ORIGINAL ARTICLE

Characterization of equine inflammasomes and their regulation

Huijeong Ahn[1] · Jeongeun Kim[1] · Hansae Lee[1] · Eunsong Lee[1] · Geun-Shik Lee[1,2]

Abstract

Inflammasome, a cytosolic multi-protein complex, assembly is a response to sensing intracellular pathogenic and endogenic danger signals followed by caspase-1 activation, which maturates precursor cytokines such as interleukin (IL)-1β. Most inflammasome research has been undertaken in humans and rodents, and inflammasomes in veterinary species have not been well-characterized. In this study, we observed the effects of well-known inflammasome activators on equine peripheral blood monocytes (PBMCs). The NLRP3 inflammasome triggers include ATP, nigericin, aluminum crystals, and monosodium urate crystals, and NLRP3 activation induces IL-1β secretion in a dose-dependent manner. Activators of NLRC4 and AIM2 inflammasomes include cytosolic flagellin and dsDNA, and their activation induces IL-1β secretion. The bacterial inflammasome triggers *Salmonella* Typhimurium and *Listeria monocytogenes* also induce IL-β releases. To elucidate the role of potassium efflux as an upstream signal of NLRP3 inflammasome activation, equine PBMCs were treated with blockers of potassium efflux in the presence of NLRP3 triggers. As a result, the IL-1β secretion stemming from equine NLRP3 inflammasome activation was not completely attenuated by the inhibition of potassium efflux. Taken together, the results indicate that equine PBMCs normally secrete IL-1β in response to well-known inflammasome activators, although equine NLRP3 inflammasome activation might not be dependent on potassium efflux.

Keyword Horse · *Equus ferus caballus* · Inflammasome · Cytokine · Interleukin-1β

Introduction

Cytosolic pathogen- and damage-associated molecular pattern molecules (PAMPs and DAMPs) lead to the assembly of inflammasomes, multi-protein complexes in myeloid and epithelial cells, which activate caspase-1 to maturate interleukin (IL)-1β and IL-18. Inflammasomes have been implicated in the pathogenesis of a wide range of diseases in humans and mice, and they have been investigated as potential therapeutic targets in attempts to cure those diseases (Swanson et al.

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2019). Initially inflammasomes were simply known as regulators of the secretion of IL-1β and IL-18 through caspase-1 activation in innate immune cells, but it has been recently reported that they are active in governing between innate and adaptive immunities in host immune homeostasis (Ciraci et al. 2012; Evavold and Kagan 2018; Swanson et al. 2019). An inflammasome consists of a sensor, an adaptor protein (ASC [apoptosis-associated speck-like protein containing a caspase recruitment domain]), and an effector (caspase-1) and is named according to the identity of the sensor. The most well-known sensors are NLRP3 (nucleotide-binding oligomerization domain [NOD]-like receptor [NLR] pyrin domain-containing protein 3), NLRC4 (NLR caspase activation and recruitment domain [CARD]-containing protein 4), and AIM2 (absent in melanoma 2). NLRP3 senses several signals including those related to potassium efflux, calcium flux, lysosomal disruption, mitochondrial dysfunction, and metabolic changes (Swanson et al. 2019). The NLRC4 complex with NAIPs (NLR family, apoptosis inhibitory proteins) interacts with bacterial ligands such as flagellin and the type 3 secretion system (T3SS) (Duncan and Canna 2018), whereas AIM2 detects dsDNA derived from host,

bacteria, or virus (Lugrin and Martinon 2018). NLRP3 has three domains, PYD (pyrin domain), NACHT (neuronal apoptosis inhibitor protein [NAIP] class 2 transcription activator [C2TA] of the heterokaryon incompatibility [HET-E] and telomerase-associated protein 1 [TP1]), and LRR (leucine-rich repeat). Upon stimulation, NLRP3 induces self-oligomerization via NACHT–NACHT interaction, and recruits ASC and caspase-1 through PYD–PYD and CARD–CARD binding (Swanson et al. 2019). Caspase-1 is activated by proximity-induced self-cleavage and matures cytokines of the IL-1 family (Swanson et al. 2019).

In past decades, inflammasomes have been studied in humans and rodents; recently, however, the inflammasomes of veterinary species have received research attention. Among livestock, porcine inflammasomes have been relatively well-characterized because pigs are an animal model used in the study of human diseases and xenotransplantation. Inflammasome-related genes such as NLRP3, ASC, and caspase-1 are expressed in the lymph nodes of pigs, and the inflammasome triggers observed in humans and rodents are reported to provoke IL-1β secretion in porcine peripheral blood monocytes (Dawson et al. 2017; Kim et al. 2014b; Tohno et al. 2011). Porcine inflammasomes are activated by PAMPs, such as porcine reproductive and respiratory syndrome virus (PRRSV) and the swine fever virus, and by DAMPs, including hypoxia and atherosclerosis, and such activations are similar to the inflammasome responses in humans and rodents (Angelis et al. 2014; Islam et al. 2016; Li et al. 2013; Vrentas et al. 2018). In cattle, viral and bacterial pathogens do not only lead to inflammasome activation resulting in IL-1β secretion, but human- and rodent-identified NLRP3 triggers also result in activation (Di Virgilio et al. 2017; Vrentas et al. 2018). Moreover, gene expressions of NLRP3, ASC, and caspase-1 have been detected in sheep and goats, although the mechanisms of IL-1β secretion and inflammasome activation have not been elucidated (Zhang et al. 2017). As well, it has been reported that canine NLRP3 inflammasomes are activated by pathogens, and IL-1β secretion has been observed in dogs suffering from myocardial ischemia and inflammatory bowel diseases (Hu et al. 2015; Jalilian et al. 2012; Vrentas et al. 2018). Based on recent results, inflammasome activation in veterinary species is, for the most part, similar to that of humans and mice, but there are some exceptions or differences (Vrentas et al. 2018). For example, although pigs do not possess the NLRC4 and AIM2 genes in their chromosomes, the NLRC4 and AIM2 triggers, flagellin and dsDNA transfection, successfully lead to the secretion of IL-1β in porcine peripheral blood monocytes (Ahn et al. 2018c; Dawson et al. 2017).

