2012

Activation of the damage-associated molecular pattern receptor P2X7 induces interleukin-1B release from canine monocytes

Iman Jalilian
University Of Wollongong

Michelle Peranec
University Of Wollongong

Belinda L. Curtis
Albion Park Veterinary Hospital, Australia

Aine Seavers
Oak Flats Veterinary Clinic, Australia

Mari Spildrejorde
University of Wollongong, mgs840@uow.edu.au

See next page for additional authors

Activation of the damage-associated molecular pattern receptor P2X7 induces interleukin-1B release from canine monocytes

Abstract

P2X7, a damage-associated molecular pattern receptor and adenosine 5'-triphosphate (ATP)-gated cation channel, plays an important role in the activation of the NALP3 inflammasome and subsequent release of interleukin (IL)-1β from human monocytes; however its role in monocytes from other species including the dog remains poorly defined. This study investigated the role of P2X7 in canine monocytes, including its role in IL-1β release. A fixed-time flow cytometric assay demonstrated that activation of P2X7 by extracellular ATP induces the uptake of the organic cation, YO-PRO-1^{2+}, into peripheral blood monocytes from various dog breeds, a process impaired by the specific P2X7 antagonist, A438079. Moreover, in five different breeds, relative P2X7 function in monocytes was about half that of peripheral blood T cells but similar to that of peripheral blood B cells. Reverse transcription-PCR demonstrated the presence of P2X7, NALP3, caspase-1 and IL-1β in LPSprimed canine monocytes. Immunoblotting confirmed the presence of P2X7 in LPS-primed canine monocytes. Finally, extracellular ATP induced YO-PRO-1^{2+} uptake into and IL-1β release from these cells, with both processes impaired by A438079. These results demonstrate that P2X7 activation induces the uptake of organic cations into and the release of IL-1β from canine monocytes. These findings indicate that P2X7 may play an important role in IL-1β-dependent processes in dogs.

Keywords

receptor, pattern, molecular, associated, induces, p2x7, interleukin, activation, 1, release, canine, monocytes, damage, CMMB

Disciplines

Life Sciences | Physical Sciences and Mathematics | Social and Behavioral Sciences

Publication Details

Jalilian, I., Peranec, M., Curtis, B. L., Seavers, A., Spildrejorde, M., Sluyter, V. amp; Sluyter, R. (2012). Activation of the damage-associated molecular pattern receptor P2X7 induces interleukin-1B release from canine monocytes. Veterinary Immunology and Immunopathology, 149 (1-2), 86-91.

Authors

Iman Jalilian, Michelle Peranec, Belinda L. Curtis, Aine Seavers, Mari Spildrejorde, Vanessa Sluyter, and Ronald Sluyter

Activation of the damage-associated molecular pattern receptor P2X7 induces interleukin-1β release from canine monocytes

Iman Jalilian[a,b,†,‡], Michelle Peranec[a,b,‡], Belinda L Curtis[c], Aine Seavers[d], Mari Spildrejorde[a,b], Vanessa Sluyter[a,b], Ronald Sluyter[a,b,*]

[a]School of Biological Sciences, University of Wollongong, Wollongong, NSW 2522, Australia

[b]Illawarra Health and Medical Research Institute, Wollongong, NSW 2522, Australia

[c]Albion Park Veterinary Hospital, Albion Park, NSW 2527, Australia

[d]Oak Flats Veterinary Clinic, Oak Flats, NSW 2529, Australia

[*]Corresponding author at: School of Biological Sciences, Illawarra Health and Medical Research Institute, University of Wollongong, Wollongong, NSW 2522, Australia. Tel.: +61 2 4221 5508; fax: +61 2 4221 8130.

Email address: rsluyter@uow.edu.au (R. Sluyter).

[†]Current address: Department of Pharmacology, School of Medical Sciences, Faculty of Medicine, University of New South Wales, Sydney, NSW 2052, Australia

[‡]These authors contributed equally to this work.

Abstract

P2X7, a damage-associated molecular pattern receptor and adenosine 5'-triphosphate (ATP)-gated cation channel, plays an important role in the activation of the NALP3 inflammasome and subsequent release of interleukin (IL)-1β from human monocytes, however its role in monocytes from other species including the dog remains poorly defined. This study investigated the role of P2X7 in canine monocytes, including its role in IL-1β release. A fixed-time flow cytometric assay demonstrated that activation of P2X7 by extracellular ATP induces the uptake of the organic cation, YO-PRO-1^{2+}, into peripheral blood monocytes from various dog breeds, a process impaired by the specific P2X7 antagonist, A438079. Moreover, in five different breeds, relative P2X7 function in monocytes was about half that of peripheral blood T cells but similar to that of peripheral blood B cells. Reverse transcription-PCR demonstrated the presence of P2X7, NALP3, caspase-1 and IL-1β in LPS-primed canine monocytes. Immunoblotting confirmed the presence of P2X7 in LPS-primed canine monocytes. Finally, extracellular ATP induced YO-PRO-1^{2+} uptake into and IL-1β release from these cells, with both processes impaired by A438079. These results demonstrate that P2X7 activation induces the uptake of organic cations into and the release of IL-1β from canine monocytes. These findings indicate that P2X7 may play an important role in IL-1β-dependent processes in dogs.

