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Extracellular ATP activates P2X7R-NF-κB (p65) pathway to promote the maturation of bone marrow-derived dendritic cells of mice

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ABSTRACT

The maturation state of dendritic cell (DC) plays an important role in immune activities. Previously we had found that NF-κB (p65) pathway could promote DC maturation and subsequent immune effects. But the upstream mechanism of this pathway was still unclear. Extracellular adenosine triphosphate (ATP) activating its receptor P2X7R has recently been considered as the fourth signal to activate T lymphocytes. Here we aimed to find out the connection between P2X7R and NF-κB (p65) pathway in DC maturation. Results showed that the expression of P2X7R and the intracellular ATP levels were increased along with the maturation of DC. P2X7R agonist stimulated the morphological changes of DCs into the appearance of mature DCs, and promoted the expression of NF-κB (p65), as well as the release of IFN-γ and IL-12. Whereas, P2X7R inhibitor had the opposite influences. Co-immunoprecipitation assay confirmed the binding of P2X7R and NF-κB (p65). Our study suggested that extracellular ATP could promote DC maturation and release of inflammatory cytokines through the binding of P2X7R and NF-κB (p65). This is the first study to show the P2X7R-NF-κB (p65) pathway in DC. Interference with this pathway may be able to regulate immune responses in areas like infectious diseases, inflammation, transplantation, tumor and autoimmune diseases. In addition, intracellular ATP level could be a new indicator of the maturation state of DC.

1. Introduction

Antigen presenting cells (APCs) mainly include dendritic cells (DCs) and macrophages. The maturation state of DC could play an important role in immune activities [1]. Regulatory DC (DCreg) is a subgroup of DC, which could induce immune tolerance by inducing anergy of T lymphocytes, production of regulatory T lymphocytes, apoptosis of activated T lymphocytes and immune deviation [2]. DCregs generally are immature DCs [3–5]. Thus intervening the maturation of DC could be a way to induce immune tolerance.

Nuclear factor-kappa B (NF-κB) family is composed of p50, p52, Rel, Rel-A (p65) and Rel-B. The main biological function of NF-κB is induced by p65 phosphorylation [6]. Our research group had previously found that TLR2/MyD88/NF-κB (p65) pathway could promote DC maturation, T lymphocytes proliferation and the release of inflammatory cytokines such as interleukin (IL)-1β; and the inhibition of TLR2/MyD88/NF-κB (p65) pathway significantly inhibited DC maturation, improved corneal allograft tolerance and prolonged the survival duration of grafts in rats [7–9]. However, the upstream mechanism of NF-κB (p65) pathway to promote DC maturation was still unclear.

The activation of T lymphocytes plays a pivotal role in adaptive immunity. It's known that this process requires three signals: first is the specific binding of T cell receptor (TCR) to the major histocompatibility complex (MHC) -antigen peptide complex; second is the costimulatory signal, which is the costimulatory molecules on the surface of APCs such as CD80 and CD86; third is the related cytokines secreted by T lymphocytes, such as IL-2. Interestingly, in addition to these three traditional pathways, recent studies have recognized the fourth signal, which is extracellular adenosine triphosphate (ATP) acting as autocrine stimuli by activating its receptor P2X7R [10].

ATP is a high-energetic phosphoric acid compound, which releases energy when hydrolyzed and is the most direct energy source in organisms [11]. During inflammation and injury, the level of extracellular ATP could increase significantly [10]. Extracellular ATP and its metabolites, such as adenosine diphosphate, adenosine monophosphate and adenosine, produce purinergic signals mainly by stimulating purinergic receptors (PR), thus exerting their biological effects [12].

PR includes P1R and P2R. ATP can affect the functions of various

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kinds of immune cells by activating P2R. P2R includes P2XR and P2YR, while P2XR has seven subtypes as P2X1R-P2X7R [13]. P2X7R is one of the most important subtypes of PR, and plays a significant role in inflammation [14]. P2X7R could induce the activation of cascade signals, proliferation and activation of lymphocytes, apoptosis of cells and release of cytokines. The combining of ATP and P2X7R could induce the initiation of inflammation and the secretion of pro-inflammatory cytokines (IL-1β, IL-18, TNF-α, IL-23) in DCs, increase the secretion of IL-2 and the proliferation of the effector T lymphocytes, and lead to apoptosis of regulatory T lymphocytes [15].

Some studies have shown that P2X7R leads to NF-κB signaling in APCs such as monocytes and macrophages [16,17]. However, there has been no such study in DCs. Therefore, we aimed to find out the connection between P2X7R and NF-κB (p65) pathway in DC maturation. DC plays a pivotal role in the immune system. This study not only could contribute to understanding the pathological mechanisms of infectious diseases, inflammation, transplantation, tumor, autoimmune diseases, etc., but also could inspire new effective measures for immune regulation.