Thus, further characterization of inflammasomes in veterinary species can provide the information needed to elucidate veterinary diseases and develop potential veterinary therapeutics that target inflammasome-mediated veterinary diseases (Swanson et al. 2019; Vrentas et al. 2018). To date, however, equine inflammasomes have not been widely studied. In the present study, we characterize equine inflammasome activation by using NLRP3, NLRC4, and AIM2 inflammasome triggers that are well-established in humans and rodents. In addition, presence of an upstream signaling event, potassium efflux, associated with equine NLRP3 inflammasome activation was confirmed by using blockers, and the results were compared with those from human and murine inflammasomes.

Materials and Methods

Cell preparation

Equine peripheral blood monocytes (PBMCs) were isolated from blood freshly drawn from a Manchurian horse (*Equus ferus caballus*; 2-year-old male) by using lymphocyte separation medium (LSM; Thermo Fisher Scientific, Waltham, MA, USA). Briefly, blood was drained into anticoagulant EDTA-containing tubes (1.5 mg/mL of blood) and moved to the laboratory. The blood was then mixed with the same volume of phosphate-buffered saline (PBS) in 50 mL centrifuge tubes (SPL Life Science Co., Gyeonggi-do, Republic of Korea), and the same volume of LSM was carefully added into the bottom of the tube (blood: PBS: LSM = 1: 1: 1). After centrifuging at $500 \times g$ at room temperature (RT) for 30 min, the blood-LSM interphase containing mononuclear cells was harvested, placed in a new tube, and washed with PBS to eliminate residual LSM. Cell pellets, mixed with red blood cells lysis buffer (iNtRON Biotech., Sengnam-si, Republic of Korea), were collected by centrifuging at $500 \times g$ at RT. The obtained PBMCs were maintained in DMEM (Dulbecco's modified Eagle's medium; Capricorn Scientific, Ebsdorfergrund, Germany) containing 10% fetal bovine serum (FBS; Capricorn Scientific) and antibiotics (100 U/mL of penicillin and 100 μg/mL of streptomycin; Capricorn Scientific) at 37 °C in a 5% CO_2 atmosphere. Further experiments were conducted within one day from when the PBMCs were isolated.

To obtain murine bone marrow-derived macrophages (BMDMs), marrow progenitor cells were collected from the tibias and femurs of C57BL/6 mice (Narabio Co., Seoul, Republic of Korea) and differentiated into macrophages in DMEM with 30% L929 cell-conditioned media that contained granulocyte/macrophage colony-stimulating factor, 10% FBS, and antibiotics for 7 days at 37 °C in a 5% CO_2 atmosphere (Ahn et al. 2019, 2014, 2015; Kim et al. 2014a). Human monocyte-like cells (THP-1) were obtained from the Korea Cell Line Bank (Seoul, Republic of Korea) and cultured in RPMI (Roswell Park Memorial Institute 1640; Capricorn Scientific) media containing 10% FBS and antibiotics (Ahn et al. 2018b). The THP-1 cells were differentiated into macrophage-like phenotypes by treating with 12-myristate 13-acetate (PMA, 200 nM; InvivoGen, San Diego, CA.

USA) overnight (Kim et al. 2019). Animal experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Kangwon National University (IACUC; approval KW-190319-3 for mice and KW-191028-1 for horses).

Inflammasome activation and inhibition

Equine PBMCs (2×10^6 cells / well), murine BMDMs (1×10^6 cells / well) and THP-1 (1×10^6 cells / well) were plated in 12-well plates (SPL Life Science), and then primed with lipopolysaccharide (LPS, 1 μg/mL; #L4130, Sigma-Aldrich, St. Louis, MI, USA) in RPMI media containing 10% of FBS and antibiotics. After 3 h of LPS priming, cells were treated with adenosine triphosphate (ATP; InvivoGen) for 1 h, nigericin (NG; Tocris Bioscience, Bristol, UK) for 1 h, aluminum potassium sulfate (Alum; Daejeung Chemicals & Materials Co., Gyeonggi-do, Republic of Korea) for 3 h, monosodium urate crystals (MSU; U2875, Sigma-Aldrich), prepared as described previously (Martinon et al. 2006), for 6 h, *Salmonella enterica* serovar Typhimurium (*Salmonella*, optical density at 600 nm [OD600] = 1.2) for 1 h, flagellin (InvivoGen) with Lipofectamine 2000 (10 μL/mL; Invitrogen, Carlsbad, CA, USA) for 1 h, *Listeria monocytogenes* (*Listeria*, OD600 = 1.2) for 6 h, or double-stranded DNA (dsDNA) with jetPRIME™ (2 μL/mL; Polyplus-transfection Inc., Illkirch, France) for 1 h (Ahn et al. 2017a, b; Lee et al. 2016). For inflammasome inhibition assessment, LPS-primed cells were treated with glibenclamide (Glibe, 150 μM; Santa Cruz Biotech., Santa Cruz, CA, USA), extracellular high potassium solution (KCl, 50 mM; Biosesang, Seoul, Republic of Korea), or Z-VAD-FMK (10 μg/mL; R&D System, Minneapolis, MN, USA) in the presence of ATP (5 mM) or NG (40 μM) for 1 h (Ahn et al. 2018a). Cellular supernatant (Sup) and lysate (Lys) were prepared for use in further analyses.

