Acta Physiologica Sinica, April 25, 2005, 57 (2): 193-198
http://www.actaps.com.cn

Research Paper

Effects of various inducers on the expression of P2X7 receptor in human peripheral blood mononuclear cells

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Abstract: Regulation of P2X7 receptor expression is of interest because activation of this receptor by extracellular ATP triggers a wide variety of cell functions in leukocytes. However, its expression and modulation in human peripheral blood mononuclear cells (PBMC) and monocytes remain unclear. RT-PCR was used to detect the constitutive level of P2X7 receptor and the levels upon stimulation with bacteria, bacterial product, mitogen and various cytokines in human PBMC and monocytes. P2X7 receptor mRNA was detected in PBMC and monocytes. P2X7 receptor expression in PBMC was up-regulated by interleukin-2, -4, -6 (IL-2, IL-4, IL-6) tumour necrosis factor-α (TNF-α), lipopolysaccharide (LPS) and heat-inactivated *Staphylococcus aureus* Cowan strain Ⅰ (SAC). However, interferon-γ (IFN-γ), granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF) and phytohemagglutinin-M (PHA-M) had little effect on the expression of P2X7 receptor. Furthermore, LPS and M-CSF could up-regulate P2X7 receptor expression in monocytes, while IFN-γ, TNF-α and GM-CSF had weak effects, but pretreatment with these inducers could not further enhance LPS-stimulated P2X7 receptor expression in monocytes. The results obtained demonstrate that inflammatory stimuli drive P2X7 expression, thus supporting the hypothesis that P2X7 receptor may play a role in the inflammatory responses against bacteria infection, which need further verification.

Key words: P2X7 receptor; cytokines; peripheral blood mononuclear cells; monocytes

不同诱导因子对人外周血单个核细胞 P2X7 受体表达的作用

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摘　要：ATP 激活 P2X7 受体可产生一系列的白细胞功能反应，因此 P2X7 受体的表达调控引起我们的兴趣。然而 P2X7 受体在正常人外周血单个核细胞(peripheral blood mononuclear cells, PBMC)、单核细胞中的表达调控机制尚未阐明。本文用半定量 RT-PCR 方法检测多种细胞因子、细菌抗原、丝裂原对 P2X7 受体表达的诱导作用，探索 P2X7 受体的诱导表达模式。结果表明，单个核细胞和单核细胞可检出 P2X7 受体的表达；白细胞介素 2、4、6 (interleukin-2、-4、-6, IL-2、IL-4、IL-6)、肿瘤坏死因子 α (tumour necrosis factor-α, TNF-α)等细胞因子和金黄色葡萄球菌 Cowan Ⅰ株(*Staphylococcus aureus* Cowan strain Ⅰ, SAC)、脂多糖(lipopolysaccharide, LPS)能上调 PBMC 的 P2X7 受体表达，而 γ 干扰素(interferon-γ, IFN-γ)、粒-巨噬细胞集落刺激因子(granulocyte-macrophage colony-stimulating factor, GM-CSF)、巨噬细胞集落刺激因子(macrophage colony-stimulating factor, M-CSF)和植物血凝素(phytohemagglutinin-M, PHA-M)等则没有作用； LPS 和 M-CSF 可以提高单核细胞的 P2X7 受体表达，IFN-γ、TNF-α、GM-CSF 作用较弱，但是这些因子的预处理并不能增强 LPS 对 P2X7 受体表达的诱导。炎症因子促进 P2X7 受体的表达，提示 P2X7 受体可能在对抗细菌感染的免疫反应中起一定作用，这有待于进一步研究。

关键词：P2X7 受体；细胞因子；外周血单个核细胞；单核细胞
中图分类号：Q256

Received 2004-08-19　Accepted 2004-10-20
This work was supported by the National Natural Science Foundation of China (No. 30100072).
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Intercellular communication is critical for multi-cellular organisms. Nucleotides are newly identified members carrying signals in the communications between cells through binding P2 membrane receptors and triggering specific intracellular signal transduction. The P2 receptors are divided into two structure distinct subclasses: the P2X receptors, which are two-transmembrane purinergic receptor channels, and the P2Y receptors, which are seven-transmembrane G-protein coupled receptors. The P2X7 receptor, which has unique characteristics among P2X subtypes, is a newly identified member. Binding of extracellular ATP to P2X7 receptor induces cation channel opening imparting significant permeability to Ca^{2+} and pore formation with changes in the plasma membrane potential [1-2].

