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P2X4 receptor regulates P2X7 receptor-dependent IL-1β and IL-18 release in mouse bone marrow-derived dendritic cells

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ABSTRACT

Activation of P2X7 receptor of dendritic cells plays a significant role in inflammation through production of cytokines such as IL-1β, and recent studies have suggested structural and functional interactions of P2X7 receptor with P2X4 receptor in macrophages. However, it is unknown whether P2X4 receptor modulates P2X7 functions in dendritic cells. Here, we present evidence that expression of P2X4 receptor is required for P2X7 receptor-dependent IL-1β and IL-18 release in mouse bone marrow-derived dendritic cells (BMDCs). We confirmed expression of both P2X7 receptor and P2X4 receptor in BMDCs. Treatment of BMDCs with 3 mM ATP caused a transient, P2X4-dependent elevation, or spike, of intracellular Ca^{2+} level $[Ca^{2+}]_i$, followed by the sustained P2X7-dependent increase of $[Ca^{2+}]_i$. We performed knockdown of P2X4 receptor in BMDCs by transfection with short hairpin RNA targeting this receptor. The ATP-induced initial peak of $[Ca^{2+}]_i$ was decreased in P2X4-knockdown cells (P2X4-KD). Further, we found that ATP-induced IL-1β and IL-18 release from LPS-primed BMDCs was suppressed by pretreatment with P2X7 antagonist A438079 or P2X4 antagonist TNP–ATP. The P2X7-dependent IL-1β and IL-18 release was significantly lower in P2X4-KD cells. Chelation of intracellular Ca^{2+} also caused suppression of ATP-induced IL-1β and IL-18 release. These results suggest that P2X4 receptor-induced Ca^{2+} influx is required for effective production of IL-1β and IL-18 via activation of P2X7 receptor in BMDCs. We conclude that co-expression of P2X4 receptor with P2X7 receptor in dendritic cells leads to enhancement of inflammation through facilitation of P2X7-dependent release of pro-inflammatory cytokines.

1. Introduction

Dendritic cells (DCs) are the most potent antigen-presenting cells in the immune system [1]. These cells are distributed widely in the body and regulate both immunity and immune tolerance. They are specialized for capturing and processing antigens, and then presenting antigenic peptides to naive T lymphocytes to initiate antigen-specific immune responses [2]. Moreover, DCs produce several pro-inflammatory cytokines, including IL-1β and IL-18, which play a significant roles in induction of inflammation in a variety of diseases [3,4].

Extracellular ATP is able to evoke physiological responses in a wide spectrum of tissues via binding to P2 receptors. P2 receptors have classified into two major groups; ligand-gated ion channel P2X receptors and metabotropic G protein-coupled P2Y receptors [5]. These receptors and their ligand (extracellular ATP) play important roles in cell signaling, modulation of cell growth, differentiation and induction of cell death [6,7].

P2X7 receptor is the seventh member of the P2X receptor subfamily, and is expressed in immune cells, including macrophages, dendritic cells, and T cells [6,8,9]. P2X7 receptor is unique among the P2X receptors in that it requires high concentrations (millimolar range) of ATP to be activated. P2X7 receptor in unactivated immune cells shows little or no functional activity, but is upregulated and becomes functional in response to lipopolysaccharide (LPS) and other inflammatory stimuli [10]. Activation of P2X7 receptor induces Ca^{2+} influx, non-selective large pore formation, caspase-1 activation, cytokine production and cell death [7–14]. P2X7-dependent activation of caspase-1 and K^+ efflux lead to production of inflamasome-dependent cytokines, such as IL-1β and IL-18 [9,14]. The effects of extracellular ATP and activated P2X7 receptor

Abbreviations: BAPTA–AM, 1,2-bis(2-aminophenoxy)ethane-*N,N,N,N*-tetraacetic acid acetoxymethyl ester; BzATP, 2′&3′-*O*-(4-benzoyl)benzoyl-ATP; $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration; shRNA, short hairpin RNA.

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on DC function have been studied extensively. Activation of P2X7 receptor in murine and human DCs causes DC maturation and production of pro-inflammatory cytokines such as IL-1β [11–13,15,16]. It is noteworthy that P2X7-dependent IL-1β release from DCs plays a critical role in contact hypersensitivity [17].