Immunoblotting

Sup and Lys samples were analyzed by performing SDS-PAGE (10% or 16%) in a gel running system (Mini-PROTEAN® TETRA Handcast system; BIO-RAD, Hercules, CA, USA). The obtained gels were transferred onto a polyvinylidene difluoride membrane (Thermo Fisher Scientific), and the membranes were then blocked with 3% skim milk and probed with anti-human IL-1β sera (AF-201-NA, R&D Systems) or anti-mouse IL-1β sera (AF-401-NA, R&D Systems) overnight at 4 °C (Ahn and Lee 2017; Han et al. 2017). In addition, the membrane was probed with horseradish peroxidase (HRP) secondary antibody (#sc-2020, Santa Cruz Biotechnology) for 1 h and then visualized by applying a chemiluminescence solution (WESTSAVE

STAR™, Abfrontier, Seoul, Republic of Korea) and a chemiluminescence imaging system (EZ-Capture II, ATTO Technology, Tokyo, Japan). The membranes were stripped and re-probed with anti-actin sera (#sc-1615, Santa Cruz Biotechnology). Band intensity was determined by using image analyzing software (CS analyzer version 3, ATTO Technology).

Statistical analysis

Statistical analyses were conducted by applying one-way ANOVA (Tukey's multiple comparisons test) for multiple groups and nonparametric t-tests (Mann-Whitney test) for two groups by using GraphPad software (version 6, GraphPad Software, San Diego, CA, USA). The obtained *p*-values are included in the figures.
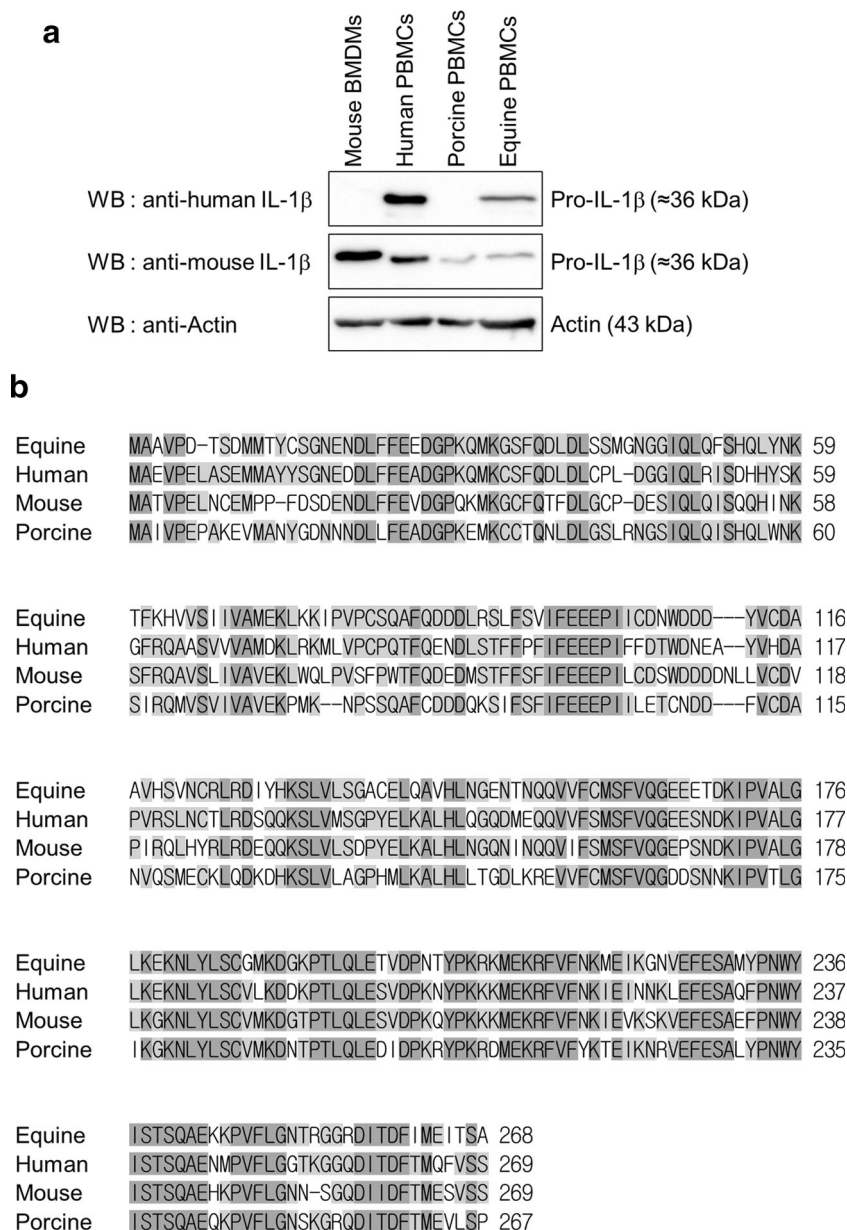
Results