The expression of P2X7 receptor has been reported in various cells in immune system including monocyte/macrophages, dendritic cells, mast cells, mesangial, and microglial cells, *etc*. Activation of P2X7 receptor by extracellular ATP triggers a wide variety of cell functions in leukocytes, including granule secretion, superoxide production, adhesion molecule expression, processing/release of cytokines, proliferation, differentiation, death through either necrosis or apoptosis, and activation of multiple downstream signaling events [3-6].

The survival, proliferation and function of immune cells are tightly governed by the complex regulating network through intercellular communications. It has been reported that lipopolysaccharide (LPS) and interferon-γ (IFN-γ) synergistically up-regulate P2X7 receptor expression and function in human THP-1 cell line of monocytic lineage [7]. However, the modulation of P2X7 receptor expression in human peripheral blood mononuclear cells (PBMC) and monocytes remains unclear. Therefore, in this study, we tested the effects of various bacterial products, cytokines on P2X7 receptor expression in normal human PBMC and monocytes.

1 MATERIALS AND METHODS

1.1 *Cytokines and reagents*

LPS (*Escherichia coli* serotype 026:B6) and PHA (PHA-M) were from Sigma. Formalin-fixed, heat-inactivated *Staphylococcus aureus* Cowan strain Ⅰ (SAC, Pansorbin) was from Calbiochem (San Diego, CA). TNF-α, M-CSF, rhIL-2 and rhIL-6 were products of Peprotech (London, UK). GM-CSF, IFN-γ and rhIL-4 were from R&D Systems, Inc. (Minneapolis, MN).

1.2 *Cell isolation and cell culture*

U937 is a human leukemic cell line and HEK293 is a human embryonic kidney cell line. Blood samples were collected from 6 healthy donors originating from Tianjin Blood Center. PBMC were isolated by standard Ficoll-Paque gradient centrifugation. Monocytes were isolated by centrifugation on a continuous Percoll density gradient (Pharmacia, Piscataway, NJ) [8]. Monocyte enrichment was confirmed by Giemsa and non-specific esterase staining (85% to 90%). All cells were maintained in RPMI-1640 medium (Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, Invitrogen), glutamine (2 mmol/L), penicillin (100 U/ml) and streptomycin (100 μg/ml), at 37 ºC in a humidified, 95% air/5% carbon dioxide atmosphere. PBMC and monocytes were cultured in polystyrene tubes (Simport Plactics Ltd., Canada) immediately after isolation.

1.3 *Stimulations*

PBMC were stimulated with the following stimuli for 4, 12 or 24 h: LPS (1 μg/ml), SAC (0.0075%, *W/V*), IL-2 (1 000 U/ml), IL-4 (100 U/ml), IL-6 (200 U/ml), IFN-γ (100 ng/ml), TNF-α (100 ng/ml), GM-CSF (100 ng/ml), M-CSF (100 ng/ml), PHA-M (10 μg/ml). Monocytes were treated with the same amounts of LPS, GM-CSF, M-CSF, TNF-α, or IFN-γ for 4 h. In priming experiments, monocytes were first pretreated with IFN-γ (100 ng/ml), GM-CSF (100 ng/ml) or M-CSF (100 ng/ml) for 12 h before treated with LPS (1 μg/ml) for another 4 h. At the end of each experiment, cells were harvested for analysis of P2X7 receptor expression by RT-PCR. Cells without any stimulus were also included in each experiment as control. All culture reagents were free of endotoxin (<0.1 EU/ml).

