Activation of nucleotide-binding domain-like receptor containing protein 3 inflammasome in dendritic cells and macrophages by *Streptococcus sanguinis*

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Summary

Streptococcus sanguinis is frequently isolated from the blood of patients with infective endocarditis and contributes to the pathology of this disease through induction of interleukin (IL)-1β responsible for the development of the disease. However, the mechanism of IL-1β induction remains unknown. In this study, *S. sanguinis* activated a murine dendritic cell (DC) to induce IL-1β and this activity was attenuated by silencing the mRNAs of nucleotide-binding domain-like receptor containing protein 3 (NLRP3) and caspase-1.

S. sanguinis induced IL-1β production in murine bone marrow-derived macrophage (BMM), but this activity was significantly reduced in BMMs from NLRP3-, apoptosis-associated speck-like protein containing a caspase-recruitment domain-, and caspase-1-deficient mice. DC phagocytosed *S. sanguinis* cells, followed by the release of adenosine triphosphate (ATP). The ATP-degradating enzyme attenuated the release of ATP and IL-1β. The inhibitors for ATP receptor reduced IL-1β release in DC.

These results strongly suggest that *S. sanguinis* has the activity to induce IL-1β through the NLRP3 inflammasome in macrophage and DC and interaction of purinergic receptors with ATP released is involved in expression of the activity.

Introduction

More than 700 bacterial species have been identified in the human oral microbiota (Jenkinson *et al.*, 2005). Among these bacteria, streptococci are the most abundant and the initial colonizers in the formation of dental plaque (Hamada *et al.*, 1980, Aas *et al.*, 2005, Jenkinson *et al.*, 2005). While most oral streptococci are beneficial for establishing healthy microbial communities, they have the potential to cause lifethreatening infective endocarditis (IE) when they enter the bloodstream after certain dental procedures or oral injuries, such as those that may occur while chewing or tooth brushing (Douglas *et al.*, 1993, Moreillon *et al.*, 2004). IE caused by oral streptococci is thought to occur when blood-borne bacteria adhere to preformed cardiac vegetations composed of platelets and fibrin that formed in response to an injury (Moreillon *et al.*, 2004, Bashore *et al.*, 2006). Among these oral streptococci, *Streptococcus sanguinis* has been most frequently isolated from the blood of patients with IE (Douglas *et al.*, 1993, Di Filippo *et al.*, 2006). Although the pathogenesis of IE is complex and poorly understood, the proinflammatory cytokine interleukin (IL)-1 has been shown to play a role (Veltrop *et al.*, 1999, Hahn *et al.*, 2007). The clotting system, in which tissue factors (TFs) play a key role, has to be activated for endocardial vegetations to form (Camerer *et al.*, 1996, Steffel *et al.*, 2006). It has been reported that IL-1 and bacteria such as *S. sanguinis*, *Staphylococcus aureus*, and *Staphylococcus epidermidis* have a synergistic effect on the induction of TF-dependent procoagulant activity of endothelial cells (Veltrop *et al.*, 1999, Hahn *et al.*, 2007). IL-1 consists of IL-1α and IL-1β. IL-1β induces TF in endothelial cells about 10 times more strongly than IL-1α, and IL-1β produced by monocytes plays important roles in the upregulation of endothelial

TF (Wharram *et al.*, 1991, Napoleone *et al.*, 1997).

Caspase-1, which is activated by an inflammasome, processes proIL-1β to IL-1β. Inflammasomes are intracellular multiprotein complexes comprising nucleotide-binding domain-like receptor (NLR), the adaptor protein apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC), and procaspase-1. Several types of NLRs are involved in inflammasome activation (Mariathasan *et al.*, 2007, Martinon *et al.*, 2009, Davis *et al.*, 2011, Lamkanfi *et al.*, 2012, Latz *et al.*, 2013, Lamkanfi *et al.*, 2014). Active caspase-1 is required for the processing and subsequent release of active IL-1β. In addition, the inflammasome activation can lead to host cell death, called pyroptosis, in certain types of cells (Mariathasan *et al.*, 2007, Bergsbaken *et al.*, 2009, Lamkanfi *et al.*, 2010, Miao *et al.*, 2011). This mechanism might be important in restricting the intracellular replication of invasive bacterial pathogens.

The mechanism by which *S. sanguinis* induces IL-1β production in mammalian cells is poorly understood. Therefore, this study sought to elucidate the pathogenesis of *S. sanguinis* in IE. The result of this study address whether *S. sanguinis* can activate the intracellular sensor inflammasome to induce IL-1β production in innate immune cells such as dendritic cells (DCs) or macrophages and, if so, what kinds of inflammasomes are activated.

Results

IL-1β production and caspase-1 activation by XS106 cells, a murine dendritic cell, in response to S. sanguinis

The activation and release of IL-1β requires two distinct signals; The first signal is triggered by pattern recognition receptors such as Toll-like receptors (TLRs), leading to the synthesis of proIL-1β and NLRs; and the second signal is triggered by environmental stimulators (alum, asbestos, silica etc.), endogenous danger stimulators called danger-associated molecular patterns (DAMPs)(ATP, monosodium urate etc.), and microbial stimulators (pore-forming toxins, RNA etc.) which trigger the assembly of NLR, ASC and procaspase-1(Martinon *et al.*, 2006, Kanneganti *et al.*, 2007, Mariathasan *et al.*, 2007, Dostert *et al.*, 2008, Hornung *et al.*, 2008, Kool *et al.*, 2008, Li *et al.*, 2008, Cassel *et al.*, 2009, Davis *et al.*, 2011, Franchi *et al.*, 2012, Lamkanfi *et al.*, 2012, Latz *et al.*, 2013, Ratsimandresey *et al.*, 2013, Vladimer *et al.*, 2013, Lamkanfi *et al.*, 2014). Therefore, expression of TLR2 and TLR4 in XS106 cells, which are representative sensors for bacterial pathogen-associated molecular patterns (PAMPs), was examined at first. We found that XS106 cells express TLR2 but not TLR4 (supplement Fig. 1), which is responsible for the recognition of PAMPs of Gram-positive bacteria such as peptidoglycans and lipoproteins. Therefore, the next experiment was carried out to examine whether *S. sanguinis* cells induce production of IL-1β in XS106 cells. XS106 cells were stimulated with live *S. sanguinis* cells and the amounts of IL-1β released into the culture supernatant were measured. Live *S. sanguinis* cells induced IL-1β release by the cells in dose- and time-dependent manners (Fig. 1A) and the activation of caspase-1 (p20) (Fig. 1B). However, *S. sanguinis* grew when they were incubated with XS106 cells (Fig. 1C), changing the MOI (multiplicity of infection). Therefore, XS106 cells were stimulated with *S. sanguinis* cells in the presence of penicillin and streptomycin. Hereafter, B/T ratio (ratio of bacteria cell to XS106 cell number) is used instead of MOI, because *S. sanguinis* cells lose the viability

by these antibiotics. However, we confirmed that these antibiotics did not disrupt but keep *S. sanguinis* cells and kept a chain of round cells like intact streptococci (data not shown). *S. sanguinis* cells induced IL-1β production by XS106 cells in dose- and time-dependent manners even in the presence of these antibiotics (Fig. 1D, E). Therefore, the following experiments in which DCs and macrophages were stimulated with *S. sanguinis* were performed in the presence of these antibiotics. Although the culture supernatant of *S. sanguinis* also induced IL-1β production by XS106 cells (Fig. 1F), the activity of *S. sanguinis* cells was studied in the following experiments.

Involvement of caspase-1 in IL-1β-inducing activity and cell death

Mature IL-1β secretion is induced after proIL-1β processing by caspase-1, which is activated by inflammasomes (Mariathasan *et al.*, 2007, Martinon *et al.*, 2009, Davis *et al.*, 2011, Lamkanfi *et al.*, 2012, Latz *et al.*, 2013, Lamkanfi *et al.*, 2014). Therefore, in order to initially clarify the involvement of caspase-1 in the IL-1β-inducing activity of *S. sanguinis*, the pan-caspase inhibitor z-VAD-fmk or the caspase-1 inhibitor z-YVAD-fmk was used. Both z-VAD-fmk and z-YVAD-fmk attenuated the IL-1β-inducing activity in a dose-dependent manner (Fig. 2A). Furthermore, transfection of caspase-1-specific siRNA downregulated caspase-1 mRNA by approximately 50% (Fig. 2B) and the IL-1β-inducing activity was reduced by approximately 40% (Fig. 2C). These results strongly suggest that caspase-1 is involved in the IL-1β production.

Inflammasome activation triggers an inflammatory caspase-1-dependent cell death termed pyroptosis (Bergsbaken *et al.*, 2009, Lamkanfi *et al.*, 2010, Miao *et al.*, 2011);

Pyroptosis is morphologically similar to necrosis, which is characterized by cell expansion and lysis (Lamkanfi *et al.*, 2010). To initially determine whether *S. sanguinis* cells could induce cell death in XS106 cells, extracellular lactate dehydrogenase (LDH) was measured after stimulation with *S. sanguinis* cells. They induced cell death in XS106 cells in a dose-dependent manner (Fig. 3A). Therefore, XS106 cells were stained with PI and annexin-V to evaluate the mode of cell death and analyzed by flow cytometry. The percentages of swelling cell (Fig. 3B) and PI-stained cell (Fig. 3C) populations were increased relative to non-stimulated cells. Thus, *S. sanguinis* cells induced necrosis-like, but not apoptosis-like, cell death in XS106 cells. In addition, we confirmed whether the cell death was downregulated by silencing caspase-1 mRNA (Fig. 3D and 3E), because pyroptosis is dependent on caspase-1 as described above. The cell death was considered to be pyroptosis because it was accompanied by IL-1β production as described above.

Involvement of NLRP3 in IL-1β-inducing activity

The inflammasome is a complex consisting of procaspase-1, ASC and NLR (Mariathasan *et al.*, 2007, Martinon *et al.*, 2009, Davis *et al.*, 2011, Lamkanfi *et al.*, 2012, Latz *et al.*, 2013, Lamkanfi *et al.*, 2014). NLRP3 is a representative cytosolic NLR that recognizes various bacterial pathogens (Franchi *et al.*, 2012, Vladimer *et al.*, 2013). Therefore, we hypothesized that *S. sanguinis* also activates the NLRP3 inflammasome to induce IL-1β production. In order to determine the involvement of NLRP3 in the IL-1β-inducing activity, we established transfectants that stably and strongly expressed GFP after transection with the GFP-containing plasmid expressing

NLRP3-specific or non-specific siRNA. These transfectants were examined for the production of IL-1β after stimulation with *S. sanguinis* cells. NLRP3 mRNA expression in the transfectant containing the NLRP3 specific plasmid was significantly downregulated by approximately 30% than that in the transfectants containing the non-specific plasmid (Fig. 4A). The IL-1β-inducing activity of the transfectant with the NLRP3 specific plasmid was also significantly downregulated relative to that with the non-specific plasmid (Fig. 4B), suggesting that NLRP3 is involved in the IL-1β-inducing activity of *S. sanguinis*.

