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Abstract

Binding of extracellular adenosine 5'-triphosphate (ATP) or lipopolysaccharide (LPS) to the damage-associated molecular pattern receptor P2X7 or the pathogen-associated molecular pattern receptor Toll-like receptor (TLR)4, respectively, can induce the release of the pleiotropic cytokine interleukin (IL)-1 β in humans and mice. However, the release of IL-1 β in dogs remains poorly defined. Using a canine IL-1 β enzyme-linked immunosorbent assay, this study investigated whether ATP or LPS could induce IL-1 β release in a canine blood-based assay. Short-term incubations (30 min) with ATP induced IL-1 β release in LPS-primed canine blood, and this process could be near-completely impaired by the P2X7 antagonist, A438079. In contrast, ATP failed to induce IL-1 β release from blood not primed with LPS. ATP-induced IL-1 β release was observed with LPS-primed blood from eight different pedigrees or cross breeds. Long-term incubations (24 h) with LPS induced IL-1 β release in canine blood in a concentration-dependent manner. This process was not altered by co-incubation with A438079. LPS-induced IL-1 β release was observed with blood from 10 different pedigrees or cross breeds. These results demonstrate that both extracellular ATP and LPS can induce IL-1 β release in dogs, and that ATP- but not LPS-induced IL-1 β release in blood is dependent on P2X7 activation. These findings support the role of both P2X7 and TLR4 in IL-1 β release in dogs.

Keywords

interleukin, induce, lipopolysaccharide, triphosphate, blood, canine, 5, adenosine, extracellular, release, 1, CMMB

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Extracellular adenosine 5'-triphosphate and lipopolysaccharide induce interleukin-1 β release in canine blood

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Abstract

Binding of extracellular adenosine 5'-triphosphate (ATP) or lipopolysaccharide (LPS) to the damage-associated molecular pattern receptor P2X7 or the pathogen-associated molecular pattern receptor Toll-like receptor (TLR)4, respectively can induce the release of the pleiotropic cytokine interleukin (IL)-1 β in humans and mice. However, the release of IL-1 β in dogs remains poorly defined. Using a canine IL-1 β enzyme-linked immunosorbent assay, this study investigated whether ATP or LPS could induce IL-1 β release in a canine blood-based assay. Short-term incubations (30 min) with ATP induced IL-1 β release in LPS-primed canine blood, and this process could be near-completely impaired by the P2X7 antagonist, A438079. In contrast, ATP failed to induce IL-1 β release from blood not primed with LPS. ATP-induced IL-1 β release was observed with LPS-primed blood from eight different pedigrees or cross breeds. Long-term incubations (24 h) with LPS induced IL-1 β release in canine blood in a concentration-dependent manner. This process was not altered by co-incubation with A438079. LPS-induced IL-1 β release was observed with blood from 10 different pedigrees or cross breeds. These results demonstrate that both extracellular ATP and LPS can induce IL-1 β release in dogs, and that ATP- but not LPS-induced IL-1 β release in blood is dependent on P2X7 activation. These findings support the role of both P2X7 and TLR4 in IL-1 β release in dogs.

Keywords: damage-associated molecular pattern receptor; purinergic receptor; pathogen-associated molecular pattern receptor; Toll-like receptor; interleukin-1 β ; dog

Abbreviations: ATP, adenosine 5'-triphosphate; ELISA, enzyme-linked immunosorbent assay; IL, interleukin; LPS, lipopolysaccharide; PBMCs, peripheral blood mononuclear cells; TLR, Toll-like receptor.

1. Introduction

Interleukin (IL)-1 β (or IL-1F2) is a pleiotropic cytokine predominately produced by monocytes and macrophages, and has key roles in immunity, inflammation, hematopoiesis and metabolism (Dinarello, 2011). As a result, IL-1 β has emerged as a promising therapeutic target for treating various local and systemic inflammatory conditions in humans (Dinarello et al., 2012). IL-1 β is also expressed in dogs (Jalilian et al., 2012b), however knowledge of IL-1 β in canine biology is largely limited to a small number of mRNA expression studies, where IL-1 β has been reported to be up-regulated in either infectious, musculoskeletal, cardiac or respiratory disorders (Chen et al., 2012; Kiczak et al., 2008; Maccoux et al., 2007; Unver et al., 2006). More recently, increased IL-1 β release has been observed in canine inflammatory disorders of the bowel and eye (Maeda et al., 2012; Wichayacoop et al., 2009). Nevertheless very little is known about the mechanisms involved in IL-1 β release in dogs. Thus, further information regarding canine IL-1 β and its release is required to understand the role of this cytokine in canine biology and to translate any potential therapeutic benefits of targeting IL-1 β in humans to dogs.

