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LSECs express functional NOD1 receptors: A role for NOD1 in LSEC maturation-induced T cell immunity in vitro

Shunmei Huang[a], Jun Wu[a], Xiaoyan Gao[a], Shi Zou[a], Liwen Chen[a], Xilang Yang[a], Chan Sun[a], Yanqin Du[a], Bin Zhu[a], Jia Li[a], Xuecheng Yang[a], Xuemei Feng[a], Chunchen Wu[d], Chunwei Shi[e], Baoju Wang[a], Yinping Lu[a], Jia Liu[a], Xin Zheng[a], Feili Gong[c], Mengji Lu[b,**], Dongliang Yang[a,*]

[a] Department of Infectious Diseases, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, China
[b] Institute of Virology, University Hospital of Essen, Essen, Germany
[c] Department of Immunology, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China
[d] State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, China
[e] Department of Pathogen Biology, School of Basic Medicine, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China

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ABSTRACT

Liver sinusoidal endothelial cells (LSECs) are organ resident APCs capable of antigen presentation and subsequent tolerization of T cells under physiological conditions. In this study, we investigated whether LSEC pretreatment with NOD-like receptor (NLR) agonists can switch the cells from a tolerogenic to an immunogenic state and promote the development of T cell immunity. LSECs constitutively express NOD1, NOD2 and RIPK2. Stimulation of LSECs with DAP induced the activation of NF-κB and MAP kinases and upregulated the expression of chemokines (CXCL2/9, CCL2/7/8) and cytokines (IFN-γ, TNF-α and IL-2). Pretreatment of LSECs with DAP induced significantly increased IFN-γ and IL-2-production by HBV-stimulated $CD8^+$ T cells primed by DAP-treated LSECs. Consistently, a significant reduction in the HBV DNA and HBsAg level occurred in mice receiving T cells primed by DAP-treated LSECs. MDP stimulation had no impact on LSECs or HBV-stimulated $CD8^+$ T cells primed with MDP-treated LSECs except for the upregulation of PD-L1. DAP stimulation in vitro could promote LSEC maturation and activate HBV-specific T cell responses. These results are of particular relevance for the regulation of the local innate immune response against HBV infections.

1. Introduction

NOD-like receptors (NLRs), germline-encoded pattern recognition receptors (PRRs), play a crucial role in many inflammatory diseases in humans, highlighting their significant immunologic role (Coutermarsh-Ott et al., 2016). Toll-like receptors (TLRs) are a group of transmembrane receptors that are able to recognize a large variety of microbial-associated molecular patterns (MAMPs) from different pathogenic microorganisms and induce the activation of the innate immune system. NLRs recognize specific pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) or sense changes in the intracellular environment following virus infection (Coutermarsh-Ott et al., 2016; Caruso et al., 2014; Philpott et al., 2014). The NOD protein family comprises more than 20 members identified in humans and more than 30 in mice, including NOD1 and NOD2. NOD1 specifically recognizes γ-d-glutamyl-meso-diaminopimelic acid (iE-DAP) released from predominantly gram-negative bacterial organisms (Travassos et al., 2010; Moreira and Zamboni, 2012; Kim et al., 2008). NOD2 recognizes uramyl dipeptide (MDP), a peptidoglycan (PGN) motif widely distributed among both gram-positive and gram-negative

Abbreviations: HBV, Hepatitis B virus; LSECs, liver sinusoidal endothelial cells; NLR, NOD-like receptors; TLR, toll-like receptors; DAP, diaminopimelic acid; RIPK2, receptor-interacting serine-threonine protein kinase 2; NF-κB, nuclear factor-κB; MAPK, mitogen-activated protein kinase; IFN-α, alpha interferon; PRR, pattern recognition receptor; MAMPs, microbial-associated molecular patterns; PAMPs, pathogen-associated molecular patterns; DAMPs, damage-associated molecular patterns; HI, hydrodynamic injection; NOD, nucleotide-binding oligomerization domain; IFN, interferon; IL, interleukin; TNF-α, tumour necrosis factor alpha; APCs, antigen-presenting cells