IL-1β-inducing activity toward bone marrow-derived macrophages from C57BL/6, caspase-1$^{-/-}$, NLRP3$^{-/-}$, or ASC$^{-/-}$ mice

As mentioned above, the NLRP3 inflammasome appears to be involved in the IL-1β-inducing activity of *S. sanguinis* toward XS106 cells. The NLRP3 inflammasome is a protein complex comprising NLRP3, ASC and procaspase-1 (Mariathasan *et al.*, 2007, Sutterwala *et al.*, 2014). In order to further confirm that *S. sanguinis* activates the NLRP3 inflammasome, IL-1β production by bone marrow-derived macrophages (BMMs) from C57BL/6 (B6) mice and caspase-1-, NLRP3-, or ASC-deficient mice (caspase-1$^{-/-}$, NLRP3$^{-/-}$, and ASC$^{-/-}$, respectively) in response to *S. sanguinis* cells was examined. *S. sanguinis* cells induced production of IL-1β by BMMs from B6 mice in a dose dependent manner, whereas the activity toward BMMs from caspase-1$^{-/-}$ and ASC$^{-/-}$ mice was almost completely lost and the activity toward BMMs from NLRP3$^{-/-}$ mice was significantly, but not completely, attenuated (Fig. 5A). In addition, heat-killed cells of *S. sanguinis* also revealed the activity toward BMMs from B6 mice in a dose

dependent manner, whereas the activity toward BMMs from caspase-$1^{-/-}$, $NLRP3^{-/-}$ and $ASC^{-/-}$ mice was almost completely lost (Fig. 5B). These results strongly suggest that the IL-1β-inducing activity of *S. sanguinis* is modulated by at least the NLRP3 inflammasome activation and one of the active entities is heat-stable.

Detection of active caspase-1 in BMMs from B6 mice

The activation of the NLRP3 inflammasome leads to the processing of procaspase-1 to active caspase-1. Therefore, the next experiment was carried out to detect active caspase-1 by western blotting using anti-caspase-1 antibody (Ab). However, we failed to detect the band of active caspase-1 under the assay conditions used (data not shown). We thought that this failure was attributed to the level of the IL-1β-inducing activity and the detection sensitivity of the western blotting. LPS priming was used before stimulation with *S. sanguinis* cells to increase the activity, because LPS priming before stimulation with the NLRP3 inflammasome activators is often used to detect active caspase-1 by western blotting. In addition, LPS priming upregulated the expression of the Gram-positive bacterial sensor TLR2 but not TLR4 (Fig. 6A). After LPS priming we used ATP, a strong NLRP3 inflammasome activator, as a positive control. As a result, it was found that the IL-1β-inducing activity (Fig. 6B) at a B/T ratio of 40 was increased by approximately 4-fold relative to that obtained by stimulation with *S. sanguinis* alone (Fig. 5), although the activity was much lower than that produced by LPS-ATP stimulation (Fig. 6C). Therefore, we tried to detect active caspase-1 under this assay condition and found that both active caspase-1 as well as mature IL-1β was detected in LPS-*S. sanguinis* at a B/T ratio of 80 and LPS-ATP stimulation by western blotting (Fig.

6D, E).

Involvement of phagocytosis in IL-1β-inducing activity

It has been reported that phagocytosis of particles such as silica, asbestos, bacteria and fungi is essential for NLRP3 inflammasome activation and mature IL-1β production (Martinon *et al.*, 2006, Hornung *et al.*, 2008, Gross *et al.*, 2009, Shimada *et al.*, 2010). Therefore, we first investigated whether XS106 cells phagocytose *S. sanguinis* cells. The cells did phagocytose FITC-labeled *S. sanguinis* cells (Fig. 7A, B). Cytochalasin D (Cyt-D), an inhibitor of actin polymerization, inhibited phagocytosis of *S. sanguinis* and reduced the IL-1β-inducing activity (Fig. 7C, D), suggesting that phagocytosis of the organism plays an essential role in expression of the IL-1β-inducing activity of *S. sanguinis*.

Release of ATP by XS106 cells in response to S. sanguinis

ATP released from damaged cells has been shown to activate the NLRP3 inflammasome through the receptor P2X7 (P2X7R) for ATP (Iyer *et al.*, 2009, Riteau *et al.*, 2012). Furthermore, it has been reported that ATP and its metabolic derivatives, ADP and UTP, are involved in particle-induced IL-1β production by the NLRP3 inflammasome through multiple purinergic signal by both P2XR and P2YR (Riteau *et al.*, 2012). Therefore, we examined if stimulation with *S. sanguinis* cells induced ATP release. Stimulation of XS106 cells with *S. sanguinis* cells at B/T ratios of 0, 4, and 40 induced ATP release (Fig. 8A) and IL-1β production (Fig. 8B) by XS106 cells in time- and

dose-dependent manners. The amount of ATP released at a B/T ratio of 40 peaked at 18 h, but the amount of ATP decreased drastically after 18 h (Fig. 8A). This drastic decline was considered to be due to the degradation of released ATP by an ecto-ATPase (Joseph *et al.*, 2003) of *S. sanguinis* cells. A small amount of ATP was also released when *S. sanguinis* cells were incubated for 7 h in the absence of XS106 cells, but the amount of ATP released gradually decreased to zero after 15 h (Fig. 8A). These declines in ATP were also considered to be due to an ecto-ATPase of *S. sanguinis*. The next experiment was carried out to investigate if the IL-1β-inducing activity is mediated through the interaction of extracellular ATP with P2X7R. It was found that both the P2XR and P2YR inhibitor oxidized ATP (oATP) and the P2X7R selective inhibitor (KN-62) significantly downregulated IL-1β production in a dose-dependent manner (Fig. 8C, D), suggesting that signaling pathways mediated by P2XRs including P2X7R and/or P2YR are involved in the activity. The ATP- and ADP-degrading enzyme apyrase did not only downregulate the level of extracellular ATP but also IL-1β production (Fig. 8E, F), strongly suggesting that extracellular ATP released by XS106 cells in response to *S. sanguinis* cells is involved in the IL-1β-inducing activity. As described above, we obtained the results suggesting that phagocytosis of *S. sanguinis* cells by XS106 cells plays an essential role in modulation of the IL-1β-inducing activity of *S. sanguinis* (Fig. 7C, D). To further confirm the involvement of ATP in the IL-1β-inducing activity, therefore, we investigated if the actin polymerization inhibitor Cyt-D, which reduced the activity (Fig. 7D), attenuated the ATP release. Indeed, Cyt-D reduced the ATP release in a dose-dependent manner (Fig. 8G).

Discussion

S. sanguinis is a member of the Mitis group of streptococci (Facklam, 2002). The species name (formerly *sanguis*) originates from streptococcal strains isolated from patient with IE (White *et al.*, 1946). *S. sanguinis* is isolated most frequently from the blood of IE patients (Douglas *et al.*, 1993). Numerous lines of evidence have been accumulated that the organism plays pathogenic roles in IE (Herzberg *et al.*, 1983, Erickson *et al.*, 1990, Herzberg *et al.*, 1992, Veltrop *et al.*, 1999, Ohashi *et al.*, 2000, Kerrigan *et al.*, 2002, Herzberg *et al.*, 2005, Plummer *et al.*, 2005, Das *et al.*, 2009, Turner *et al.*, 2009, Okahashi *et al.*, 2010, Do *et al.*, 2011, Zhu *et al.*, 2011, Chen *et al.*, 2012). IL-1 and Tissue factor play important roles in the pathogenesis of IE, although the pathogenesis is complex and poorly understood (Drake *et al.*, 1984, Bancsi *et al.*, 1996, Veltrop *et al.*, 1999, Veltrop *et al.*, 2001, Steffel *et al.*, 2006, Hahn *et al.*, 2007). IL-1, especially IL-1β, from monocytes has been reported to induce tissue factor in the endothelial cells (Wharram *et al.*, 1991, Napoleone *et al.*, 1997, Veltrop *et al.*, 2001). In addition, it has been reported that *S. sanguinis* cells induce IL-1β production in human monocytes (Banks *et al.*, 2002, Hahn *et al.*, 2007, Okahashi *et al.*, 2011). However, the mechanism by which *S. sanguinis* induces IL-1β in monocytes still remains unknown. Therefore, this study was designed to clarify the mechanism. The activation and release of IL-1β requires formation of an inflammasome complex. Several types of NLRs are involved in inflammasome activation (Mariathasan *et al.*, 2007, Davis *et al.*, 2011, Lamkanfi *et al.*, 2012, Lamkanfi *et al.*, 2014). Of these NLRs, the NLRP3 inflammasome is most extensively studied (Latz *et al.*, 2013). Therefore, we thought that *S. sanguinis* cells also activated the NLRP3 inflammasome and found that *S.*

sanguinis cells induced IL-1β and pyroptosis in XS106 cells (Fig. 1 and Fig. 3). The IL-1β-inducing activity of the organism was downregulated by the caspase-1 inhibitor and by silencing caspase-1 and NLRP3 mRNAs (Fig. 2 and Fig. 4). These results suggest that *S. sanguinis* activates the NLRP3 inflammasome in XS106 cells to induce IL-1β production. This was further confirmed by drastic downregulation of the activity in BMMs from procaspase-1-, and ASC- and NLRP3-deficient mice and B6 mice (Fig. 5).

The NLRP3 inflammasome is activated by a diverse set of soluble or particulate stimuli from microbes that are collectively referred to as PAMPs (Franchi *et al.*, 2012, Ratsimandresey *et al.*, 2013, Vladimer *et al.*, 2013), by DAMPs such as monosodium urate (Martinon *et al.*, 2006) or by environmental stimulators such as asbestos (Cassel *et al.*, 2008, Dostert *et al.*, 2008, Hornung *et al.*, 2008), and alum (Eisenbarth *et al.*, 2008, Hornung *et al.*, 2008, Kool *et al.*, 2008, Li *et al.*, 2008). However, the mechanism by which these diverse and structurally and chemically unrelated stimuli activate the NLRP3 inflammasome remains unclear. Microbial activators include Gram-positive and -negative bacteria, fungi, viruses and protozoa (Franchi *et al.*, 2012, Ratsimandresey *et al.*, 2013, Vladimer *et al.*, 2013), but the mechanism that causes activation of the NLRP3 inflammasome is also still controversial. Numerous lines of study reveal that phagocytosis of particulate stimuli such as monosodium urate, silica, asbestos and alum play important roles in the activation of the NLRP3 inflammasome (Martinon *et al.*, 2006, Cassel *et al.*, 2008, Dostert *et al.*, 2008, Eisenbarth *et al.*, 2008, Hornung *et al.*, 2008). In addition, Riteau *et al.* (Riteau *et al.*, 2012) demonstrate that internalization of these particulate stimulators triggers the extracellular release of endogenous ATP. The extracellular ATP is recognized by the purinergic receptor P2X7, which is required for

the secretion of mature IL-1β. ATP, ADP and UTP act upon multiple purinergic receptors to activate IL-1β production through the NLRP3 inflammasome. Therefore, we first investigated whether XS106 cells phagocytose *S. sanguinis* cells. We found that the cell did phagocytose *S. sanguinis* cells (Fig. 7A, B) and that Cyt-D, an inhibitor of actin polymerization, inhibited the phagocytosis of *S. sanguinis* and reduced the IL-1β-inducing activity (Fig. 7C, D). These results suggest that phagocytosis of the organism is required for expression of the IL-1β-inducing activity. On the basis of the findings of Riteau *et al.* (Riteau *et al.*, 2012), the next experiment was carried out to determine whether stimulation of XS106 cells with the organism induces ATP release. Expectedly, we found that the stimulation induced extracellular ATP release in time- and dose-dependent manners (Fig. 8A). In addition, we found that both the P2XR and P2YR inhibitor oATP and the P2X7R selective inhibitor KN-62 significantly downregulated IL-1β production in a dose-dependent manner (Fig. 8C, D) and that the ATP- and ADP-degrading enzyme apyrase inhibited ATP release and reduced IL-1β production (Fig. 8E, F). The actin polymerization inhibitor Cyt-D inhibited ATP release as well as IL-1β release in a dose-dependent manner (Fig. 8G). These results strongly suggest that the interaction of purinergic receptors with ATP released by phagocytosis of *S. sanguinis* cells and its derivatives including ADP plays a key role in the IL-1β-inducing activity.

