Original Article
Synergistic effect of toll-like receptor 4 and thymic stromal lymphopoietin on the maturation of murine dendritic cells

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Abstract: Objectives: To investigate whether there was a synergistic effect of toll-like receptor (TLR) 4 and thymic stromal lymphopoietin (TSLP) on dendritic cells (DCs) activation. Methods: The maturation of bone marrow dendritic cells (BMDCs) cultured in the presence of lipopolysaccharide (LPS), LPS plus TLR4-myeloid differentiation protein (MD)-2, LPS plus TSLP receptor (TSLPR)-immunoglobulin (Ig) G, or LPS plus TLR4-MD2 combined with TSLPR-Ig G, was detected. CD80 and CD86 expression was analyzed by flow cytometry (FCM). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to assess the expression of TSLP and TLR4. Besides, TSLP concentrations of culture supernatants were determined using enzyme-linked immunosorbent assay (ELISA). Protein levels of mitogen-activated protein kinase (MAPK), phosphorylation extracellular regulated protein kinases (pERK) 1/2, and signal transducers and activators of transcription (STAT) 3 were measured by Western blotting. Results: CD80+ and CD86+ cell percentages were significantly decreased by TLR4-MD2, TSLPR-Ig G, and TLR4-MD2 plus TSLPR-Ig G. The mRNA levels of TLR4 could be effectively reduced by TSLPR-Ig G ($P < 0.05$), but TSLP level was not affected by TLR4-MD2. However, the TSLP concentrations were effectively decreased by TLR4-MD2 compared with LPS group. Moreover, the protein levels of MAPK and pERK1 were significantly reduced by TSLPR-Ig G, and the protein levels of MAPK and STAT3 were significantly reduced by TLR4-MD2. Conclusion: These data indicate that TLR4 combined with TSLP could exert synergistic action on BMDCs maturation. The synergistic effect may be regulated by MAPK signaling pathway.

Keywords: Toll-like receptor 4, thymic stromal lymphopoietin, synergistic effect, dendritic cell

Introduction

Dendritic cells (DCs) are the most potent antigen-presenting cells (APC), which exerts a profound influence on innate and adaptive immune response toward microbial pathogens [1]. It has been well demonstrated that recognition of microbial pathogens is important for initiating innate immune responses [2]. The pattern-recognition strategy, also called pathogen-associated molecular patterns (PAMPs), is mediated by a limited set of conserved molecular patterns [3]. PAMPs are detected by germline-encoded pattern recognition receptors (PRRs) that initiate several signaling programs and ultimately lead to host defensive responses. Many kinds of PRRs are expressed by DCs [4]. In addition, PRR signaling simultaneously induces the DCs maturation, contributing to the second line of host defense, which is so-called adaptive immunity.

In recent years, toll-like receptors (TLRs), the first identified PRRs, play a critical role in the antigen recognition and responses to microbial pathogens [5, 6]. Multiple PAMPs can be detected by TLRs, for example, lipopolysaccharide (LPS) can be detected by TLR4 and its coreceptor myeloid differentiation protein (MD)-2 [7], bacterial lipoproteins and lipoteichoic acids can be detected by TLR2 [8]. Also, TLRs have been reported to be crucial initiators of innate and adaptive immune response [9-11]. Besides, they are also considered as the check-points in regulating immunity when the host encounters antigens. Recent studies have reported that
TLR ligands are involved in the DCs maturation [12], which is characterized by upregulating surface major histocompatibility complex (MHC)-peptide complexes and costimulatory molecules (CD80 and CD86) during infection [4]. Among the TLRs, soluble TLR4, particularly TLR4 agonists (TLR4-MD-2) is regarded as the most promising inducers of DC maturation [13]. Furthermore, the cytokine thymic stromal lymphopoietin (TSLP) has been reported to be involved with the initiation and progression of allergic inflammation by activation of DCs [14]. TSLP is a type I cytokine that can be found in skin, gut, lungs, and thymus [15]. TSLP signal is mediated via a TSLP receptor (TSLPR) that consists of heterodimer of the IL-7 receptor α chain and the TSLPR chain. TSLP plays important roles in the polarization of DCs to drive the production of T helper (Th) 2 cytokine [16] though many signal pathway, such as transducer and activator of transcription (STAT), mitogen-activated protein kinase (MAPK), NF-κB, etc. [17, 18].

Both TLR4 and TSLP signaling pathway are involved in DCs activation and ultimately induce airway inflammatory reaction. Therefore, we hypothesized that there might be a cross talk between TLR4 and TSLP signaling pathway that could co-regulate DCs activation. In this present study, we used specific inhibition of TLR4 (TLR4-MD-2) and TSLPR (TSLPR-immunoglobulin (Ig) G to observe the bone marrow dendritic cells (BMDCs) activation induced by LPS, as well as the underling mechanism.