2. Material and methods

2.1. Generation of bone marrow-derived DC

The bone marrow cells were harvested from the femur and tibia of female C57BL/6 mice (aged 6–8 weeks) then treated as previous studies [9]. The cells were incubated with RPMI1640 (Hyclone Inc.) medium containing 10% fetal bovine serum (Gibco Inc.) and 1% Penicillin-Streptomycin (Thermo Fisher Inc.) in 10 × 10 cm dishes, with a density of about 2×10^6/ml. Recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 stimulating factors (Peprotch Inc.) were added (both final concentrations were 50 ng/ml) to stimulate the differentiation and maturation of DCs. On the 3rd day, half amount of the median was changed. On the 5th day, the cells were seeded in the needed plates for following experiments. On the 6th day, the cells have become immature DCs. On the 8th day, the cells have become mature DCs. The morphology of the cells was observed under light microscope.

2.2. Grouping

The cells were divided into 5 groups as 6d, 8d, LPS, Bz and Ox. According to our previous methods [7–9], different maturation states of DCs were set as 6d group (immature DC), 8d group (mature DC) and LPS group (enhanced mature DC). 6d group were the cells harvested on the 6th day. 8d group were the cells harvested on the 8th day. LPS group was treated with lipopolysaccharide (LPS) (Sigma Inc.). Bz group was treated with P2X7R agonist, 2′,3′-(4-benzoyl-benzoyl)-ATP (BzATP) (Apexbio Inc.), which is a potent analog of ATP. Ox group was treated with P2X7R inhibitor, oxidized ATP (OxATP) (Sigma-Aldrich Inc.). LPS group, Bz group and Ox group were all treated on the 6th day and harvested on the 8th day. The dosages of LPS, BzATP and OxATP were determined by pre-experiments and referring to other studies [9,18]. In pre-experiments, LPS was used in 0.4 μg/mL, 0.8 μg/mL and 1.2 μg/mL; BzATP was used in 1×10^{-6}, 5×10^{-6} and 1×10^{-5} respectively; OxATP was used in 1×10^{-5}, 5×10^{-5} and 1×10^{-4} respectively. The most effective dosage was determined to be 0.4 μg/mL in LPS, 1×10^{-6} M in BzATP and 1×10^{-4} M in OxATP, which were applied in the rest of the study.

2.3. Western blot (WB)

The collected cells were lysed in RIPA buffer in the presence of protease inhibitors (Selleck Chemicals Inc.). Protein concentrations were tested by the Brandford method (Bioss Inc.). Samples were denatured in protein loading buffer (Bio-RAD Inc.) at 100 °C for 5 min. 50 μg total protein extracts were subjected to SDS-PAGE on 8% gels and transferred to nitrocellulose membranes, then blocked with 5% BSA power in TBST for 1 h. Blots were first incubated with a primary antibody, as the purified anti-mouse P2X7R antibody (rat IgG2b, κ isotype), purified anti-NF-κB (p65) antibody (rabbit polyclonal IgG isotype) and GAPDH at 4 °C overnight (Biolegend Inc.). After that, the blots were washed twice in TBST for 10 min, then incubated with secondary antibody, as the HRP conjugated goat ati-rat IgG Abbkine Inc.) and rabbit IgG-heavy and light chain antibody (Bethyl Laboratories Inc.) for 1 h. The proteins were detected by a chemiluminescence kit (Millipore Inc.). Three replicates were set and the experiments were performed three times.

2.4. Reverse transcription–quantitative real-time PCR (RT-qPCR)

The total RNA of the cells were extracted by TRIzol reagent (Beyotime Biotechnology Inc.). The primers of P2X7R and NF-κB (p65) were synthesized (Sangon Biotech Inc.). The sequence of P2X7R upstream and downstream primers were respectively GACAAACAAAGTC ACCCGGAT (5′-3′) and CGCTCACCAAAGCAAAGCTAAT (5′-3′). The sequence of NF-κB (p65) upstream and downstream primers were GAAGGGCGTGTTTGACAAGGA (5′-3′) and GCATCCCGAACAAGAGAC AGAAT (5′-3′) respectively. β-actin was used as reference. SyBR green method, PrimeScript RT reagent Kit with gDNA Eraser (Takara Bio Inc.) and fluorescent quantitative PCR instrument (Bio-Rad Inc.) were used to perform RT-qPCR. Bio-Rad CFX manager software (Bio-Rad Inc.) was used to analyze RNA levels. The results were determined using the comparative threshold cycle method. Three replicates were set and the experiments were performed three times.

2.5. Co-immunoprecipitation (Co-IP)

Pierce immuno Co-Immunoprecipitation Kit (Thermo Fisher Inc.) was used for Co-IP assay. The proteins were extracted from the collected cells and tested for concentration. 5 μL IgG antibody was added to each protein extracts. At the same time, 30 μL Protein A/G Magnetic Beads (Med Chem Express Inc.) was added to each tube, shaken for 30 min, then centrifuged at 2500 rpm/min for 5 min at 4 °C to obtain the supernatants. Repeat the shaking and centrifuging steps. Then the Brandford method (Bioss Inc.) was used for protein quantification. Each 1 mg protein was added with 2μg NF-κB (P65) antibody and incubated on the shaking table at 4 °C for 4 h. Then the magnetic rack was used to absorb and the supernatants were removed. 1 mL PBS was added in each tube to wash the Protein A/G and repeated for 3 times. 40 μL 1 × sample buffer was added in each tube and boiled for 10 min. The product was assayed in WB method with the sample size of 5 μL.