It has been reported that *Streptococci* induce IL-1β production by monocytes/macrophages. For example, exposure of *S. pneumoniae* to the human monocytic THP-1 cells induced ATP release, which was blocked by oATP (Hoegen *et al.*, 2011). However, the ATP release was not inhibited by Cyt-D, suggesting that the ATP release is not involved in phagocytosis of the bacterium. In addition, Harder *et al.*

have reported that caspase-1 activation in response to *S. pyogenes* infection proceeds independently of P2X7R using P2X7R-deficient mice, although they did not measure ATP release (Harder *et al.*, 2009). Taken together, this is the first study to show that a *Streptococcus* species induces the production of IL-1β in dendritic cells through activation of the NLRP3 inflammasome, in which ATP released by phagocytosis of the bacterium plays a key role, although there are some reports that an oral gram-negative bacterium, *Porphyromonas gingivalis*, activates an inflammasome through the interaction of ATP and purinerigic receptors (Yilmaz *et al.*, 2010, Hung *et al.*, 2013).

Thus, the present finding that *S. sanguinis* activates monocytes to induce IL-1β production through activation of the NLRP3 inflammasome explains a previously unknown pathological role of *S. sanguinis* in IE.

Experimental procedures

Bacterium

S. sanguinis ATCC 10556 was grown in brain heart infusion broth (BD Biosciences, San Jose, CA, USA). Cultures were incubated at 37°C and centrifuged at 8,000 × *g* for 15 min at the late log-phase. The cell pellets were washed three times with sterilized PBS, suspended in PBS to make aliquots, and then stored at −80°C.

Mice

Sex-matched 8-week-old B6 mice were purchased from CLEA (Tokyo, Japan) and

maintained in specific pathogen-free conditions at the animal facility of the Graduate School of Dental Medicine, Hokkaido University. Caspase-1$^{-/-}$, NLRP3$^{-/-}$, and ASC$^{-/-}$ of the same genetic background were maintained in specific pathogen-free conditions at the animal facility of the Graduate School of Medicine, University of the Ryukyus.

All experiments were performed in accordance with the regulations of the Animal Care and Use Committees of both universities.

Cell culture

XS106, a murine DC line, kindly provided by Professor Akira Takashima (University of Texas Southwestern, Dallas, TX, USA), is a long-established DC line derived from the epidermis of newborn A/J mice (Mohan *et al.*, 2005). XS106 cells were cultured in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% FBS (Invitrogen, Carlsbad, CA), 2 mM L-glutamine, 1 mM sodium pyruvate (Sigma), 10 mM HEPES buffer (Sigma), 100 U/ml penicillin G, 100 μg/ml streptomycin (Sigma), 50 μM 2-ME (Sigma), 1% non-essential amino acids (Sigma), 0.5 ng/ml murine recombinant GM-CSF (Pepro Tech, Rocky Hill, NJ, USA), and 5% culture supernatant derived from NS47 fibroblast cells (hereafter referred to as XS medium)(Mohan *et al.*, 2005).

Femurs and tibias prepared from caspase-1$^{-/-}$, NLRP3$^{-/-}$ or ASC$^{-/-}$ mice at the University of the Ryukyus were sent to Hokkaido University. Femurs and tibias from B6 mice were prepared at Hokkaido University.

Bone marrow cells were obtained from femurs and tibias as follows. The ends of the bones were cut off, and marrow tissue was eluted by irrigation with RPMI 1640

medium containing 10% FBS, 100 U/ml penicillin G, and 100 μg/ml streptomycin. Cells were suspended by pipetting and washed by centrifugation. The cells were cultured in a non-tissue culture plastic 10-cm petri dish in RPMI 1640 medium containing 10% FBS, 100 U/ml penicillin G, 100 μg/ml streptomycin, and cell conditioned medium (i.e., culture supernatants derived from L929 fibroblast cells). After 7–9 days of culture, macrophages loosely adhered to the dishes were harvested by using cold PBS and used as BMMs (Celada *et al.*, 1984).

Expression of TLR2 or TLR4 by flowcytometry

For TLR2 expression, XS106 cells or BMMs were stained at room temperature for 1 h with Alexa Fluor 488-conjugated anti-TLR2 mAb (T2.5; BioLegend, San Diego, CA, USA) or Alexa Fluor 488-conjugated isotype-matched mouse IgG (MOPC-21; BioLegend). For TLR4 expression, PE-conjugated isotype-matched mouse IgG (P3; eBioscience, San Diego, CA, USA) and PE-conjugated anti-TLR4 mAb (UT41; eBioscience) were used. Expression of TLR2 or TLR4 after stimulation with or without 1 μg/ml of ultrapure *E. coli* LPS (InvivoGen, San Diego, CA, USA) was then analyzed by a FACSCalibur flow cytometer (BD Biosciences). Data for 20,000 cells falling within the appropriate forward and side light scatter gates were collected from each sample. Data were analyzed using FlowJo software (Tree Star Inc., Ashland, OR, USA).

Measurement of IL-1β

XS106 cells were added to a 24-well plate at 3×10^5 cells per well in 500 μl XS

medium and incubated at 37°C for 16 h at a 5% CO_2 atmosphere. The cultures were centrifuged at 400 × *g* for 5 min, and the cells were washed with supplement-free RPMI 1640 basal medium. The cells were resuspended in 300 μl RPMI 1640 basal medium and incubated at 37°C for various periods of time at several MOI or B/T ratios in the absence or presence of 100 U/ml penicillin G and 100 μg/ml streptomycin. The following chemicals were applied to the cells: z-VAD-fmk, z-YVAD-fmk, Cyt-D, oATP, KN-62, apyrase and NAc. All of these chemicals were obtained from Sigma. The cells were resuspended in 300 μl RPMI 1640 basal medium in the absence or presence of various concentrations of these chemicals and incubated at 37°C for 24 h at a B/T ratio of 40. Concentrations of these chemicals used are indicated in figures and figure legends.

BMMs from B6, caspase-$1^{-/-}$, NLRP$3^{-/-}$ or ASC$^{-/-}$ mice were added to a 24-well plate at 4×10^5 cells per well in 500 μl RPMI 1640 medium containing 10% FBS. The cells were resuspended in 300 μl RPMI 1640 basal medium and incubated at 37°C for 12 h with *S. sanguinis* cells at B/T ratios of 4 and 40 in the presence of 100 U/ml penicillin G and 100 μg/ml streptomycin. BMMs are also treated with heat-killed cells (treated for 10 min in boiling water) of *S. sanguinis* at B/T ratios of 4 and 40 in the absence of 100 U/ml penicillin G and 100 μg/ml streptomycin.

The amounts of IL-1β in cell culture supernatants were measured using an ELISA kit (BD OptEIA™ Set Mouse IL-1β, BD Biosciences). The cell culture supernatants were subjected to SDS-PAGE to distinguish mature IL-1β from proIL-1β, and proteins were transferred to a PVDF membrane (Bio-Rad, Hercules, CA, USA). These proteins were reacted with the appropriate polyclonal Ab against IL-1β (R&D Systems, Minneapolis, MN, USA).

Cell death assay

XS106 cells or the caspase-1-silencing XS106 cells as described below were added to a 24-well plate at 3×10^5 cells per well in 500 μl XS medium and incubated at 37°C for 16 h in a 5% CO_2 atmosphere. The cultures were centrifuged at 400 × *g* for 5 min, and the cells were washed with RPMI 1640 basal medium. The cells were resuspended in 300 μl RPMI1640 basal medium and incubated at 37°C for 24 h at a B/T ratio of 40 in the presence of 100 U/ml penicillin G and 100 μg/ml streptomycin. The cells were stained with PI (propidium iodide) and annexin-V using an Annexin-V-FLUOS Staining Kit (Roche, Mannheim, Germany) and subsequently analyzed by a FACSCalibur flow cytometer (BD Bioscience). Data for 30,000 cells falling within the appropriate forward and side light scatter gates were collected from each sample. Data were analyzed using FlowJo software (Tree Star Inc.).

XS106 cells were added to a 24-well plate at 3×10^5 cells per well in 500 μl XS medium and incubated at 37°C for 16 h at a 5% CO_2 atmosphere. The cultures were centrifuged at 400 × g for 5 min, and the cells were washed with supplement-free RPMI 1640 basal medium. The cells were resuspended in 300 μl RPMI 1640 basal medium and incubated at 37°C for 24 h at several B/T ratios in the presence of 100 U/ml penicillin G and 100 μg/ml streptomycin. LDH content in the supernatants was measured using a CytoTox 96 kit (Promega, Madison, WI, USA), according to the manufacturer's instructions. Cytotoxicity was calculated against the maximum release of LDH, which was obtained by cell lysis with 0.09% Triton X-100. The percentage of cytotoxicity was calculated as LDH released in [tested sample (A490)/maximum LDH

release (A490)] × 100.

RNA interference

Caspase-1 was silenced in XS106 cells as follows. siRNAs specific for caspase-1 and a non-targeting siRNA control (Applied Biosystems, Carlsbad, CA, USA) at final concentrations of 2 μM were transfected into XS106 cells (2×10^6 cells/0.1 ml Opti-MEM) by electroporation using a two-step electroporator (CUY21 Pro-Vitro, NEPA GENE, Tokyo, Japan) at 175 V for 3 ms. The cells were cultured in XS106 medium at 37°C for 40 h to reach 100% confluence in a 10-cm dish. The cells collected from the dish were subsequently inoculated onto a 24-well plate at 3×10^5 cells per well. After a 16-h incubation, the cells were stimulated at 37°C for 24 h at B/T ratios of 4 and 40.

NLRP3 was silenced in XS106 cells as follows. The GFP-containing plasmids psiRNA-mNLRP3 and psiRNA-LucGL3 (InvivoGen), which express an NLRP3-specific and a nonspecific siRNA, respectively, were transfected into XS106 cells by electroporation as described above. Transfectants stably expressing NLRP3-specific or nonspecific siRNA were established by selection in the presence of 100 μg/ml Zeocin (Invitrogen) at 37°C for 2 weeks in a 10-cm dish.