Since the P2X7 subtype differs from other members of the family in having a very long cytoplasmic C-terminal tail, and a low affinity for ATP, it has been widely assumed that P2X7 does not form heteromeric assemblies with other members of the P2X family. However, recent evidence has indicated a structural interaction between P2X7 and P2X4 receptors [18,19]. P2X4 receptor, which is highly permeable to calcium [20], is more homologous to P2X7 receptor (~40%) than are the other P2X receptor subtypes at the amino acid sequence level. P2X4 receptor is markedly up-regulated by LPS due to activation of Toll-like receptors [21]. It is abundantly expressed in activated microglia [22,23] and macrophages [24], and is also expressed in DCs [25]. Recent work has demonstrated that P2X4 receptor influences inflammasome signaling associated with IL-1β processing in neurons [26], and thus P2X4 receptor has become a focus of attention in efforts to control inflammatory responses. Previously, we have shown that decreased P2X4 expression results in suppression of the initial ATP-induced Ca^{2+} influx and P2X7 receptor-mediated inflammatory functions, such as release of IL-1β, release of high mobility group box-1 (HMGB1), and cell death in RAW 264.7 mouse macrophages [27,28]. Thus, there is increasing evidence pointing to major roles of P2X7 and P2X4 receptors in inflammatory functions in various cells, but it is still unknown whether P2X4 receptor is involved in P2X7 receptor-dependent events in DCs.

In this study, in order to further elucidate the mechanisms of inflammatory functions in DCs, we investigated the role of P2X4 receptor in P2X7 receptor-dependent release of IL-1β and IL-18, which are inflamasome-dependent pro-inflammatory cytokines. We show here that decrease of P2X4 expression results in suppression of P2X7-dependent IL-1β and IL-18 release in DCs. Our findings indicate that P2X4 receptor has an important role in the regulation of DC-mediated inflammation via modulation of P2X7-mediated inflammatory functions.

2. Materials and methods

2.1. Animals

Male C57BL/6 mice were purchased from Sankyo Labo Service (Tokyo, Japan) and used at 6 weeks of age. They were housed in plastic cages with paper chip bedding and bred in rooms kept at a temperature of 23 ± 2 °C and a relative humidity of 55 ± 10% under a 12 h light–dark cycle. They were allowed free access to tap water and normal diet, CE-2 (CLEA Co, Tokyo, Japan). The mice were treated and handled according to the Guiding Principles for the Care and Use of Laboratory Animals of the Japanese Pharmacological Society and with the approval of Tokyo University of Science's Institutional Animal Care and Use Committee.

2.2. Generation of DC from bone marrow

Bone marrow from a 6 week-old male C57BL/6 mouse was used. The cell suspension was obtained by flushing the femur with 2 ml of PBS. A single cell suspension was made by pipetting and filtering the cells through a sterile strainer (70 mm). Cells were then plated at a density of 2×10^5 cells/ml in conditioned medium (10 ng/ml GM–CSF) at 37 °C. Cultures were fed fresh conditioned medium every 3–4 days. Non-adherent cells were discarded at day 4. First passages of DC-enriched cultures were performed at day 7. Adherent DCs were collected and used for experiments.

2.3. Measurement of intracellular calcium level

Cells were washed twice, then loaded with the Ca^{2+}-sensitive fluorescent dye Fluo-4AM (Invitrogen, Carlsbad, CA) in Ca^{2+}-free RPMI 1640-based buffer for 30 min at 37 °C, and washed twice with Ca^{2+}-free RPMI 1640-based buffer. Cells were then suspended in Ca^{2+}-free RPMI 1640-based buffer. The samples were analyzed using a fluorescence spectrometer (F-2500, Hitachi) with laser excitation at 495 nm and emission at 518 nm.

