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TLR4-independent and PKR-dependent interleukin 1 receptor antagonist expression upon LPS stimulation

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ABSTRACT

Dendritic cells (DCs) induce innate immune responses by recognizing bacterial LPS through TLR4 receptor complexes. In this study, we compared gene expression profiles of TLR4 knockout ($TLR4^{neg}$) DCs and wild type ($TLR4^{pos}$) DCs after stimulating with LPS. We found that the expression of various inflammatory genes by LPS were TLR4-independent. Among them, interleukin 1 receptor antagonist (IL-1rn) was of particular interest since IL-1rn is a potent natural inhibitor of proinflammatory IL-1. Using RT-PCR, real-time PCR, immunoblotting and ELISA, we demonstrated that IL-1rn was induced by DCs stimulated with LPS in the absence of TLR4. 2-Aminopurine, a pharmacological PKR inhibitor, completely abrogated LPS-induced expression of IL-1rn in $TLR4^{neg}$ DCs, suggesting that LPS-induced TLR4-independent expression of IL-1rn might be mediated by PKR pathways. Considering that IL-1rn is a physiological inhibitor of IL-1, TLR4-independent and PKR-dependent pathways might be crucial in counter-balancing proinflammatory effector functions of DCs resulted from TLR4-dependent activation by LPS.

1. Introduction

The incidence of sepsis increases by 1.5% annually [1]. Various symptoms of sepsis patients generally come from the activation of the innate immune system by bacterial endotoxin (LPS). LPS activates many types of cells involved in the innate and adaptive immune systems [2,3]. Dendritic cells (DCs) and macrophages play key roles in the activation of a host's innate immune responses. They release an array of proinflammatory cytokines and chemokines upon LPS stimulation, such as IL-1 [4], IL-6 [5,6], TNF-α [7], IL-12 [8], IFN-γ [9], MIP-1 [10] and RANTES [11], etc. Overproduction of these proinflammatory mediators is a hallmark of sepsis. Numerous trials targeting LPS and proinflammatory mediators such as TNF-α, have shown promising outcomes in animal models, but failed for sepsis patients [12]. To ameliorate systemic inflammation, anti-IL-1 antibody has been tried in numerous clinical settings of sepsis [13]. However, this strategy showed low efficacy for the sepsis patients [12]. Better understanding of LPS-mediated immune activation might expedite the development of novel therapeutics for sepsis.

Signal transduction pathways by LPS are mediated by receptor complexes consisting of TLR4, CD14 and MD2 [14]. Interaction of LPS with its receptor complexes results in the production of proinflammatory mediators [15]. In addition, it has been reported that LPS interacts with various surface receptors other than TLR4 (such as CD55, chemokine receptor (CXCR4) and integrin CD11/CD18 as well as heat shock proteins), indicating further complexity of the signal transduction pathways mediated by LPS [16]. Incidentally, we found that expression of $I\text{-}A^b$ molecule on $TLR4^{neg}$ DCs was not completely abrogated upon LPS stimulation. This prompted us to investigate molecules modulated by LPS in the absence of TLR4.

Since molecules contaminated in LPS preparation might be responsible for activation of DCs, we used highly purified LPS derived from *Escherichia coli* and *Salmonella*. The phenol–chloroform–petroleum ether (PCP) extraction method has been used to isolate LPS from bacteria. However, it was shown that a high percentage of PCP-purified LPS preparations contain significant levels of protein contaminants with endotoxin-like activity [14]. A modified phenol–water extraction procedure was developed to obtain protein-free LPS from the original PCP-extracted preparations [17]. The repurified LPS was known to contain no detectable protein and to stimulate normal C3H/OuJ macrophages to secrete TNF-α [17] and IL-6 [18]. However, it lost >99% of its activity in stimulating TNF-α secretion by TLR4 mutant C3H/HeJ macrophages. Based on the analysis of repurified LPS, contaminants in 1.0 mg/ml of LPS were proteins (0.28%) and nucleic acids (0.62%). Since we used 200 ng/ml of LPS, the proteins and nucleic acids in LPS used in this study were diluted 5000-fold, resulting in lower than 560 pg/ml of proteins and 1.2 ng/ml of nucleic acids. For these reasons, the effect of protein and nucleic acid contaminations

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might be negligible for TLR4-independent activation of DCs in this study.