Detection of IL-1β secretion in derived equine PBMCs

Activation of inflammasomes finally leads to the secretion of IL-1β p17, referred to as the active form of IL-1β, following by activation of caspase-1 (Ahn et al. 2018d). Thus secretion of the active form of IL-1β is a well-known indication of inflammasome activation and its presence should be detected by immunoblot analysis rather than via ELISA because anti-IL-1β cannot distinguish between the cleavage form (p17) and the pro-form (p36) of IL-1β (Kim et al. 2014b). Initially, we tested which anti-sera against human and mouse IL-1β were appropriate for the detection of equine IL-1β (Fig. 1A). Macrophages or monocytes of mouse, human, porcine, and equine were treated with LPS to induce pro-IL-1β expression and the cellular lysates were then subjected to immunoblotting. Although equine IL-1β was well detected by each antiserum, anti-human IL-1β sera showed a stronger band intensity than that of anti-mouse IL-1β. We also analyzed the sequence similarity of the amino acids of human, mouse, porcine, and equine IL-1β. The sequences of equine IL-1β were 66.67%, 64.15%, and 62.78% similar to those of human, mouse, and porcine IL-1β, respectively (Fig. 1B). Based on those results, human anti-IL-1β serum was selected for use in further studies.

Equine NLRP3 inflammasomes

NLRP3 is a well-characterized inflammasome because it is involved in a variety genetic, infectious, and metabolic diseases; however, the equine NLRP3 inflammasome has not yet been characterized (Ahn et al. 2018d; Guo et al. 2015; Vrentas et al. 2018). In this study, we characterized NLRP3 inflammasome activation in equine PBMCs by applying several well-known NLRP3 activation triggers: ATP, NG, Alum,

Fig. 1 Detection of IL-1β expression in equine PBMCs. **A**, Mouse BMDMs, human PBMCs, porcine PBMCs, and equine PBMCs were primed with LPS (1 μg/mL) for 3 h, and the cellular lysates were immunoblotted with the indicated anti-IL-1β sera. The lysate samples for human and porcine PBMCs were obtained from those in a previous study (Kim et al. 2014b). **B**, Amino acid alignment of equine (GenBank ID: NP_001075995), human (NP_000567), murine (NP_032387), and porcine (NP_999220) IL-1β precursors was analyzed by using the EMBL-EBI web-tool (http://www.ebi.ac.uk/Tools). The numbers presented indicate the amino acid positions. Gray indicates the same sequence as that of the equine, and dark gray reflects sequences with the same amino acids between the four species

a

	Mouse BMDMs	Human PBMCs	Porcine PBMCs	Equine PBMCs	
WB : anti-human IL-1β					Pro-IL-1β (≈36 kDa)
WB : anti-mouse IL-1β					Pro-IL-1β (≈36 kDa)
WB : anti-Actin					Actin (43 kDa)

b

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Equine    MAAVPD-TSDMMTYCSGNENDLFFEEDGPKQMKGSFQDLDLSSMGNGGIQLQFSHQLYNK   59
Human     MAEVPELASEMMAYYSGNEDDLFFEADGPKQMKCSFQDLDLCPL-DGGIQLRISDHHYSK   59
Mouse     MATVPELNCEMPP-FDSDENDLFFEVDGPQKMKGCFQTFDLGCP-DESIQLQISQQHINK   58
Porcine   MAIVPEPAKEVMANYGDNNNDLLFEADGPKEMKCCTQNLDLGSLRNGSIQLQISHQLWNK   60

Equine    TFKHVVSIIVAMEKLKKIPVPCSQAFQDDDLRSLFSVIFEEEPIICDNWDDD----YVCDA   116
Human     GFRQAASVVVAMDKLRKMLVPCPQTFQENDLSTFFPFIFEEEPIFFDTWDNEA--YVHDA    117
Mouse     SFRQAVSLIVAVEKLWQLPVSFPWTFQDEDMSTFFSFIFEEEPILCDSWDDDDNLLVCDV   118
Porcine   SIRQMVSVIVAVEKPMK--NPSSQAFCDDDQKSIFSFIFEEEPIILETCNDD----FVCDA   115

Equine    AVHSVNCRLRDIYHKSLVLSGACELQAVHLNGENTNQQVVFCMSFVQGEEETDKIPVALG   176
Human     PVRSLNCTLRDSQQKSLVMSGPYELKALHLQGQDMEQQVVFSMSFVQGEESNDKIPVALG   177
Mouse     PIRQLHYRLRDEQQKSLVLSDPYELKALHLNGQNINQQVIFSMSFVQGEPSNDKIPVALG   178
Porcine   NVQSMECKLQDKDHKSLVLAGPHMLKALHLLTGDLKREVVFCMSFVQGDDSNNKIPVTLG   175