1.4 *RT-PCR*

Total cellular RNA was extracted using TRIzol reagent (Invitrogen) following the manufacturer's instructions and dissolved in diethylpyrocaronate (DEPC)-treated water before visualized on 1% agarose gel for the assessment of quality by measuring the intensity ratio of 28S and 18S RNA. The mRNA in 1 μg total cellular RNA was reverse transcribed in the system containing 0.5 μg oligo $(dT)_{18}$ primer, 40 U RNasin (TaKaRa Biotech, Japan) and 200 U M-MLV reverse transcriptase (Invitrogen) following the standard protocol. The two primers for P2X7 receptor were: 5'-TCTGCAAGATGTCAAGGGC-3' (1286-1304 in exon 12), and 5'-TCACTCTTCGGAAACTCTTTCC-3' (1780-1759 in exon 13) [9]. The primers for GAPDH were 5'-TGAAGGTCGGAGTCAACGGATTTGG-3' and 5'-CATGTGGGCCATGAGGTCCACCAC-3'. Two microliters of cDNA were amplified in a 50-μl reaction mixture containing 50 mmol/L KCl, 10 mmol/L Tris-HCl, 2 mmol/L $MgCl_2$, 0.2 mmol/L of each deoxyribonucleoside

triphosphates, 0.4 μmol/L primers and 1 U *Ex Taq* polymerase (TaKaRa Biotech) with hot-start PCR as follows: 95 ºC for 5 min followed by 26 (GAPDH) or 35 (P2X7 receptor) cycles: denaturation at 95 ºC for 45 s, annealing at 57 ºC (GAPDH) or 52 ºC (P2X7 receptor) for 45 s, extension at 72 ºC for 1 min, and final incubation at 72 ºC for 10 min for fully extension. Furthermore, cDNA samples with strongest amplification were serially diluted, and close correlation between the amount of product and initial cDNA was seen after PCR analysis. To further confirm that the PCR products were P2X7 receptor fragments, Pyrobest DNA polymerase (TaKaRa Biotech) was used in PCR reaction and the fragments were sequenced by ABI Prism 310 (Perkin Elmer) with the same set of primers. Ten microliters of each PCR product were visualized by electrophoresis on a 1.5% agarose gel stained with ethidium bromide, and quantified by densitometry using a Kodak digital camera equipped with Kodak image software. Results were expressed as positive index (PI), corrected for GAPDH, calculated according to the formula:

PI = (densitometrical units of P2X7 receptor) / (densitometrical units of GAPDH).

1.5 *Measurements of intracellular* Ca^{2+}

Intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) was determined as previously described [10]. Cell suspension was incubated at 37 ºC for 20 min with 3 μmol/L Fura-2/AM before washed twice with Locke's solution (mmol/L: NaCl 154, KCl 5.6, $CaCl_2$ 2.2, $MgCl_2$ 1.2, glucose 10, Hepes 5, pH 7.4), and finally resuspended at the concentration of $1\times10^6\sim2\times10^6$ cells/ml. Before analysis, aliquots of cell suspensions (1 ml) were centrifuged at 200 *g* for 1 min, and cells were resuspended in 2 ml pre-warmed (37 ºC) fresh Locke's solution before transferred into a 10 mm×10 mm quartz cuvette placed in the thermostat-regulated sample chamber of a dual excitation beam spectrophotometer (F-4500, Hitachi, Tokyo) with continuous stirring. The fluorescence intensities at 510 nm were simultaneously recorded when excited at 340 and 380 nm. At the end of each measurement, Triton X-100 was added to obtain maximal fluorescence and then excess EGTA was added to obtain minimal fluorescence. $[Ca^{2+}]_i$ was calculated using F4500 Intracellular Cation Measurement System (version 1.02) software, with a K_d value of 224 nmol/L for the Fura-2-Ca^{2+} equilibrium [11]. Sulfinpyrazone (250 mmol/L) was added to all solutions to prevent dye leakage.

