Mice Plasmacytoid Dendritic Cells Were Activated by Lipopolysaccharides Through Toll-Like Receptor 4/Myeloid Differentiation Factor 2

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Plasmacytoid dendritic cells (pDCs) are known to respond to viral infections. However, the activation of pDCs by bacterial components such as lipopolysaccharides (LPS) has not been well studied. Here, we found that pDCs, conventional dendritic cells (cDCs), and B cells express high levels of toll-like receptor 4 (TLR4), a receptor for LPS. Moreover, LPS could effectively bind to not only cDCs but also pDCs and B cells. Intraperitoneal administration of LPS promoted activation of splenic pDCs and cDCs. LPS treatment led to upregulation of interferon regulatory factor 7 (IRF7) and induced production of interferon-alpha (IFN-α) in splenic pDCs. Furthermore, LPS-dependent upregulation of co-stimulatory molecules in pDCs did not require the assistance of other immune cells, such as cDCs. However, the production levels of IFN-α were decreased in cDC-depleted splenocytes, indicating that cDCs may contribute to the enhancement of IFN-α production in pDCs. Finally, we showed that activation of pDCs by LPS requires the TLR4 and myeloid differentiation factor 2 (MD2) signaling pathways. Thus, these results demonstrate that the gram-negative component LPS can directly stimulate pDCs via TLR4/MD2 stimulation in mice.

Keywords: lipopolysaccharide, plasmacytoid dendritic cell, conventional dendritic cell, toll-like receptor 4, myeloid differentiation factor 2
INTRODUCTION

Lipopolysaccharides (LPS) are lipid polysaccharides present in the outer membrane of gram-negative bacteria and are known to stimulate the immune system (1, 2). Amongst the three structural domains, lipid A (also known as the endotoxin) is primarily responsible for the immunostimulatory activity of LPS (3, 4). LPS is a classical pathway-associated molecular pattern (PAMP) that can be recognized by innate immune cells through the toll-like receptor 4 (TLR4) (5). Upon interacting with LPS, TLR4 forms a heterodimer with an extracellular adaptor glycoprotein named myeloid differentiation factor 2 (MD2) and induces two distinct signaling cascades (6, 7). The first signaling pathway depends on myeloid differentiation primary response 88 (MyD88) and induces to the secretion of inflammatory cytokines by activating nuclear transcription factor κB (NF-κB) in innate immune cells, whereas the second pathway is independent of MyD88 and mediates interferon regulatory factor 3 (IRF3) activation to induce type-I interferon (IFN) responses (8, 9).

TLR4 is the crucial receptor of the mammalian innate immune system and can be expressed by various types of immune cells (10). Moreover, it is highly expressed by antigen-presenting cells (APCs) such as macrophages, dendritic cells (DCs), and B cells (11). Numerous studies have reported that stimulation with LPS induces the activation of these APCs. To elaborate, murine B cells show stronger cell proliferation, cytokine secretion, and class switch recombination in response to LPS stimulation (12, 13). Whereas in case of macrophages, the TLR4 stimulation promotes to the activation of these cells, which leads to the secretion of inflammatory cytokines in the macrophages (14, 15). Furthermore, after sensing LPS via TLR4, DCs not only undergo maturation and migration but also show improved regulation of the adaptive immune responses (16, 17).

DCs are professional APCs that capture antigens and then process and present them to T cells (18–21). They can be divided into two major subsets: plasmacytoid DCs (pDCs), which specialize in antiviral defense by producing interferon-α (IFN-α), and conventional DCs (cDCs), which are essentially responsible for antigen-presentation and T-cell activation (22–24). Although it is still controversial, the pDCs may be more efficient at presenting endogenous antigens rather than exogenous antigens, such as viral proteins (25). By utilizing pattern-recognition receptors (PRRs), such as TLR7 and TLR9 that bind to viral nucleic acids, pDCs detect virus invasion and produce large amounts of IFN-α (26). However, fewer studies have focused on the response of pDCs against bacterial infection and the expression of TLR4 in the surface of pDCs, and on the effect of TLR4 ligands on pDC activation.