Using the repurified LPS, we investigated gene expression profiles of $TLR4^{neg}$ DCs by microarray. Microarray data revealed that $TLR4^{neg}$ DCs have five clusters of genes distinct from $TLR4^{pos}$ DCs upon LPS stimulation. Among them, interleukin 1 receptor antagonist (IL-1rn) was of particular interest since it is an endogenous antagonist of IL-1, one of the most critical proinflammatory cytokines in the development of sepsis.

In this study, we report that IL-1rn was transcriptionally and translationally induced by DCs stimulated with LPS in the absence of TLR4, and the TLR4-independent expression was dependent on PKR pathway. These results imply that the balance maintaining the steady-state conditions for DCs might be partly regulated by TLR4-dependent expression of proinflammatory cytokines and TLR4-independent expression of anti-inflammatory cytokines such as IL-1rn.

2. Experimental procedures

2.1. Animals and cells

C57BL/6 (B6) mice and $Rag2^{neg}$ mice were obtained from Taconic Farms, Inc. (Hudson, NY). TLR4 knockout ($TLR4^{neg}$) mice were kindly provided by Dr. S. Akira (Osaka University, Osaka, Japan). Rag2 and TLR4 double knockout ($Rag2^{neg}TLR4^{neg}$) mice were generated by breeding $Rag2^{neg}$ mice with $TLR4^{neg}$ mice. In this study, TLR^{pos} DCs were prepared from $Rag2^{neg}$ mice and $TLR4^{neg}$ DCs were from $Rag2^{neg}TLR4^{neg}$ mice. Primer sequences used for genotyping are listed in Table 1. Mice were maintained in a specific pathogen-free environment at the Center for Animal Resource Development, Seoul National University College of Medicine. All animals were used in accordance with the Policy and Regulation for the Care and Use of Laboratory Animals (Laboratory Animal Center, Seoul National University, Korea).

2.2. Reagents

LPSs derived from *E. coli* 0111:B4 (eLPS) or *Salmonella minnesota* R595 (sLPS) are highly purified and protein-free extracts [17]. We purchased them from List Biological Laboratories (Campbell, CA). Protease inhibitors were obtained from Roche (Indianapolis, IN). Goat polyclonal anti-IL-1rn antibody (clone M-20, sc-8482) and donkey anti-goat IgG-HRP (sc-2020) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). 2-Aminopurine (2-AP) was obtained from Sigma (St. Louis, MO).

2.3. Microarray

To see if the signaling was induced by LPS in the absence of TLR4, we first activated $TLR4^{neg}$ DCs or $TLR4^{pos}$ DCs with eLPS and compared the gene expression profile with that of resting $TLR4^{neg}$ DCs or resting $TLR4^{pos}$ DCs, respectively. We examined gene expression profiles of DCs stimulated with LPS by microarray assay. Briefly, we stimulated $TLR4^{pos}$ or $TLR4^{neg}$ DCs with eLPS (100 ng/ml) for 18 h and extracted total RNA. Total cellular RNA was obtained using RNeasy protect mini kit according to the manufacture's procedure (Qiagen, Valencia, CA), and then amplified using the RNA amplification kit (Applied Biosystems/Ambion, Austin, TX) according to the manufacturer's instructions. Amplified RNA from medium-treated DCs or LPS-treated DCs was labeled with Cy3 and Cy5, respectively. We utilized a custom service hybridization of labeled RNA to mouse microarray chip and scanning (NIAID, NIH, Bethesda). For analysis, GenPix pro software and clustering software (mAdB in NIAID) were used.

Table 1
Primer sequences.