Keywords: damage-associated molecular pattern receptor; purinergic receptor; inflammasome; interleukin-1β; monocyte; dog

Abbreviations: ATP, adenosine 5'-triphosphate; DAMP, damage-associated molecular pattern; MFI, mean fluorescence intensity; RT, reverse transcription.

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-characterised DAMP is extracellular adenosine 5'-triphosphate (ATP), which mediates its effects through the activation of the P2X7 purinergic receptor, a trimeric ATP-gated cation channel (Bours, et al., 2011; Wiley, et al., 2011). Activation of P2X7 by extracellular ATP causes the flux of Ca^{2+}, Na^{+} and K^{+}, as well as the uptake of organic cations such as ethidium^{+} and YO-PRO-1^{2+} (Jarvis and Khakh, 2009). Furthermore, P2X7 activation induces various downstream events including the NALP3 inflammasome-dependent maturation of IL-1β, and its subsequent release from various myeloid cell types (Di Virgilio, 2007). This event, at least in monocytes, requires the prior activation of cells with the TLR4 ligand, LPS, which results in the up-regulation and assembly of the NALP3 inflammasome, as well as the synthesis of IL-1β (Mehta, et al., 2001; Bauernfeind, et al., 2009). Due to this and other properties of P2X7 activation, this receptor plays important roles in human health and disease (Sluyter and Stokes, 2011), and thus may have similar roles in canine health and disease.

The presence of functional P2X7 on human and murine cell types is well established, but little is known about P2X7 in other mammalian species including the dog. We have previously demonstrated that peripheral blood monocytes, lymphocytes and erythrocytes from English Springer Spaniels express functional P2X7 (Sluyter, et al., 2007; Shemon, et al., 2008; Stevenson, et al., 2009). In particular, we have previously shown that the relative amount of P2X7 function in monocytes is about half that of canine T cells but similar to that of B cells (Stevenson, et al., 2009). Similar studies in other dog breeds however are lacking, and given that distinct phenotypic traits exist between breeds it remains necessary to examine P2X7 in

additional breeds. Moreover, it remains unknown if P2X7 activation can induce the release of IL-1β from canine monocytes, and there is a general paucity of studies examining IL-1β release from canine myeloid cell types. Given the importance of P2X7 in human health and disease (Sluyter and Stokes, 2011), new knowledge about this receptor and events downstream of its activation in the dog is necessary to establish and understand the role of P2X7 in canine health and disease. Therefore, we investigated P2X7 in canine monocytes, and in particular its role in the release of IL-1β.

2. Materials and methods

2.1. Materials

Ficoll-Paque™ PLUS was from GE Healthcare Biosciences (Uppsala, Sweden). A438079 was from Tocris Bioscience (Ellisville, MO). ATP and LPS (*Escherichia Coli* serotype 055:B5) were from Sigma Chemical Co (St Louis, MO). YO-PRO®-1 iodide solution, RPMI-1640 medium, L-glutamine and ExoSAP-IT were from Invitrogen (Grand Island, NJ). FCS was from Bovogen Biologicals (East Keilor, Australia). BigDye Terminator v3.1 was from Applied Biosystems (Carlsbad, CA).

2.2. Canine monocytes

Peripheral blood was collected into VACUETTE® lithium heparin tubes (Greiner Bio-One, Frickenheisen, Germany) from either pedigree or cross breed dogs with informed, signed consent of pet owners, and with the approval of the University of Wollongong Ethics Committee (Wollongong, Australia). PBMCs were isolated from buffy coats using Ficoll-

Paque™ density centrifugation as described (Stevenson, et al., 2009). To study LPS-primed monocytes, PBMCs in complete culture medium (RPMI-1640 medium containing 2 mM L-glutamine and 10% FCS) were incubated for 2 h at 37°C/5% CO_2, 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 100 ng/ml LPS.

2.3. J774 cells

J774 cells (American Type Culture Collection, Rockville, MD), a murine macrophage cell line, were maintained in complete culture medium at 37°C/5% CO_2.

2.4. Reverse transcription PCR

Total RNA was isolated using the RNeasy® Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Reverse transcription (RT)-PCR was performed using Superscript® III One-Step RT-PCR System Platinum Taq DNA polymerase (Invitrogen) according to the manufacturer's instructions using primer pairs (GeneWorks, Hindmarsh, Australia) specific for P2X7, NALP3, caspase-1 or IL-1β 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.

2.5. Immunoblotting

Immunoblotting of whole cell lysates was performed using a rabbit anti-rat P2X7 polyclonal Ab (Alomone Labs, Jerusalem, Israel) as described (Constantinescu, et al., 2010). Immunoblotting demonstrated that this Ab binds a protein corresponding to the predicted size of glycosylated P2X7 in HEK-293 cells transfected with canine P2X7 cDNA but not in mock-transfected HEK-293 cells (Spildrejorde and Sluyter, unpublished results).