Our previous research showed that monophosphoryl lipid A (MPLA) induces the activation of pDCs and has a synergistic effect on anti-PD-L1-antibody-mediated anti-cancer immunity (27). MPLA is a detoxified form of LPS that stimulates TLR4 and leads to the activation of immune cells. However, the molecular details of the MPLA dependent activation of pDCs have not been studied thus far. Therefore, we hypothesized that pDCs may express considerable levels of TLR4 and that LPS may stimulate pDCs either directly or indirectly, as a result of the cytokines expressed by other immune cells. In the following study, we treated mice with LPS and characterized the molecules responsible for LPS-dependent activation of pDCs.

MATERIALS AND METHODS

Mice

Female C57BL/6 mice (6 to 8 weeks) were obtained from Korea Orient Bio Inc. (Gyeonggi-do, Korea) and Shanghai Public Health Clinical Center (SPHCC, Shanghai, China). TLR2-knockout (KO), TLR4-KO, and B6.129P2-Ly96-KO (MD2-KO) mice were provided by SPHCC. The mice were maintained either in the Laboratory Animal Center of SPHCC or at Yeungnam University, under 50–60% humidity and at 20–22°C. This study was approved by the Ethics of Animal Experiments Committee of Yeungnam University (2020–015) and SPHCC (2018-A049-01).

Reagents and Antibodies

LPS (O111:B4) and FITC-conjugated LPS were purchased from Sigma-Aldrich (St. Louis, MO, USA). TLR4 Agonist-Ultrapure LPS (055:B5) and CpG-1826 were obtained from Invivogen (San Diego, CA, USA). The following fluorescence-conjugated antibodies (Abs) were provided by BioLegend (San Diego, CA, USA) and were used for flow cytometry analysis: anti-B220 (RA3-6B2), anti-CD11c (N418), anti-CD3 (17A2), anti-CD317 (927), anti-CD40 (3/23), anti-CD80 (16-10A1), anti-CD86 (GL-1), anti-IRF7 (MNGPKL), and anti-TLR4 (SA15-21). Anti-IFN-α (RMMA-1) Ab was purchased from pbl Assay Science (Piscataway, NJ, USA). Anti-class I major histocompatibility complex (MHC) Abs (28–8–6) and anti-class II MHC (M5/114.15.2) Abs were purchased from eBioscience (San Diego, CA, USA).

Analysis of Mouse pDCs and cDCs

pDC and cDC activation was analyzed as described elsewhere (27, 28). The spleens were harvested after intraperitoneal (i.p.) administration of 0.1 mg/kg LPS or 10 mg/kg CpG to C57BL/6 mice and were then digested with 2% FBS, collagenase IV, and DNase containing digestion buffer for 20 min at 37°C. After filtering with 100-nm nylon mash, the cells were resuspended in 5 ml of Histopaque-1077 (Sigma-Aldrich) and layered over 3 ml of fresh Histopaque-1077, and 1 ml of FBS was then added above on the top. The cells were centrifuged at 1700 × g for 10 min to harvest the leukocytes (<1.077 g/cm³). Leukocytes were

Abbreviations: LPS, lipopolysaccharide; cDC, conventional dendritic cell; pDC, plasmacytoid dendritic cell; TLR4, toll-like receptor 4; IFN-α, interferon-alpha; IL, interleukin; TNF, tumor necrosis factor; MD2, myeloid differentiation factor 2; IRF7, interferon regulatory factor 7; Ab, antibody; MyD88, myeloid differentiation primary response 88; NF-κB, nuclear transcription factor-κB; P8S, phosphate buffered saline; APCs, antigen-presenting cells; MHC, major histocompatibility complex; CTL, cytotoxic T lymphocyte; PRRs, pattern-recognition receptors; MPLA, monophosphoryl lipid A; KO, knockout.
incubated with unlabeled isotype control Abs and Fc-block Abs for 15 min and then stained with anti-CD11c, anti-CD317, and lineage Abs such as anti-CD3 (17A2), anti-CD49b (DX5), anti-CD90.1 (OX-7), anti-B220 (RA3-6B2), anti-Gr-1 (RB68C5), and anti-TER-119 (TER-119). In addition, the cells were stained with anti-CD40 (3/23), anti-CD80 (16-10A1), anti-CD86 (GL-1), anti-class I MHC (28–8–6), and anti-class II MHC (M5/114.15.2) Abs to determine cell activation. Following a second wash with PBS to remove the unbound Abs, the cells were resuspended in 50 μg/ml 4’,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) containing PBS. The cells were analyzed using a Novocyte flow cytometer (ACEA Biosciences Inc., San Diego, CA, USA) after gating out DAPI-positive cells as dead cells. The cDCs and pDCs in splenocytes were identified in live leukocytes by flow cytometry and defined as lineage CD11c+ cells and CD317+B220+ cells, respectively.