IL-1 β is not constitutively expressed in monocytes, but is upregulated following exposure of cells to pro-inflammatory mediators including lipopolysaccharide (LPS) (Dinarello, 1996), a process referred to as priming. In humans and mice, LPS binds to the pathogen-associated molecular pattern receptor Toll-like receptor (TLR)4 to induce IL-1 β expression and synthesis in monocytes (Hoshino et al., 1999; Medzhitov et al., 1997). Incubation with LPS also induces the expression of NALP3 (or NLRP3) (Bauernfeind et al., 2009), which assembles with caspase-1 to form the NALP3 inflammasome to mediate IL-1 β maturation and release (Agostini et al., 2004). Although LPS appears sufficient to induce the slow (24 h) release of IL-1 β (Lepe-Zuniga and Gery, 1984), a second signal, such as activation

of the damage-associated molecular pattern receptor P2X7 by its ligand extracellular adenosine 5'-triphosphate (ATP), is required for the rapid (30 min) release of IL-1 β following its synthesis (Grahames et al., 1999; Solle et al., 2001). P2X7 activation induces IL-1 β release from LPS-primed canine monocytes (Jalilian et al., 2012a) and in LPS-primed canine blood (Roman et al., 2009), however it remains unknown if LPS priming is required for P2X7-induced IL-1 β release in dogs. In human monocytes, LPS can induce the slow release of IL-1 β by both P2X7-dependent (Netea et al., 2009; Piccini et al., 2008) and P2X7-independent mechanisms (Ward et al., 2010). LPS can also induce IL-1 β release from canine peripheral blood mononuclear cells (PBMCs) (Baggio et al., 2005), but whether this process is dependent on P2X7 activation remains to be established. Blood-based assays are commonly used to study cytokine release *ex vivo*, as they require fewer manipulations compared to assays that use purified blood leukocytes, and thereby limit the inadvertent activation of cells. Therefore using a blood-based assay the current study investigated first, if canine P2X7-induced IL-1 β release requires LPS priming and second, if LPS is sufficient to induce canine IL-1 β release and if so, whether this process involves P2X7 activation.

2. Materials and methods

2.1. Materials

RPMI-1640 medium and penicillin-streptomycin-glutamine were from Invitrogen (Grand Island, NJ). LPS (*Escherichia Coli* serotype 055:B5), ATP (BioXtra) and bovine serum albumin (fatty acid and globulin free) were from Sigma Chemical Co (St Louis, MO). A438079 was from Tocris Bioscience (Ellisville, MO).

2.2. Blood-based IL-1 β release assay

Peripheral blood was collected into VACUETTE® lithium heparin tubes (Greiner Bio-One, Frickenheisen, Germany) from either 11 pedigree or four cross breed dogs with informed, signed consent of pet owners, and with the approval of the University of Wollongong Ethics Committee (Wollongong, Australia). Eleven of the animals were healthy at the time of blood collection, while four presented with minor health problems (an Alaskan Malamute with a fractured carnassial tooth, a Maltese and Shih Tzu cross with dental disease, a Labrador Retriever with otitis externa and a Shar Pei with bilateral otitis externa). All animals had no evidence of fever at the time of blood collection.