2.6. ATP assays

ATP Assay Kit (Beyotime Biotechnology Inc.) was used to detect intracellular ATP. The cells were collected and treated with ATP lysing reagent, then centrifuged at 4 °C 12,000*g* for 5 min to obtain the supernatants. 100 mL ATP test reagent was added to the test wells and placed at room temperature for 5 min so that the background ATP could be completely depleted. Standard wells were set according to the concentration gradient to calculate the standard curve. The concentrations were calculated from relative light unit (RLU) values, which were measured by luminometer (Bio-Rad Inc.). Three replicates were set and the experiments were performed three times.

2.7. Enzyme-linked immuno sorbent assay (ELISA)

Double antibody sandwich ELISA kits (SenBeiJia Biological Technology Inc.) were applied to detect the cytokine levels of interferon-γ (IFN-γ) and IL-12 in cell supernatants. The cells were first centrifuged for 20 min (2000 rpm). Then the supernatants were collected for test. Blank well and standard wells were set according to the

concentration gradient to calculate the standard curve. The absorbance at 450 nm was measured by a multifunctional microplate spectrophotometer (Biotech Inc.). Three replicates were set and the experiments were performed three times.

2.8. Data analysis

Graphpad Prism 7.00 software and SPSS 20.0 software were used to produce the graphs and analyze the data. The continuous variables were presented as mean ± standard deviation (SD). The data were compared among the groups using one-way ANOVA with post-hoc test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. DC morphology

By the 6th day of the differentiation process of DCs, the cells volume has expanded. Most cells appeared clear edges, while some have developed spiculate spikes on their surface. Some cells adhered to the wall, while some grew in suspension. These were accorded with the pattern of immature DCs (Fig. 1A). By the 8th day, the cell boundary was blurred with dendritic spikes on their surface. The cells have gradually left the walls. These were accorded with the pattern of mature DCs (Fig. 1B). LPS group showed more suspended cells in various shapes, with dendritic spikes on their surface. These were accorded with the pattern of enhanced mature DCs (Fig. 1C). The cells in Bz group presented an appearance similar with 8d group and LPS group (Fig. 1D). The cells in Ox group presented an appearance similar with 6d group (Fig. 1E).

3.2. Protein and mRNA expression of P2X7R in different groups

One-way ANOVA test showed that there were significant differences of P2X7R expression both in protein and mRNA levels among the groups (both $P < 0.01$). The post-hoc comparison among each group is shown in the figure (Fig. 2).

WB results showed that the target/GAPDH ratio of RLU values increased gradually in order as 6d group (0.47 ± 0.05), 8d group (0.84 ± 0.05), and LPS group (1.22 ± 0.04). Both 8d group and LPS group were significantly higher than 6d group (both $P < 0.01$) (Fig. 2A). RT-qPCR results also showed that the relative P2X7R mRNA level increased gradually in order as 6d group (1.00 ± 0.00), 8d group (1.88 ± 0.43), and LPS group (3.14 ± 0.50). Both 8d group and LPS group were significantly higher than 6d group ($P < 0.05$ and $P < 0.01$ respectively) (Fig. 2B). These results indicated that the protein and mRNA expression of P2X7R could elevate as DCs become more mature, which suggested ATP could be involved in the maturation process of DC.

In addition, comparing with 8d group, the protein expression of P2X7R was significantly promoted in Bz group (1.27 ± 0.04) and reduced in Ox group (0.52 ± 0.07) (both $P < 0.01$) (Fig. 2A). Comparing with 8d group, the mRNA expression of P2X7R was also promoted in Bz group (3.35 ± 0.38) ($P < 0.01$). Although Ox group (0.52 ± 0.07) was not significantly lower than 8d group, it's significantly lower than LPS group ($P < 0.01$) (Fig. 2B). These results suggested that the stimulation of P2X7R agonist and inhibitor were both effective.

3.3. Protein and mRNA expression of NF-κB (p65) in different groups

One-way ANOVA test showed that there were significant differences of NF-κB (p65) expression both in protein and mRNA levels among the groups (both $P < 0.01$). The post-hoc comparison among each group is shown in the figure (Fig. 2).

WB results of NF-κB (p65) protein showed that the target/GAPDH ratio of RLU values increased gradually in order as 6d group (0.46 ± 0.06), 8d group (0.81 ± 0.02), and LPS group (0.98 ± 0.11). Both 8d group and LPS group were significantly higher than 6d group (both $P < 0.01$) (Fig. 2A). The relative NF-κB (p65) mRNA level increased gradually in order as 6d group (1.00 ± 0.00), 8d group (1.53 ± 0.19), and LPS group (1.98 ± 0.27). Both 8d group and LPS