Genes		Primer sequence	Length of amplified fragments (bp)
mβ-Actin	Forward	5′-GAGCACAGCTTCTTTGCAGC-3′	266
	Backward	5′-GGGGTACTTCAGGGTCAGGA-3′	
mTLR4	Forward	5′-AGGACTGGGTGAGAAATG-3′	414
	Backward	5′-GATTCGAGGCTTTTCCATC-3′	
mRAG2	Forward	5′-ATGTCCCTGCAGATGGTAA-3′	246
	Backward	5′-ATGTCCCTGCAGATGGTAA-3′	
Neomycin	Forward	5′-AGGTGAGATGACAGGAGA-3′	273
	Backward	5′-CTTGGGTGGAGAGGCTAT-3′	
IL-1rn	Forward	5′-AACCACCAGGGCATCACATA-3′	150
	Backward	5′-CCTCTTGCCGACATGGAATA-3′	

2.4. Activation of bone marrow-derived DCs

Bone marrow-derived DCs were purified from $Rag2^{neg}$ ($TLR4^{pos}$) mice or $Rag2^{neg}TLR4^{neg}$ ($TLR4^{neg}$) mice. Briefly, bone marrow from the femurs and tibias of mice was flushed with complete IMDM [IMDM (Invitrogen, Carlsbad, CA) supplemented with 50 nM 2-mercaptoethanol (ME) (Invitrogen), 10% heat-inactivated FBS, 2 mM L-glutamine, antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin and 50 μg/ml gentamycin), recombinant mouse GM-CSF (0.75 ng/ml) and mouse IL-4 (1.5 ng/ml; PeproTech, Rocky Hill, NJ)]. Cells were washed three times with complete IMDM, suspended in medium at 10^6 cells/ml and plated in 24-well culture plates. Half of the medium was replaced every other day with an equal volume of complete IMDM. At day 6 of culture, immature DCs were stimulated with 200 ng/ml of LPS for an additional 18 h.

2.5. RT-PCR

Total cellular RNA was obtained using RNeasy protect mini kit according to the manufacture's procedure (Qiagen, Valencia, CA). Total RNA (0.5 μg) was reverse transcribed using Omniscript RT kit (Qiagen). After incubation at 37 °C for 1 h, the samples were stored at −20 °C until use. For end-point RT-PCR, cDNA was amplified in a final volume of 20 μl containing 200 nM primers, 17 μl of Platinum PCR supermix (Invitrogen) for 30 cycles of 94 °C for 30 s, 59.2 °C for 30 s, and at 72 °C for 1 min. Intensity of the PCR product run on 1.5% agarose gel was normalized to that of β-actin using Image-J software (NIH, Bethesda, MD). For real-time RT-PCR analysis, cDNA was amplified in a final volume of 20 μl containing 200 nM primers, 10 μl of 2× SYBR Green PCR mastermix (Applied Biosystems) using Applied Biosystems 7500 Real-Time PCR system. Threshold cycle number was determined using the software provided by the manufacturer (Applied Biosystems, Foster City, CA). The expression level of mouse IL-1rn was calculated by the $2^{-\Delta\Delta C_T}$ method [19] and then normalized to that of mouse β-actin. Primer sequences used for RT-PCR are listed in Table 1.

2.6. Immunoblotting

Whole cell lysates (WCL) of DCs stimulated with LPS for 18 h were prepared by lysing with mammalian cell lysis buffer (20 mM Tris–Cl (pH 7.4), 1 mM EDTA, 1% NP-40, protease inhibitor). WCL was loaded onto a 15% SDS–PAGE, followed by transfer onto a PVDF membrane (Amersham Biosciences, Piscataway, NJ). The membrane was incubated with goat anti-IL-1rn antibody in TBST (TBS containing 5% skim milk and 0.1% Tween 20), followed by donkey anti-goat IgG-HRP. An immunoblot was developed by use of Fuji LAS 2000 imaging system (Fujifilm, Tokyo, Japan) and Supersignal West Chemilluminescence reagents (Pierce, Rockford, IL).