The release of IL-1 β from cells in blood was performed as described (Perregaux et al., 2000), with minor modification. Briefly, 100 μ L of canine blood and 100 μ L of RPMI-1640 medium (containing 10 mM HEPES, 100 U/mL penicillin, 100 μ g/mL streptomycin and 2 mM L-glutamine) were combined into flat-bottomed 96-well plates (Greiner Bio-One). In the first series of experiments, diluted blood was incubated in the absence or presence of 0.1 μ g/mL LPS for 2 h at 37°C/5% CO₂, and then in the absence or presence of 6 mM ATP for a further 30 min. In some experiments, 50 μ M A438079 was added during the final 15 min of the 2 h LPS incubation before ATP addition. In the second series of experiments, diluted blood was incubated with 0-10 μ g/mL LPS in the absence or presence of 50 μ M A438079 for 24 h at 37°C/5% CO₂. Samples from each series were centrifuged at 700 $\times$ g for 10 min, and the cell-free supernatants stored at -80°C until required. The amount of IL-1 β in cell-free supernatants was quantified using a Canine IL-1 β VetSet™ enzyme-linked immunosorbent assay (ELISA) Development Kit and ELISA Accessory Pack (both Kingfisher Biotech, St. Paul, MN) with 4% bovine serum albumin in Dulbecco's phosphate-buffered saline used as the Assay Diluent as per the manufacturer's instructions.

2.3. Statistical analysis

Differences between groups were compared using a one-way analysis of variance (using Tukey's post test).

3. Results and discussion

3.1 P2X7 activation induces IL-1 β release in canine blood and requires priming with LPS

Our group and one other have previously demonstrated that P2X7 activation induces canine IL-1 β release from LPS-primed monocytes (Jalilian et al., 2012a) or in LPS-primed blood (Roman et al., 2009), however it remains unknown if LPS priming is required for P2X7-induced IL-1 β release in dogs. Therefore, to confirm that P2X7 activation induces IL-1 β release in blood, LPS-primed blood was incubated in the absence or presence of the P2X7 antagonist A438079 (Nelson et al., 2006) before 30 min incubation in the absence or presence of ATP, and measurement of IL-1 β in cell-free supernatants by ELISA. Consistent with previous findings (Roman et al., 2009), ATP in the absence of P2X7 antagonist induced IL-1 β release in LPS-primed blood, which was on average four-fold greater than that of LPS-primed blood incubated in the absence of ATP, which was minimal (Fig. 1A). In LPS-primed blood, pre-incubation with A438079 impaired ATP-induced IL-1 β release by $97 \pm 9\%$ (mean $\pm$ standard deviation) compared to ATP-induced IL-1 β release in the absence of A438079 (Fig. 1A). In contrast, A438079 in the absence of ATP had no significant effect on IL-1 β release compared to IL-1 β release in the absence of both A438079 and ATP (Fig. 1A). Collectively,

these results indicate that ATP induces IL-1 β release in LPS-primed canine blood and that this process is mediated by P2X7 activation.

To determine whether P2X7-induced IL-1 β release in canine blood requires LPS priming, blood was pre-incubated in the absence or presence of LPS for 2 h before 30 min incubation in the absence or presence of ATP. Similar to above (Fig. 1A), ATP induced IL-1 β release in LPS-primed blood, which was on average five-fold greater than that of LPS-primed blood in the absence of ATP, which again was minimal (Fig. 1B). Notably, ATP was unable to increase IL-1 β release in blood in the absence of LPS compared to blood incubated in the absence of both LPS and ATP, and was significantly lower than ATP-induced IL-1 β release in LPS-primed blood (Fig. 1B). Thus, these results indicate that LPS-priming is necessary for P2X7-induced IL-1 β release in canine blood.

Combination of the above IL-1 β release data from LPS-primed blood incubated in the absence or presence of ATP (Fig. 1A and B), with results from four other dogs (three Labrador Retrievers, and a Maltese and Shih Tzu cross), demonstrates that P2X7 activation induces IL-1 β release in LPS-primed blood from eight pedigrees or cross breeds other than the Beagle (Fig. 1C). On average, ATP induced a four-fold increase in IL-1 β release in blood compared to blood incubated in the absence of ATP (Fig. 1C). Similar to our previous findings with LPS-primed canine monocytes (Jalilian et al., 2012a), P2X7-induced IL-1 β release in LPS-primed blood varied between dogs (Fig. 1). The cause of this variation remains unknown, but may be due to differences in TLR4 signalling, P2X7 activation, NALP3 inflammasome stimulation and IL-1 β release. For example, single nucleotide polymorphisms in the canine *TLR4* gene have been associated with inflammatory bowel disease in German Shepherds (Kathrani et al., 2010), suggesting that differences in LPS priming as a result of such polymorphisms may also influence IL-1 β production. Alternatively, single nucleotide polymorphisms in the human *P2RX7* gene can significantly modulate P2X7-induced IL-1 β release in LPS-primed human

monocytes (Sluyter et al., 2004; Stokes et al., 2010), suggesting that the canine *P2RX7* gene may also encode for polymorphisms that modify P2X7-induced IL-1 β release in dogs. It is unlikely that health status contributed to the variation in P2X7-induced IL-1 β release between dogs, as all dogs had no evidence of fever at the time of blood collection, and the four dogs with minor health issues had IL-1 β release measurements equivalent to at least one healthy dog.