Equine    LKEKNLYLSCGMKDGKPTLQLETVDPNTYPKRKMEKRFVFNKMEIKGNVEFESAMYPNWY   236
Human     LKEKNLYLSCVLKDDKPTLQLESVDPKNYPKKKMEKRFVFNKIEINNKLEFESAQFPNWY   237
Mouse     LKGKNLYLSCVMKDGTPTLQLESVDPKQYPKKKMEKRFVFNKIEVKSKVEFESAEFPNWY   238
Porcine   IKGKNLYLSCVMKDNTPTLQLEDIDPKRYPKRDMEKRFVFYKTEIKNRVEFESALYPNWY   235

Equine    ISTSQAEKKPVFLGNTRGGRDITDFIMEITSA   268
Human     ISTSQAENMPVFLGGTKGGQDITDFTMQFVSS   269
Mouse     ISTSQAEHKPVFLGNN-SGQDIIDFTMESVSS   269
Porcine   ISTSQAEQKPVFLGNSKGRQDITDFTMEVLSP   267
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and MSU (Hornung et al. 2008; Mariathasan et al. 2006; Martinon et al. 2006). Equine PBMCs were primed with LPS to induce the expression of inflammasome components and then treated with increasing dosages of each NLRP3 activation trigger, as indicated in Fig. 2A. The results reveal that the LPS-primed PBMCs showed up-regulation of pro-IL-1β expression in the cellular lysates. In addition, all NLRP3 activation triggers dose-dependently induced IL-1β secretion in the cellular supernatant (Fig. 2B). Based on Fig. 2B bar graphs, the band intensities of IL-1β p17 secretion were significantly increased by treatment with the NLRP3 activation triggers. Thus, common NLRP3 activation triggers (ATP, NG, Alum, and MSU) elicit equine IL-1β secretion, thereby implying that equine NLRP3 inflammasomes are similar to those in other species.

Equine NLRC4 and AIM2 inflammasomes

Two other inflammasomes, NLRC4 and AIM2, are mainly activated by pathogens and regarded as gatekeepers for intracellular infection. Based on previous study, *Salmonella* Typhimurium induces assembly of the NLRC4 inflammasome and *Listeria monocytogenes* triggers activation of the AIM2 inflammasome (Diamond et al. 2017; Guo et al. 2015; Tsuchiya et al. 2010; Zhao et al. 2011). In this study, equine PBMCs were primed with LPS and then inoculated with *Salmonella* or *Listeria* to activate the NLRC4 or AIM2 inflammasomes (Fig. 3A). As expected, both bacterial treatments lead to the secretion of IL-1β in the supernatant. Moreover, the IL-1β p17 band intensities in the bacteria-inoculated samples were significantly greater than those of the non-inoculated samples. In addition, flagellin or dsDNA

Fig. 2 Effect of NLRP3 activation triggers on equine PBMCs. **A**, Schematic diagram of the experimental procedure. Briefly, equine PBMCs were primed with LPS (1 µg/mL) for 3 h and then treated with NLRP3 triggers for the indicated time. Cellular supernatants (Sup) and lysates (Lys) were then analyzed by immunoblotting. **B**, LPS-primed equine PBMCs treated with the indicated concentration of the NLRP3 triggers ATP, NG, Alum, or MSU. Sup and Lys were immunoblotted with anti-IL-1β sera. The lower bar graphs present the mean ± SD intensities of IL-1β p17 normalized to that of actin in Lys. Immunoblotting images are representative of two independent experiments
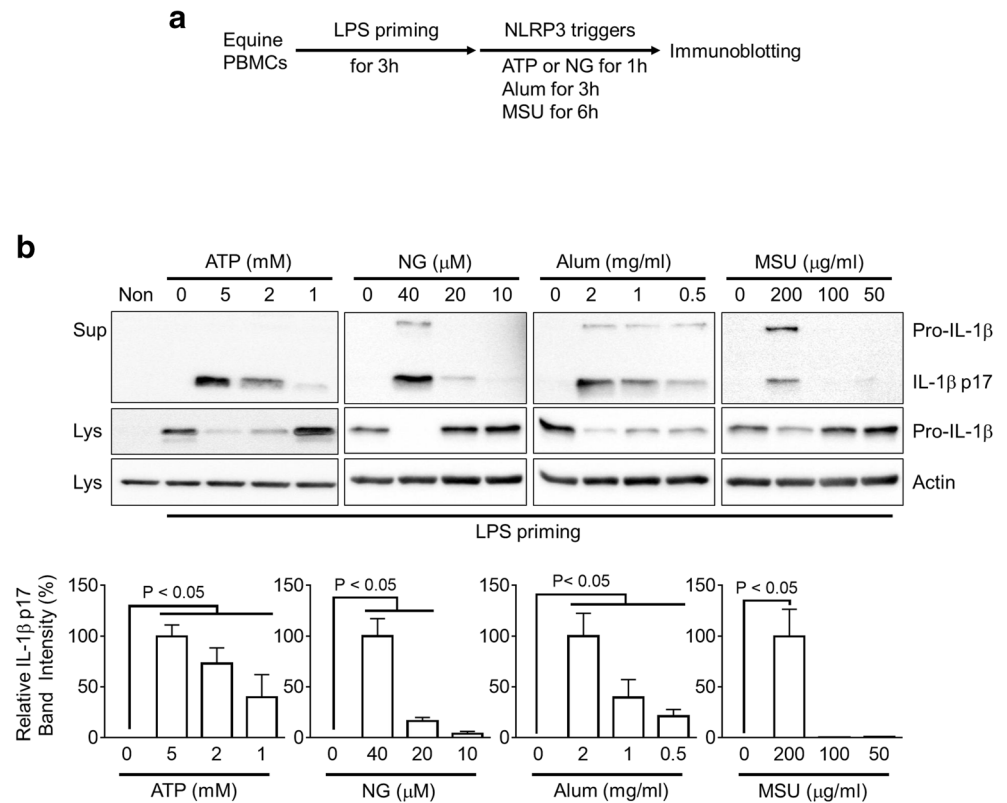
were intracellularly introduced into LPS-primed equine PBMCs to activate NLRC4 or AIM2 inflammasome (Fig. 3B). Similar to the bacteria treatment results, flagellin and dsDNA provoked IL-β secretion in dose-dependent manners. Thus, various well-known NLRC4 and AIM2 activation triggers induced IL-1β secretion.