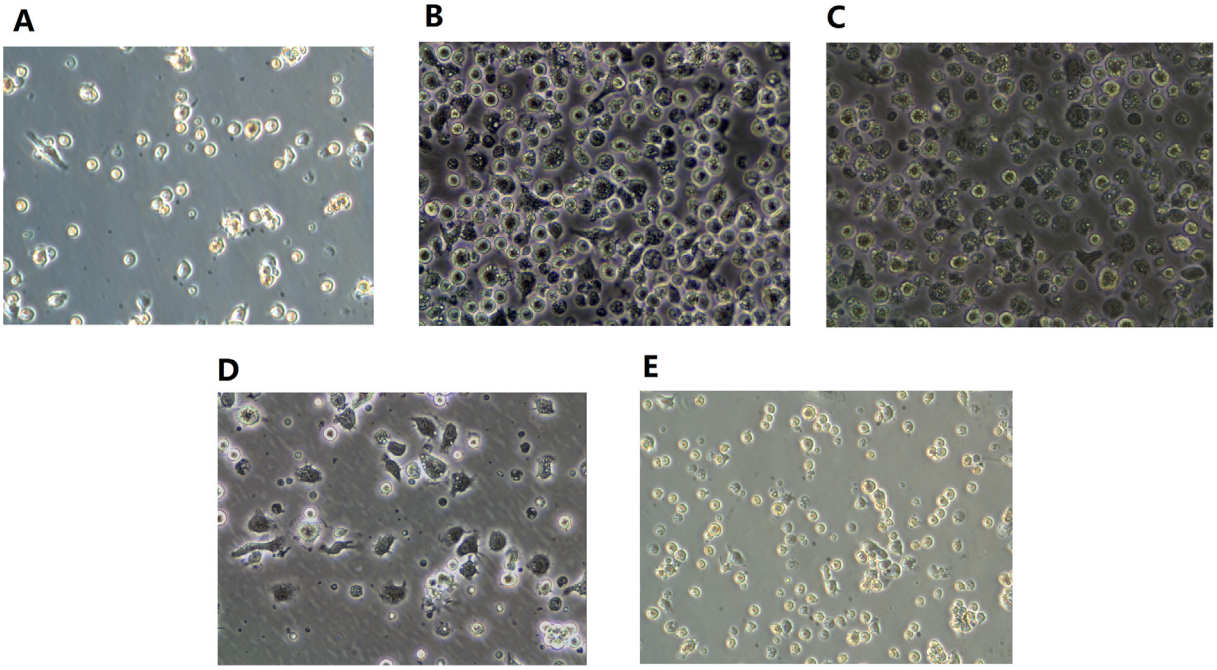


Fig. 1. Morphology of dendritic cells (DCs) under 20× microscope. (A) The morphology of 6d group DCs, which were harvested on the 6th day. (B) The morphology of 8d group DCs, which were harvested on the 8th day. (C) The morphology of LPS group DCs, which were treated with 0.4 μg/mL lipopolysaccharide (LPS). (D) The morphology of Bz group DCs, which were treated with 1×10^{-6} M P2X7R agonist 2′,3′- (4-benzoyl-benzoyl)-ATP (BzATP). (E) The morphology of Ox group DCs, which were treated with 1×10^{-4} M P2X7R inhibitor oxidized ATP (OxATP).