2.6. YO PRO 1^{2+} uptake assay

ATP-induced YO-PRO-1^{2+} uptake into PBMCs suspended in NaCl medium (145 mM NaCl, 5 mM KCl, 5 mM glucose, 0.1% BSA, 10 mM HEPES, pH 7.4) was determined using a fixed-time flow cytometric assay as described (Gadeock, et al., 2010). To confirm that ATP-induced YO-PRO-1^{2+} uptake was mediated by P2X7, cells were pre-incubated in the absence or presence of 10 μM A438079 for 15 min. Following ATP incubation, cells were then labelled with PerCP/Cy5.5- or allophycocyanin-conjugated anti-human/canine CD14 mAb (clone M5E2) (BioLegend, San Diego, CA). To assess YO-PRO-1^{2+} uptake in lymphocytes, cells were labelled with murine anti-canine CD21-like (clone CA2.1D6) or CD3 (clone CA17.2A12) mAb (AbD Serotec, Oxford, United Kingdom), and allophycocyanin-conjugated donkey anti-murine IgG (eBioscience, San Diego, CA). Data was acquired using a LSR II flow cytometer (BD) and the mean fluorescence intensity (MFI) of YO-PRO-1^{2+} uptake determined using FlowJo software (Tree Star, Ashland, OR).

2.7. IL 1β release assay

ATP-induced IL-1β release from canine monocytes was performed as previously described for human monocytes (Sluyter, et al., 2004). Briefly, PBMCs in complete culture

medium were incubated in 24-well plates (0.625 x 10^6 cells/well) for 2 h at 37°C/5% CO_2. Plates were washed and the plastic-adherent cells incubated for a further 4 h in complete culture medium containing 100 ng/ml LPS. The plastic-adherent cells were then pre-incubated in the absence or presence of 50 μM A438079 in RPMI-1640 medium containing 0.1% bovine serum albumin for 15 min, followed by incubation in the absence or presence of 5 mM ATP (0.5 ml/well) for 30 min. Following ATP incubation, samples were centrifuged (11,000 x *g* for 30 s) and cell-free supernatants stored at -20°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.

2.8. Statistical analysis

Differences between treatments were compared using either the unpaired Student's t-test or ANOVA (using Tukey's post test) for single or multiple comparisons, respectively. Errors are expressed as SDs.

3. Results and discussion

3.1. P2X7 activation induces organic cation uptake into peripheral blood monocytes

To determine if P2X7 activation induces the uptake of an organic cation into monocytes from additional dog breeds to English Springer Spaniels, ATP-induced YO-PRO-1^{2+} (375 Da) uptake into peripheral blood monocytes from two Staffordshire Bull Terriers and one Bull Terrier was examined in the absence or presence of A438079, which impairs human,

monkey, rat and murine P2X7 (Donnelly-Roberts, et al., 2009; Bradley, et al., 2011). In the absence of A438079, ATP induced YO-PRO-1^{2+} uptake into peripheral blood monocytes from all three dogs (Fig. 1A). Pre-incubation with this antagonist impaired ATP-induced YO-PRO-1^{2+} uptake by 93 ± 13% (Fig. 1A). Thus, P2X7 activation induces the uptake of YO-PRO-1^{2+} into canine peripheral blood monocytes, and the presence of functional P2X7 receptors on peripheral blood monocytes is not restricted to one breed.

To determine the relative pattern of P2X7 function in leukocytes from various dog breeds other than English Springer Spaniels, ATP-induced YO-PRO-1^{2+} uptake into peripheral blood monocytes from five different breeds was examined. As above (Fig. 1A), ATP induced YO-PRO-1^{2+} uptake into peripheral blood monocytes from each dog (Fig. 1B). ATP also induced YO-PRO-1^{2+} uptake into peripheral blood B and T cells from each dog, with the average amount of uptake three-fold greater in T cells compared to monocytes and B cells (range fold increase of 2.7-4.5 and 1.7-3.8, respectively) (Fig. 1B). Thus, a relative pattern of P2X7 function similar to that observed for English Springer Spaniels (Stevenson, et al., 2009) is also seen between peripheral blood monocytes, B cells and T cells from five other dog breeds (Australian Bulldog, Border Collie, Bull Terrier, Jack Russell Terrier and Staffordshire Bull Terrier). The difference between canine monocytes, B cells and T cells most likely relates to the amount of P2X7 expression, as cell-surface P2X7 expression correlates with P2X7 function in human leukocytes (Gu, et al., 2000). Moreover, the consistent pattern of P2X7 function between leukocyte subsets from the various breeds suggests that the regulation of P2X7 expression is highly conserved within dogs. The pattern of P2X7 function between leukocyte subsets from humans is also similar however, in contrast to dogs, P2X7 function is five-fold higher in human peripheral blood monocytes compared to human peripheral blood B or T cells (Gu, et al., 2000; Stevenson, et al., 2009). Whether these differences between the two species are of physiological significance remain unknown. Nevertheless, despite these species

dissimilarities, it appears likely that functional P2X7 receptors are expressed on peripheral blood monocytes, B cells and T cells from all dog breeds.

3.2 P2X7 activation induces organic cation uptake into and IL 1β release from LPS primed monocytes

To determine if P2X7 can induce the release of IL-1β from canine monocytes, the presence of mRNA transcripts for P2X7 and IL-1β, as well as for the NALP3 inflammasome components, NALP3 and caspase-1, were examined in LPS-primed canine monocytes. RT-PCR demonstrated the presence of mRNA transcripts for all four molecules (Fig. 2A), with transcripts corresponding to the predicted size (Table 1) for each molecule (results not shown). Moreover, with the exception of IL-1β (due to its small size of 64 bp), the identity of each transcript was confirmed by sequencing (results not shown). No PCR products were observed in the water control (Fig. 2A).

