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## Functional expression of the damage-associated molecular pattern receptor P2X7 on canine kidney epithelial cells

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### Abstract

Epithelial cells are important in inflammation and immunity. In this study, we examined if Madin-Darby canine kidney (MDCK) epithelial cells express functional P2X7 receptors, which bind the damage-associated molecular pattern extracellular adenosine 5'-triphosphate (ATP). Reverse transcription (RT)-PCR and immunoblotting revealed the expression of P2X7 in MDCK cells. A flow cytometric assay demonstrated that ATP or 2'(3')-O-(4-benzoylbenzoyl)ATP induced ethidium(+) uptake into MDCK cells, and that this process was impaired by the P2X7 antagonists KN-62 and A438079. RT-PCR also demonstrated the presence of Toll-like receptor 4, NALP3, caspase-1, interleukin-1 $\beta$  and interleukin-18 in MDCK cells, as well as in positive control LPS-primed canine monocytes. In conclusion, the MDCK epithelial cell line expresses functional P2X7, as well as Toll-like receptor 4 and molecules associated with the NALP3 inflammasome. This cell line may help elucidate the role of these molecules in kidney epithelial cells and renal disorders in dogs and humans.

### Keywords

kidney, canine, p2x7, receptor, pattern, molecular, associated, damage, expression, cells, functional, epithelial

### Disciplines

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# Functional expression of the damage-associated molecular pattern receptor P2X7 on canine kidney epithelial cells

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## Abstract

Epithelial cells are important in inflammation and immunity. In this study, we examined if Madin-Darby canine kidney (MDCK) epithelial cells express functional P2X7 receptors, which bind the damage-associated molecular pattern extracellular adenosine 5'-triphosphate (ATP). Reverse transcription (RT)-PCR and immunoblotting revealed the expression of P2X7 in MDCK cells. A flow cytometric assay demonstrated that ATP or 2'(3')-O-(4-benzoylbenzoyl)ATP induced ethidium<sup>+</sup> uptake into MDCK cells, and that this process was impaired by the P2X7 antagonists KN-62 and A438079. RT-PCR also demonstrated the presence of Toll-like receptor 4, NALP3, caspase-1, interleukin-1 $\beta$  and interleukin-18 in MDCK cells, as well as in positive control LPS-primed canine monocytes. In conclusion, the MDCK epithelial cell line expresses functional P2X7, as well as Toll-like receptor 4 and molecules associated with the NALP3 inflammasome. This cell line may help elucidate the role of these molecules in kidney epithelial cells and renal disorders in dogs and humans.

**Keywords:** purinergic receptor; TLR4; inflammasome; cytokine; epithelial cell; dog

**Abbreviations:** ATP, adenosine 5'-triphosphate; BzATP, 2'(3')-O-(4-benzoylbenzoyl)ATP; DAMP, damage-associated molecular pattern; MDCK, Madin-Darby canine kidney; MFI, mean fluorescence intensity; PAMP, pathogen-associated molecular pattern; RT, reverse transcription; TLR4, Toll-like receptor 4.

## 1. Introduction

Damage-associated molecular patterns (DAMPs) play important roles in inflammation and immunity by functioning as signals of cell damage, stress or death during infection, injury or disease (Kono and Rock, 2008; Chen and Nuñez, 2010). One of the best-characterized DAMP is extracellular adenosine 5'-triphosphate (ATP) which mediates its effects through the activation of purinergic receptors particularly P2X7, a trimeric ligand-gated cation channel (Bours, et al., 2011; Wiley, et al., 2011). Activation of P2X7 by extracellular ATP or the most potent P2X7 agonist 2'(3')-O-(4-benzoylbenzoyl)ATP (BzATP) causes the flux of Ca<sup>2+</sup>, Na<sup>+</sup> and K<sup>+</sup>, as well as the uptake of organic cations such as ethidium<sup>+</sup> (Jarvis and Khakh, 2009). Furthermore, P2X7 activation induces various downstream events including the NALP3 inflammasome/caspase-1-dependent maturation of IL-1 $\beta$  and IL-18, and their subsequent release from various myeloid cell types (Di Virgilio, 2007). This event, at least in monocytes, requires the prior activation of cells with the Toll-like receptor 4 (TLR4) ligand, LPS, a well-characterized pathogen-associated molecular pattern that induces the up-regulation and assembly of the NALP3 inflammasome, as well as the synthesis of IL-1 $\beta$  and IL-18 (Mehta, et al., 2001; Bauernfeind, et al., 2009).