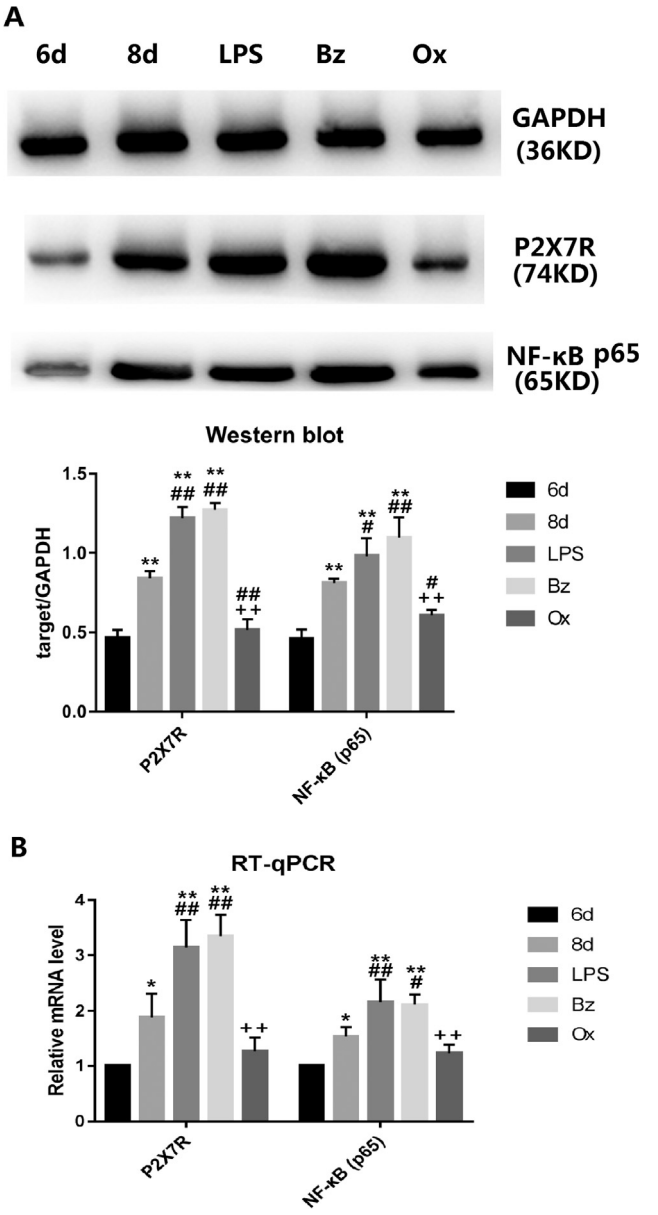


Fig. 2. The protein and mRNA expression of P2X7R and NF-κB (p65) in different groups. 6d group is dendritic cells (DCs) harvested on the 6th day; 8d group is DC harvested on the 8th day; LPS group is DCs treated with 0.4 μg/mL lipopolysaccharide (LPS); Bz group is DCs treated with 1×10^{-6} M P2X7R agonist 2′,3′- (4-benzoyl-benzoyl)-ATP (BzATP); Ox group is DCs treated with 1×10^{-4} M P2X7R inhibitor oxidized ATP (OxATP). One-way ANOVA with post-hoc test results with significant difference were presented as * ($p < 0.05$) and ** ($p < 0.01$) comparing to 6d group; # ($p < 0.05$) and ## ($p < 0.01$) comparing to 8d group; + ($p < 0.05$) and + + ($p < 0.01$) comparing to LPS group. Three replicates were set and the experiments were performed three times. (A) Western blot results of P2X7R and NF-κB (p65) protein levels were shown as the target/GAPDH ratio of relative light unit (RLU) values. (B) RT-qPCR results of P2X7R and NF-κB (p65) relative mRNA levels.

group were significantly higher than 6d group ($P < 0.05$ and $P < 0.01$ respectively) (Fig. 2B). These results indicated that the protein and mRNA expression of NF-κB (p65) could elevate as DCs become more mature.

Moreover, comparing with 8d group, the protein expression of NF-κB (p65) was promoted in Bz group (1.10 ± 0.13) and reduced in Ox group (0.61 ± 0.04) ($P < 0.01$ and $P < 0.05$ respectively) (Fig. 2A). Comparing with 8d group, the mRNA expression of NF-κB (p65) was also promoted in Bz group (2.30 ± 0.23) ($P < 0.05$). Although Ox

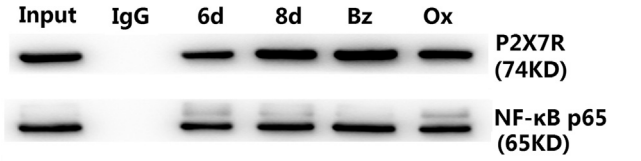


Fig. 3. Co-immunoprecipitation (Co-IP) results of P2X7R and NF-κB (p65). 6d group is dendritic cells (DCs) harvested on the 6th day; 8d group is DC harvested on the 8th day; Bz group is DCs treated with 1×10^{-6} M P2X7R agonist 2′,3′- (4-benzoyl-benzoyl)-ATP (BzATP); Ox group is DCs treated with 1×10^{-4} M P2X7R inhibitor oxidized ATP (OxATP). After adding the NF-κB (p65) antibody, P2X7R protein was detected in the immunoprecipitates of NF-κB (p65) in all the above groups, which confirmed the combining of P2X7R and NF-κB (p65) protein.

group (1.23 ± 0.15) was not significantly lower than 8d group, it's significantly lower than LPS group ($P < 0.01$) (Fig. 2B). These results indicated that ATP could be related with the regulation of NF-κB (p65) expression.

3.4. Interaction between P2X7R and NF-κB (p65) protein

Co-IP assay results showed that, after adding the NF-κB (p65) antibody, P2X7R protein were detected in the immunoprecipitates of NF-κB (p65), which confirmed the combining of P2X7R and NF-κB (p65) protein (Fig. 3).

3.5. Intracellular ATP levels in different groups

One-way ANOVA test of ATP assay results showed that there were significant differences among the groups ($P < 0.01$). The post-hoc comparison among each group is shown in the figure (Fig. 4).

The intracellular ATP concentration increased gradually in order as 6d group (2.15 ± 0.25 μM), 8d group (2.45 ± 0.04 μM), and LPS group (2.97 ± 0.38 μM), which indicated that the intracellular ATP levels were increased along with the maturation of DC. LPS group was significantly higher than 6d group ($P < 0.05$). In addition, comparing with 8d group, the intracellular ATP concentration was promoted in Bz group (3.77 ± 0.76 μM) and reduced in Ox group (1.57 ± 0.31 μM) ($P < 0.01$ and $P < 0.05$ respectively), suggesting the stimulation of P2X7R agonist and inhibitor were both effective.

3.6. Supernatant inflammatory cytokine levels in different groups

One-way ANOVA test of ELISA results showed that there were significant differences in both IFN-γ and IL-12 levels among the groups (both $P < 0.01$). The post-hoc comparison among each group is shown in the figure (Fig. 5).

The supernatant concentration of IFN-γ increased gradually in order as 6d group (143.11 ± 6.98 ng/L), 8d group (205.35 ± 4.00 ng/L), and LPS group (248.92 ± 9.16 ng/L). Both 8d group and LPS group were significantly higher than 6d group (both $P < 0.01$) (Fig. 5A). IL-12 level also increased gradually in order as 6d group (4.96 ± 0.13 ng/L), 8d group (10.59 ± 0.26 ng/L), and LPS group (11.78 ± 0.38 ng/L). Both 8d group and LPS group were significantly higher than 6d group (both $P < 0.01$) (Fig. 5B). IFN-γ and IL-12 are inflammatory cytokines that could be elevated as DCs become mature. Thus these results reflected the maturation process of DCs.