Mechanistic study of equine NLRP3 inflammasome activation

NLRP3 inflammasomes are activated by several intracellular signaling molecules and proteins. In particular, potassium efflux has been reported to be a key mediator of NLRP3 inflammasome activation (Munoz-Planillo et al. 2013). To characterize equine NLRP3 inflammasome activation, we applied the potassium efflux inhibitors, glibenclamide (Glibe) and an extracellular high potassium solution (KCl) (Di et al. 2018; Fernandes-Alnemri et al. 2010; Lamkanfi et al. 2009). As shown in Fig. 4A and Supplementary Figure 1, equine PBMCs primed with LPS were treated with Glibe or KCl in the presence of ATP, an NLRP3 trigger. As expected, ATP led to the secretion of equine IL-1β and the pan-caspase inhibitor (Z-VAD-FMK) completely blocked that IL-1β secretion. The KCl treatment inhibited the ATP-mediated IL-1β secretion but not completely. We undertook the same experiments in mouse BMDMs and the human THP-1 cells. In mouse macrophages (Fig. 4B), ATP mediated the secretion of IL-1β, and

treatment with Glibe or KCl blocked the ATP-induced IL-1β secretion. In addition, Z-VAD-FMK blocked the ATP-mediated IL-1β secretion. As shown in Fig. 4C, human THP-1 cells produced the active form of IL-1β in response to treatment with NG, an NLRP3 activation trigger. Moreover, Glibe, KCl, and Z-VAD-FMK treatments inhibited IL-1β secretion in THP-1 cells. Based on those results, compared to human and mouse cell responses, the equine NLRP3 inflammasome activation response might be less dependent on potassium efflux stimulation.

Discussion

In this study, we characterized equine inflammasome activation and its upstream mechanism. Inflammasome activation can be divided into two distinct steps: priming and activation (Ahn et al. 2018d; Lee 2013). During the priming step, equine monocytes were up-regulated to the p36 pro-form of IL-1β by LPS treatment. The LPS-primed equine monocytes secreted the p17 active form of IL-1β in response to exposure to inflammasome activators (ATP, NG, Alum, MSU, *Salmonella*, *Listeria*, flagellin, and dsDNA) at the activation step. To characterize the intracellular mechanism, a common pathway, potassium efflux, in the activation of NLRP3 inflammasomes was blocked by glibenclamide or high KCl solution at the activation step. The results indicated that
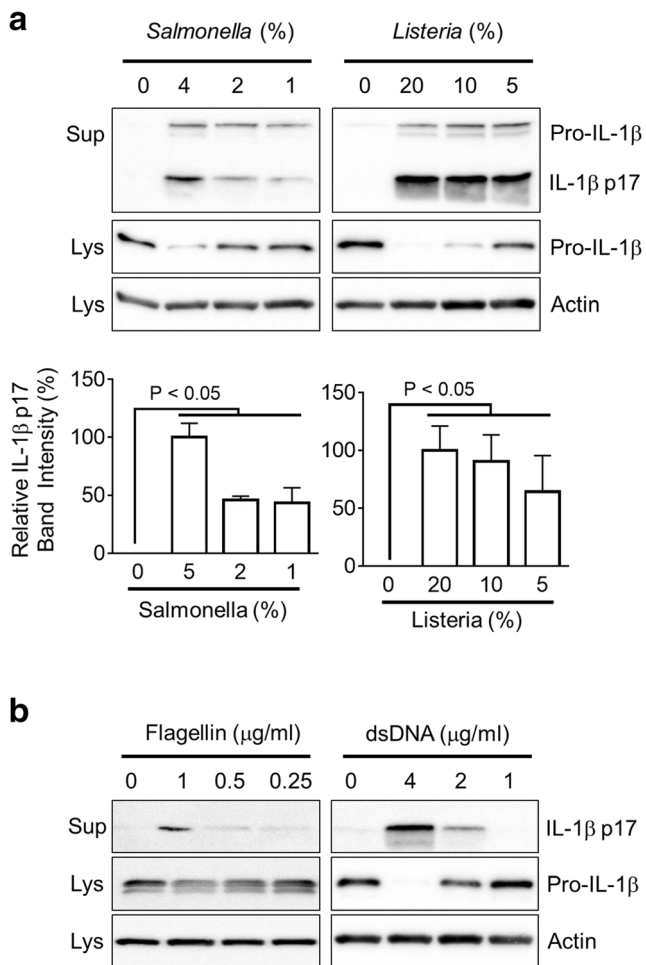
Fig. 3 Effect of NLRC4 or AIM2 activation triggers on equine PBMCs. **A**, LPS-primed equine PBMCs were inoculated with *Salmonella* (*Salmonella enterica* serovar Typhimurium) or *Listeria* (*Listeria monocytogenes*) as indicated. IL-1β secretion was analyzed by immunoblotting. The band intensities of the active form of IL-1β p17 are presented in the lower bar graphs (mean ± SD). **B**, Equine PBMCs were primed with LPS and then flagellin or dsDNA was introduced into the cytoplasm. IL-1β secretion was analyzed by immunoblotting. Immunoblotting images are representative of two independent experiments