To determine if LPS-primed canine monocytes express functional P2X7, the presence of P2X7 protein was examined by immunoblotting. Murine J774 macrophages, which express P2X7 (Coutinho-Silva, et al., 2005), were used as a positive control. Immunoblotting with an anti-P2X7 Ab demonstrated the presence of a major band at 75 kDa, the predicted size of glycosylated P2X7, in both cell types (Fig. 2B). Minor bands were also observed at 60 and 68 kDa in LPS-primed canine monocytes and J774 cells, respectively (Fig. 2B). These minor bands most likely represent a degradation product and non-glycosylated P2X7, respectively.

To confirm that P2X7 was functional in LPS-primed canine monocytes, ATP-induced YO-PRO-1^{2+} uptake was examined in the absence or presence of A438079. In the absence of A438079, ATP induced YO-PRO-1^{2+} uptake into LPS-primed canine monocytes (Fig. 2C). Pre-incubation with this antagonist impaired ATP-induced YO-PRO-1^{2+} uptake by 97 ± 4% (Fig.

2C). A438079 in the absence of ATP had no significant effect on YO-PRO-1^{2+} uptake compared to YO-PRO-1^{2+} uptake in the absence of both A438079 and ATP (results not shown). Thus, LPS-primed canine monocytes from two pure breeds (Australian Kelpie and two Labradors) and from one cross breed (Border Collie cross Australian Cattle Dog) express functional P2X7 receptors.

Finally, to determine if P2X7 activation induces IL-1β release from LPS-primed canine monocytes, cells were pre-incubated in the absence or presence of A438079 prior to incubation in the absence or presence of ATP, and the amount of IL-1β in cell-free supernatants measured using a canine IL-1β ELISA. In the absence of A438079, ATP induced IL-1β release from LPS-primed canine monocytes (Fig. 2D). Pre-incubation with this antagonist impaired ATP-induced IL-1β release by 97 ± 9% (Fig. 2D). A438079 in the absence of ATP had no significant effect on IL-1β release compared to IL-1β release in the absence of both A438079 and ATP (results not shown). Thus, P2X7 activation induces the release of IL-1β from LPS-primed canine monocytes. To the best of our knowledge, the P2X7-induced IL-1β release from canine monocytes has not been previously reported, however others, using peripheral blood from Beagles, have shown that P2X7 activation induces IL-1β release in a whole-blood assay (Roman, et al., 2009). Collectively, these observations are consistent with findings with human monocytes or whole blood (Perregaux, et al., 2000; Mehta, et al., 2001; Sluyter, et al., 2004; Bauernfeind, et al., 2009), and indicate that P2X7 may also play an important role in IL-1β-dependent processes in dogs.

3.3. P2X7 and the NALP3 inflammasome in canine health and disease

P2X7 has an established role in inflammation and immunity (Bours, et al., 2011; Wiley, et al., 2011), and related disorders that include inflammatory arthritis, and neuropathic and

inflammatory pain (Labasi, et al., 2002; Chessell, et al., 2005). The action of P2X7 in these circumstances is largely attributed to its release of IL-1β from monocytes and macrophages, although other inflammatory mediators may also be involved (Hughes, et al., 2007). Nonetheless, P2X7 is attracting considerable interest as a therapeutic target in humans (Donnelly-Roberts and Jarvis, 2007; Arulkumaran, et al., 2011), and it is likely that compounds that target P2X7 will have similar therapeutic benefits in dogs. The results also support the use of the dog as a suitable model to study the role of P2X7 in health and disease, as well as to test the therapeutic efficacy and other pharmacological parameters of P2X7 antagonists before commencing human clinical trials. In regard to this latter point, dogs have been used to test the bioavailability and half-life of potential P2X7 antagonists for use as therapeutics in humans (Abberley, et al., 2010; Duplantier, et al., 2011).

3.4. Conclusion

The current study demonstrates that functional P2X7 receptors are present in peripheral blood monocytes from various breeds, and that the relative P2X7 function in these cells is about half that of peripheral T cells but similar to that of peripheral blood B cells. Moreover, the study demonstrates that LPS-primed canine monocytes produce mRNA transcripts for P2X7 and IL-1β, as well as for the NALP3 inflammasome components, NALP3 and caspase-1. Finally, the study demonstrates that these cells express P2X7 protein, and that activation of this receptor induces the uptake of the organic cation, YO-PRO-1^{2+}, and the release of IL-1β. Collectively, these results show that dogs express functional P2X7 receptors, and that this receptor may play important roles in canine health and disease, and thus may represent a promising therapeutic target in dogs.

Conflict of interest

The authors have no conflict of interest to declare.

Acknowledgements

We gratefully acknowledged helpful advice from Jason McArthur and Mark Dowton (both University of Wollongong), and excellent technical assistance by Margaret Phillips (University of Wollongong) and the staff of the Illawarra Health and Medical Research Institute. This work was kindly supported by grants from the American Kennel Club Canine Health Foundation and the Canine Research Foundation. The sponsors did not have any influence in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication. The contents of this publication are solely the responsibility of the authors and do not necessarily represent the views of the funding sources.