* Corresponding author at: Department of Infectious Diseases, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Jiefang Avenue 1277, 430030, Wuhan, China.
** Corresponding author at: Institute of Virology, University Hospital of Essen, Virchowstraße 179, D-45147 Essen, Germany.
E-mail addresses: hshmfy2005@163.com (S. Huang), junwu77@hust.edu.cn (J. Wu), gaoxiaoyan19870308@163.com (X. Gao), d201578179@hust.edu.cn (S. Zou), chenlw07@taikangre.com (L. Chen), yxl1512107@163.com (X. Yang), zhi.qingshui@163.com (C. Sun), m201575404@hust.edu.cn (Y. Du), 2014xh0843@hust.edu.cn (B. Zhu), leejia@hust.edu.cn (J. Li), xcyang@hust.edu.cn (X. Yang), fxm109@163.com (X. Feng), wucc@wh.iov.cn (C. Wu), chunweishi@hust.edu.cn (C. Shi), bjwang73@163.com (B. Wang), yinpinglu@163.com (Y. Lu), jialiu77@hust.edu.cn (J. Liu), xin11@hotmail.com (X. Zheng), flgong@163.com (F. Gong), mengji.lu@uni-due.de (M. Lu), dlyang@hust.edu.cn (D. Yang).

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bacteria, as well as DNA and RNA virus genomes (Sabbah et al., 2009; Kim et al., 2011). Recent studies using a HEK293 transfection system, dendritic cells and hepatocytes demonstrated that NOD1 and NOD2 associate with receptor-interacting protein kinase 2 (RIPK2)/RICK via CARD-CARD interactions, which allow RIPK2 to associate with TRAF6/TAK1, subsequently leading to the activation of NF-κB and MAPK and the upregulation of inflammatory mediators such as interleukin (IL)-6 and tumour necrosis factor alpha (TNF-α) (Tukhvatulin et al., 2011; Scott et al., 2010; Hasegawa et al., 2008a; Lupfer et al., 2014). Moreover, following the recognition of viral ssRNA genomes in 293 cells and A549 cells, NOD2 utilized the adaptor protein MAVS (mitochondrial antiviral signalling) to activate IRF3, leading to the production of interferon-β (IFN) (Sabbah et al., 2009). However, the immune responses elicited by Nod stimulation in non-parenchymal liver cells are poorly characterized.

The liver is a sentinel organ in a unique position to induce tolerance rather than immunity towards antigens that are presented locally to T cells (Knolle and Limmer, 2001; Knolle and Thimme, 2014; Kern et al., 2010a). The immune response in the liver is regulated by the unique hepatic microenvironment rich in gut-derived food and bacterial degradation products and by liver resident cells with unique functions, such as liver sinusoidal endothelial cells (LSECs), hepatocytes, Kupffer cells (KCs) and DCs, which are involved in antigen-specific tolerance induction (Breous et al., 2009; Crispe, 2003). Among these different cell populations, LSECs are particularly important because they are strategically located in the liver sinusoids to interact with passenger leukocytes, which constitute a unique organ-resident cell population that bears APC function, induces CD8 + T-cell tolerance, and takes up more hepatotropic viruses (such as HBV and hepatitis C virus) from the blood than other hepatic cells during viral infection (Limmer et al., 2000; Kern et al., 2010b). Tolerance induction by LSECs can be broken by different regulatory mechanisms, such as exogenous IL-2 and promotion of CD28 signalling, which can overcome PD-L1-mediated inhibitory signals, interfere with LSEC-induced T cell tolerance, and induce IFN-γ production by Th1 cells (Schurich et al., 2010). Palmitoyl-3-cysteine-serine-lysine-4 (P3C; TLR1/2 ligand) pretreatment of LSECs induces proliferation and IFN-γ production of allogeneic or Ag-specific $CD4^+$ T or $CD8^+$ T cells mediated by IL-12 production (Liu et al., 2013). Viral infection with murine cytomegalovirus caused functional maturation of antigen-presenting LSECs and was sufficient to promote antigen-specific differentiation into effector $CD8^+$ T cells, independent of CD80/86, CD40/CD40 L, or IL-12 (Kern et al., 2010b; Bottcher et al., 2011). However, it is still unclear whether the ligation of NLRs can cause the functional maturation of LSECs and revert their suppressive properties to induce T cell immunity.

In this study, we sought to determine whether LSECs express functional NOD receptors and whether NOD expression is altered in response to specific NOD ligands. We found that LSECs could respond to NOD1 ligands to activate NF-κB and MAPK, resulting in LSEC maturation and T cell activation, which mediated anti-HBV function both in vitro and in vivo.