equine PBMCs showed a lower response level to the pathway blockage than those of human THP-1 cells and mouse BMDMs. Taken together, the results show that the NLRP3, NLRC4, and AIM2 equine inflammasomes are activated by activation triggers well-established in other species including human and mouse. However, the lower response of the potassium efflux pathway indicates that it may not be a key pathway in the activation of equine inflammasomes.

Before establishment of the inflammasome concept, it was reported that ATP and NG elicited IL-1β maturation and secretion (Perregaux and Gabel 1994). It was subsequently revealed that ATP activated the NLRP3 inflammasome through potassium efflux (Mariathasan et al. 2006). NG, a bacterial toxin derived from *Streptomyces hygroscopicus*, acts as a potassium ionophore and decreases intracellular potassium

levels resulting in NLRP3 inflammasome activation similar to that of ATP (Mariathasan et al. 2006). To date, several NLRP3 activation triggers have been reported. Among them, Alum can activate NLRP3 inflammasomes and is currently used as a vaccine adjuvant (Hornung et al. 2008). As well, MSU crystals, which can induce acute gout inflammation, can induce the secretion IL-1β via NLRP3 inflammasome activation (Martinon et al. 2006). Although NLRP3 inflammasomes are involved in genetic, metabolic, and infectious diseases, NLRC4 and AIM2 inflammasomes have been mainly associated with reactions to pathogens (Guo et al. 2015). *Salmonella enterica* serovar Typhimurium, a Gram-negative bacteria related to food- and water-borne diseases, activates both NLRP3 and NLRC4 inflammasomes (Diamond et al. 2017). Intracellular flagellin and T3SS derived from *Salmonella* Typhimurium activate NLRC4 inflammasomes through the recognition of NAIP5 (Diamond et al. 2017). Extracellular flagellin interacts with toll-like receptor 5, whereas cytosolic flagellin is recognized by NLRC4/NAIP5 following inflammasome activation (Zhao et al. 2011). *Listeria monocytogenes*, a Gram-negative bacteria associated with listeriosis and lethal food-borne infections, contains components that can activate NLRP3, NLRC4, and AIM2 inflammasomes (Tsuchiya et al. 2010). Among various inflammasomes, *Listeria*-mediated inflammasome activation is strongly dependent on AIM2 (Tsuchiya et al. 2010). AIM2 also recognizes dsDNA derived from infected bacteria and/or damaged cells or mitochondria (Man et al. 2016). Various previously well-characterized activation triggers of NLRP3, NLRC4, and AIM2 inflammasomes were shown in the present study to successfully elicit IL-1β maturation and secretion in equine PBMCs. We also obtained similar results in porcine PBMCs (Ahn et al. 2018c; Kim et al. 2014b). Taken together, equine inflammasome activation might be identical with that in humans, mice, and pigs.

Although equine inflammasome activation has not been widely reported, there are several reports on the role of equine IL-1β, one of the final substrates of inflammasome activation, in arthritis (Colbath et al. 2018; Lasarzik et al. 2016; Ross et al. 2012). Recombinant IL-1β has been injected into the joints of horse to induce a model of arthritis (Colbath et al. 2018; Ross et al. 2012). In addition, synovial levels of IL-1β have been monitored as a biomarker of arthritis (Lasarzik et al. 2016). Based on these reports, inflammasome activation triggers might be replaced by recombinant IL-1β in efforts to induce arthritis, and synovial levels of inflammasome components could be useful as biomarkers of arthritis.