References

Abberley, L., Bebius, A., Beswick, P.J., Billinton, A., Collis, K.L., Dean, D.K., Fonfria, E., Gleave, R.J., Medhurst, S.J., Michel, A.D., Moses, A.P., Patel, S., Roman, S.A., Scoccitti, T., Smith, B., Steadman, J.G., Walter, D.S. 2010. Identification of 2-oxo-N-(phenylmethyl)-4-imidazolidinecarboxamide antagonists of the $P2X_7$ receptor. Bioorg. Med. Chem. Lett. 20, 6370-6374.

Arulkumaran, N., Unwin, R.J., Tam, F.W. 2011. A potential therapeutic role for P2X7 receptor (P2X7R) antagonists in the treatment of inflammatory diseases. Expert Opin. Investig. Drugs 20, 897-915.

Bours, M.J., Dagnelie, P.C., Giuliani, A.L., Wesselius, A., Di Virgilio, F. 2011. P2 receptors and extracellular ATP: a novel homeostatic pathway in inflammation. Front. Biosci. (Schol. Ed.) 3, 1443-1456.

Bradley, H.J., Browne, L.E., Yang, W., Jiang, L.H. 2011. Pharmacological properties of the rhesus macaque monkey P2X7 receptor. Br. J. Pharmacol. 164, 743-754.

Chen, G.Y., Nuñez, G. 2010. Sterile inflammation: sensing and reacting to damage. Nat. Rev. Immunol. 10, 826-837.

Chessell, I.P., Hatcher, J.P., Bountra, C., Michel, A.D., Hughes, J.P., Green, P., Egerton, J., Murfin, M., Richardson, J., Peck, W.L., Grahames, C.B., Casula, M.A., Yiangou, Y., Birch, R., Anand, P., Buell, G.N. 2005. Disruption of the P2X7 purinoceptor gene abolishes chronic inflammatory and neuropathic pain. Pain 114, 386-396.

Constantinescu, P., Wang, B., Kovacevic, K., Jalilian, I., Bosman, G.J., Wiley, J.S., Sluyter, R. 2010. P2X7 receptor activation induces cell death and microparticle release in murine erythroleukemia cells. Biochim. Biophys. Acta 1798, 1797-1804.

Coutinho-Silva, R., Ojcius, D.M., Górecki, D.C., Persechini, P.M., Bisaggio, R.C., Mendes, A.N., Marks, J., Burnstock, G., Dunn, P.M. 2005. Multiple P2X and P2Y receptor subtypes in mouse J774, spleen and peritoneal macrophages. Biochem. Pharmacol. 69, 641-655.

Di Virgilio, F. 2007. Liaisons dangereuses: $P2X_7$ and the inflammasome. Trends Pharmacol. Sci. 28, 465-472.

Donnelly-Roberts, D.L., Jarvis, M.F. 2007. Discovery of P2X7 receptor-selective antagonists offers new insights into P2X7 receptor function and indicates a role in chronic pain states. Br. J. Pharmacol. 151, 571-579.

Donnelly-Roberts, D.L., Namovic, M.T., Han, P., Jarvis, M.F. 2009. Mammalian P2X7 receptor pharmacology: comparison of recombinant mouse, rat and human P2X7 receptors. Br. J. Pharmacol. 157, 1203-1214.

Duplantier, A.J., Dombroski, M.A., Subramanyam, C., Beaulieu, A.M., Chang, S.P., Gabel, C.A., Jordan, C., Kalgutkar, A.S., Kraus, K.G., Labasi, J.M., Mussari, C., Perregaux, D.G., Shepard, R., Taylor, T.J., Trevena, K.A., Whitney-Pickett, C., Yoon, K. 2011. Optimization of the physicochemical and pharmacokinetic attributes in a 6-azauracil series of P2X7 receptor antagonists leading to the discovery of the clinical candidate CE-224,535. Bioorg. Med. Chem. Lett. 21, 3708-3711.

Gadeock, S., Tran, J.N., Georgiou, J.G., Jalilian, I., Taylor, R.M., Wiley, J.S., Sluyter, R. 2010. TGF-β1 prevents up-regulation of the P2X7 receptor by IFN-γ and LPS in leukemic THP-1 monocytes. Biochim. Biophys. Acta 1798, 2058-2066.

Gu, B.J., Zhang, W.Y., Bendall, L.J., Chessell, I.P., Buell, G.N., Wiley, J.S. 2000. Expression of $P2X_7$ purinoceptors on human lymphocytes and monocytes: evidence for nonfunctional $P2X_7$ receptors. Am. J. Physiol. Cell. Physiol. 279, C1189-C1197.

Hughes, J.P., Hatcher, J.P., Chessell, I.P. 2007. The role of $P2X_7$ in pain and inflammation. Purinergic Signal. 3, 163-169.

Jarvis, M.F., Khakh, B.S. 2009. ATP-gated P2X cation channels. Neuropharmacology 56, 208-215.

Kono, H., Rock, K.L. 2008. How dying cells alert the immune system to danger. Nat. Rev. Immunol. 8, 279-289.