Total RNA was extracted from the washed cells using the ReliaPrep™ RNA Cell Miniprep System (Promega), and cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). For real-time PCR analysis, PCR amplification was performed in the presence of a TaqMan probe using a StepOne Real-Time PCR system (Applied Biosystems). Specific primers for caspase-1, NLRP3,

and GAPDH were purchased from Applied Biosystems. The cycling threshold (C_T) value was defined as the number of PCR cycles in which the fluorescence signal exceeded the detection threshold. The normalized amount of target mRNA (Nt) was calculated from the C_T value obtained for both target and GAPDH mRNAs according to the following equation: $Nt = 2^{Ct(GAPDH) - Ct(target)}$. Relative mRNA expression was obtained by setting Nt to 1 in samples transfected with non-specific siRNA in each experiment.

Detection of mature caspase-1

XS106 cells were added to a 6-well plate at 1.2×10^6 cells per well in 2 ml XS medium and incubated at 37°C for 16 h at a 5% CO_2 atmosphere. The cultures were centrifuged at 400 × g for 5 min, and the cells were washed with supplement-free RPMI 1640 basal medium. The cells were resuspended in 1 ml RPMI 1640 basal medium and incubated at 37°C for 24 h at B/T ratios of 0.4 or 4. The cells (one well) were lysed in cell lysis buffer [(25 mM Tris-HCl pH7.5, 150 mM NaCl, 1% (w/v) IGEPAL® CA-630 (Sigma), complete protease inhibitors (Roche)] and combined with the supernatant (one well) precipitated with 6% TCA. The samples were treated with SDS sample buffer and submitted to SDS-PAGE analysis. Proteins were transferred to a PVDF membrane and reacted with mouse anti-mouse caspase-1 p20 Ab (AdipoGen, USA).

BMMs from B6 mice were added to a 6-well plate at 2×10^6 cells per well in 2 ml RPMI 1640 medium containing 10% FBS and incubated at 37°C for 4 h with 1 μg/ml ultrapure *Escherichia coli* LPS (InvivoGen). The cells were resuspended in 1 ml RPMI 1640 basal medium and incubated at 37°C for 12 h with *S. sanguinis* cells at a B/T ratio of 80 in the presence of 100 U/ml penicillin G and 100 μg/ml streptomycin. The cells

(one well) were lysed in cell lysis buffer and combined with the supernatant (three wells) precipitated with 6% TCA. The samples were treated with SDS sample buffer and submitted to SDS-PAGE analysis. Proteins were transferred to a PVDF membrane and reacted with rabbit anti-mouse caspase-1 Ab (sc-514, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-mouse IL-1β Ab.

BMMs from B6 mice were added to a 6-well plate at 1.6×10^6 cells per well in 2 ml RPMI 1640 medium containing 10% FBS and incubated at 37°C for 4 h with 1 μg/ml ultrapure *E. coli* LPS. The cells were resuspended in 1 ml RPMI 1640 basal medium and incubated at 37°C for 1 h with 5 mM ATP. The cells (one well) were lysed in cell lysis buffer and combined with the supernatant (one well) precipitated with 6% TCA. The samples were treated with SDS sample buffer and submitted to SDS-PAGE analysis. Proteins were transferred to a PVDF membrane and reacted with rabbit anti-mouse caspase-1 Ab or anti-mouse IL-1β Ab.

Phagocytosis

Cell suspensions (10^9 cfu/ml) of *S. sanguinis* were treated at 95° C for 5 min and then incubated at 37° C for 1 h with a solution (0.5 mg/ml) of FITC (Sigma) in 0.1 M carbonate buffer (pH 9.5). The FITC-conjugated cells were washed three times with PBS and resuspended with PBS to a concentration of 1×10^{10} cfu/ml.

XS106 cells were added to a 24-well plate at 3×10^5 cells per well in 500 μl XS medium and incubated at 37°C for 16 h at a 5% CO_2 atmosphere. The cultures were centrifuged at 400 × *g* for 5 min, and the cells were washed with supplement-free RPMI 1640 basal medium. The cells were resuspended in 300 μl RPMI 1640 basal medium

and incubated at 37°C for 1 h with FITC-conjugated cells at B/T ratios of 40 and 100 in the absence or presence of Cyt-D (0.5 μM and 1 μM). After the cells had been washed three times with PBS, they were suspended in PBS containing 0.2% trypan blue to quench fluorescence caused by the binding of bacteria to surface of the cells. The level of phagocytosis was analyzed by a FACSCalibur flow cytometer. Data for 20,000 cells falling within appropriate forward and side-light scatter gates were collected from each sample. Data were analyzed using FlowJo software.

Intracellular localization of S. sangunis cells in XS106 cells

XS106 cells were grown on poly-l-lysine-coated coverglasses. After a 6-h incubation, the medium was removed, and the cells were resuspended in 1 ml RPMI 1640 basal medium and incubated at 37°C for 1 h with FITC-conjugated *S. sanguinis* cells at a B/T ratio of 100. After the cells had been washed three times with PBS, they were suspended in RPMI 1640 containing Alexa Fluor 594-conjugated concanavalin A (50 μg/ml; Invitrogen) and incubated at room temperature for 20 min. After the cells had been washed three times with PBS, they were fixed for 15 min at room temperature by 4% paraformaldehyde phosphate buffer solution (Wako, Tokyo, Japan). The cells were washed three times with PBS containing 10 mM glycine and after finally washed one time with PBS, they were sealed in SlowFade® Gold Antifade Mountant with DAPI (Thermo Fisher Scientific, Tokyo, Japan). Confocal images were taken by a confocal laser scannning microscopy system (Nikon A1 and Ti-E) equipped with a Plan Apo VC x60 objective lens (NA 1.40, Nikon, Tokyo, Japan) and processed through the Huygens Essential Deconvolution software (SVI, Hilversum, Natherlands).

Measurement of extracellular ATP

XS106 cells were added to a 24-well plate at 3×10^5 cells per well in 500 µl XS medium and incubated at 37°C for 16 h at a 5% CO_2 atmosphere. The cultures were centrifuged at 400 × g for 5 min, and the cells were washed with supplement-free RPMI 1640 basal medium. The cells were resuspended in 300 µl RPMI 1640 basal medium and incubated at 37°C for various periods of time with several B/T ratios in the presence of 100 U/ml penicillin G and 100 µg/ml streptomycin. The extracellular ATP in cell culture supernatant was quantified using an ATP Bioluminescence Assay Kit CLS II (Roche) according to the manufacturer's instructions. Luminescence was measured with a Wallac 1420 ARVOsx (PerkinElmer, Inc., Waltham, MA, USA) and the ATP concentration was determined using a standard curve.

Statistical analysis

The statistical analyses were performed using Student's t test. Differences were considered significant at a P value of < 0.05.

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The authors have no financial conflicts of interest.

Figure legends

Fig.1. IL-1β production by XS106 cells in response to *S. sanguinis*

XS106 cells were stimulated at 37°C for various periods of time with live cells of *S. sanguinis* at various MOIs (0, 0.4, and 4) and the IL-1β production (A) was measured by ELISA and western blotting. After a 24-h stimulation, p20 fragment of active caspase-1 was measured by western blotting (B). The growth of *S. sanguinis* in the reaction mixture was measured (C). The IL-1β production by XS106 cells was measured at B/T ratios of 0, 0.4, 4, and 40 in the presence of penicillin G/streptomycin (pen/str) (D). The time course of the IL-1β production was measured at a B/T ratio of 40 (E). The activity of culture supernatants of *S. sanguinis* to induce IL-1β production in XS106 cells after dilutions (1:4 and 1:2) with PBS was measured using BHI broth as a medium control (F). The amounts of total IL-1β released into the conditioned medium were measured by ELISA and western blotting. The results are expressed as the mean ± SD of duplicate or triplicate assays of a representative experiment. All of the experiments were repeated at least twice and similar results were obtained. mIL-1β was evaluated according to molecular weight (mIL-1β: 17.5 kDa, proIL-1β: 31 kDa) of the bands detected by western blotting.

Student's T test: *, $0.01 < P < 0.05$; **, $0.01 < P < 0.001$; ***, $P < 0.001$.

Fig. 2. Involvement of caspase-1 in the IL-1β-inducing activity of *S. sanguinis*

XS106 cells were stimulated at 37°C for 1 h in the presence of z-VAD-fmk or z-YVAD fmk and then for another 24 h with *S. sanguinis* cells at a B/T ratio of 40. The IL-1β-inducing activity was expressed as the relative activity (%) calculated as (the activity in the presence of the inhibitor/the activity in the absence of the inhibitor) x 100.

The results are expressed as the mean ± SD of duplicate assays of a representative experiment (A). XS106 cells were transfected with caspase-1–specific siRNA or non-targeting (control) siRNA. Relative mRNA expression was determined by real-time PCR analysis. The results are expressed as the mean ± SD of triplicate assays of a representative experiment (B). The transfected cells were stimulated at 37^{o} C for 24 h with *S. sanguinis* at various B/T ratios (0, 4, and 40). Total IL-1β released into the culture supernatant was measured by ELISA and western blotting. The results are expressed as the mean ± SD of duplicate assays of a representative experiment (C). All of the experiments were repeated at least twice and similar results were obtained. Student's T test; *, $0.01 < P < 0.05$; ***, $P < 0.001$.

Fig. 3. Cell death of XS106 cells induced by *S. sanguinis*

XS106 cells were stimulated at 37°C for 24 h with *S. sanguinis* cells at various B/T ratios (0, 0.4, 4, and 40). Cell death (%) was evaluated by LDH release (A). The percentages of swelling cells (B) and necrotic cells (C) were analyzed by flow cytometry; the former by FSC and the latter by PI-stained cells. XS106 cells were transfected with caspase-1–specific siRNA or non-targeting (control) siRNA. The transfected cells were stimulated at 37^{o} C for 24 h with *S. sanguinis* at a B/T ratio of 40. The percentages of necrotic cells were analyzed by flow cytometry (D). The difference of cell death (%) was calculated as (the PI stained cell population in the presence of *S. sanguinis* cells) – (PI stained cell population in the absence of *S. sanguinis* cells) (E). The results are expressed as the mean ± SD of duplicate assays of a representative experiment. All of the experiments were repeated at least twice and similar results were obtained.

Student's T test; *, $0.01 < P < 0.05$.

Fig. 4. Effects of silencing NLRP3 mRNA on the IL-1β-inducing activity of *S. sanguinis*

XS106 cells were transfected with psiRNA-mNLRP3 and psiRNA-LucGL3 containing GFP-containing plasmids, which express NLRP3-specific and nonspecific siRNA, respectively, by electroporation. Transfectants stably expressing NLRP3-specific or nonspecific siRNA were established by selection in the presence of Zeocin. Relative mRNA expression was determined by real-time PCR analysis. The results are expressed as the mean ± SD of triplicate assays of a representative experiment (A). The transfectants were stimulated at 37^{o} C for 24 h with *S. sanguinis* at various B/T ratios (0, 4, and 40). Total IL-1β released into the culture supernatant was measured by ELISA and western blotting. The results are expressed as the mean ± SD of duplicate assays of a representative experiment (B). All of the experiments were repeated at least twice and similar results were obtained.
Student's T test: **, $0.01 < P < 0.001$; ***, $P < 0.001$.