2 RESULTS

2.1 PBMC and monocytes express P2X7 receptor mRNA and functional P2X7 receptor

RT-PCR was used to show the expression of P2X7 receptor mRNA in PBMC and monocytes from healthy donors. U937 cells were used as positive control while HEK293 cells were used as negative control [12]. The amplified fragment was within the C-terminal intracellular region with the expected size of 495 bp, which was unique from other P2X receptors. The PCR conditions and the number of PCR cycles for each primer pair (35 cycles for P2X7 receptor, 26 cycles for GAPDH,) were chosen according to preliminary experiments, in which PCR product was detectable in an amount directly proportional to the quantity of starting cDNA. A typical result was shown in Fig.1. DNA sequencing analysis confirmed that the amplified fragments shared 100% homology with published P2X7 receptor sequence. Signal intensities of the specific bands were normalized in relation to the GAPDH internal control to eliminate fluctuations as a result of the visible quantities of RNA loaded. The expression level (PI) was defined as the OD ratio of P2X7 receptor specific band to GAPDH.

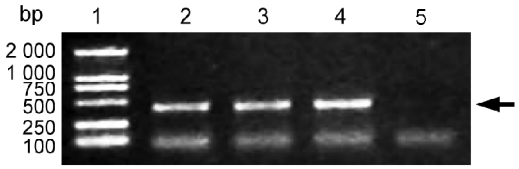

Fig. 1. Expression of P2X7 receptor in human PBMC and monocytes. The expression of P2X7 receptor mRNA in different kinds of cells was analyzed by RT-PCR detailed in *Materials and Methods*. Lane 1, DNA marker DL2000; lane 2, PBMC; lane 3, monocytes; lane 4, U937 cells; lane 5, HEK293 cells.

As P2X7 receptor mRNA was detected in human PBMC and monocytes, it's interesting to know whether these receptors were functional. We chose PBMC as the model to study whether the expressed P2X7 receptor were functional. To confirm that the $[Ca^{2+}]_i$ increase was mediated by P2X7 receptor other than other members of P2X family, we stimulated the cells with 100 μmol/L BzATP, the specific, complete and most potent agonist for the P2X7 receptor. When cells were maintained in Ca^{2+}-free medium, as indicated in Fig. 2, BzATP failed to elicit $[Ca^{2+}]_i$ increase; however, $[Ca^{2+}]_i$ increase could be observed when Ca^{2+} was reintroduced to the final concentration of 3 mmol/L. Humphreys *et al.* [13] suggested that isoquinolines interact with residues in the amino-terminal half (containing the large extracellular loop) of the human P2X7 receptor. KN-62 potently inhibited BzATP-gated Ca^{2+} influx in several leukocyte cell lines (THP-1, BAC1.2f5, and BW5147) that

natively express the human or murine P2X7 receptor mRNA. In our experiment, BzATP stimulated $[Ca^{2+}]_i$ influx could be markedly inhibited by KN-62 (3 μmol/L) (data not shown). Moreover, $[Ca^{2+}]_i$ increase could also be detected when PBMC were treated with ATP (data not shown). Together, these results suggested that PBMC cells expressed functional P2X7 receptor, which mediated the extracellular calcium-dependent, ATP and BzATP-stimulated $[Ca^{2+}]_i$ increase.