The role of P2X7 in inflammation and immunity is largely attributed to its expression on myeloid and lymphoid cells, but there is emerging evidence that P2X7 on epithelial cells is also involved in these responses. P2X7 activation prevents chlamydial infection in cervical epithelial cells (Darville, et al., 2007), while kidney epithelial P2X7 is involved in inflammation and renal injury (Goncalves, et al., 2006; Taylor, et al., 2009). Moreover, P2X7 activation induces caspase-1-dependent IL-1 $\beta$  release from intestinal epithelial cells, implicating a role for the NALP3 inflammasome in this process (Cesaro, et al., 2010). As a result of these and other observations, P2X7 is attracting considerable interest as a therapeutic target in kidney

and other disorders (Arulkumaran, et al., 2011). The role of P2X7 on epithelial cells from the kidney and other tissues however remains poorly understood.

The presence of functional P2X7 on human and rodent cell types is well established, but little is known about P2X7 in other mammalian species. We have previously demonstrated that peripheral blood monocytes, lymphocytes and erythrocytes from English Springer Spaniels and other breeds express functional P2X7 (Sluyter, et al., 2007; Shemon, et al., 2008; Stevenson, et al., 2009; Jalilian, et al., 2012), however similar studies in other canine cell types are lacking. Given the importance of P2X7 in human health and disease (Sluyter and Stokes, 2011), new knowledge about this receptor in the dog is necessary to establish and understand the role of P2X7 in canine health and disease.

Therefore, using molecular, immunochemical and pharmacological approaches we investigated whether the Madin-Darby canine kidney (MDCK) epithelial cell line, originally derived from a Cocker Spaniel, expresses functional P2X7 receptors. Moreover, we investigated whether this cell line also expresses TLR4 and molecules associated with the NALP3 inflammasome.

## 2. Materials and methods

### 2.1. Materials

RPMI-1640 medium, L-glutamine and ExoSAP-IT were from Invitrogen (Grand Island, NJ). FCS was from Bovogen Biological (East Keilor, Australia). Ficoll-Paque™ PLUS was from GE Healthcare Biosciences (Uppsala, Sweden). Ethidium bromide was from Amresco (Solon, OH). BigDye Terminator v3.1 was from Applied Biosystems (Carlsbad, CA). ATP, BzATP, LPS (*Escherichia Coli* serotype 055:B5) and nigericin were from Sigma Chemical Co (St Louis, MO).

KN-62 and A438079 were from Alexis Biochemicals (Lausen, Switzerland) and Tocris Bioscience (Ellisville, MO), respectively.

## *2.2. Cells*

MDCK (European Collection of Cell Cultures, Porton Down, UK) and RAW264.7 (American Type Culture Collection, Rockville, MD) cells were maintained in complete culture medium (RPMI-1640 medium containing 2 mM L-glutamine and 10% FCS) at 37°C/5% CO<sub>2</sub>. For PBMC isolation, peripheral blood was collected into VACUETTE® lithium heparin tubes (Greiner Bio-One, Frickenheisen, Germany) from either pedigree or crossed breed dogs with informed, signed consent of pet owners, and with the approval of the University of Wollongong Ethics Committee (Wollongong, Australia). PBMCs were then isolated from buffy coats using Ficoll-Paque™ density centrifugation. PBMCs in complete culture medium were then incubated for 2 h at 37°C/5% CO<sub>2</sub>, the non-adherent cells were removed by gently washing twice with PBS, and the plastic-adherent cells incubated for a further 4 h in complete culture medium containing 0.1 µg/ml LPS.

## *2.3. Detection of mRNA and protein*

Total RNA was isolated using the RNeasy® Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Equal amounts of RNA for each cell type were amplified by reverse transcription (RT)-PCR using the MyTaq™ One-Step RT-PCR Kit (Bioline, Sydney Australia) according to the manufacturer's instructions using primer pairs (GeneWorks, Hindmarsh, Australia) specific for various canine mRNA transcripts (Table 1). The identity of each transcript was confirmed by sequencing of Exo-SAP-IT purified

amplicons using the above primer pairs with BigDye Terminator and an Applied Biosystems 3130xl Genetic Analyzer.

