepatocytes cultured overnight and performed quantitative PCR using validated specific primers for NOD1, NOD2, and RIP2 as well as Western blot analysis for NOD1, NOD2, and RIP2. Hepatocytes highly expressed both NOD1 and NOD2 mRNA and protein at baseline (Fig. 1A). Similarly RIP2 expression was also easily detected (Fig. 1A). Not all NLRs were expressed. IPAF, an NLR family member known to specifically recognize intracellular flagellin, was not expressed in hepatocytes (Fig. 1A).

We then wanted to determine whether expression of NOD1, NOD2, or RIP2 in hepatocytes changed over time after stimulation with LPS (TLR4-ligand), C12-iEDAP (NOD1 ligand), or MDP (NOD2 ligand). NOD1 and NOD2 mRNA expression increased significantly between 1 and 4 h after stimulation with 100 ng/ml LPS (Fig. 1B, left). However, the functional significance of this increase is hard to appreciate as the expression of both NOD1 and NOD2 is already high. However, there was a large increase in mRNA expression of RIP2 by 4 h after LPS stimulation (Fig. 1B, right), with levels nearly five times those at baseline. A similar pattern of change in expression of RIP2 mRNA was also seen after stimulation of hepatocytes with C12-iEDAP (Fig. 1C), suggesting that regulation of expression is not specific to one ligand or stimulus. NOD1 and NOD2 expression did not change, however, after stimulation for 24 h with C12-iEDAP. These data may suggest that regulation of NOD1 and NOD2 signaling in hepatocytes is primarily through regulation of levels of RIP2. There was no significant increase in expression of NOD1, NOD2, or RIP2 up to 24 h after stimulation of hepatocytes with MDP (Fig. 1D).

We then determined whether NOD1, NOD2, and RIP2 expression could be detected in mouse liver at baseline and at 24 h after intraperitoneal injection of LPS, MDP, or C12-iEDAP. No increase in expression of NOD1, NOD2, and RIP2 was measured in liver after LPS, C12-iEDAP, or MDP compared with baseline expression (data not shown). Even at 48 h no significant difference in NOD1, NOD2, or RIP2 mRNA expression was determined. The discrepancy between changes of expression in hepatocytes and whole liver may reflect different patterns of NOD1, NOD2, and RIP2 expression in different liver cell types following specific stimuli.