Intracellular Cytokine Staining

Intracellular cytokine production was analyzed as described previously (29, 30). C57BL/6 mice were injected i.p. with PBS, 0.1 mg/kg LPS, and 10 mg/kg CpG. Twelve hours after the injection, splenocytes were harvested and incubated with 2 μM monensin solution (BioLegend) for 2 h. After washing with PBS, the cells were stained with surface Abs followed by labeling with the Zombie Violet Fixable Viability Kit (BioLegend) at 25°C for 20 min to remove dead cells. The cells were fixed with a fixation buffer (BioLegend) at 4°C for 20 min and then stained with intracellular staining Abs in permeabilization buffer (BioLegend) at 25°C for 15 min. After washing with PBS, the cells were analyzed using a Novocyte flow cytometer (ACEA Biosciences Inc.). IFN-α and IRF7 expression levels were analyzed in CD317+B220+ pDCs.

ELISA

The IFN-α concentration in serum or cultured media was measured in triplicates using ELISA kits from BioLegend. For the serum concentration of IFN-α, the mice received PBS, 0.1 mg/kg LPS, and 10 mg/kg CpG. Twelve hours after the injection, blood sera were harvested from the mice. IFN-α concentration in the cultured media was analyzed from LPS-stimulated enriched pDCs, splenocytes, or cDC-depleted splenocytes 12 h after LPS stimulation.

Isolation of pDCs

The pDCs were isolated from splenocytes using a pDC isolation kit (Miltenyi Biotec, Auburn, CA, USA). The pDC isolation purity was determined via flow cytometry, and the purity of CD317+B220+ pDCs was higher than 90%.

Depletion of cDCs

The cDCs in splenocytes were stained with an anti-CD11c-biotin Ab (BioLegend). The cells were then stained with a microbead-conjugated anti-biotin Ab (Miltenyi Biotec) for 15 min. The CD11c+ cDCs were removed by negative selection using an LD column (Miltenyi Biotec). The efficacy of CD11c+ cDC depletion was >98%.

Statistical Analysis

All data are expressed as mean ± standard error of the mean (SEM). One- or two-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test and Mann-Whitney U-test were used for the analysis of datasets with the help of SPSS software (IBM, Armonk, NY, USA). p < 0.05 was considered to be statistically significant.

RESULTS

LPS Binds to pDCs

To identify TLR4-expressing APCs in splenocytes, we gated TLR4+ and MHC class II+ cells. The TLR4+MHC class II+ cell population included CD11c+ cDCs, B220+ B cells, and CD317+B220+ pDCs (Figure 1A). Although, the TLR4 expression levels in pDCs was lower than that in cDCs and B cells (Figure 1B). In addition, we observed that FITC-conjugated LPS could efficiently bind to pDCs, cDCs, and B cells (Figure 1C). Thus, our data indicate that pDCs express considerable levels of TLR4 on their surface, and that LPS can bind to pDCs in mouse splenocytes.

LPS Induces the Upregulation of Activation Markers in pDCs

After establishing that LPS is able to bind to pDCs, we next examined whether LPS can induce the in vivo activation of these cells. C57BL/6 mice were treated i.p. with PBS, LPS (0.1 mg/kg), and CpG (10 mg/kg), and the splenic pDCs and cDCs in the live leukocytes were defined as B220+CD317+ and lineage CD11c+ cells, respectively (Figure 2A). LPS administration induced the upregulation of CD40, CD80, CD86, and class I and II MHC expression in both pDCs and cDCs, 12 h after injection (Figures 2B, C). LPS was able to upregulate the co-stimulatory molecules with a higher efficacy than CpG, a positive control for pDC activation (Figures 2B, C). In the mouse in vitro study, LPS exerted a considerably higher effect on the induction of pDC and cDC activation than CpG (Figure S1). The highest levels of co-stimulatory molecules in pDCs were recorded 12 h after LPS treatment, while those in cDCs peaked 18 h after LPS treatment (Figure S2). However, the expression of MHC class I and II in both pDCs and cDCs increased dramatically 3 h after LPS treatment, and there after decreased gradually (Figure S2). In addition, we examined whether LPS can induce the activation of liver and thymic pDCs and found that LPS treatment dramatically upregulated the expression levels of co-stimulatory molecules and class I and II MHC in both liver and thymic pDCs (Figure S3). In conclusion, our data suggest that treatment with LPS induces activation of pDCs in mice in vivo.