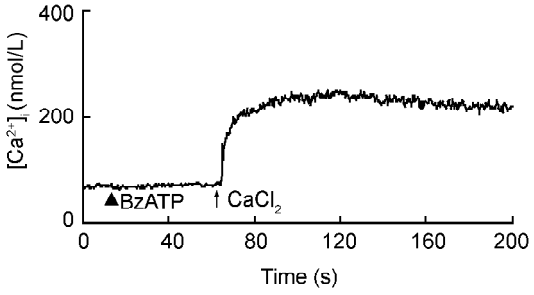

Fig. 2. P2X7 receptor-mediated $[Ca^{2+}]_i$ increase in PBMC upon stimulation. Cells were preloaded with Fura-2, washed, and resuspended in Ca^{2+}-free Locke's solution. $[Ca^{2+}]_i$ was detected by spectrophotometer (F-4500, Hitachi) as described in *Materials and Methods*. BzATP and $CaCl_2$ were added to the final concentration of 100 μmol/L and 3 mmol/L, respectively, at the time points indicated by arrows: ▲, BzATP; ↑, $CaCl_2$. The figure represented the typical results from three independent experiments.

2.2 Modulation of P2X7 receptor expression in normal PBMC upon various stimuli

All PBMC samples without stimulation clearly expressed P2X7 receptor as showed in Fig. 1. When cells were treated with various cytokines (IL-2, IL-4, IL-6, TNF-α, IFN-γ, M-CSF, GM-CSF), LPS, SAC and T cell mitogen PHA-M as stimuli for 4 h, different expression patterns were observed (Fig. 3*A*). IL-2, TNF-α, LPS and SAC could induce 2.0-folds, 1.5-folds, 2.0-folds and 2.8-folds increase of P2X7 receptor, respectively, while IL-4 and IL-6 could induce 1.2-folds and 0.9-fold increase of P2X7 receptor at mRNA level, respectively. However, IFN-γ, GM-CSF, M-CSF and PHA-M had little effect on P2X7 receptor expression. No further enhancement of P2X7 receptor expression could be detected when cells were treated with these stimuli for 12 or 24 h. Instead, the enhancements decreased significantly at the time point of 24 h when compared with the results of 4 h. (Fig. 3*B*)

2.3 Modulation of P2X7 receptor expression in monocytes upon various stimuli

The basal level of P2X7 receptor mRNA expression in

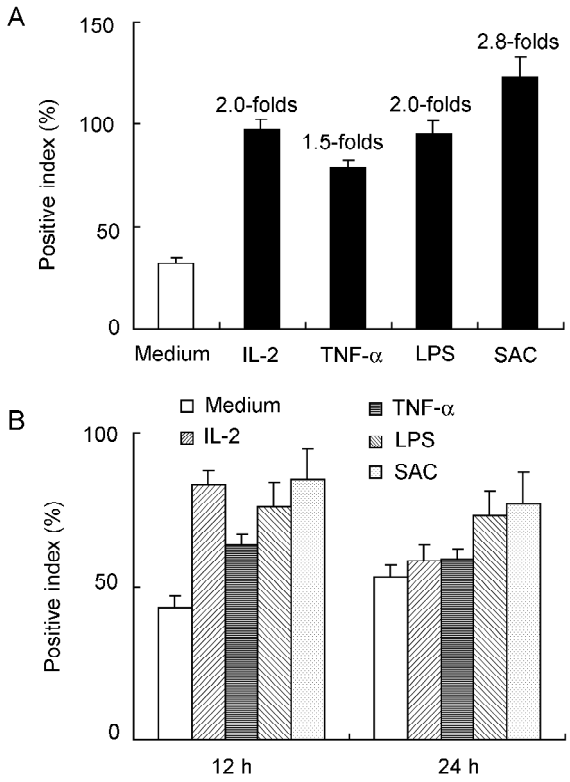

Fig. 3. Effects of different stimuli on P2X7 receptor expression in PBMC. PBMC were cultured with medium alone (control) or treated with IL-2 (1 000 U/ml), TNF-α (100 ng/ml), LPS (1 μg/ml) or SAC (0.0075%, *W/V*) for 4 h (*A*) or 12 and 24 h (*B*) before semiquantitative RT-PCR analysis. mean±SD, *n*=3. The P2X7 receptor positive index of samples was quantified by fluorography and normalized to GAPDH. The extent of significant increase (fold) was indicated.