Equal amounts of protein for whole cell lysates were separated by SDS-PAGE, transferred to nitrocellulose membrane and immunoblotting was performed using a rabbit anti-mouse P2X7 polyclonal Ab (Alomone Labs, Jerusalem, Israel) as described (Constantinescu, et al., 2010). Mean fluorescence intensity (MFI) of cell-surface TLR4 expression was detected using PE-conjugated murine anti-human TLR4 (clone HTA125) and IgG<sub>2a</sub> isotype mAbs (both eBioscience, San Diego, CA) and flow cytometry as described (Gadeock, et al., 2010).

#### *2.4. Ethidium<sup>+</sup> uptake*

Nucleotide-induced ethidium<sup>+</sup> uptake into cells suspended in either sucrose (280 mM sucrose, 5 mM KCl, 10 mM *N*-methyl-D-glucamine, 10 mM glucose, 0.1% BSA, 10 mM HEPES, pH 7.4) or NaCl (145 mM NaCl, 5 mM KCl, 5 mM glucose, 0.1% BSA, 10 mM HEPES, pH 7.4) medium was determined using a fixed-time flow cytometric assay as described (Gadeock, et al., 2012).

#### *2.5. IL-1 $\beta$ release*

MDCK cells were incubated overnight in 24-well plates ( $2 \times 10^5$  cells/0.5 ml/well) at 37°C/5% CO<sub>2</sub>, and then in the absence or presence of LPS (as indicated) for 24 h. Alternatively, MDCK cells were incubated overnight in 24-well plates ( $1 \times 10^5$  cells/well) at 37°C/5% CO<sub>2</sub> and then in the absence or presence of 10  $\mu$ g/ml LPS for 4 or 24 h. These cells were then pre-incubated in RPMI-1640 medium (containing 0.1% bovine serum albumin) or

in sucrose medium (containing 2 mM Ca<sup>2+</sup> and 1 mM Mg<sup>2+</sup>) (0.3 ml/well) at 37°C/5% CO<sub>2</sub> for 15 min, followed by 60 min incubation in the absence or presence of 3 or 5 mM ATP or 20 μM nigericin. Following incubations, samples were centrifuged (11,000 x *g* for 30 s) and cell-free supernatants stored at -80°C until required. The amount of IL-1β in cell-free supernatants was quantified using a Canine IL-1β VetSet™ ELISA Development Kit Kingfisher Biotech (St. Paul, MN) according to the manufacturer's instructions.

## *2.6. Statistical analysis*

Quantitative data are presented as mean ± SD. Differences between treatments were compared using ANOVA for multiple comparisons (with Tukey's post test).

## **3. Results and discussion**

### *3.1. MDCK cells express functional P2X7 receptors*

RT-PCR was first used to determine if MDCK cells express P2X7. Based on our recent observations (Jalilian, et al., 2012), LPS-primed canine monocytes were used as a positive control. Both MDCK cells and LPS-primed canine monocytes produced P2X7 mRNA (Fig. 1A). Transcripts corresponded to the predicted size (240 bp) for P2X7 and sequencing confirmed the identity of each as P2X7 (results not shown). Immunoblotting with a rabbit anti-murine P2X7 Ab was then used to confirm the presence of P2X7 in MDCK cells. This Ab recognizes an extracellular epitope of murine P2X7 (residues 132-152), which is 100% identical to the corresponding sequence of canine P2X7. Murine RAW264.7 cells, which we have previously shown to express P2X7 (Constantinescu, et al., 2010), were used as a positive control.

Immunoblotting revealed the presence of a major band at 75 kDa, the predicted size of glycosylated P2X7, in both MDCK and RAW264.7 cells (Fig. 1B).