3.2 LPS incubation is sufficient for IL-1 β release in canine blood

The above data (Fig. 1), indicates that incubation with LPS for 2.5 h induces negligible to low amounts of IL-1 β release in canine blood. In contrast, 24 h incubation with 1 μ g/mL LPS induces IL-1 β release from isolated canine PBMCs (Baggio et al., 2005). Therefore, to determine if incubation with LPS alone is sufficient to induce IL-1 β release in canine blood, blood was incubated with increasing concentrations of LPS for 24 h, before measurement of IL-1 β in cell-free supernatants by ELISA. In the absence of LPS, 24 h incubation resulted in negligible to low amounts of IL-1 β release (Fig. 2A), similar to that observed after 2.5 h (Fig. 1B). In contrast, 24 h incubation with LPS induced IL-1 β release in a concentration-dependent manner, with an average 16-fold increase in IL-1 β release between no LPS and the highest LPS concentration (10 μ g/mL) tested (Fig. 2A).

Previous studies with human monocytes have implicated a role for ATP release and the autocrine activation of P2X7 in LPS-induced IL-1 β release (Netea et al., 2009; Piccini et al., 2008). Therefore, blood was incubated with 0.1 μ g/mL LPS, the same concentration used for the P2X7 studies above (Fig. 1), in the absence or presence of A438079. In contrast to the near-complete inhibition of ATP-induced IL-1 β release by A438079 above (Fig. 1A), this antagonist was unable to prevent LPS-induced IL-1 β release over 24 h (Fig. 2B). A438079 in

the absence of LPS also had no significant effect on IL-1 β release compared to IL-1 β release in the absence of both A438079 and LPS (Fig. 2B). Collectively, these results indicate that LPS is sufficient for IL-1 β release in canine blood and that this process does not involve ATP release and the autocrine activation of P2X7 as observed for LPS-induced IL-1 β release from human monocytes (Netea et al., 2009; Piccini et al., 2008). Nevertheless the possibility remains that A438079 has limited efficacy in 24 h incubations. This however is unlikely, as this same antagonist has been shown in our laboratory to prevent P2X7-induced death in lymphoid and erythroid cells over the same time period (Constantinescu et al., 2010; Farrell et al., 2010). Of note, incubation of human monocytes for 24 h with TLR agonists, including LPS, can result in P2X7-independent IL-1 β release (Ward et al., 2010), further supporting the concept that 24 h incubation with LPS is sufficient for IL-1 β release in canine blood and that this process occurs independently of P2X7.

Finally, combined data from blood primed with 0.1 μ g/mL LPS (Fig. 2A and B), or data from blood primed with 10 μ g/mL LPS (Fig. 2A) combined with results using blood (primed with 10 μ g/mL LPS) from four other dogs (three Labrador Retrievers, and a Maltese and Shih Tzu cross), demonstrates that 24 h incubation with LPS induces IL-1 β release in blood from 10 pedigrees or cross breeds (Fig. 2C and D). On average, 0.1 and 10 μ g/mL LPS induced a 12- and 13-fold increase in IL-1 β release in blood compared to blood incubated in the absence of LPS (Fig. 2C). Similar to P2X7-induced IL-1 β release in LPS-primed blood (Fig. 1), LPS-induced IL-1 β release varied between dogs (Fig. 2). Thereby supporting the concept that differences in TLR4 signalling, NALP3 inflammasome stimulation and IL-1 β release exist between dogs. Again it is unlikely that health status contributed to the variation in LPS-induced IL-1 β release between dogs, as all dogs had no evidence of fever at the time of blood collection, and the three dogs with minor health issues had IL-1 β release measurements equivalent to at least one healthy dog.