freshly isolated monocytes was similar to that of PBMC. However, LPS could induce 1.4-folds increase of P2X7 receptor mRNA expression in monocytes (Fig. 4*A*), M-CSF could induce 1.0-fold increase, while IFN-γ, TNF-α, and GM-CSF could weakly up-regulate P2X7 receptor expression after 4 h stimulation (Fig. 4*B*). Then monocytes were first treated with IFN-γ, GM-CSF or M-CSF for 12 h, followed by LPS stimulation for 4 h to see whether the pretreatment of these cytokines could enhance the effects of LPS. However, no significant enhancement of P2X7 receptor mRNA expression could be observed (Fig. 5) compared with cells without cytokine pretreatment.

3 DISCUSSION

P2X7 receptor is of special interest for its unique structure and characteristics among P2X receptors. Of the P2X receptor subtypes, only P2X7 receptor is associated with

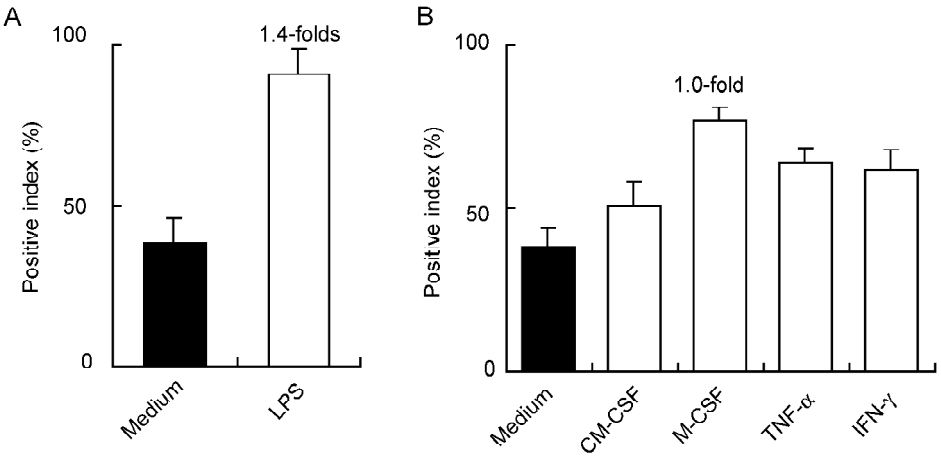

Fig. 4. Effects of different stimuli on P2X7 receptor expression in monocytes. Monocytes from healthy donors were cultured with medium alone (control) or treated with LPS (1 μg/ml), GM-CSF (100 ng/ml), M-CSF (100 ng/ml), TNF-α (100 ng/ml), or IFN-γ (100 ng/ml) for 4 h before semiquantitative RT-PCR analysis. mean±SD, n=3. The P2X7 receptor positive index of samples was quantified by fluorescence intensity and normalized to GAPDH. The extent of significant increase (fold) was indicated.

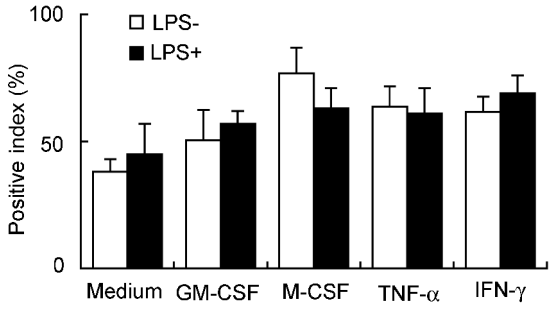

Fig. 5. Effects of different stimuli on LPS-induced up-regulation of P2X7 receptor expression in human monocytes. Monocytes from healthy donors were pretreated with IFN-γ (100 ng/ml), GM-CSF (100 ng/ml),TNF-α (100 ng/ml), or M-CSF (100 ng/ml) for 12 h, then cultured with or without LPS (1 μg/ml) for additional 4 h before semiquantitative RT-PCR analysis. mean±SD, n=3. The P2X7 receptor positive index of samples was quantified by fluorescence intensity and normalized to GAPDH.

the formation of a nonselective pore that is permeable to both inorganic ions and small (<900 Da) organic molecules. While the functional role of these pores is unclear, analysis of the regulation of P2X7 receptor expression may provide important clues about its role in leukocyte physiology for further study.