LPS Induces IFN-α Production in pDCs

Since it is well known that activated pDCs produce IFN-α (31–33), we studied IFN-α production in LPS activated pDCs and observed an increase in the levels of intracellular IFN-α (Figure 3A). The concentration of IFN-α in serum was also significantly increased in LPS-treated mice in comparison to the control mice (Figure 3B). In addition, LPS treatment also led to a
remarkable increase in IFN-α regulatory protein interferon regulatory factor 7 (IRF7) levels in pDCs (Figure 3C). Although the effect of LPS on IFN-α production was lower than that of CpG, the increase in IFN-α production in LPS-treated pDCs was significant (Figures 3A, B). These data suggest that LPS can promote IFN-α production in mice pDCs.

**LPS Directly Upregulates the Surface Activation Markers in pDCs**

Since cDCs can mediate the activation of other immune cells (18, 27, 34), we tried to ascertain if cDCs were required for the LPS-dependent activation of pDCs. The splenocytes were depleted of cDCs and then treated with 0.1 mg/ml LPS (Figure S5). In both total splenocytes (+cDCs) and cDC-depleted splenocytes (−cDCs), LPS treatment led to a significant increase in the expression of co-stimulatory molecules, and MHC class I and II (Figure 4A). Next, we also examined the effect of LPS on isolated pDCs (Figure S6) and found that LPS promoted their activation (Figure 4B). These data indicate that the increased expression of activation markers in pDCs by LPS does not require interaction with cDCs. In addition, LPS stimulation led to an increased IFN-α production in isolated pDCs, total splenocytes (+cDCs) and cDC-depleted splenocytes (−cDCs) (Figure 4C). Moreover, LPS-activated total splenocytes showed greater IFN-α production than isolated pDCs and cDC-depleted splenocytes (Figure 4C). Thus, these data suggest that LPS directly induces upregulation of co-stimulatory molecules in pDCs without interacting with other cells, especially cDCs. However, IFN-α production in pDCs in response to LPS may be influenced by the activation of cDCs.

**LPS-Induced Activation of pDCs Require TLR4 and MD2**

TLR4 and MD2 are the key receptors that are required in LPS-induced activation of cDCs (6, 7). To determine if this was also the case for LPS-stimulated pDCs, we i.p. injected 0.1 mg/kg LPS in C57BL/6, TLR4-KO, and MD2-KO mice. We observed that FITC-conjugated LPS was unable to bind to the pDCs in TLR4-KO and MD2-KO mice (Figure 5A). Moreover, LPS treatment did not lead to an increase in the serum concentration of IFN-α in TLR4-KO and MD2-KO mice (Figure 5B). The IRF7 expression levels were not increased in TLR4-KO and MD2-KO pDCs in response to LPS (Figure 5C). Furthermore, LPS did not affect the expression of co-stimulatory molecules and class I and II MHC in the pDCs of TLR4-KO and MD2-KO mice (Figure 5D). LPS from Sigma-Aldrich used in this study could stimulate TLR4 as well as TLR2. We confirmed this result using ultrapure LPS and data showed similar effects on the activation of pDCs by ultrapure LPS as well as that from Sigma-Aldrich (Figure S4). Moreover, LPS promoted the upregulation of these molecules in the pDCs of TLR2-KO mice (Figure S7). Therefore, these data suggest that LPS-induced pDC activation is dependent on the TLR4/MD2 pathway.
DISCUSSION

Being a member of the DC family, pDCs can serve as a connecting link between the innate and adaptive immune system (35). Moreover, pDCs typically act as sensors of viral infections by producing large amounts of type I IFN and generating strong antiviral responses (24, 26). However, when compared to cDCs, due to the low expression of MHC and costimulatory molecules, pDCs are not efficient at presenting antigens and mediating T cell activation. They become potent APCs upon proper stimulation with TLR ligands, such as the TLR9 agonist CpG and TLR7 agonist imiquimod (36, 37). In addition, it has been demonstrated that human pDCs express TLR1/2. The TLR1 mechanism contributes to the upregulation