3.3 Conclusion

The current study indicates that both extracellular ATP and LPS induce canine IL-1 β release in blood. Although the cell type was not identified in our study, it is likely that IL-1 β was released from monocytes, the main producers of IL-1 β in human blood (Allen et al., 1992). Thus, the possibility remains that the variation in ATP- or LPS-induced IL-1 β release between dogs reflects differences in blood monocyte counts, which were unavailable at the time of study. It is also likely that both ATP- and LPS-induced IL-1 β release required activation of the NALP3 inflammasome. We have previously shown that both NALP3 and caspase-1 transcripts are present in canine monocytes (Jalilian et al., 2012a), while others have shown that the caspase inhibitor z-VAD impairs ATP-induced IL-1 β release in LPS-primed blood of Beagles (Roman et al., 2009). The current study also indicates that both extracellular ATP and LPS can induce IL-1 β release in blood from various breeds including the Shar Pei. Of note, the Shar Pei can suffer from a recurring fever-like condition similar to that of familial Mediterranean fever (Hayem, 2013), a human condition that arises from a mutation in the inflammasome-related protein pyrin and results in elevated IL-1 β (Mansfield et al., 2001). Although the amount of IL-1 β release in this Shar Pei, which had no reported history of recurring fever, was within the range to that of the other dogs studied, to the best of our knowledge this is the first observation of IL-1 β production in this breed, *albeit* in only one dog.

In conclusion, these results demonstrate that both extracellular ATP and LPS can induce IL-1 β release in dogs, and that ATP- but not LPS-induced IL-1 β release in blood is mediated by P2X7. These findings support the role of both P2X7 and TLR4 in IL-1 β release in dogs. Finally, these results suggest that further study of IL-1 β as a potential therapeutic target in dogs is warranted.

Conflict of interest

The authors have no conflict of interest to declare.

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Figure legends

Fig. 1. P2X7 activation induces IL-1 β release in canine blood and requires priming with LPS. (A) Blood diluted with an equal volume of RPMI-1640 medium was incubated in the presence of 0.1 μ g/mL LPS for 2 h at 37°C. During the final 15 min of this 2 h incubation, samples were incubated in the absence or presence of 50 μ M A438079 (A438). Samples were then incubated for a further 30 min in the absence or presence of 6 mM ATP. (B) Blood diluted with an equal volume of RPMI-1640 medium was incubated in the absence or presence of 0.1 μ g/mL LPS for 2 h at 37°C, and then for a further 30 min in the absence or presence of 6 mM ATP. (C) Blood diluted with an equal volume of RPMI-1640 medium was incubated in the

presence of 0.1 µg/mL LPS for 2 h at 37°C, and then for a further 30 min in the absence or presence of 6 mM ATP. (A-C) Following a total incubation of 2.5 h, cell-free supernatants were collected and the amount of IL-1β release determined by ELISA. Symbols represent individual dogs; bars represent group means; **P*<0.05, ****P*<0.001 compared to Control; ††*P*<0.01 compared to (A) LPS and ATP, or (B) LPS or ATP alone. (C) Maltese X Shih Tzu, Maltese and Shih Tzu cross; Staffordshire Bull Terrier X, Staffordshire Bull Terrier and Australian Kelpie cross.

Fig. 2. LPS incubation is sufficient for IL-1β release in canine blood. (A, C and D) Blood diluted with an equal volume of RPMI-1640 medium was incubated in the absence or presence of LPS (as indicated) for 24 h at 37°C. (B) Blood diluted with an equal volume of RPMI-1640 medium was co-incubated in the absence or presence of 0.1 µg/mL LPS and 50 µM A438079 (A438) for 24 h at 37°C. (A-D) Following 24 h incubation, cell-free supernatants were collected and the amount of IL-1β release determined by ELISA. Symbols represent individual dogs; bars represent group means; **P*<0.05, ***P*<0.01, ****P*<0.001 compared to Control; †*P*<0.01 compared to A438079 alone. (C and D) Boxer X Bull Terrier, Boxer and Bull Terrier cross; Bull Mastiff X, Bull Mastiff cross; Maltese X Shih Tzu, Maltese and Shih Tzu cross; Staffordshire Bull Terrier X, Staffordshire Bull Terrier and Australian Kelpie cross.