The presence of P2X7 receptor could be identified at mRNA, protein and functional levels. In this study, we first showed that all the PBMC and monocytes samples from 6 donors expressed P2X7 receptor at mRNA level. Then we chose PBMC as the model and analyzed the change of intracellular free calcium concentration as the functional marker to confirm the presence of P2X7 receptor in these cells. Since several P2 receptors may co-express on the same cell type, the $[Ca^{2+}]_i$ increase by other activated P2 subtypes upon ATP stimulation should be excluded. We found that $[Ca^{2+}]_i$ increase could be observed in PBMC upon stimulation of both ATP and BzATP, which is the specific, complete and most potent agonist of P2X7 receptor. Furthermore, the P2X7 receptor specific inhibitor KN-62 could block the process. Hence, we conclude that these cells express functional P2X7 receptor.

It was reported that LPS and IFN-γ could synergistically up-regulate P2X7 receptor mRNA in human THP-1 cell line [7]. LPS binding to leukocytes triggers a wide variety of responses aimed at defending the host against bacterial infection, including induction of genes encoding cytokines, adhesion molecules, and enzymes that produce inflammatory mediators [14-16]. In our study, we found that SAC and LPS could induce a peak increase of P2X7 receptor expression in PBMC at mRNA level after 4 h stimulation. Thus, it appears that SAC and LPS are potent inducers of P2X7 receptor expression in PBMC. The ability of bacteria and bacterial products to induce P2X7 receptor expression strongly suggests that P2X7 receptor plays a role in determining the early immune response to bacterial infection. Our results showed that other cytokines including IL-2, TNF-α, IL-4 and IL-6 are also potent inducers of P2X7 receptor expression, but IFN-γ, GM-CSF, M-CSF and PHA-M have little effect on P2X7 receptor expression. This is the first report of a panel of cytokines

on the modulation P2X7 receptor expression in PBMC.

Then monocytes were isolated and treated with both LPS and TNF-α for 4 h to see whether they could effectively up-regulate P2X7 receptor expression in monocytes. On the contrary to our hypothesis, relatively low increase of P2X7 receptor transcript could be observed in monocytes, which suggested that the accumulation of P2X7 receptor mRNA in PBMC by stimulation with LPS and TNF-α might mainly be contributed by other cell types (T and/or B lymphocytes) than monocytes. Moreover, we found that priming with IFN-γ, GM-CSF or M-CSF could not result in significant increase of P2X7 receptor expression by LPS stimulation in monocytes.

How LPS induces the expression of P2X7 receptor is of interest. Since LPS is a potent inducer of a variety of cytokines in lymphocytes and monocytes, certain cytokines are possibly secreted in response to LPS stimulation. Hence, there raises two possible mechanisms: One is that LPS directly induces P2X7 receptor expression; another is that LPS induces one or several of these cytokines, which ultimately regulate P2X7 receptor expression. However, our data showed that LPS was more potent than the cytokines tested, suggesting that the mechanism by which LPS up-regulates the P2X7 receptor seems not through these secondary inflammatory cytokines, at least not through any of the single cytokine, and whether through other pathways, awaits further study to elucidate.

In summary, in this paper, we show that the expression and function of P2X7 receptor are constitutive in normal human PBMC and monocyte/macrophage lineage. P2X7 receptor expression can be up-regulated by SAC, LPS and several cytokines.

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