Labasi, J.M., Petrushova, N., Donovan, C., McCurdy, S., Lira, P., Payette, M.M., Brissette, W., Wicks, J.R., Audoly, L., Gabel, C.A. 2002. Absence of the P2X7 receptor alters leukcoyte function and attenuates an inflammatory response. J. Immunol. 168, 6436-6445.

Maccoux, L.J., Salway, F., Day, P.J.R., Clements, D.N. 2007. Expression profiling of select cytokines in canine osteoarthritis tissues. Vet. Immunol. Immunopathol. 118, 59-67.

Roman, S., Cusdin, F.S., Fonfria, E., Goodwin, J.A., Reeves, J., Lappin, S.C., Chambers, L., Walter, D.S., Clay, W.C., Michel, A.D. 2009. Cloning and pharmacological characterization of the dog P2X7 receptor. Br. J. Pharmacol. 158, 1513-1526.

Shemon, A.N., Sluyter, R., Stokes, L., Manley, P.W., Wiley, J.S. 2008. Inhibition of the human $P2X_7$ receptor by a novel protein tyrosine kinase antagonist. Biochem. Biophys. Res. Commun. 365, 515-520.

Sluyter, R., Shemon, A.N., Wiley, J.S. 2004. Glu^{496} to Ala polymorphism in the $P2X_7$ receptor impairs ATP-induced IL-1β release from human monocytes. J. Immunol. 172, 3399-3405.

Sluyter, R., Shemon, A.N., Hughes, W.E., Stevenson, R.O., Georgiou, J.G., Eslick, G.D., Taylor, R.M., Wiley, J.S. 2007. Canine erythrocytes express the $P2X_7$ receptor: greatly increased function compared to human erythrocytes. Am. J. Physiol. Regul. Integr. Comp. Physiol. 293, R2090-R2098.

Sluyter, R., Stokes, L. 2011. Significance of P2X7 receptor variants to human health and disease. Recent Pat. DNA Gene Seq. 5, 41-54.

Stevenson, R.O., Taylor, R.M., Wiley, J.S., Sluyter, R. 2009. The $P2X_7$ receptor mediates the uptake of organic cations in canine erythrocytes and mononuclear leukocytes: comparison to equivalent human cell types. Purinergic Signal. 5, 385-394.

Wiley, J.S., Sluyter, R., Gu, B.J., Stokes, L., Fuller, S.J. 2011. The human P2X7 receptor and its role in innate immunity. Tissue Antigens 78, 321-332.

Figure legends

Fig. 1. P2X7 activation induces organic cation uptake into peripheral blood monocytes. (A) Canine PBMCs (from a Bull Terrier or two Staffordshire Bull Terriers) in NaCl medium were pre-incubated in the absence (Control) or presence of 10 μM A438079 at 37°C for 15 min. PBMCs were then incubated with 1 μM YO-PRO-1^{2+} in the absence or presence of 1 mM ATP at 37°C for 5 min. (B) Canine PBMCs (from various breeds as indicated) in NaCl medium containing 1 μM YO-PRO-1^{2+} were incubated in the absence or presence of 1 mM ATP at 37°C for 5 min. (A,B) Incubations were stopped by $MgCl_2$ solution and centrifugation, the PBMCs labelled with (A,B) anti-CD14 mAb, or (B) anti-CD21-like or anti-CD3 mAb, and the MFI of YO-PRO-1^{2+} uptake into monocytes, B cells and T cells, respectively measured by flow cytometry. ATP-induced YO-PRO-1^{2+} uptake was defined as the difference in the MFI of YO-PRO-1^{2+} uptake in the presence and absence of ATP for each group. Symbols represent individual dogs; bars represent group mean; $^{**}P<0.01$, $^{***}P<0.001$ compared to (A) Control or (B) monocytes or B cells.