Fig. 5. IL-1β production by BMMs from B6, caspase-1$^{-/-}$, NLRP3$^{-/-}$, or ASC$^{-/-}$ mice in response to *S. sanguinis*

BMMs were stimulated at 37°C for 12 h with *S. sanguinis* cells (A) in the presence of penicillin and streptomycin or with heat-killed cells of *S. sanguinis* (B) in the absence of these antibiotics at B/T ratios of 0, 4, and 40. Total IL-1β released into the culture supernatant was measured by ELISA and western blotting. The results are expressed as the mean ± SD of triplicate assays of a representative experiment. All of the

experiments were repeated at least twice and similar results were obtained.
Student's T test: *, $0.01 < P < 0.05$; **, $0.01 < P < 0.001$; ***, $P < 0.001$.

Fig. 6. Detection of active caspase-1 in BMM from B6 mice

BMM from B6 mice were incubated at 37°C for 4 h with ultrapure *E. coli* LPS (1 μg/ml) and the expression of TLR2 or TLR4 was examined by flow cytometry using anti-TLR2 mAb, anti-TLR4 mAb and their isotype controls (A). The numbers in histograms express the ratio of mean fluorescence intensity of cells stained with isotype control to that with anti-TLR2 or anti-TLR4 mAb. For IL-1β-inducing activity, the cells were then stimulated at 37°C for 12 h and 1 h with *S. sanguinis* cells at B/T ratios of 0, 4, 40, and 80 (B) and with ATP (C), respectively. Total IL-1β released into the culture supernatant was measured by ELISA. The results are expressed as the mean ± SD of duplicate assays of a representative experiment. All of the experiments were repeated at least twice and similar results were obtained. For the detection of active caspase-1 (D) and IL-1β (E) by western blotting, the cells were resuspended in RPMI 1640 basal medium and incubated at 37°C for 12 h at a B/T ratio of 80.
Student's T test: *, $0.01 < P < 0.05$; **, $0.01 < P < 0.001$

Fig. 7. Phagocytosis of *S. sanguinis* by XS106 cells

XS106 cells were incubated at 37°C for 1 h with the FITC-conjugated *S. sanguinis* cells at B/T ratios of 0, 40 and 100 in the absence or presence of Cyt-D. The level of phagocytosis at a B/T raio of 40 was analyzed by flow cytometry (A). Intracellular localization of *S. sangunis cells* in XS106 cells was analyzed by confocal microscopy. Arrows indicate FITC-conjugated *S. sanguinis* cells incorporated (B). The mean

fluorescence intensities of XS106 cells incubated in the absence or presence of Cyt-D (0.5 μM and 1 μM) were obtained by flow cytometry (C). Total IL-1β released into the culture supernatant at a B/T ratio of 40 was measured by ELISA. The results are expressed as the mean ± SD of triplicate assays of a representative experiment (D). The experiment was repeated at least twice and similar results were obtained.

Student's T test: *, $0.01 < P < 0.05$.

Fig. 8. Release of ATP by XS106 cells in response to *S. sanguinis*

XS106 cells were stimulated for various periods of time (7, 15, 18, and 24 h) with *S. sanguinis* at B/T ratios of 0, 4, and 40. The amounts of extracellular ATP (A) or IL-1β (B) in the culture supernatant were measured. XS106 cells were stimulated at 37°C for 1 h in the presence of oATP (0, 100, and 500 μM) (C) or KN-62 (0, 1, and 10 μM) (D) and then for another 24 h with *S. sanguinis* at a B/T ratio of 40. The IL-1β-inducing activity was expressed as the relative activity (%) calculated as (the activity in the presence of the inhibitor/the activity in the absence of the inhibitor) x 100. XS106 cells were stimulated for various periods of time (7, 15, 18, and 24 h) with *S. sanguinis* at B/T ratios of 0 and 40 in the absence or presence of apyrase (0.4, and 4 U/ml). The amounts of extracellular ATP (E) or IL-1β (F) in the culture supernatant were measured. XS106 cells were stimulated for 18 h with *S. sanguinis* cells at a B/T ratio of 40 in the absence or presence of Cyt-D (0.5, 1.0 μM) and the amount of ATP released in the culture supernatant was measured (G). The results are expressed as the mean ± SD of duplicate or triplicate assays of a representative experiment. All of the experiments were repeated at least twice and similar results were obtained.

Student's T test: *, $0.01 < P < 0.05$, **, $0.01 < P < 0.001$, ***, $P < 0.001$.

Supplement　Fig. 1.

Expression of TLR2 or TLR4 on the cell surface of XS106 cells was examined by flow cytometry using anti-TLR2 mAb, anti-TLR4 mAb and their isotype controls.

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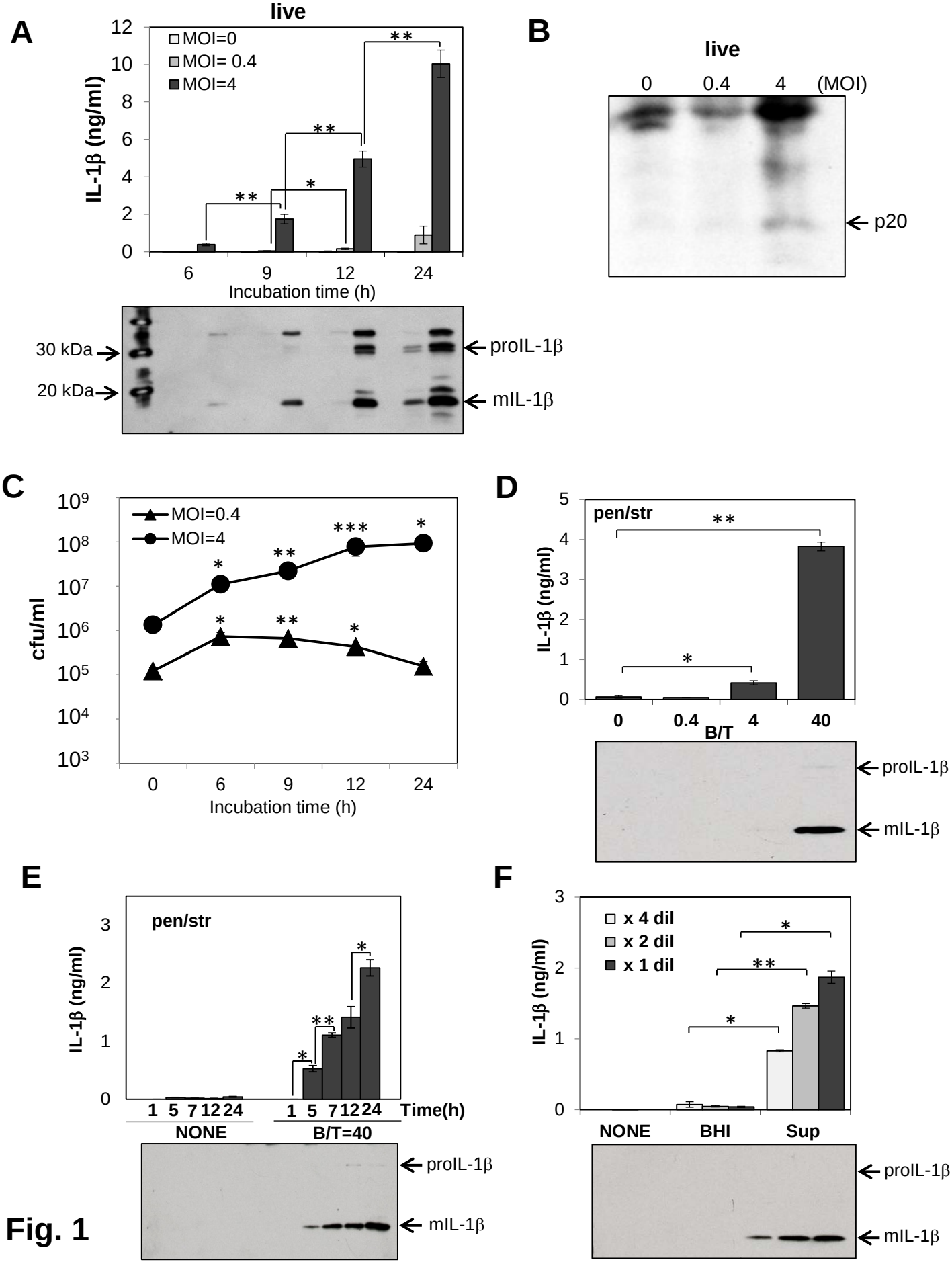
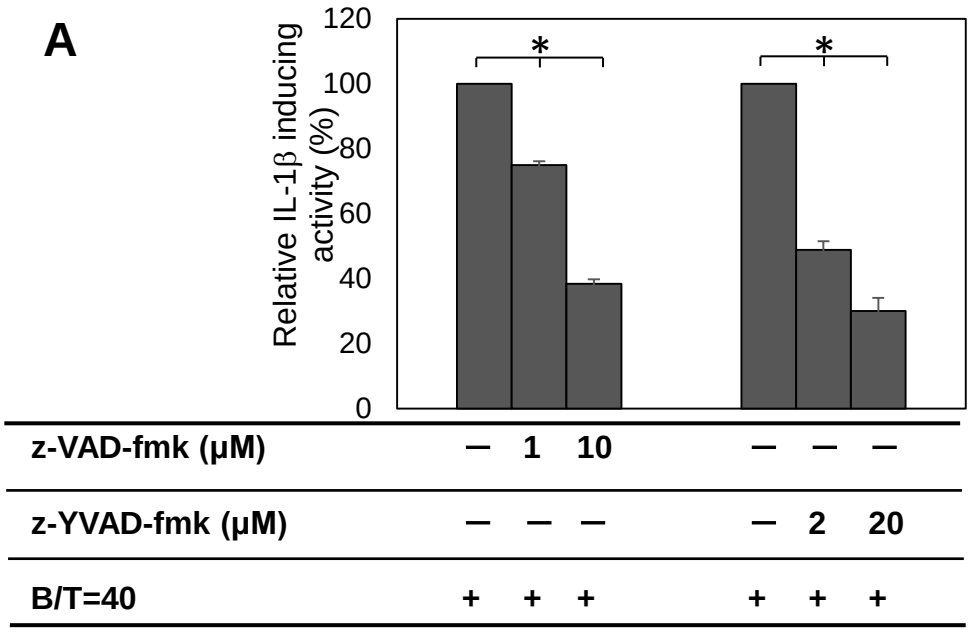
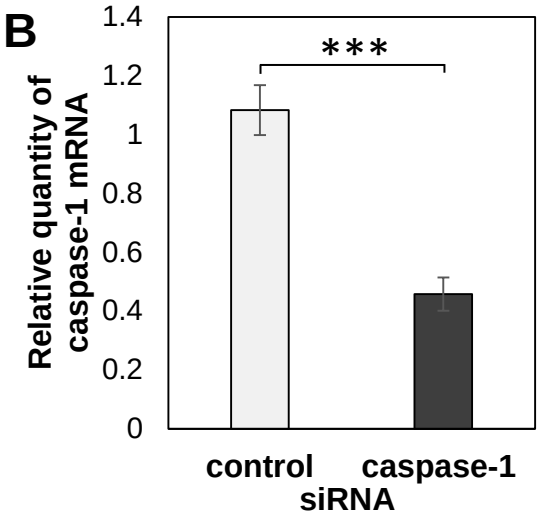