Moreover, comparing with 8d group, the IFN-γ level was promoted Bz group (319.05 ± 6.51 ng/L) and reduced in Ox group (158.05 ± 15.88 ng/L) (both $P < 0.01$) (Fig. 5A). Comparing with 8d group, the IL-12 level was also promoted in Bz group (12.74 ± 0.24 ng/L) and reduced in Ox group (8.05 ± 0.19 ng/L) (both $P < 0.01$) (Fig. 5B). These results indicated that ATP could be related with the regulation of DC maturation.

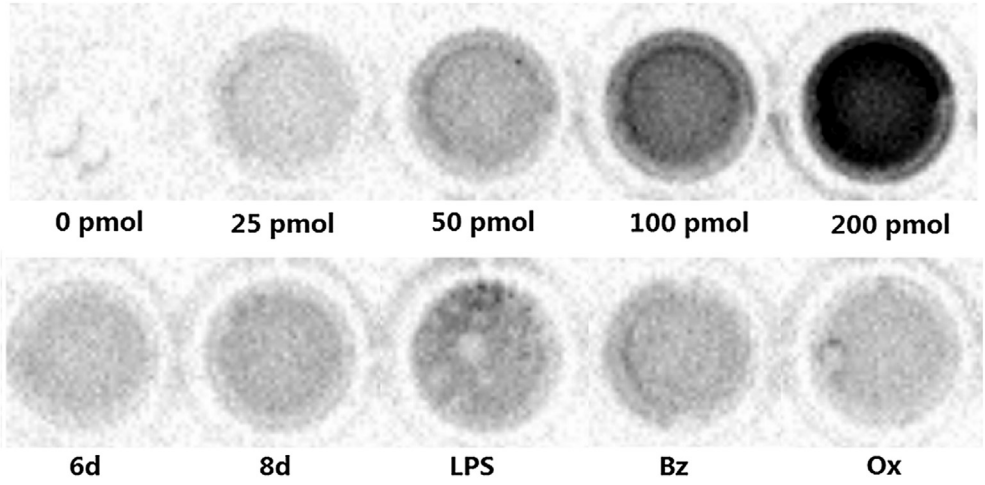
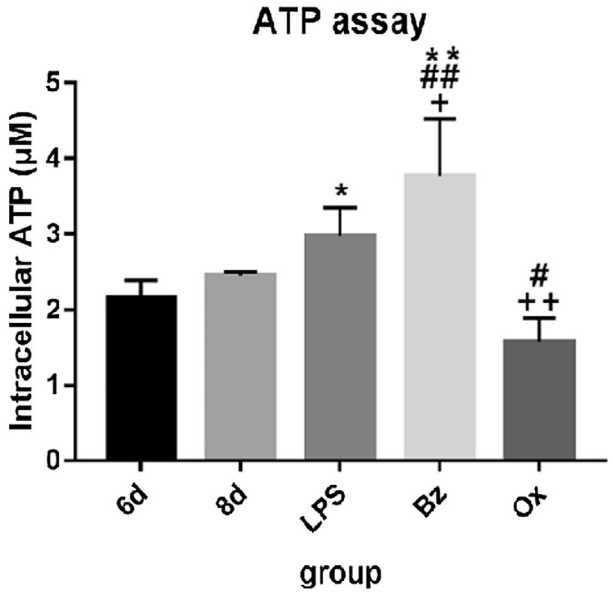


Fig. 4. ATP assay results of intracellular ATP concentration. The concentrations of intracellular ATP were calculated from relative light unit (RLU) values, and by comparing with the standard wells (0 pmol, 25 pmol, 50 pmol, 100 pmol and 200 pmol). 6d group is dendritic cells (DCs) harvested on the 6th day; 8d group is DC harvested on the 8th day; LPS group is DCs treated with 0.4 μg/mL lipopolysaccharide (LPS); Bz group is DCs treated with 1×10^{-6} M P2X7R agonist 2′,3′- (4-benzoyl-benzoyl)-ATP (BzATP); Ox group is DCs treated with 1×10^{-4} M P2X7R inhibitor oxidized ATP (OxATP). One-way ANOVA with post-hoc test results with significant difference were presented as * ($p < 0.05$) and ** ($p < 0.01$) comparing to 6d group; # ($p < 0.05$) and ## ($p < 0.01$) comparing to 8d group; + ($p < 0.05$) and ++ ($p < 0.01$) comparing to LPS group. Three replicates were set and the experiments were performed three times.