Next we used a flow cytometric ethidium<sup>+</sup> uptake assay to determine if MDCK cells express functional P2X7. Preliminary experiments demonstrated that 5 min incubation of MDCK cells suspended in NaCl medium with 1 mM ATP or 200  $\mu$ M BzATP, the most potent P2X7 agonist (Jarvis and Khakh, 2009), failed to induce ethidium<sup>+</sup> uptake into these cells, despite significant ethidium<sup>+</sup> uptake into RAW264.7 cells in NaCl medium after 5 min ATP or BzATP incubation (results not shown). Sucrose medium facilitates P2X7-induced cation uptake in other cell types (Michel, et al., 1999; Gadeock, et al., 2012). Therefore, the ability of ATP and BzATP to induce ethidium<sup>+</sup> uptake into MDCK cells suspended in sucrose medium was assessed. Both 1 mM ATP and 200  $\mu$ M BzATP induced significant ethidium<sup>+</sup> uptake into MDCK cells within 5 min, as well as at later time-points (Fig. 1C). To determine whether the ATP-induced ethidium<sup>+</sup> uptake into MDCK cells was mediated by P2X7, cells suspended in sucrose medium were pre-incubated in the absence or presence of the P2X7 antagonists, KN-62 and A438079, which impair human, monkey, and murine P2X7 (Donnelly-Roberts, et al., 2009; Bradley, et al., 2011). Pre-incubation of cells with 1  $\mu$ M KN-62 or 10  $\mu$ M A438079 inhibited 1 mM ATP-induced ethidium<sup>+</sup> uptake by approximately 60% and 92%, respectively (Fig. 1D). In contrast, KN-62 or A438079 did not significantly affect ethidium<sup>+</sup> uptake in the absence of ATP (Fig. 1D).

Collectively, the above results demonstrate that the MDCK epithelial cell line expresses functional P2X7 receptors. The expression of P2X7 in MDCK cells however is relatively low, with functional responses only observed in sucrose medium, a medium known to potentiate P2X7-mediated responses (Michel, et al., 1999; Gadeock, et al., 2012). MDCK cells express various P2Y purinergic receptors (Insel, et al., 2001), however to the best of our knowledge the current study is the first to demonstrate the expression of any P2X purinergic receptor in

these cells. The presence of P2X7, *albeit* at relatively low amounts, in MDCK cells indicates caution should be exercised when studying these cells following transfection with recombinant P2X7.

The low amount of P2X7 expression in MDCK epithelial cells is consistent with the low to negligible amounts of P2X7 detected in human and murine kidney epithelium (Turner, et al., 2007). Kidney epithelial P2X7 however is up-regulated in various renal disease states including unilateral ureteral obstruction (Goncalves, et al., 2006) and glomerulonephritis (Turner, et al., 2007). These studies indicate a potential pro-inflammatory or pro-apoptotic role for P2X7 in these conditions. Although differences exist between established cell lines and primary cells, the identification of P2X7 in MDCK cells suggests that these cells may serve as a potential model to further investigate P2X7 in kidney epithelial physiology and pathophysiology. It remains to be determined if the relative amount of P2X7 on MDCK cells is altered by time post-seeding or inflammatory stimuli.

### *3.2. MDCK cells express TLR4, as well as molecules associated with the NALP3 inflammasome*

NALP3 inflammasome activation, and the subsequent maturation and release of IL-1 $\beta$  and IL-18 from LPS-primed monocytes are well-described events downstream of P2X7 activation (Di Virgilio, 2007). Therefore, RT-PCR was used to determine if MDCK cells express TLR4, as well as the NALP3 inflammasome-associated molecules, NALP3, caspase-1, IL-1 $\beta$  and IL-18. Both MDCK cells and LPS-primed canine monocytes produced TLR4, NALP3, caspase-1, IL-1 $\beta$  and IL-18 mRNA (Fig. 2A), with transcripts corresponding to the predicted size (Table 1) for each molecule (results not shown). The identity of each transcript, with the exception of IL-1 $\beta$  due to its small size of 64 bp, was also confirmed by sequencing (results not shown).

Immunolabeling with a murine anti-human TLR4 mAb, which detects canine TLR4

(Burgener and Jungi, 2008), and flow cytometry was used to confirm the presence of TLR4 in MDCK cells. Flow cytometric analysis demonstrated cell-surface TLR4 expression on MDCK cells with a MFI of  $11.1 \pm 0.9$  ( $n=3$ ) (Fig. 2B). Previous studies have shown that MDCK cells express TLR4 (Sun, et al., 2007), while incubation with LPS can induce Erk1/2 phosphorylation in these cells (Liévin-Le Moal, et al., 2011). Collectively, the current and previous studies demonstrate that the MDCK epithelial cell line express functional TLR4. TLR4 has an emerging role in renal disease. In particular, TLR4 activation induces pro-inflammatory cytokine and chemokine expression in kidney epithelial cells, and plays a significant role in the inflammation and associated damage in kidney ischemia/reperfusion injury (Wu, et al., 2007). Given that DAMPs, such as HMGB1, biglycan and hyaluronan, can also activate TLR4 and are released during renal ischemia/reperfusion injury (Wu, et al., 2007) it will be of interest to see if these DAMPs can stimulate TLR4 on MDCK cells.