Materials and methods

Animals

Forty female BALB/c mice (4-6 weeks, Slac Laboratory Animal Co. Ltd., Shanghai, China) weighing 18-22 g were used. Each animal was maintained under specific-pathogen-free (SPF) conditions and provided with standard diet and tap water ad libitum before the procedure. The ambient temperature was maintained at 20-22°C and relative humidity was 45-65% with a 12:12 h light-dark cycle. The animal care and use was approved by local Ethics Committee and was complied with the ethical standards.

Generation of BMDC

The generation of BMDC was based on a previously described method [19]. Bone marrow was isolated from femurs and tibias of each mouse. These bones were harvested, flushed by phosphate buffer saline (PBS), minced and digested. Single cell suspensions were prepared. After centrifugation at 1000 g for 10 min, cells were maintained in RPMI1640 (Gibco, Grand Island, NY) with 10% fetal calf serum (FCS) supplemented with 10,000 U/L penicillin (Gibco), 10 g/L streptomycin (Gibco) and 50 μm 2-mercaptoethanol (Gibco). Cells (2 × 106) were placed in sterile Petri dishes in the above cultural media supplemented with 10 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF; R&D Systems, Minneapolis, USA) and 1 ng/mL IL-4 for seven days. Medium was changed using fresh cultural medium at days 3 and 6. After culture, DCs were purified with anti-CD11c-coated microbeads (Miltenyi-Biotec, Auburn, CA, USA). After 7 days, adhering cells were harvested and the cells (1 × 106) were seeded in 24-well plates for 24 h before the experiments.

Experimental designs

The cells were randomly assigned to five groups: (1) control group, the cells were maintained only in cultural medium; (2) LPS group, the cells were maintained in cultural medium supplemented with 10 μg/L LPS (R&D Systems, Minneapolis, USA); (3) LPS + TLR4-MD-2 group, the cells were maintained in cultural medium supplemented with 10 μg/L LPS and 40 mg/L TLR4-MD-2 (R&D Systems, Minneapolis, USA); (4) LPS + TSLPR-immunoglobulin G (Ig) G group, the cells were maintained in cultural medium supplemented with 10 μg/L LPS and 200 ng/mL TSLPR-Ig G (R&D Systems, Minneapolis, USA); (5) LPS + TLR4-MD-2 + TSLPR-Ig G group, the cells were maintained in cultural medium supplemented with 10 μg/L LPS, 40 mg/L TLR4-MD-2 and 200 ng/mL TSLPR-Ig G. After culture for 24 h, DCs were removed and re-suspended in 5% FCS in PBS for further analysis. In addition, supernatants were collected and frozen at -70°C until use.

Flow cytometry (FCM) analysis for phenotypic markers

Cells in each group were collected, washed twice with PBS containing 3% fetal bovine serum (FBS), centrifuged at 300 g for 5 min. The cells (2 × 105) were incubated in 96-well plates for 30 min at 4°C and stained with phy-
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coerythrin (PE)-CD80 (10 μl/mL, clone IG10, rat IgG2ακ; Pharmingen), CD86 (10 μl/mL, clone GL1, rat IgG2ακ; Pharmingen) mAbs, or isotype-matched negative control mAbs. The staining cells (at least 10 000 cells) were run on a fluorescence activated cell sorter (FACS) Calibur (Becton Dickinson, San Jose, CA, USA). The data were analyzed using CellQuest software (Becton Dickinson, San Jose, CA, USA).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from cells using TRIZOL Reagent (Life Technologies, Rockville, MD) according to the manufacturer’s instructions. First strand complementary DNA (cDNA) was synthesized using SuperScript™ II (Bio-Rad, Hercules, CA). QRT-PCR were carried out using a LightCycler™ 480 Instrument (Roche Applied Science, Basel, Switzerland) using a LightCycler 480 Probes Master kit (Roche Applied Science) following the manufacturer’s instructions. Glyceraldehyde-3-phosphatedehydrogenase (GAPDH) was used as an internal control. The relative mRNA expression levels of the genes were determined using 2-ΔΔCT method.

The primers sequences used were as follows: TSLP: upstream, 5'-CCCAGGCT ATTCGGAAACTCA-3', downstream, 5'-ACGCCACAATCCTTGT AAAT-3'; TLR4: upstream, 5'-CGGATGGCAACATTTAGAATTAGT-3', downstream, 5'-TGAT TGAGACTGTAATCAAGAACC-3'. GAPDH: upstream, 5'-GATGATATCGCCCGCTCGT-3, downstream, 5'-GTAGATGGGCACAGTGTGGGTG-3'.