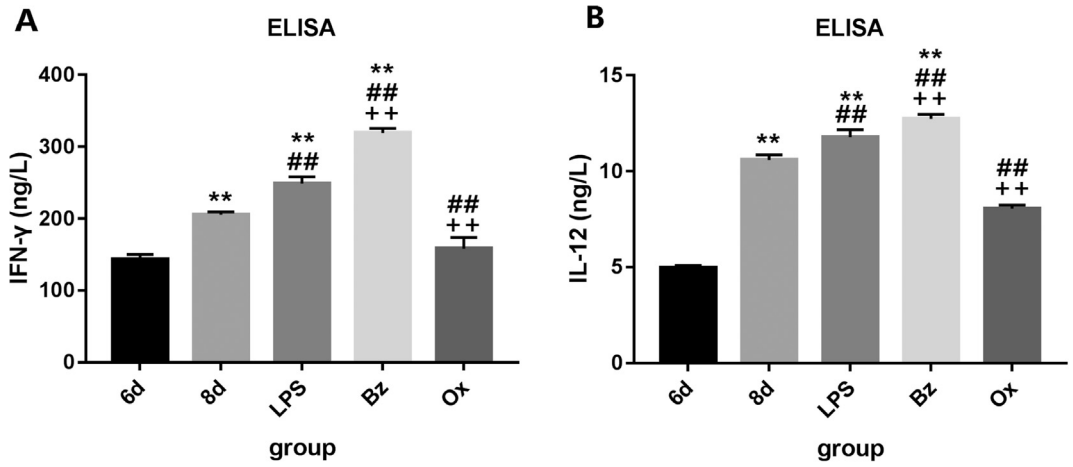


Fig. 5. The ELISA results of supernatant inflammatory cytokines in different groups. 6d group is dendritic cells (DCs) harvested on the 6th day; 8d group is DC harvested on the 8th day; LPS group is DCs treated with 0.4 μg/mL lipopolysaccharide (LPS); Bz group is DCs treated with 1×10^{-6} M P2X7R agonist 2′,3′- (4-benzoyl-benzoyl)-ATP (BzATP); Ox group is DCs treated with 1×10^{-4} M P2X7R inhibitor oxidized ATP (OxATP). One-way ANOVA with post-hoc test results with significant difference were presented as * ($p < 0.05$) and ** ($p < 0.01$) comparing to 6d group; # ($p < 0.05$) and ## ($p < 0.01$) comparing to 8d group; + ($p < 0.05$) and ++ ($p < 0.01$) comparing to LPS group. Three replicates were set and the experiments were performed three times. (A) ELISA results of the supernatant concentration of interferon-γ (IFN-γ). (B) ELISA results of the supernatant concentration of interleukin-12 (IL-12).

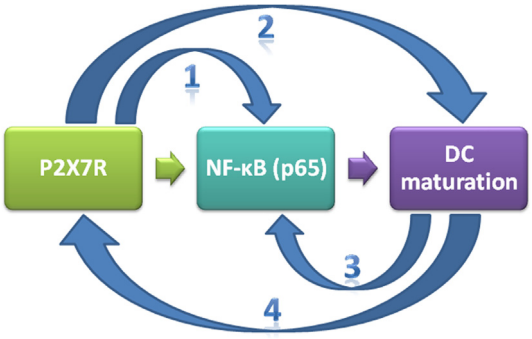


Fig. 6. Diagram of main results and conclusions. Previously we had found that NF-κB (p65) pathway could promote the maturation of dendritic cells (DCs) and subsequent immune effects. But the upstream mechanism was unclear. This study mainly suggested that extracellular ATP could promote DC maturation and release of inflammatory cytokines through the binding of P2X7R and NF-κB (p65). Results of this study showed: (1) P2X7R agonist stimulated-DCs expressed increased protein and mRNA levels of NF-κB (p65). P2X7R inhibitor had the opposite effects. Co-immunoprecipitation assay confirmed the binding of P2X7R and NF-κB (p65). (2) P2X7R agonist stimulated-DCs had morphological changes towards the appearance of mature DCs and elevated the supernatant levels of inflammatory cytokines interferon-γ (IFN-γ) and interleukin-12 (IL-12). P2X7R inhibitor had the opposite effects. (3) The more mature DCs expressed the higher protein and mRNA levels of NF-κB (p65). (4) The more mature DCs expressed the higher protein and mRNA levels of P2X7R, and with higher intracellular ATP levels.

4. Discussion

Results of this study showed that the expression of P2X7R and the intracellular ATP levels were increased along with the maturation of DC. BzATP stimulated the morphological changes of DCs towards the appearance of mature DCs, and promoted the expression of NF-κB (p65), as well as the release of IFN-γ and IL-12. Whereas, OxATP had the opposite influences. Co-IP assay confirmed the binding of P2X7R and NF-κB (p65) (Fig. 6).

ATP is one of the most important metabolites in cells and widely exists in the metabolic intermediate processes of sugar, protein and fat. Extracellular ATP is involved in many biological behaviors such as cell survival, proliferation, differentiation, migration and so on. ATP is also a kind of endogenous damage-associated molecular pattern (DAMP), which plays a particular role in the immunity, exerting as an extracellular signal of the inflammatory responses. The concentration of extracellular ATP is very low at rest, but it increases substantially in the case of tissue damage or inflammation. This is because ATP can be released by regulatory exocytosis, traumatic cell lysis, and passive exudation of injured cells [19,20]. Therefore, the concentration of extracellular ATP can be greatly increased in circumstances like infectious diseases, inflammation, transplantation, tumor, autoimmune diseases etc. In addition, the stimulation of TCR could induce calcium influx during immune rejection responses, which could further stimulate the increase of extracellular ATP levels [21]. In order to understand better the immune mechanisms of the above circumstances, it is important to study the effects of extracellular ATP and its receptor P2X7R in the immune cells, especially in DCs, as DC plays a significant role in immune activities.