The above results also demonstrate that MDCK cells express NALP3, caspase-1, IL-1 $\beta$  and IL-18 mRNA. To the best of our knowledge the expression of NALP3, caspase-1 and IL-18 have not been directly observed in MDCK cells, although pharmacological blockade of caspase-1 prevents apoptosis in MDCK cells (Feldenberg, et al., 1999) indirectly supporting the presence of this enzyme in these cells. In contrast, IL-1 $\beta$  mRNA has been previously demonstrated in MDCK cells (Gröne, et al., 2002). Despite various attempts however we were unable to detect IL-1 $\beta$  in cell-free supernatants from MDCK cells using a commercially available IL-1 $\beta$  ELISA kit (minimum assay sensitivity of 0.053 ng/ml). IL-1 $\beta$  was not detected in supernatants from MDCK cells following 24 h incubation, while 24 h incubation of MDCK cells with 0.1-10  $\mu$ g/ml LPS also failed to induce detectable IL-1 $\beta$  release (results not shown). Moreover, neither 60 min incubation with 3 mM ATP, 5 mM ATP nor 20  $\mu$ M nigericin could induce detectable IL-1 $\beta$  release from MDCK cells primed with 10  $\mu$ g/ml LPS for 4 or 24 h, or from unprimed MDCK cells (results not shown). This result contrasts our recent observations

with LPS-primed canine monocytes, in which 30 min incubation with 5 mM ATP induced IL-1 $\beta$  release (Jalilian, et al., 2012). The inability of ATP to induce IL-1 $\beta$  from MDCK cells could not be solely attributed to the low amount of P2X7 on MDCK cells, as nigericin, which induces IL-1 $\beta$  release independently of P2X7 activation (Solle, et al., 2001; Sluyter, et al., 2004), also failed to induce IL-1 $\beta$  from MDCK cells. Thus, it is likely that IL-1 $\beta$  and/or other NALP3 inflammasome-associated molecules are present at low amounts in MDCK cells, and that the LPS-priming conditions employed in this study are insufficient to activate or up-regulate these molecules to cause detectable IL-1 $\beta$  release.

The presence of NALP3 in MDCK cells is noteworthy. Recent evidence using knockout mice indicates that kidney epithelial-expressed NALP3, independently of inflammasome activation and cytokine release, mediates the inflammation and associated tissue damage during renal ischemia/reperfusion injury (Shigeoka, et al., 2010). Whether the autocrine release of ATP and subsequent activation of P2X7 is involved in this process *in vivo* remains unknown, however MDCK cells can release ATP following mechanical stimulation (Ostrom, et al., 2000), and thus may serve as a suitable model cell line to study this potential mechanism further.

### 3.3. Conclusion

In conclusion, this study demonstrates that the MDCK epithelial cell line express functional P2X7, as well as TLR4 and molecules associated with the NALP3 inflammasome, and suggests that this cell line will be useful for determining the role of these molecules in kidney epithelial physiology and pathophysiology. Moreover, further studies using healthy and diseased kidney tissue from dogs are warranted to further investigate the role of these receptors in canine renal disorders.

## Conflict of interest

The authors have no conflict of interest to declare.

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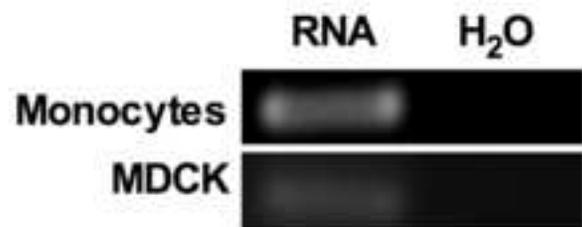
## Figure legends