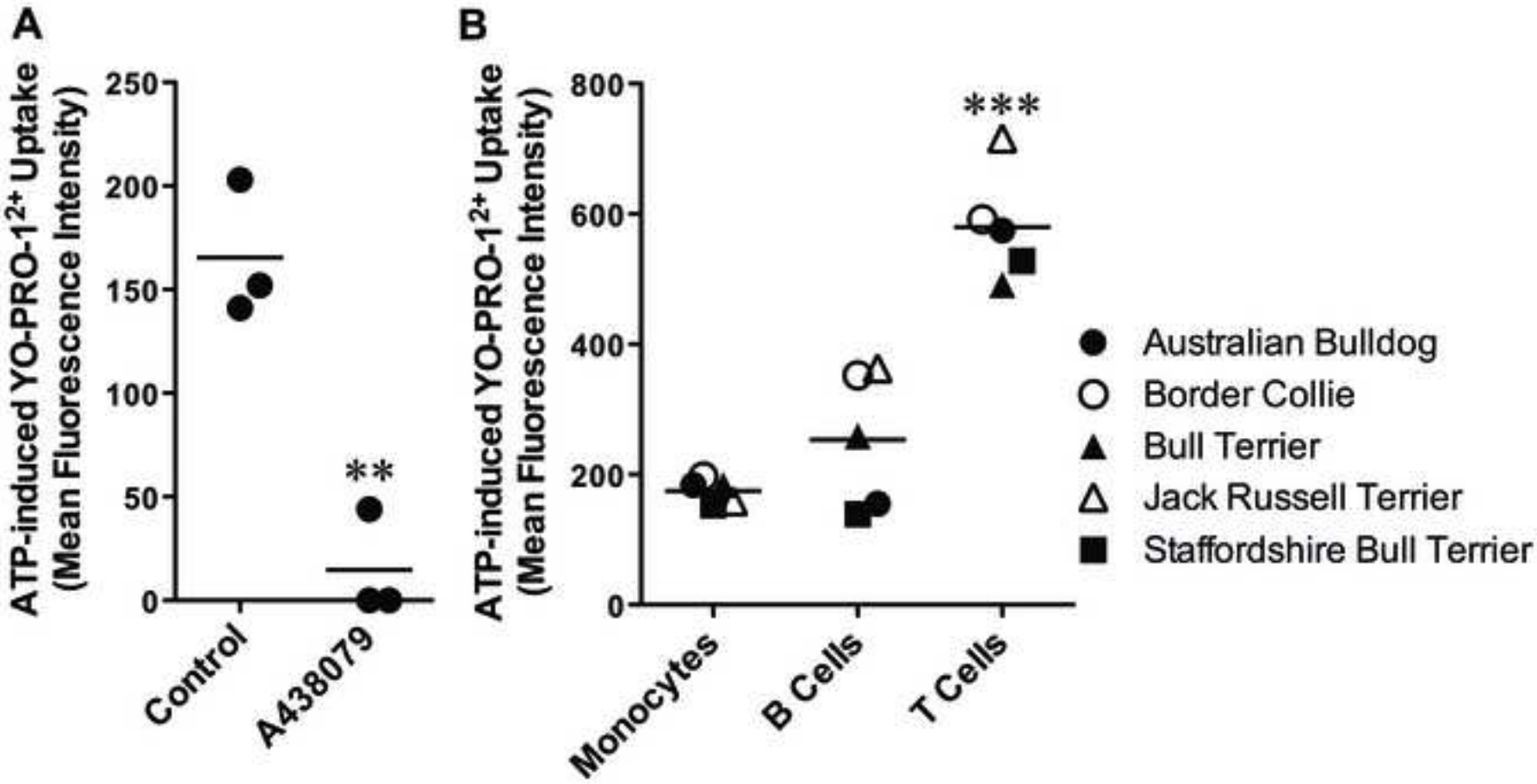
Fig. 2. P2X7 activation induces organic cation uptake into and IL-1β release from LPS-primed monocytes. (A-D) Adherent PBMCs (from various breeds or cross breeds) in complete medium containing 100 ng/ml LPS were incubated at 37°C for 4 h. (A) Isolated RNA from LPS-primed monocytes was amplified by RT-PCR using primers specific for various mRNA transcripts (as indicated) and the products examined by agarose gel electrophoresis. H_2O in place of RNA was used as a negative control. (B) Whole lysates of LPS-primed monocytes (Mon) or J774 cells were separated by SDS-PAGE, transferred to nitrocellulose membrane and probed with anti-P2X7 Ab. (C) LPS-primed monocytes in NaCl medium were pre-incubated in the absence (Control) or presence of 10 μM A438079 at 37°C for 15 min. Monocytes were