The DCs were cultured into immature DCs (6d group), mature DCs (8d group) and enhanced mature DCs (LPS group). Following this order, the DCs experienced morphologic changes, elevated release of IFN-γ and IL-12, and promoted NF-κB (p65) expression both in protein and mRNA levels. These results reflected the maturation process of DCs as predicted, because IFN-γ and IL-12 are both inflammatory cytokines, which could be elevated along with DC maturation [22]; and our research group has proved before that the expression of NF-κB (p65) could be elevated in mature DCs and the inhibition of NF-κB (p65) could prevent DCs maturation [7–9].

Results of the present study showed that, as DC became more mature, the expression of P2X7R were promoted both in protein and mRNA levels, suggesting that ATP could be involved in the maturation process of DC. P2X7R agonist BzATP was used to stimulate DC in order to simulate the cellular micro-environment of high extracellular ATP, while OxATP was applied as the inhibitor of P2X7R. Results showed that BzATP promoted the morphological changes of DCs towards the appearance of mature DCs, and promoted the expression of NF-κB (p65) both in protein and mRNA levels, as well as the release of IFN-γ and IL-12. Whereas OxATP had the opposite influences. These results indicated that the cellular micro-environment of high extracellular ATP, for example under the circumstances of injury or inflammation, could promote the maturation of DC and release of inflammatory cytokines, which could be related with the activation of NF-κB (p65).

Similar to our findings, Wilhelm et al. found that extracellular ATP-stimulated DCs increased the levels of costimulatory molecules CD80 and CD86 in vitro. Extracellular ATP initiated a series of inflammatory events in vivo, including the phosphorylation of signal transducer and activator of transcription 1 (STAT1), the production of IFN-γ, the proliferation of T lymphocytes, and the decrease of regulatory T cells. Also, the elevated expression of P2X7R during graft versus host disease (GVHD) progression increased the sensitivity of DCs to the harmful effects of ATP, thus providing a positive feedback signal. Whereas, neutralization of ATP, early blockage or gene knockout of P2X7R, could improve the survival rate [23]. Weber et al. confirmed that P2X7R-knockout mice were resistant to contact allergy. Their bone marrow-derived DCs lost the sensitivity to contact antigens, and no longer release IL-1β under LPS or ATP stimulation [24]. However, our study was the first to show that P2X7R could promote DC maturation which could be related with NF-κB (p65) pathway.

In order to further clarify the connection between P2X7R and NF-κB (p65) during DC maturation, Co-IP assay was applied. The results confirmed that there was a binding of these two proteins, which indicated that P2X7R could promote DC maturation and release of IFN-γ and IL-12 through binding to NF-κB (p65).

Recently, some other studies have also reported the relation between P2X7R and NF-κB. Liu et al. have proved in HEK293T cells and RAW264.7 cells that P2X7R was involved in the initiation of NF-κB. The co-expression of P2X7R and MyD88 enhanced the activation of NF-κB, while siRNA silencing of MyD88 almost eliminated P2X7R-initiated NF-κB activation [25]. There were also some studies using animal models to show the connection between P2X7R and NF-κB, including rat epilepticus model [26], rat arthritis model [27,28], mouse acute lung injury model [29], etc. Nevertheless, there are no researches regarding the relation between P2X7R and NF-κB in DCs. The present study is the first to demonstrate that ATP could promote DC maturation and release of inflammatory cytokines through P2X7R-NF-κB (p65) pathway.

In addition, results of our study showed that the intracellular ATP levels were increased along with the maturation of DC. This may be because some important functions of immune cells require ATP to provide energy, such as cation transport, polymer synthesis, and other important processes involved in targeting antigens [30]. In recent years, the intracellular ATP level of CD4+ T lymphocytes has become an indicator of immune function in recipients of transplantation. The response of helper T lymphocytes to mitotic stimuli could be measured by intracellular ATP levels, because ATP is necessary for most of the effective functions of immune cells [31,32]. Given the above, our results suggested that ATP may play an important role in the development of DC's immune function and intracellular ATP level could also be an indicator of the maturation state of DC.

All in all, the present study could contribute to understanding the pathological mechanisms of infectious diseases, inflammation, transplantation, tumor, autoimmune diseases etc. However, there are some limitations of this study. Here, the primary bone marrow-derived DCs of mice were used for in vitro experiments. More studies on human cells

and with in vivo experiments are required to verify the effects of ATP and P2X7R on the immune responses induced by DC. Also, more studies are needed to seek the best way to regulate ATP.

5. Conclusions

In conclusion, results of this study suggested that extracellular ATP could promote DC maturation and release of inflammatory cytokines through P2X7R-NF-κB (p65) pathway. Interference with this pathway may be able to regulate immune responses in areas like infectious diseases, inflammation, transplantation, tumor and autoimmune diseases. In addition, intracellular ATP level could be a new indicator of the maturation state of DC.

Conflicts of interest

The authors declared that there is no conflict of interest.

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