2.4. Determination of IL-1β and IL-18 production

Culture supernatant was harvested, and IL-1β and IL-18 were measured by means of ELISA. A 96-well plate was coated with purified anti-mouse IL-1β mAb (1:250) or purified anti-mouse IL-18 mAb (1:2000) (R&D Systems, Inc., Minneapolis, MN, USA) and incubated overnight at 4 °C. The wells were washed with PBS containing 0.05% Tween-20, and nonspecific binding was blocked with PBS containing 1% bovine serum albumin for 1 h at room temperature. The plate was washed, culture supernatant was added, and incubation was continued for 2 h at room temperature. The plate was washed again, and anti-mouse biotin-conjugated IL-1β mAb (1:250) or anti-mouse biotin-conjugated IL-18 mAb (1:2000) (R&D Systems, Inc.) was added for 1 h at room temperature. The plate was further washed, and avidin–horseradish peroxidase (Sigma) was added. The plate was incubated for 30 min at room temperature, then washed, and 3,3′,5,5′-tetramethylbenzidine was added. The reaction was stopped by adding 5 N H_2SO_4, and the absorption at 450 nm was measured with an ImmunoReader NJ-2000 (Nihon InterMed, Tokyo, Japan). A standard curve was established with recombinant mouse IL-1β or recombinant mouse IL-18 (R&D Systems, Inc), and the concentration of IL-1β and IL-18 were estimated by interpolation.

2.5. Immunoblotting

Equal amounts of cell lysates were dissolved in 2× sample buffer (50% glycerin, 2% SDS, 125 mM Tris, 10 mM DTT) and boiled for 10 min. Aliquots of samples containing 4 μg of protein were analyzed by 10% SDS–PAGE and transferred onto a PVDF membrane. Blots were blocked in TBST with 1% bovine serum albumin at 4 °C overnight and incubated for 1.5 h at room temperature with anti-P2X4 receptor Ab (1:300) (Alomone Labs, Jerusalem, Israel), anti-P2X7 receptor Ab (1:200) (Alomone Labs, Jerusalem, Israel) or with mouse anti-β-actin antibody (1:1000) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) to confirm equal loading. Blots were washed with TBST, incubated with goat horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody (1:20,000) (Cell Signaling Technology) or goat HRP-conjugated anti-mouse IgG antibody (1:10,000) (Santa Cruz Biotechnology) for 1.5 h at room temperature, and washed again with TBST. Specific proteins were visualized by using ECL Western blotting detection reagents (GE Healthcare, Piscataway, NJ).

2.6. Short hairpin RNA (shRNA) plasmid stable transfection

Stable transfection with shRNA was performed using the SureSilencing™ shRNA Plasmid Kit for Mouse P2X4 (SABiosciences, Frederick, MD). shRNA plasmid targeting P2X4 or the negative control shRNA plasmid was introduced into cells by lipofection using X-tremeGENE 9 DNA Transfection Reagent (Roche Applied Science).

2.7. Real-time RT-PCR

Total RNA was isolated from bone marrow-derived cells using a Fast Pure RNA kit (Takara Bio, Shiga, Japan). The first-strand cDNA

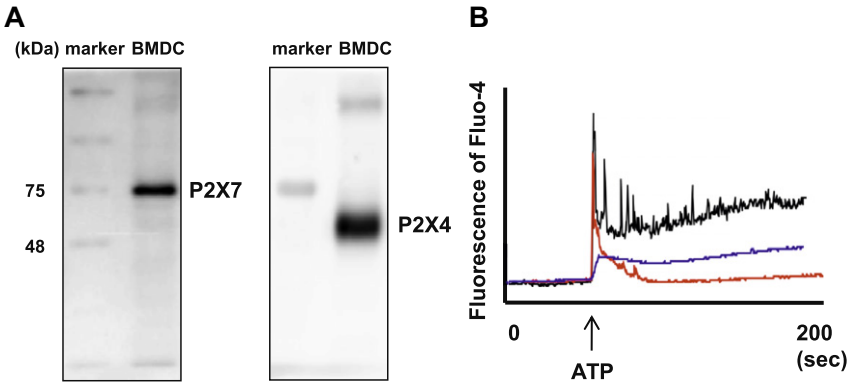

Fig. 1. Expressions of P2X4 and P2X7 receptors in BMDCs and effect of antagonists on ATP-dependent Ca^{2+} influx. (A) Expressions of P2X7 receptor (right panel) and P2X4 receptor (left panel) in BMDCs was analyzed by immunoblotting. Whole cell lysates of BMDCs were separated on 10% SDS–PAGE and immunoblotted using anti-P2X4 receptor or anti-P2X7 receptor. (B) BMDCs loaded with Fluo-4 were stimulated with 1 mM ATP in the absence (black line) or presence of 50 μM A438079 (red line) or 10 μM TNP–ATP (blue line). The fluorescence was analyzed with a fluorescence spectrometer. We obtained qualitatively similar results in three independent experiments, and typical data are presented. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