Fig. 1. MDCK cells express functional P2X7 receptors. (A) RNA, isolated from MDCK cells and LPS-primed canine monocytes, was amplified by RT-PCR using P2X7 specific primers and the products examined by agarose gel electrophoresis. H<sub>2</sub>O in place of RNA was used as a negative control. (B) Whole cell lysates from MDCK and RAW264.7 cells were separated by SDS-PAGE, transferred to nitrocellulose membrane and probed with anti-P2X7 Ab. (C) MDCK in sucrose medium were incubated with 25  $\mu$ M ethidium<sup>+</sup> in the absence or presence of 1 mM ATP or 200  $\mu$ M BzATP at 37°C for up to 30 min (as indicated). (D) MDCK in sucrose medium were pre-incubated with DMSO or 1  $\mu$ M KN-62 (*left panel*), or in the absence (Control) or presence of 10  $\mu$ M A438079 (*right panel*) at 37°C for 15 min. Cells were then incubated with 25  $\mu$ M ethidium<sup>+</sup> in the absence (Basal) or presence of 1 mM ATP at 37°C for 20 min. (C,D) Incubations were stopped by MgCl<sub>2</sub> solution and centrifugation, and the MFI of ethidium<sup>+</sup> uptake measured by flow cytometry. Results are mean  $\pm$  SD ( $n=3$ ); \* $P<0.05$  compared to corresponding basal, <sup>†</sup> $P<0.05$  compared to corresponding ATP alone.

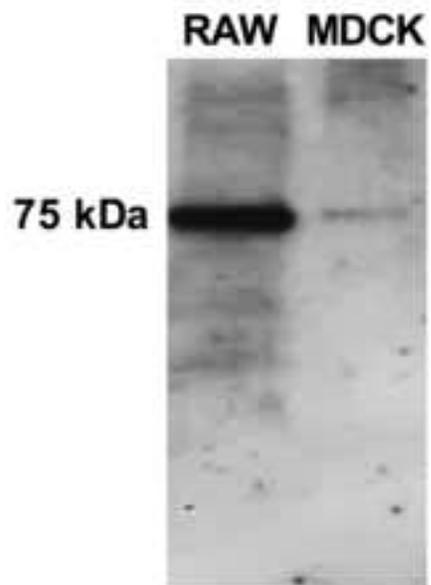
Fig. 2. MDCK cells express TLR4, as well as molecules associated with the NALP3 inflammasome. (A) RNA, isolated from MDCK cells and LPS-primed canine monocytes, was amplified by RT-PCR using primers specific for various mRNA transcripts (as indicated) and the products examined by agarose gel electrophoresis. H<sub>2</sub>O in place of RNA was used as a negative control and demonstrated no products (results not shown). (B) MDCK cells were labeled with PE-conjugated TLR4 (*solid line*) or isotype control (*shaded*) mAb, and the relative cell-surface TLR4 expression measured by flow cytometry.