A
live
MOI=0
MOI= 0.4
MOI=4
IL-1β (ng/ml)
Incubation time (h)
30 kDa
20 kDa
proIL-1β
mIL-1β
B
live
0
0.4
4
(MOI)
p20
C
MOI=0.4
MOI=4
cfu/ml
Incubation time (h)
D
pen/str
IL-1β (ng/ml)
B/T
proIL-1β
mIL-1β
E
pen/str
IL-1β (ng/ml)
1 5 7 12 24
1 5 7 12 24
Time(h)
NONE
B/T=40
proIL-1β
mIL-1β
F
x 4 dil
x 2 dil
x 1 dil
IL-1β (ng/ml)
NONE
BHI
Sup
proIL-1β
mIL-1β

Fig. 1


A
Relative IL-1β inducing activity (%)
120
100
80
60
40
20
0
*
*
z-VAD-fmk (μM) — 1 10 — — —
z-YVAD-fmk (μM) — — — — 2 20
B/T=40 + + + + + +


B
Relative quantity of caspase-1 mRNA
1.4
1.2
1
0.8
0.6
0.4
0.2
0
***
control siRNA
caspase-1 siRNA

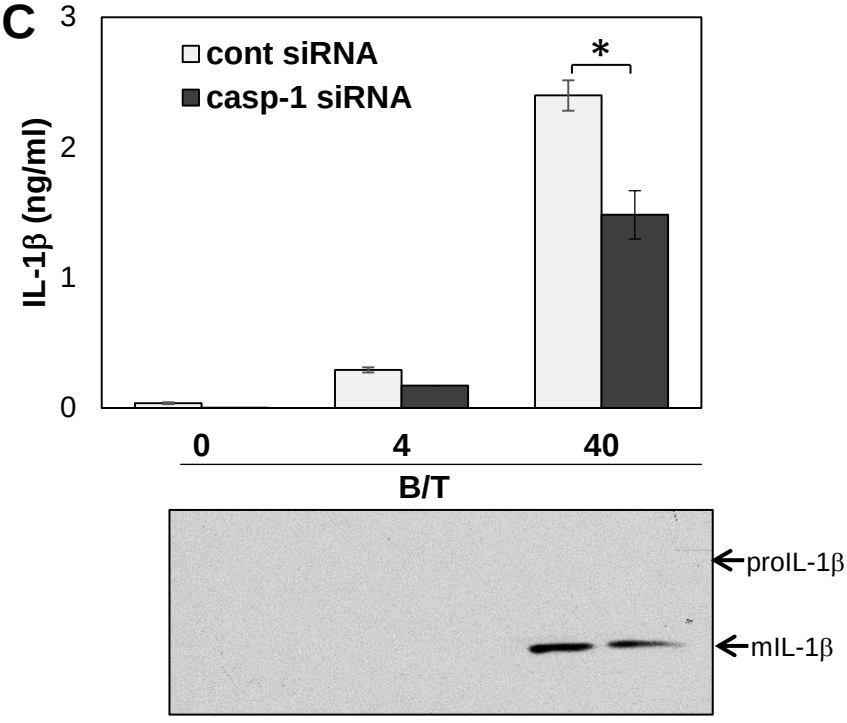

C
IL-1β (ng/ml)
3
2
1
0
cont siRNA
casp-1 siRNA
*
0
4
40
B/T
proIL-1β
mIL-1β

Fig. 2

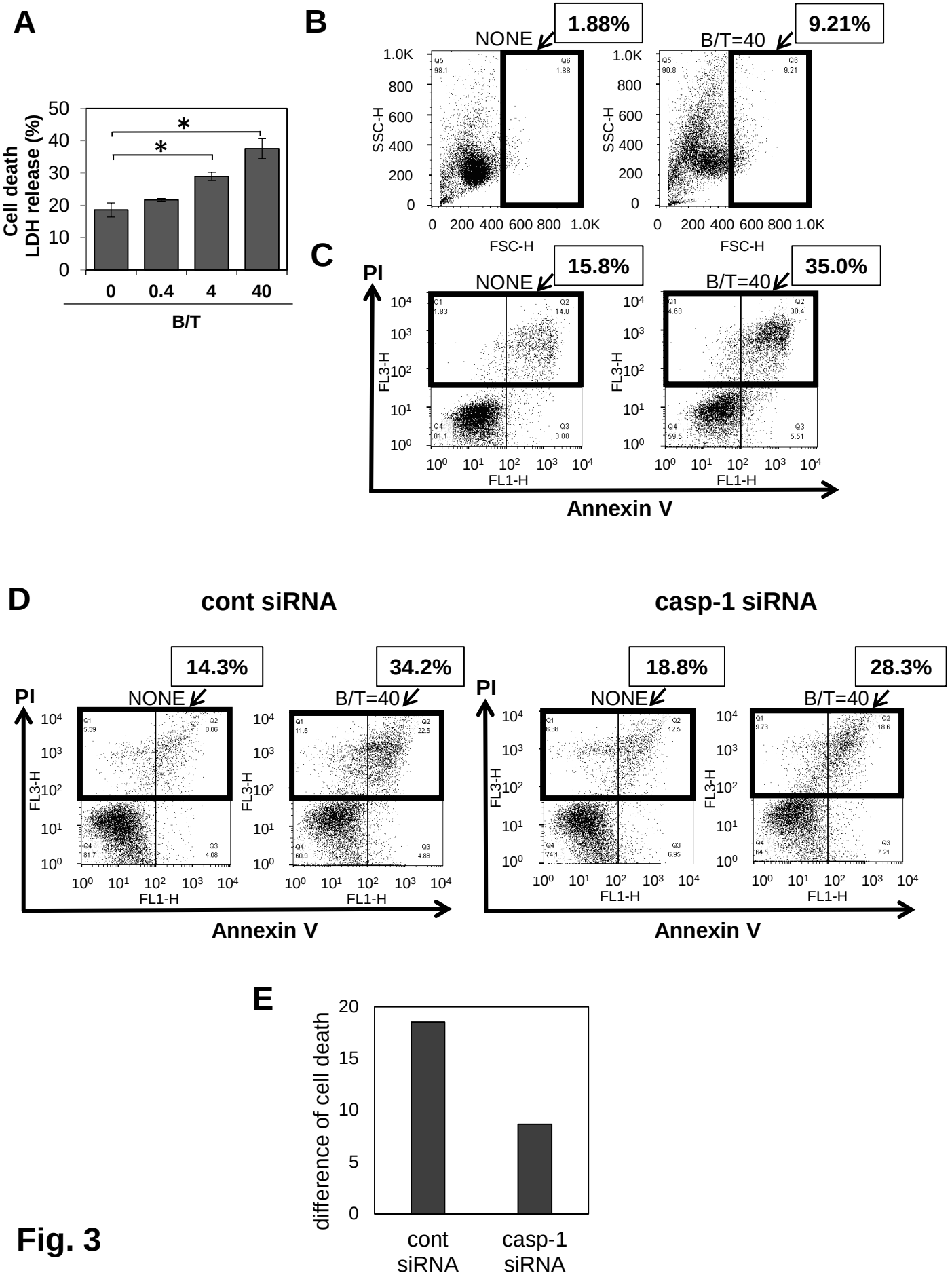

A
Cell death
LDH release (%)
50
40
30
20
10
0
*
*
0
0.4
4
40
B/T
B
NONE
1.88%
B/T=40
9.21%
1.0K
800
600
400
200
0
SSC-H
FSC-H
Q5
98.1
Q6
1.88
Q5
90.8
Q6
9.21
C
PI
NONE
15.8%
B/T=40
35.0%
FL3-H
FL1-H
Annexin V
Q1
1.83
Q2
14.0
Q4
81.1
Q3
3.08
Q1
4.68
Q2
30.4
Q4
59.5
Q3
5.51
D
cont siRNA
casp-1 siRNA
14.3%
34.2%
18.8%
28.3%
NONE
B/T=40
Q1
5.39
Q2
8.86
Q4
81.7
Q3
4.08
Q1
11.6
Q2
22.6
Q4
60.9
Q3
4.88
Q1
6.38
Q2
12.5
Q4
74.1
Q3
6.95
Q1
9.73
Q2
18.6
Q4
64.5
Q3
7.21
E
difference of cell death
20
15
10
5
0
cont
siRNA
casp-1
siRNA