was synthesized from total RNA with PrimeScript Reverse Transcriptase (Takara Bio). The cDNA was used as the template for real-time PCR analysis: reactions were performed in a Stratagene MX3000P® quantitative PCR system (Agilent Technologies, La Jolla, CA). Specific primers were designed with PrimerQuest and synthesized by Sigma Genosys. The sequences of specific primers for murine P2X4 were 5′-TCA TCC TGG CTT ACG TCA TTG GGT-3′ (sense) and 5′-CCA CAC CTT TGG CTT TGG CTT TGG TTG TCA-3′ (antisense). GAPDH mRNA was determined as a positive control. Each sample was assayed in a 20 ml amplification reaction mixture, containing cDNA, primer mixture (0.4 mM each of sense and antisense primers), and 2× GoTaq® quantitative PCR Master Mix (Promega). The amplification program consisted of 40 cycles (each cycle: 95 °C for 15 s, annealing at 60 °C for 1 min) after 95 °C for 2 min. Fluorescent products were detected at the last step of each cycle. The obtained values were within the linear range of a standard curve and were normalized with respect to GAPDH mRNA.

2.8. Statistics

Values are given as the mean ± SE. Comparison between two values was performed by means of the unpaired Student's *t*-test. Multiple groups were compared using ANOVA followed by pairwise comparisons with Bonferroni's *post hoc* analysis. Significance was defined as $P < 0.01$. Calculations were done using the Instat version 3.0 statistical package (GraphPad Software, San Diego, CA).

3. Results and discussion

We first examined the functional expression of P2X4 and P2X7 receptors in BMDCs of C57BL/6 mice. Recent studies have shown that DCs express mRNA for P2X1, P2X4, P2X5, P2X7, P2Y1, P2Y2, P2Y4, P2Y6 and P2Y11 receptors [16]. As shown in Fig. 1(A), expression of P2X7 receptor was detected in BMDCs, and P2X4 receptor was also highly expressed (Fig. 1(A). One of the hallmarks of P2X4 receptor is its very high calcium permeability [18,20], and Ca^{2+} influx is mediated by both P2X4 and P2X7 receptors. It is known that P2X4-dependent Ca^{2+} influx is acute and transient, while P2X7-dependent Ca^{2+} influx is associated with the sustained phase of $[Ca^{2+}]_i$ increase. As shown in Fig. 1(B), we examined the effect of ATP on the elevation of $[Ca^{2+}]_i$. When BMDCs were stimulated with 1 mM ATP, they showed an initial peak of $[Ca^{2+}]_i$ followed by a sustained increase. Pretreatment with A438079 (a P2X7 receptor antagonist) (Tocris Bioscience, Bristol, UK) suppressed the sustained phase, but not the initial peak of $[Ca^{2+}]_i$. On the other hand, pretreatment with TNP–ATP (an unselective P2X4 receptor antagonist) (Tocris Bioscience, Bristol, UK) suppressed the initial peak of $[Ca^{2+}]_i$, but not the sustained phase.