Western blotting

Twenty-four hours after culture, cells in each group were harvested for protein extraction. The protein concentration was determined using Bio-Rad DC protein Assay kit (Bio-Rad, Hercules, CA, USA). Proteins (20 μL) from each group were separated by a standard electrophoresis sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gel and transferred onto polyvinylidene difluoride (PVDF) or nitrocellulose membranes. Then the membranes were blocked with 2% defatted milk powder for 2 h at room temperature, washed three times with PBS, and incubated overnight at 4°C with the following primary antibodies: anti-pERK1 antibody (diluted 1:1000, Sigma, St Louis, MO, USA), anti-ERK2 antibody (1:1000, Sigma, St Louis, MO, USA), or anti-MAPK antibody (1:1000, Santa Cruz Biotechnology, CA, USA). As a loading control, β-actin (1:1000, Santa Cruz Biotechnology, CA, USA) was used. After washing, the membranes were then incubated with HRP-labelled secondary antibody (1:5000, Santa Cruz Biotechnology, CA, USA). Finally, enhanced chemiluminescence and densitometric analysis were performed.

Enzyme-linked immunosorbent assay (ELISA)

TSLP concentrations from cell culture media were quantified using a commercial enzyme immunoassay kit (Bertin Pharma, Montingy le Bretonneux, France) according to the protocol recommended by the manufacturers. Absorbance was measured at 450 nm.

Statistical analysis

The data, expressed as the mean ± standard deviation (SD), were analyzed by statistical package for the social sciences (SPSS) software (version 16.0; SPSS Inc., Chicago, IL). The collected data were compared by Student’s t test (for 2 groups) or analysis of variance (ANOVA, for ≥ 3 groups). A Student’s t test was used to statistical comparisons. Differences were considered as statistically significant when P < 0.05.

Results

Positive cell percentages of CD80 and CD86 on DCs

After culture with GM-CSF and IL-4 for 7 d, the expression of CD80 and CD86 on DCs that incubated in the presence of different drugs were analyzed by FCM. As indicated in Figure 1A and 1B, both the CD80 and CD86 positive cell percentages were significantly increased when cultured with LPS (P < 0.05). However, both the CD80 and CD86 positive cell percentages were significantly decreased when incubated with TLR4-MD2 or TSLPR-Ig G compared with the LPS group. Interestingly, we found both the CD80 and CD86 positive cell percentages were also significantly reduced by incubation with TLR4-MD2 plus TSLPR-Ig G compared with only incubated with TLR4-MD2 or TSLPR-Ig G, suggesting that there may be a synergistic effect between the two signaling pathways.
In order to confirm whether there was an interaction between the two signaling pathways, we used qRT-PCR to determine the mRNA expression levels of TLR4 and TSLP in each group. The results showed that both the mRNA expression levels of TLR4 and TSLP were significantly reduced by incubation with TLR4-MD2 plus TSLPR-Ig G compared with the LPS group ($P < 0.05$). Additionally, we found that TLR4 gene expression level can be effectively reduced by TSLPR-Ig G ($P < 0.05$), however, there were no significant differences in TSLP gene expression...
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Release of TSLP concentrations from cells

To determine the impact of different drugs on TSLP concentrations released to cell culture media, we quantified TSLP concentrations using ELISA. The results demonstrated that the concentrations of TSLP were significantly reduced by TSLPR-Ig G as expected, but the concentrations of TSLP were also effectively decreased by TLR4-MD2 and TLR4-MD2 plus TSLPR-Ig G compared with LPS group (P < 0.05) (Figure 3).

Expression of signal pathway proteins

To further explore the possible mechanism of the interaction between TSLP and TLR4, we analyzed the expression of signal pathway proteins (MAPK, pERK1/2 and STAT3) using Western blotting (Figure 4A-E). The results showed that the protein expression levels of MAPK and pERK1 were significantly reduced by TSLPR-Ig G compared with LPS group (P < 0.05). However, there were no significant differences in the protein expression levels of pERK2 and STAT3. Besides, the protein expression levels of MAPK and STAT3 were significantly reduced by TLR4-MD2, however, there were no significant differences in the protein expression levels pERK1 and pERK2. These results indicated that the MAPK related signaling pathway may be directly or indirectly regulated by both TLR4 and TSLP, leading to the regulation of inflammatory response.

Discussion

In the present study, the functional roles of TLR4 and TSLP in DCs maturation were explored, along with the possible mechanism of the interaction between TSLP and TLR4. We found that both TLR4 and TSLP could regulate the DCs maturation. Moreover, the DCs maturation was further inhibited by TLR4 in combination with TSLP, suggesting that there was a synergistic effect of TLR4 and TSLP on DCs maturation. MAPK related signaling pathway maybe contributes to the interaction between TSLP and TLR4.