Fig 1

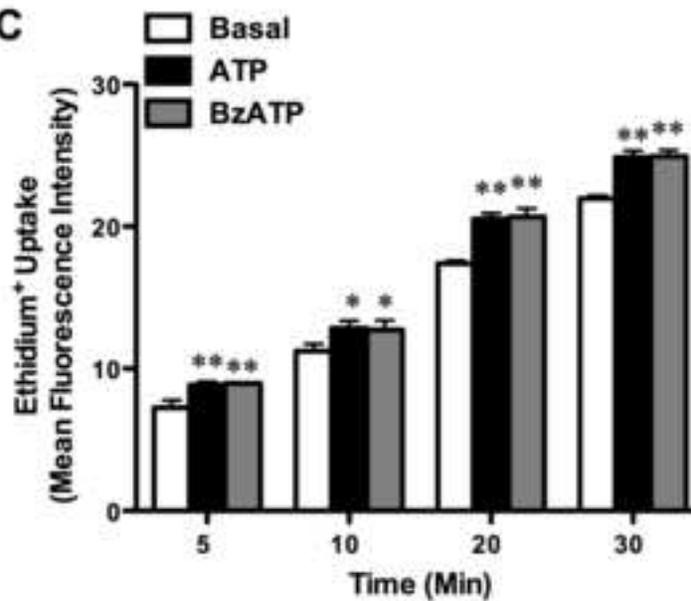
A



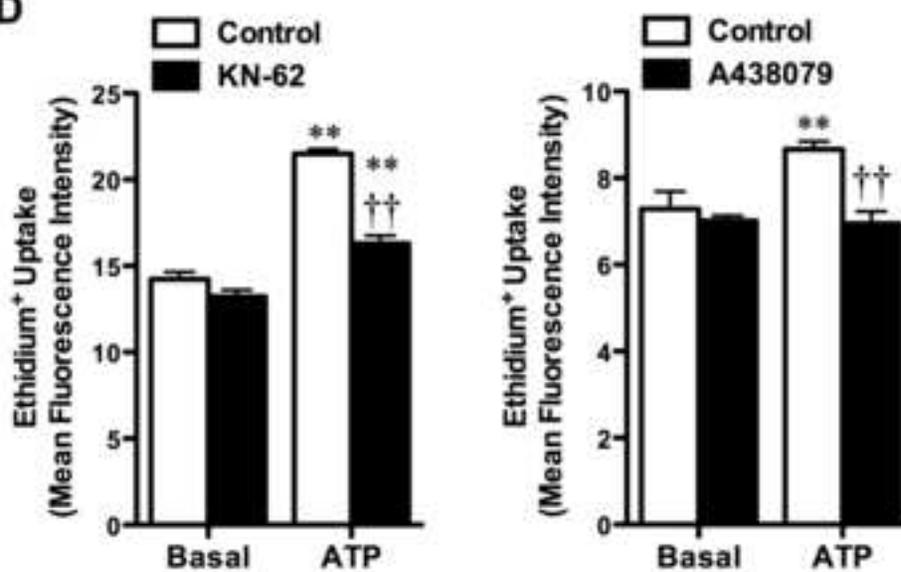
B



C

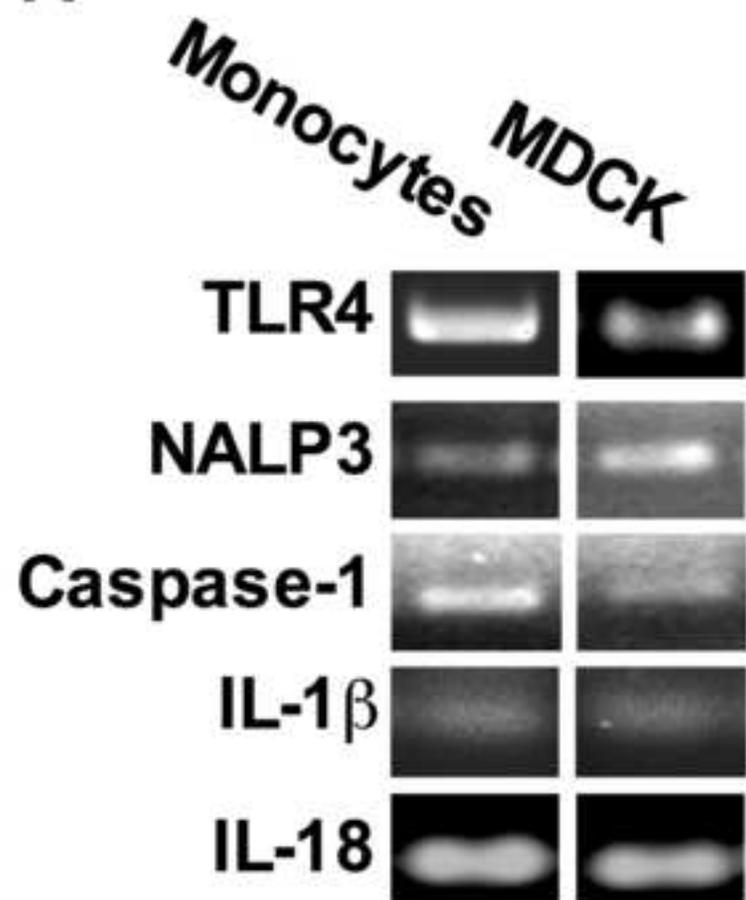


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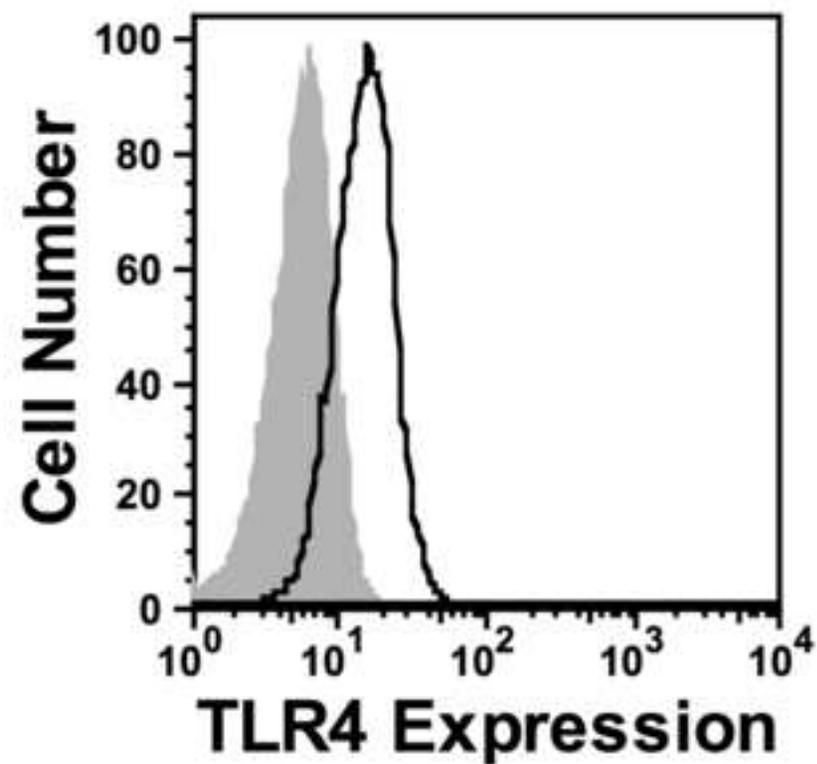


# Fig 2

**A**



**B**



**Table 1** Primers and conditions for RT-PCR

| Primer <sup>a</sup> | Forward primer 5'-3'    | Reverse primer 5'-3'   | Cycle conditions <sup>b</sup>                 | Product size (bp) | Reference                   |
|---------------------|-------------------------|------------------------|-----------------------------------------------|-------------------|-----------------------------|
| P2X7                | TGCCTCCCATCCCAGCTCCC    | GTCCTGGGAGCCAAAGCGCC   | 95°C for 10 s, 62°C for 10 s, 72°C for 30 s   | 240               | NM_001113456.1 <sup>a</sup> |
| TLR4                | GTCTGGCTGGCTTAAAGATC    | CTGCAATCTAGGATCTGGAG   | 95°C for 20 s, 52.4°C for 20 s, 72°C for 60 s | 910               | (Asahina, et al., 2003)     |
| NALP3               | CACTGTCAGCCTTTGGCAGGGT  | GTCTCCCAGGGCGTTGTGGC   | 95°C for 10 s, 63.7°C for 10 s, 72°C for 30 s | 273               | XM_843284.2 <sup>a</sup>    |