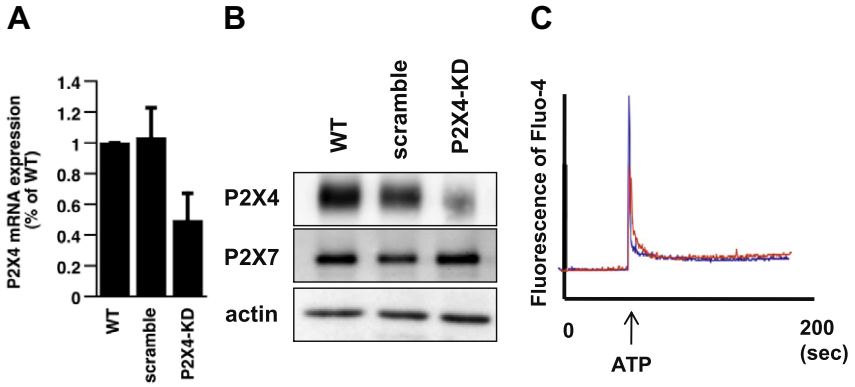

Fig. 2. Knockdown of P2X4 receptor in BMDCs. Cells was transfected with shRNA targeting P2X4 or negative control shRNA (scramble shRNA). (A) Expression levels of P2X4 mRNA in transfectants were determined by real-time RT-PCR. Optical density of P2X4 mRNA was normalized to that of GAPDH mRNA. Values are expressed as ratios to non-transfected cells (WT), and are means ± SD of three independent experiments. (B) Protein expression of P2X4 and P2X7 receptors were determined by immunoblotting. (C) BMDCs transfected with shRNA targeting P2X4 (red line) or negative control shRNA (blue line) were loaded with Fluo-4 and stimulated with 1 mM ATP, and the fluorescence was analyzed with a fluorescence spectrometer. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

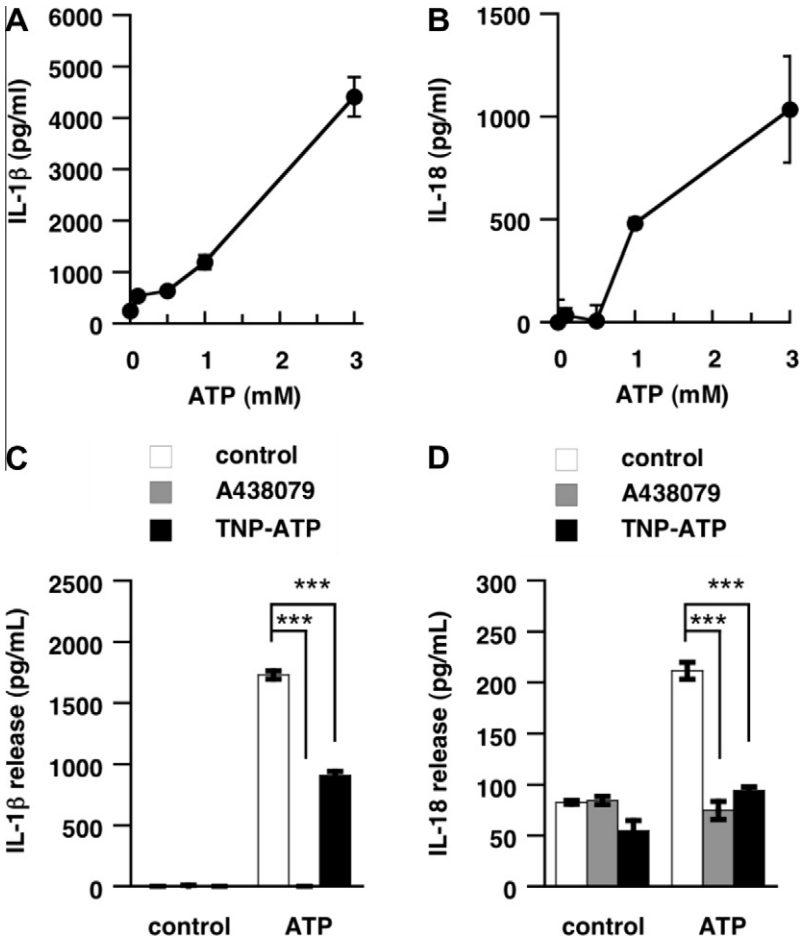

Fig. 3. ATP-induced IL-1β and IL-18 production through P2X7 receptor activation. (A and B) BMDCs were pre-incubated with LPS (1 μg/ml) for 24 h. BMDCs were incubated with the indicated dose of ATP for 6 h. (C and D) BMDCs were preincubated with 50 μM A438079 or 10 μM TNP–ATP for 30 min, and incubated with vehicle (control) or 3 mM ATP for 6 h. The culture supernatant was harvested for determination of IL-1β (A and C) or IL-18 (B and D). Concentrations of IL-1β and IL-18 in the culture supernatants were measured as described in materials and methods. Error bars indicates ± SE. Significant differences between indicated groups are indicated with ***($P < 0.001$).