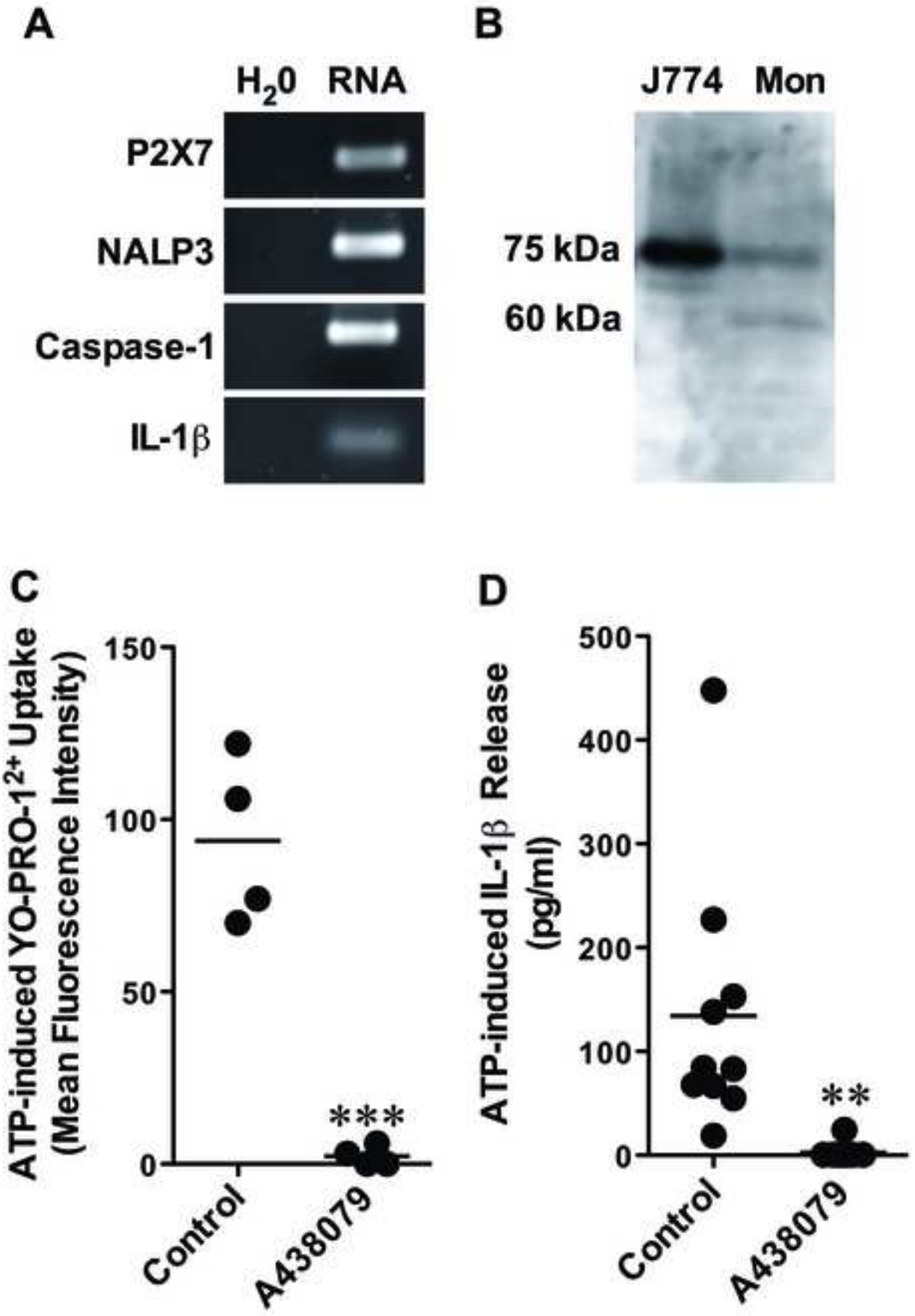
then incubated with 1 μM YO-PRO-1^{2+} in the absence or presence of 1 mM ATP at 37°C for 5 min. Incubations were stopped by $MgCl_2$ solution and centrifugation, the monocytes labelled with anti-CD14 mAb, and the MFI of YO-PRO-1^{2+} uptake measured by flow cytometry. ATP-induced YO-PRO-1^{2+} uptake was defined as the difference in the MFI of YO-PRO-1^{2+} uptake in the presence and absence of ATP. (D) LPS-primed monocytes in RPMI-1640 medium containing were pre-incubated in the absence (Control) or presence of 50 μM A438079 at 37°C for 15 min, followed by incubation in the absence or presence of 5 mM ATP for 30 min. The amount of IL-1β in cell-free supernatants was quantified using an IL-1β ELISA. ATP-induced IL-1β release was defined as difference in IL-1β concentration in the presence and absence of ATP. (C,D) Symbols represent individual dogs; bars represent group mean; $^{**}P<0.01$, $^{***}P<0.001$ compared to Control.

				Product Size (bp)
P2X7	TGCCTCCCATCCCAGCTCCC	GTCCTGGGAGCCAAAGCGCC	94° for 2 min, 30 cycles of 94° for 30 s, 59° for 30 s, 72° for 3.5 min	240
NALP3	CACTGTCAGCCTTTGGCAGGGT	GTCTCCCAGGGCGTTGTGGC	94° for 2 min, 40 cycles of 94° for 15 s, 58° for 20 s, 65° for 45 s	273
Caspase-1	ACAGACGCTGGGGCTCTCCT	CCCAGGCCCTCCAGCAGACT	94° for 2 min, 40 cycles of 94° for 15 s, 58° for 30 s, 66° for 45 s	339
IL-1β	TGCAAAACAGATGCGGATAA	GTAACTTGCAGTCCACCGATT	94° for 2 min, 40 cycles of 94° for 30 s, 47° for 30 s, 72° for 2 min	64

[a]Primers were designed for mRNA transcripts of P2X7, NALP3 and caspase-1 from the sequences, NM_001113456.1, XM_843284.2 and NM_001003125.1, respectively (http://www.ncbi.nlm.nih.gov/gene) using Primer 3 (http://frodo.wi.mit.edu/primer3/). Primers for the IL-1β mRNA transcript were previously described (Maccoux, et al., 2007).

Figure 1


A
ATP-induced YO-PRO-1^{2+} Uptake
(Mean Fluorescence Intensity)
250
200
150
100
50
0
**
Control
A438079
B
ATP-induced YO-PRO-1^{2+} Uptake
(Mean Fluorescence Intensity)
800
600
400
200
0
***
Monocytes
B Cells
T Cells
Australian Bulldog
Border Collie
Bull Terrier
Jack Russell Terrier
Staffordshire Bull Terrier


A
H20
RNA
P2X7
NALP3
Caspase-1
IL-1β
B
J774
Mon
75 kDa
60 kDa
C
ATP-induced YO-PRO-12+ Uptake
(Mean Fluorescence Intensity)
150
100
50
0
***
Control
A438079
D
ATP-induced IL-1β Release
(pg/ml)
500
400
300
200
100
0
**
Control
A438079