FIGURE 2 | LPS induced the activation of pDCs and cDCs in mice. C57BL/6 mice were treated intraperitoneally (i.p.) with 0.1 mg/kg LPS and 10 mg/kg CpG. The mice were sacrificed, and spleen was harvested 12 h after treatment. (A) Gating strategy for splenic pDCs and cDCs was shown. (B) CD40, CD80, CD86, and MHC class I and II expression levels in pDCs (upper panel) and cDCs (lower panel) were shown. (C) MFI of the indicated surface marker expression in pDCs (upper panel) and cDCs (lower panel) was shown (n = 6 mice, two-way ANOVA, mean ± SEM, **p < 0.01).
of costimulatory molecules and pro-inflammatory cytokine production in response to gram-positive bacterial lipoproteins. In contrast, type I IFN production is controlled by TLR2 stimulation (38). However, it is still controversial whether LPS, the classical TLR4 ligand, can lead to pDC activation. Although it has been previously reported that pDCs do not respond to LPS due to a lack of corresponding TLRs (39), a study in mice demonstrated that LPS can enhance the expression of costimulatory molecules in pDCs (32). Another study in humans showed that LPS could upregulate IRF-7 expression and IFN-α production in human pDCs (40). In line with a study in humans, we found that LPS upregulated IRF-7 expression and induced the in vivo production of IFN-α in mice pDCs. Future studies should determine if human peripheral blood pDCs also express TLR4 and respond to LPS treatment.

Although much remains unresolved about the interaction between pDCs and cDCs, it is known that this interaction plays an important role in immune defense (43). To elaborate, the CD40-CD40L interaction between pDCs and cDCs is necessary for IL-12 production in mouse cDCs during Listeria monocytogenes infection (44). In addition, co-culturing pDCs and cDCs had a synergistic effect on the optimal activation of both pDCs and cDCs in response to bacterial infections in human peripheral blood (45). In this study, we demonstrated that LPS induced the upregulation of co-stimulatory and class I and II MHC molecules in enriched pDCs as well as pDCs in cDC-depleted splenocytes, indicating that the upregulation of activation markers in pDCs was independent of cDCs. However, we found that LPS treatment caused a significant reduction in IFN-α levels in cDC-depleted splenocytes, indicating that cDCs may support IFN-α production in these cells. In addition, there is the possibility that cDCs can directly produce IFN-α in response to LPS (46). It is important to understand whether cDCs themselves produce IFN-α in response to LPS or indirectly promote the secretion of IFN-α by interacting with pDCs and cDCs. Therefore, a study on the interaction between cDCs and pDCs in response to LPS or other bacterial components is needed.

TLR4 has been established as a receptor for LPS (5). LPS is initially released from the outer membranes of gram-negative bacteria by the LPS binding protein (LBP). In serum, the LBP-
LPS complex transfers LPS to CD14^+ cells. LBP and CD14 help in docking LPS to the TLR4 complex, which is composed of heterodimer with MD-2 (6). The binding of LPS to the TLR4–MD-2 complex leads to activation of cells by promoting cytokine production and induces the expression of activation markers (6, 47). In contrast to cDCs, pDCs are not derived from myeloid cells and therefore do not express CD14 on their surface. As mentioned above, CD14 is important for the transfer of LPS to the TLR4-MD2 complex (Park and Lee, 2013). Therefore, even though pDCs express TLR4, CD14 is essential for the transfer of LPS to the TLR4-MD2 complex. We speculate that the LPS-induced activation of pDCs may be due to the contribution of the soluble form of CD14. To elaborate, it has been shown that the soluble forms of CD14 can deliver LPS to the TLR4-MD2 complex and contribute to immune activation (48, 49).

In conclusion, we demonstrate that mouse pDCs not only express considerable levels of TLR4 but also respond to LPS. LPS treatment induced upregulation of co-stimulatory molecules and IFN-α production in the pDCs in a TLR4-MD2 dependent manner. Thus, these data suggest that pDCs can directly react...
against LPS and may play a role in shaping the immune response against gram-negative bacterial infections.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

This study was approved by the Ethics of Animal Experiments Committee of Yeungnam University (2020-015) and Shanghai Public Health Clinical Center (2018-A049-01).

AUTHOR CONTRIBUTIONS

J-OJ designed the experiments and wrote the manuscript. WZ, E-KA, and JH participated in the experiments and data analysis. J-OJ and WZ reviewed the article. WZ and E-KA helped with manuscript writing and made important corrections to the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.727161/full#supplementary-material
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