These results indicate that the sustained phase of $[Ca^{2+}]_i$ elevation is induced by activation of P2X7 receptor, while P2X4 receptor is involved in the initial peak of $[Ca^{2+}]_i$. These results are consistent with our previous data obtained in RAW264.7 cells [27].

However, it is known that TNP–ATP also inhibits other P2X receptors (e.g., P2X2 and P2X3). Therefore, to confirm that P2X4 receptor is involved in ATP-mediated Ca^{2+} influx in BMDCs, we silenced the expression of P2X4 receptor with shRNA. In BMDCs transfected with P2X4-shRNA, P2X4 receptor mRNA expression level was decreased to 49% of that in scramble shRNA-transfected cells (negative control) (Fig. 2A). The protein expression of P2X4 receptor, but not P2X7 receptor, was also decreased, as shown in Fig. 2(B). Fig. 2(C) demonstrates that P2X4-knockdown cells showed a decrease of the initial peak of $[Ca^{2+}]_i$ induced by ATP, although the sustained phase of $[Ca^{2+}]_i$ increase was unaffected. Thus, we confirmed that P2X4-knockdown decreased the expression of P2X4 receptor and the acute-phase Ca^{2+} influx in these cells.

We have reported that decreased P2X4 expression results in suppression of P2X7 receptor-mediated inflammatory functions, such as release of HMGB1 and IL-1β, in RAW 264.7 mouse macrophages [27,28]. Therefore, we next investigated the involvement of P2X7 and P2X4 receptors in ATP-induced IL-1β and IL-18 production in LPS-primed BMDCs. As shown in Fig. 3(A) and (B), treatment with 1–3 mM ATP caused release of IL-1β and IL-18 from BMDCs. A significant increase of IL-1β and IL-18 production was observed from 3 h, reaching a plateau at 6 h after treatment with ATP (data not shown). Pretreatment with A438079 or TNP–ATP markedly decreased the ATP-induced release of IL-1β and IL-18 (Fig. 3C and D). These results indicate that both P2X7 and P2X4 receptor could be involved in IL-1β and IL-18 production in BMDCs.

It has been reported that Ca^{2+}-dependent IL-1β secretion occurs in LPS-stimulated DCs [16]. As we show in Fig. 2(C), activation of P2X4 receptor caused rapid Ca^{2+} influx. Therefore, we investigated the effect of pretreatment with an intracellular Ca^{2+} chelator, 1,2-bis(2-aminophenoxy)ethane-*N,N,N,N*-tetraacetic acid acetoxymethyl ester (BAPTA–AM) (Dojindo, Kumamoto, Japan) on ATP-induced IL-1β and IL-18 release, in order to confirm the involvement of Ca^{2+} influx. As shown in Fig. 4(A) and (B), ATP-induced release of IL-1β and IL-18 was decreased by suppression of the elevation of $[Ca^{2+}]_i$, indicating that the increase of $[Ca^{2+}]_i$ is involved in P2X7-dependent release of IL-1β and IL-18.

Although we have shown that P2X4 receptor is involved in P2X7 receptor-dependent IL-1β release via Ca^{2+} influx in RAW264.7 macrophages [27,28], it is not known whether P2X7-dependent IL-1β and IL-18 release from DCs is regulated by co-activation of P2X4 receptor. We therefore measured IL-1β and IL-18 release in P2X4-knockdown BMDC. We found that decreased P2X4 expression resulted in suppression of IL-1β and IL-18 release in response to ATP stimulation (Fig. 4C and D), suggesting that P2X4 receptor is involved in P2X7-mediated IL-1β and IL-18 production in BMDCs.