To fight the invading pathogen, DCs produce a diversity of soluble factors that direct T helper cell differentiation toward T-cell responses, including Th1, Th2, and Th17. The Th1 or Th2 immune responses induced by DCs depend on the type of signals that DC received at an immature stage [20, 21]. For example, immature DCs could be activated by microbe-derived molecules through the PRRs, such as the TLRs, which is essential for Th1 differentiation. TLRs are type I transmembrane receptors, consisting of leucine-rich repeat motifs, a transmembrane domain and a cytosolic Toll/interleukin (IL)-1 receptor homology domain. They are one of the PRRs and responsible for host defense by sensing microbial products [5]. TLR4, the first characterized TLRs, plays a crucial role in sepsis induced by gram-negative bacteria though recognition of LPS. MD2 is responsible for recognition of LPS by TLR4 because most of lipid chains of LPS interact with a hydrophobic pocket in MD2 [22]. The TLR4-MD2 complex is part of the LPS-activation cluster. In contrast, DCs instruct Th2 responses by TSLP and OX40 ligand pathway [16]. TSLP is an IL-7 like cytokine that acts via a receptor containing IL-7Rα and a TSLPR [23, 24]. The associated signals is though Janus kinase (JAK) 1 and JAK2 to mediate the activation of STAT5A and STAT5B [18]. TSLP acts on different lineages, such as DCs [25], T cells [18], mast cells [26], and NKT cells [27] etc. TSLP regulates the activation, differ-
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Figure 4. Expression of signal pathway proteins. A: Relative protein expression level of MAPK; B: Relative protein expression level of pERK1; C: Relative protein expression level of pERK2; D: Relative protein expression level of STAT3; E: Western blotting pictures. LPS, lipopolysaccharide; TLR, toll like receptor; TSLPR, thymic stromal lymphopoietin receptor; MD, myeloid differentiation protein; Ig, immunoglobulin; MAPK, mitogen-activated protein kinase; STAT, signal transducers and activators of transcription; ERK, extracellular regulated protein kinases. *P < 0.05 compared with LPS group; **P < 0.05 compared with TLR4-MD2 or TSLPR-Ig G group.

Recently, TSLP has been reported to be expressed in DCs in addition to epithelial cells, fibroblasts, keratinocytes, and mast cells etc. [30]. Both TLRs and TSLP are thought to be involved in airway inflammation [31]. Moreover, previous studies have confirmed that the production of TSLP on BMDCs could be induced by TLR signals, and this effect could be augmented by IL-4 [30, 32].

Also, the functional roles of TLR4 and TSLP on activation of DCs were explored in our study. We hypothesized that there was a cross talk between TLR4 and TSLP. To confirm the hypothesis, we first evaluated the positive cell percentages of CD80 and CD86 on BMDCs analyzed by FCM. As expected, both the positive cell percentages of CD80 and CD86 were significantly reduced by TLR4-MD2 and TSLPR-Ig G, respectively. It is noteworthy that both CD80+ and CD86+ cell percentages were markedly decreased by TLR4-MD2 in combination with TSLPR-Ig G, suggesting that there might be combined action between TLR4 and TSLP. Further, we investigated the combined action by assessing the mRNA levels of TLR4 and TSLPR. Both the mRNA levels of TLR4 and TSLPR were down-regulated by TLR4-MD2 in combination with TSLPR-Ig G. In addition to the results, we found that TLR4 gene expression level could be effectively reduced by TSLPR-Ig G; however, there were no significant differences in TSLP gene expression level by incubation with TLR4-MD2. Interestingly, the concentrations of TSLP released to cultural media were significantly reduced by TLR4-MD2. One possible reason was that TLR4 might be located in the downstream of TSLP signaling pathway. Moreover, we investigated the underlying mechanism respect to this synergistic effect between TLR4 and TSLP. The protein expression levels of MAPK, pERK1/2 and STAT3 were determined. The results demonstrated that the protein expression levels of MAPK and pERK1 were significantly reduced by TSLPR-Ig G, and the pro-
tein expression levels of MAPK and STAT3 were significantly reduced by TLR4-MD2. Therefore, MAPK related signaling pathway may be the common signaling pathway regulated by both TLR4 and TSLP, leading to the regulation of inflammatory response.

In conclusion, our study suggests that TLR4 combined with TSLP could exert synergistic action on BMDCs maturation. The underling signaling might be associated with activating MAPK signaling pathway. Both TLR4 and TSLP are involved in airway inflammation, and TLR4-MD2 in combination with TSLP-IgG might be a novel therapeutic treatment of airway inflammation, such as acute lung injury (ALI). However, further study should be carried out to confirm the clinical effect.

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Disclosure of conflict of interest

None.

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