Fig. 3

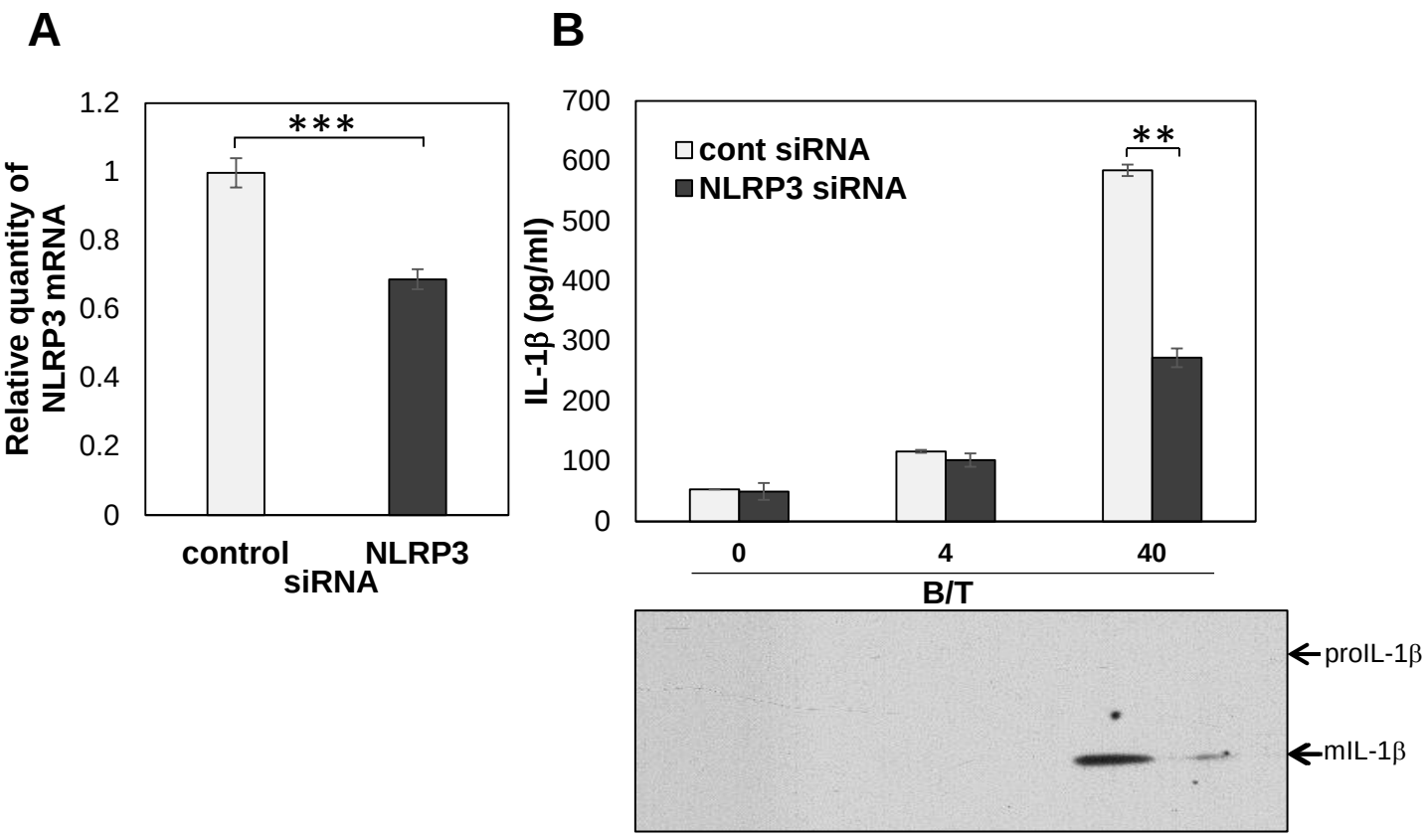
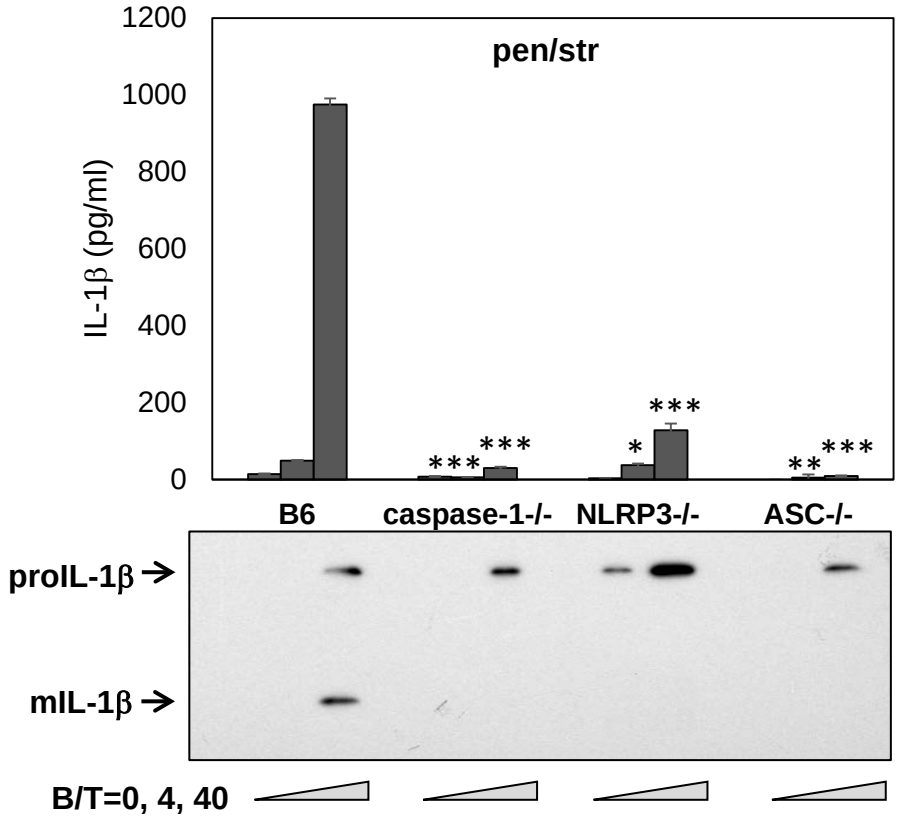

A
B
Relative quantity of NLRP3 mRNA
1.2
1
0.8
0.6
0.4
0.2
0
***
control
NLRP3 siRNA
IL-1β (pg/ml)
700
600
500
400
300
200
100
0
cont siRNA
NLRP3 siRNA
**
0
4
40
B/T
proIL-1β
mIL-1β

Fig. 4

A


pen/str
IL-1β (pg/ml)
1200
1000
800
600
400
200
0
***
***
*
***
**
***
B6
caspase-1-/-
NLRP3-/-
ASC-/-
proIL-1β
mIL-1β
B/T=0, 4, 40

B

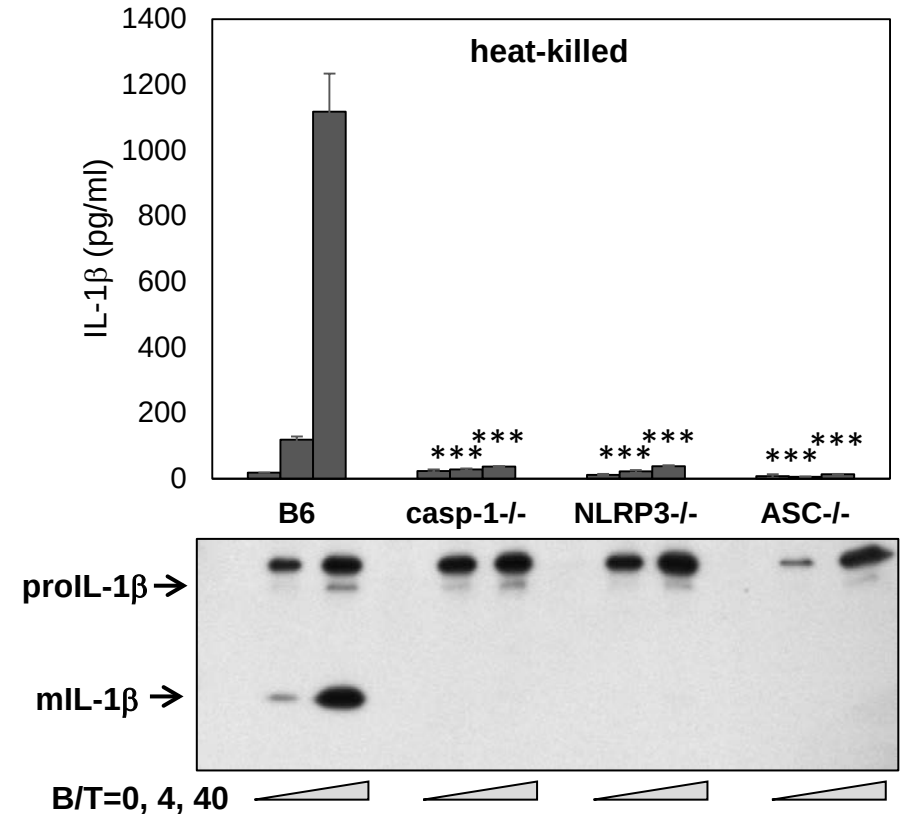

heat-killed
IL-1β (pg/ml)
1400
1200
1000
800
600
400
200
0
***
***
***
***
***
***
B6
casp-1-/-
NLRP3-/-
ASC-/-
proIL-1β
mIL-1β
B/T=0, 4, 40

Fig. 5

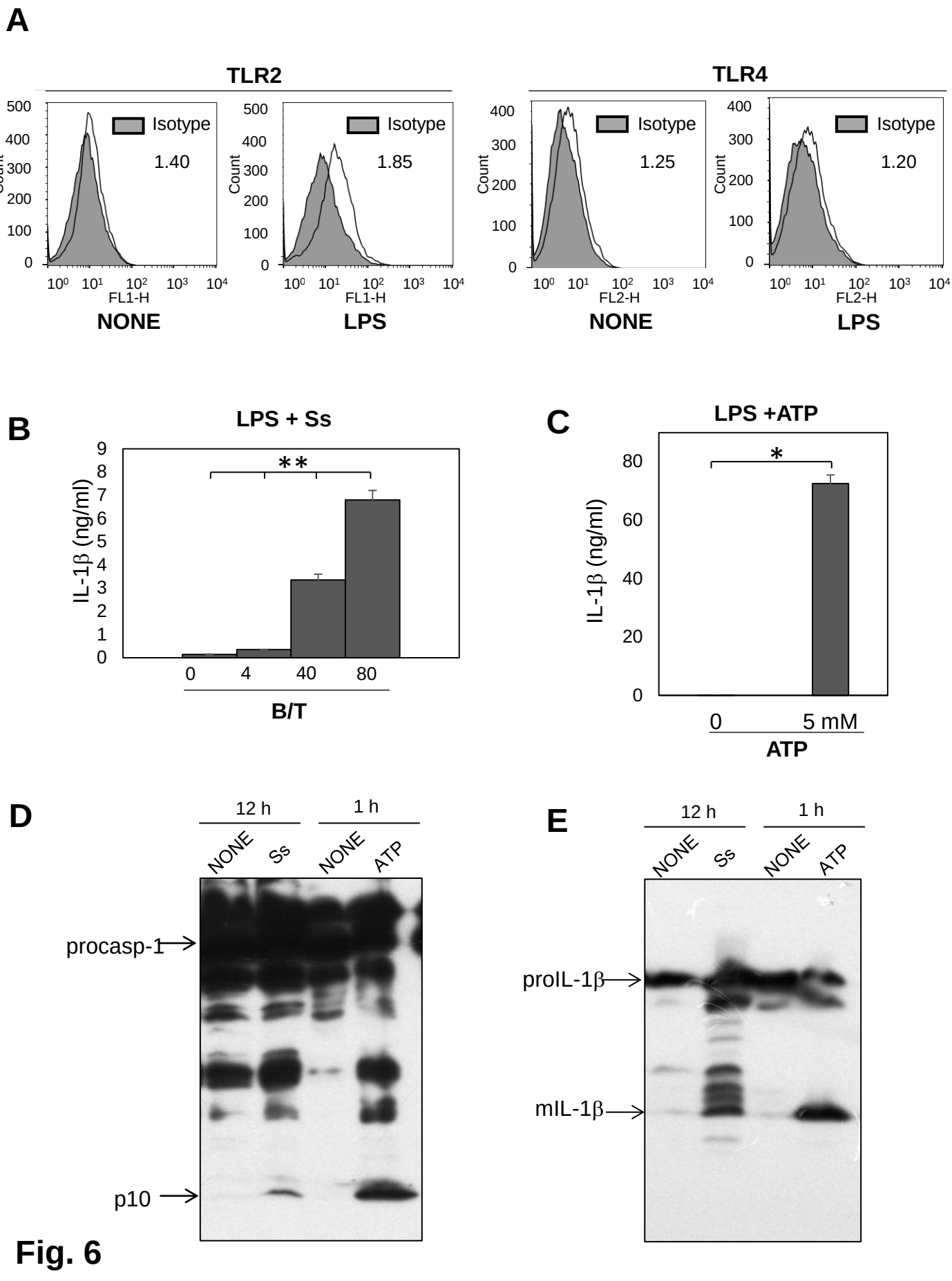

A
TLR2
TLR4
Isotype
1.40
1.85
1.25
1.20
Count
FL1-H
FL2-H
NONE
LPS
B
LPS + Ss
IL-1β (ng/ml)
**
0
4
40
80
B/T
C
LPS +ATP
*
0
5 mM
ATP
D
12 h
1 h
NONE
Ss
ATP
procasp-1
p10
E
proIL-1β
mIL-1β