The intracellular mechanistic pathways of the NLRC4 and AIM2 inflammasomes are relatively well-established when compared with that of the NLRP3 inflammasome (Munoz-Planillo et al. 2013; Swanson et al. 2019). NLRC4 and NAIPs, which directly interact with cytosolic flagellin and/or T3SS, recruit ASC and caspase-1 to activate inflammasomes
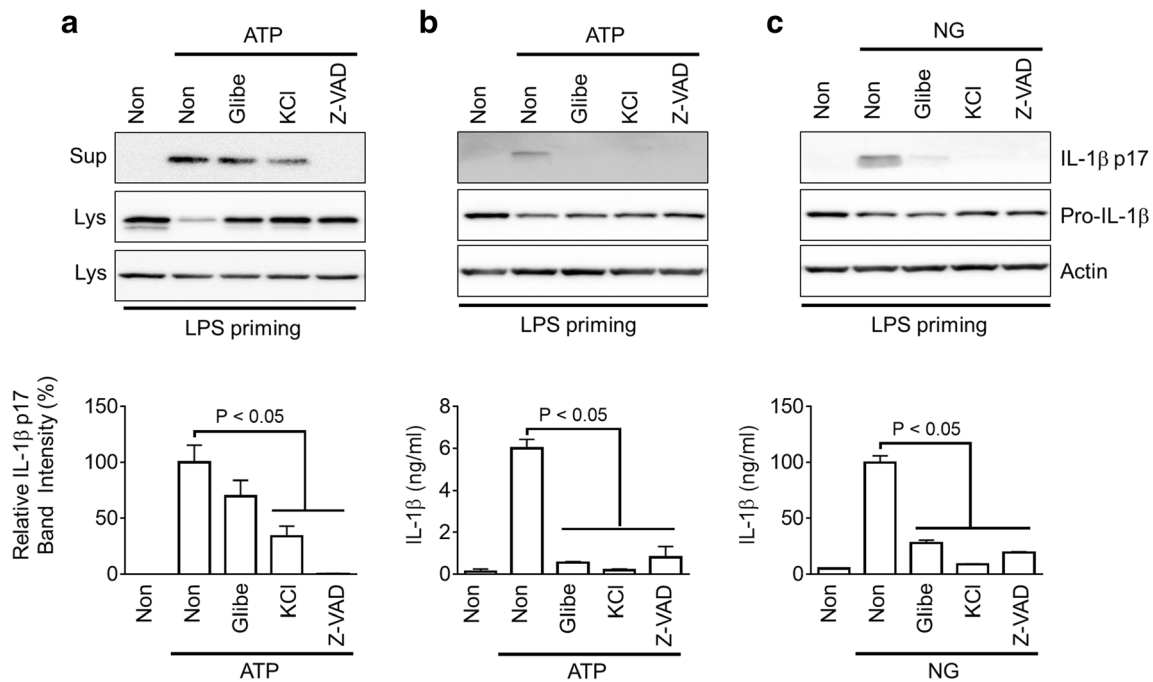
Fig. 4 Effects of potassium efflux on NLRP3 inflammasome activation. **A**, Equine PBMCs were primed with LPS and then treated with ATP (5 mM) in the presence of glibenclamide (Glibe, 150 μM), high KCl solution (KCl, 50 mM), or Z-VAD (Z-VAD-FMK, 10 μg/mL). **B**, Mouse BMDMs were primed with LPS for 3 h followed by ATP (5 mM) and Glibe, KCl, or Z-VAD treatment. **C**, THP-1 cells were pre- treated with PMA to induce differentiation into macrophage-like cells. PMA-treated THP-1 cells were then primed with LPS and treated with NG (40 μM) in the presence of Glibe, KCl, or Z-VAD. The lower bar graphs indicate the relative equine IL-1β p17 band intensities or IL-1β secretion of human and mice (mean ± SD). Immunoblotting images are representative of two independent experiments.

(Duncan and Canna 2018). The hematopoietic interferon-inducible nuclear (HIN) domain of AIM2 binds with cytosolic dsDNA and starts to assemble other inflammasome compo-nents such as ASC and caspase-1 (Jin et al. 2012). Otherwise, a direct binding pattern of NLRP3 has not been fully reported because a variety of NLRP3 activation triggers, from meta-bolic products to pathogens, activate NLRP3 inflammasomes (Munoz-Planillo et al. 2013). Thus, it has been suggested that common cytosolic signals, such as cytosolic ions changes, lysosomal disruption, mitochondrial dysregulation, and meta-bolic changes, can act as upstream signals in the activation of NLRP3 inflammasomes (Swanson et al. 2019). Among these, the potassium efflux pathway is the most fully characterized because cytosolic potassium depletion has been observed when macrophages are treated with a variety of NLRP3 acti-vation triggers (Di et al. 2018; Munoz-Planillo et al. 2013). In this study, we tested the effect of potassium efflux on the activation of equine NLRP3 inflammasomes (Fig. 4). Glibenclamide (or glyburide), used as a type 2 diabetes drug, is an inhibitor of the ATP-sensitive potassium channel and inhibits IL-1β secretion resulting from NLRP3 inflammasome activation (Lamkanfi et al. 2009). Administration of an extra-cellular high potassium solution is reported to block the secre-tion of IL-1β by inhibiting potassium efflux (Di et al. 2018; Fernandes-Alnemri et al. 2010). Unexpectedly, the disruption of potassium efflux did not inhibit IL-1β secretion following

equine NLRP3 inflammasome activation as fully as that of human and mouse NLRP3 inflammasomes. As well, we pre-viously tested the effect of glibenclamide and high KCl solu-tion on porcine NLRP3 inflammasome activation (Kim et al. 2014b) and observed that IL-1β secretion of swine PBMCs treated with NLRP3 triggers was significantly attenuated by the interruption of potassium efflux, similar to the effects on human and rodent cells (Kim et al. 2014b). Based on these findings, the potassium efflux pathway is not as tightly in-volved in the activation of equine NLRP3 inflammasome as it is in other species.

Authors' contributions Ahn, H., Kim, J., and Lee, G.-S. designed the research; Ahn, H., Kim, J., and Lee, H., performed the experiments; Ahn, H., Lee, E., and Lee, G.-S. analyzed the results; Ahn, H. and Lee, G.-S. wrote the paper; Lee, E. edited and commented on the manuscript.

Compliance with ethical standards

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