In conclusion, though it is well known that P2X7 receptor plays a critical role in release of IL-1β or IL-18 from dendritic cells, we here suggest that P2X4 receptor positively regulates P2X7-depen-

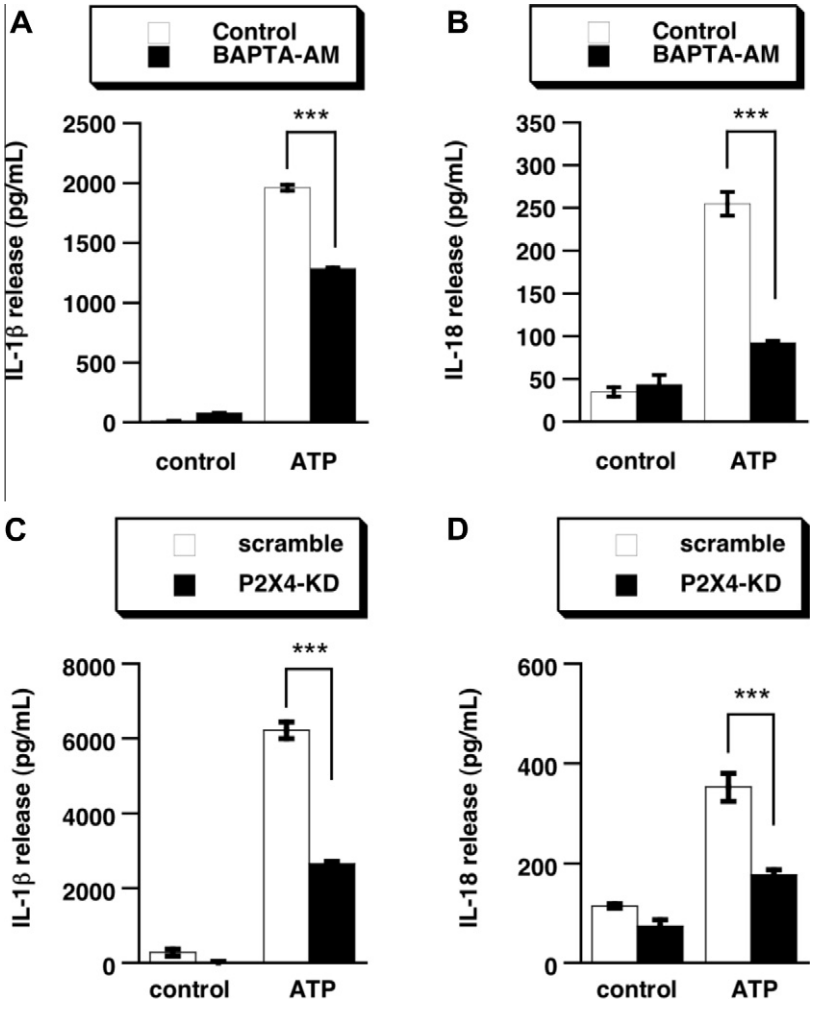

Fig. 4. Involvement of elevation of intracellular Ca^{2+} and expression of P2X4 receptor in ATP-induced IL-1β and IL-18 release. BMDCs were pre-incubated with LPS (1 μg/ml) for 24 h. (A and B) BMDCs were incubated with vehicle (control) or 3 mM ATP for 6 h in RPMI1640 buffer or RPMI1640 buffer containing BAPTA–AM (10 μM). (C and D) Cells were transfected with shRNA targeting P2X4 or negative control shRNA (scramble shRNA). Transfectants were incubated with vehicle (control) or 3 mM ATP for 6 h. At the end of incubation, supernatants were collected and IL-1β (A and C) and IL-18 (B and D) was detected by ELISA as described in materials and methods. Error bars indicates ± SE. Statistically significant differences between groups are indicated by ***($P < 0.001$), **($P < 0.01$) or *($P < 0.05$).

dent IL-1β and IL-18 release from dendritic cells. Our results may indicate that rapid and substantial initial Ca^{2+} influx via P2X4 receptor, like a spike, might be required to generate a level of $[Ca^{2+}]_i$ that exceeds a threshold value for initiation of the cascade of IL-1β and IL-18 production. This is the first study to provide evidence that P2X4 receptor has a role in the facilitation of P2X7-mediated inflammatory functions in dendritic cells. Since production of IL-1β and IL-18 from dendritic cells serves to promote inflammation, we suggest that co-expression of P2X4 and P2X7 receptors in dendritic cells would play an important role in regulating the inflammatory responses associated with a variety of immunological and pathophysiological processes.

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