2. Materials and methods

2.1. Animals

Wild-type C57BL/6 mice were obtained from Beijing HFK Bioscience CO. LTD (Beijing, China). The mice were used at 6–8 weeks of age. The animals were treated according to the Guidelines of the National Institutes of Health for Animal Care and Use. Ten micrograms of pSM2 (kindly provided by Mengji Lu from University Hospital of Essen, Essen, Germany) and pAAV-HBV1.2 (kindly provided by Professor Pei-Jer Chen from National Taiwan University College of Medicine, Taipei, Taiwan) were injected into the tail veins of 6- to 8-week-old mice in a volume of saline equivalent to 10% of the body mass of the mouse. The total volume was delivered within 5 to 8 s. The success of hydrodynamic injection (HI) was controlled by testing the levels of HBsAg and HBeAg in the serum 10 days post-HI. Mice negative for HBeAg were excluded from the experiments.

2.2. Reagents

Agonists for TLR3 (Poly(I:C)), NOD1 (C12-iE-DAP) and NOD2 (MDP) were purchased from InvivoGen (San Diego, CA).

2.3. LSEC isolation and cell culture

Isolation of LSECs and splenocytes was performed as described previously (Wu et al., 2007). The purity of the cell fractions was monitored by flow cytometry and was greater than 98% in all cases. All cell fractions contained less than 5% dead cells after the separation procedure.

2.4. Flow cytometry

Cell-surface and intracellular staining for flow cytometric analysis was performed using BD Biosciences (Heidelberg, Germany) or eBioscience (Frankfurt, Germany) reagents. Intracellular IFN-γ and IL-2 staining was performed as described (Zelinskyy et al., 2006). Data were acquired using a FACSCalibur flow cytometer (BD Biosciences) and analysed using FlowJo software (TreeStar, Ashland, OR). Cell debris and dead cells were excluded from the analysis based on scatter signals and 7-aminoactinomycin D (7AAD) fluorescence or Fixable Viability Dye eFluor 780 fluorescence.

2.5. Cytokine assays

LSECs treated without or with Poly(I:C), DAP and MDP were cultured at 2×10^6 cells/well in a total volume of 200 µl. Cell-free supernatants were collected and subjected to assays to measure IFN-γ, IL-6, IL-10, IL-2, IL-4, IL-17 A and TNF-α production using cytometric bead array (CBA) kits (BD Biosciences) and to detect IFN-α, IFN-β and TGF-β using commercially available kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

2.6. Analysis of T cell function

Allogeneic responder T cells were isolated from BALB/c mice. The Poly(I:C)-, DAP- or MDP-stimulated or unstimulated LSECs were treated with mitomycin C (50 µg/ml) at 37°C for 30 min followed by washing with medium and resuspension in an appropriate volume of culture medium. $CD8^+$ T cells were isolated from mice in a stage of acute HBV replication at 21 d post-HI. CFSE (Invitrogen, Darmstadt, Germany)-labelled allogeneic T cells or $CD8^+$ T cells were coincubated with LSECs in the presence of HBsAg or HBcAg for either 3 d or 5 d and analysed for activation/proliferation by flow cytometry. For the determination of the maturation of LSEC function, corresponding peptide-loaded LSECs were cocultured with HBV-stimulated $CD8^+$ T cells from mice in a stage of acute HBV replication for either 3 or 5d. After 5 d, intracellular IFN-γ and IL-2 staining was performed to determine the percentage of IFN-γ and IL-2-producing T cells. The ratio of T cells to LSECs/DCs was 2:1.

2.7. Western blotting

Cells were lysed in 1 x SDS loading buffer. Aliquots of proteins from cell lysates were electrophoresed on 10% (w/v) polyacrylamidegels and transferred onto nitrocellulose membranes (Schlei-cher & Schuell, Kassel, Germany). The blot was probed with antibodies for phosphorylated forms of p65 or p38 MAPKs (Cell signalling technology) or β-actin (Sigma, St. Louis, MO, USA) and HRP-conjugated secondary antibodies were applied. Then the membranes were washed with PBS with 0.1% Tween 20, incubated in LumiLight working solution (GE