Fig. 6

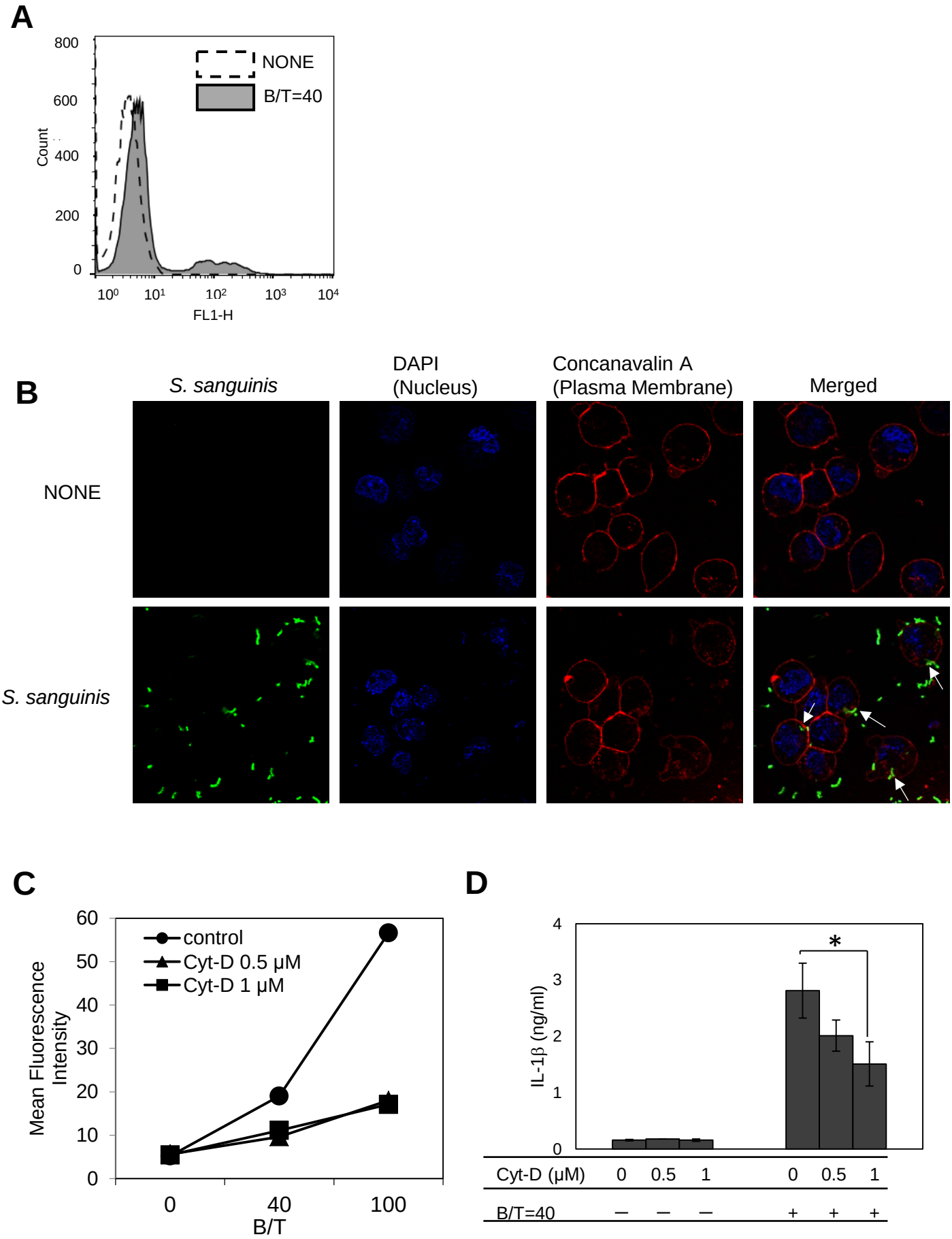

A
800
600
400
200
0
Count
NONE
B/T=40
10^0
10^1
10^2
10^3
10^4
FL1-H
B
S. sanguinis
DAPI
(Nucleus)
Concanavalin A
(Plasma Membrane)
Merged
NONE
S. sanguinis
C
60
50
40
30
20
10
0
Mean Fluorescence Intensity
control
Cyt-D 0.5 μM
Cyt-D 1 μM
0
40
100
B/T
D
4
3
2
1
0
IL-1β (ng/ml)
*
Cyt-D (μM)
0
0.5
1
0
0.5
1
B/T=40
–
–
–
+
+
+

Fig. 7

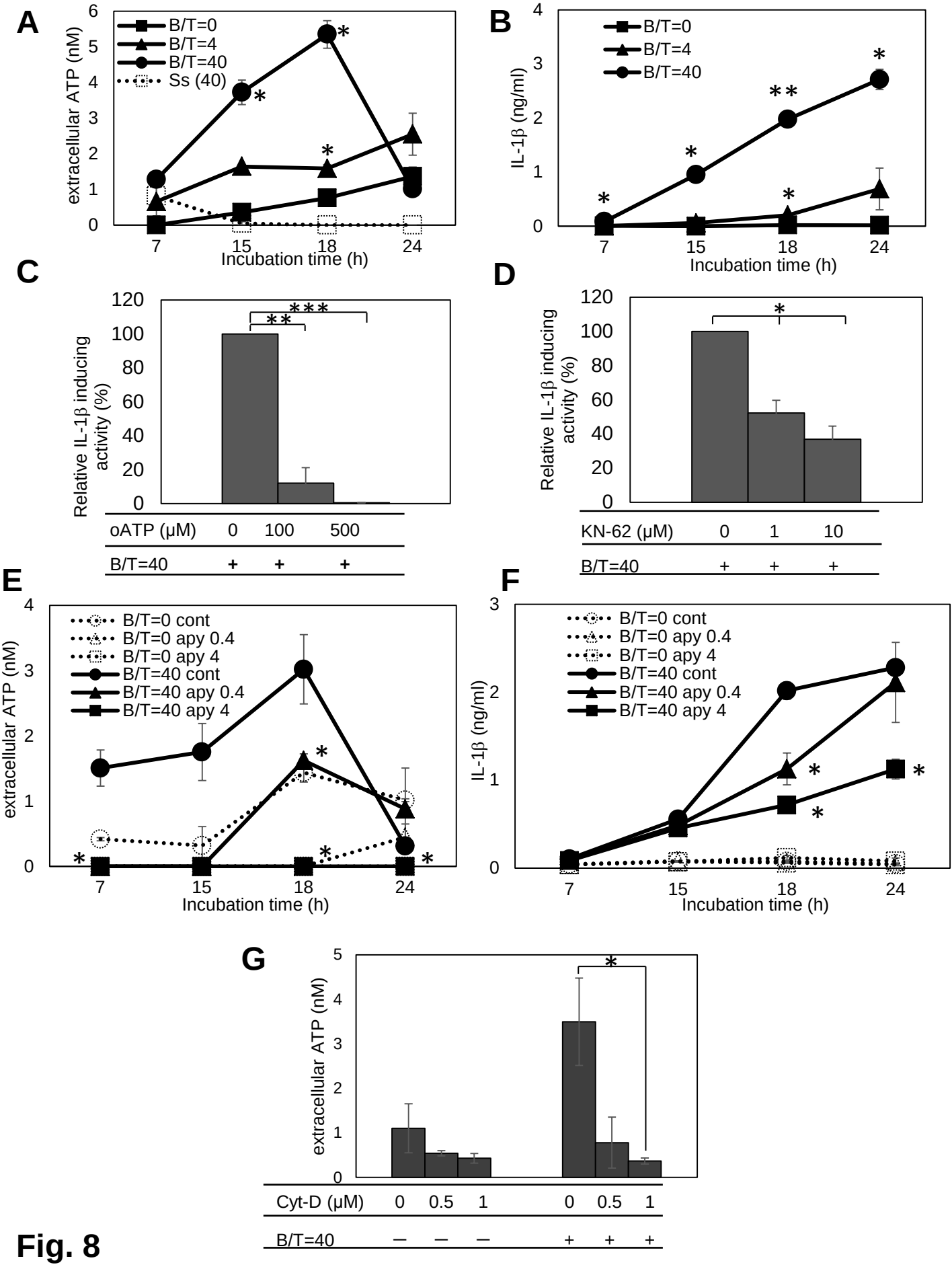
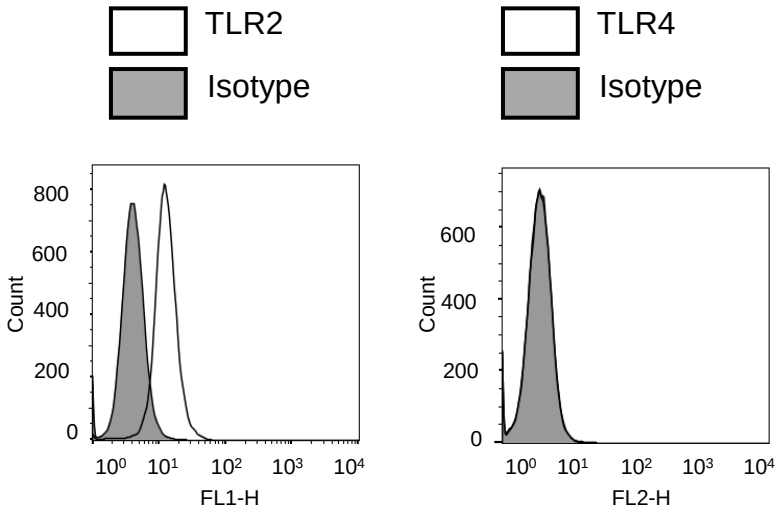

A
extracellular ATP (nM)
B/T=0
B/T=4
B/T=40
Ss (40)
Incubation time (h)
B
IL-1β (ng/ml)
B/T=0
B/T=4
B/T=40
Incubation time (h)
C
Relative IL-1β inducing activity (%)
oATP (μM) 0 100 500
B/T=40 + + +
D
Relative IL-1β inducing activity (%)
KN-62 (μM) 0 1 10
B/T=40 + + +
E
extracellular ATP (nM)
B/T=0 cont
B/T=0 apy 0.4
B/T=0 apy 4
B/T=40 cont
B/T=40 apy 0.4
B/T=40 apy 4
Incubation time (h)
F
IL-1β (ng/ml)
B/T=0 cont
B/T=0 apy 0.4
B/T=0 apy 4
B/T=40 cont
B/T=40 apy 0.4
B/T=40 apy 4
Incubation time (h)
G
extracellular ATP (nM)
Cyt-D (μM) 0 0.5 1 0 0.5 1
B/T=40 — — — + + +

Fig. 8



supplement Fig. 1