| Caspase-1           | ACAGACGCTGGGGCTCTCCT    | CCCAGGCCCTCCAGCAGACT   | 95°C for 10 s, 63.7°C for 10 s, 72°C for 30 s | 339               | NM_001003125.1 <sup>a</sup> |
| IL-1 $\beta$        | TGCAAAACAGATGCGGATAA    | GTAACCTGCAGTCCACCGATT  | 95°C for 10 s, 53°C for 10 s, 72°C for 30 s   | 64                | (Maccoux, et al., 2007)     |
| IL-18               | CCTGGAATCAGATTACTTTGGC  | GTTTTGTTCTTACAGGAGAGAG | 95°C for 10 s, 57°C for 10 s, 72°C for 30 s   | 251               | (Chamizo, et al., 2001)     |
| $\beta$ -Actin      | TGACCCAGATCATGTTTGAGACC | TCCTGCTTGCTGATCCACATCT | 95°C for 20 s, 63.6°C for 20 s, 72° for 60 s  | 700               | (Swerdlow, et al., 2006)    |

<sup>a</sup>Primers were designed from the corresponding sequences (<http://www.ncbi.nlm.nih.gov/gene>) using Primer 3 (<http://frodo.wi.mit.edu/primer3/>).

<sup>b</sup>PCR mixtures were incubated at 45°C for 20 min (except TLR4 and  $\beta$ -actin, 30 and 25 min, respectively), then at 95°C for 60 s, followed by 42 cycles as indicated, and then a final extension at 72°C for 5 min.