NLRP3 inflammasome is responsible for Hantavirus inducing interleukin-1β in THP-1 cells

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Abstract. Persistent high fever is one typical clinical symptom of hemorrhagic fever with renal syndrome (HFRS) and circulating interleukin-1β (IL-1β) is elevated throughout HFRS. The mechanisms responsible for viral induction of IL-1β secretion are unknown. In the present study, Hantaan virus (HTNV) induced the secretion of IL-1β in the human monocytic cell line THP-1. Induction of IL-1β by HTNV relies on the activation of caspase-1. Small hairpin RNA knockdown in HTNV-infected THP-1 cells indicated that nucleotide-binding domain, leucine-rich repeat containing protein 3 (NLRP3) recruits the adaptor apoptosis-associated speck-like protein and caspase-1 to form an NLRP3 inflammasome complex, crucial for the induction of IL-1β. In HTNV-infected THP-1 cells, reactive oxygen species release, but not extracellular adenosine triphosphate, was crucial for IL-1β production. In conclusion, Hantavirus induces the formation of the NLRP3 inflammasome in THP-1 cells and this may be responsible for the elevated IL-1β levels in HFRS patients.

Introduction

Hantaviruses are rodent-borne negative-stranded RNA viruses, belonging to the genus *Hantavirus*, family Bunyaviridae (1,2). Thus far, two severe human diseases caused by *Hantavirus* have been identified, and they are categorized by geographical distribution and target organ as Old World hantaviruses, which cause hemorrhagic fever with renal syndrome (HFRS) in Europe and Asia, and New World hantaviruses, which cause hantavirus pulmonary syndrome (HPS) in America (1-5). These two diseases are characterized by fever, bleeding and shock (1-5). Approximately 10,000 cases of HFRS are reported in mainland China annually, with a mortality rate of 0.1-15% (5-7). The pathogens responsible for HFRS in China are mainly Hantaan virus (HTNV) and Seoul virus (6,8). The clinical presentation of HFRS can be divided into five sequential stages; febrile, hypotensive, oliguric, diuretic and convalescent (9). High fever, thrombocytopenia and capillary leak syndrome are typical symptoms experienced throughout infection (5,9).

Although multiple hypothesizes have been developed to explain the pathogenesis of Hantavirus, the precise mechanism remains undefined (1,10-12). Numerous cytokines and chemokines, such as vascular endothelial growth factor, tumor necrosis factor-α, interleukin-6 (IL-6) and IL-8 are elevated in HFRS patients, and the induction of pro-inflammatory cytokines and chemokines is considered to play a pivotal role in the pathogenesis of Hantavirus (13-15). These cytokines increase the expression of adhesion factors on endothelial cells, enabling transmigration of leukocytes to the sites of infection and re-set the hypothalamus thermoregulatory center, causing fever. IL-1β, which is considered to be an endogenous pyrogen, is elevated in the plasma of HFRS patients (15). Recently, Zhu *et al* (16) demonstrated that IL-1β caused increased vascular permeability by activation of IL receptors and activation of the MYD88-ARNO-ARF6 cascade to disrupt vascular stability. In addition, Hottz *et al* (17) found that IL-1β expression is elevated in the platelets and platelet-derived microparticles of dengue virus-infected patients, and that the dengue virus initiates the assembly of inflammasomes, activation of caspase-1, and caspase-1-dependent IL-1β secretion. It appears that IL-1β can contribute to the pathogenesis of viral infection by inducing excessive hyperpermeability of the vascular system.

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IL-1β induction involves the formation of an inflammasome to generate bioactive caspase-1 by cleavage of pro-IL-1β (18,19). Inflammasomes are initiated by pattern-recognition receptors activated by viral or bacterial components and toxins, or even crystalline structures (18,20,21). Thus far, retinoic acid-inducible (RIG)-I-like receptors, nucleotide-binding oligomerization domain-like receptors (NLRs) and absent in melanoma 2 (AIM2)-like receptors have been found to initiate inflammasome formation, and the majority of RNA viruses are capable of initiating the nucleotide-binding domain, leucine-rich repeat containing protein 3 (NLRP3) inflammasome (22-25). NLRP3, also known as cryopyrin, NALP3 or PYPAF1, contains an N-terminal pyrin domain (PYD), a central nucleotide-binding domain and C-terminal leucine-rich repeats (26). Following activation, NLRP3 oligomerizes to form a large protein complex, and recruits the adaptor apoptosis-associated speck-like protein (ASC). ASC contains a PYD and a C-terminal caspase-recruitment domain (CARD), allowing it to interact with NLRP3 and pro-caspase-1. Pro-caspase-1 contains a pro-domain (CARD domain), a 20-kDa subunit (p20) and a 10-kDa subunit (p10), and is activated by proteolytic cleavage to generate caspase-1, which subsequently cleaves the pro-IL-1β into IL-1β, which is secreted (27,28).

The mechanisms preceding the formation of the inflammasome are not completely understood, but are characterized by three models. First, the formation of pores on the surface of cells allows extracellular NLRP3 agonists to enter the cytosol and directly activate NLRP3 (29). Second, lysosome rupture results in the release of cathepsin B, which activates NLRP3 (30). Third, all NLRP3 agonists trigger the generation of reactive oxygen species (ROS) by the mitochondria, which leads to the activation of the NLRP3 inflammasome (31-35).

The present study examined the level of IL-1β during HTNV infection of the human monocytic cell line, THP-1, and demonstrated that induction of IL-1β by HTNV was dependent on the NLRP3 inflammasome.

Materials and methods

Reagents and antibodies. Adenosine triphosphate (ATP) was obtained from Roche Diagnostics (Basel, Switzerland); MG132, Z-VAD-FMK, pyrrolidine dithiocarbamate (PDTC) and apocynin were purchased from Merck-Calbiochem (Darmstadt, Germany); and lipopolysaccharide (LPS), phorbol 12-myristate-13-acetate (PMA), Boc-D-CMK and H2DCFDA were obtained from Sigma-Aldrich (St. Louis, MO, USA). The ATP assay kit and radioimmunoprecipitation buffer (Beyotime, Shanghai, China) were used for cell lysis. Antibodies directed towards human IL-1β (#2021) were purchased from Cell Signaling Technology (Danvers, MA, USA), caspase-1 (#PAB0811) was obtained from Abnova (Taipei, Taiwan) [an additional cleaved caspase-1 antibody was obtained from Santa Cruz Biotechnology, Santa Cruz, CA, USA (sc-22163)], and actin from Beyotime. Monoclonal antibody (mAb) 1A8 [specific to the HTNV nucleocapdis protein (NP)] was prepared in our laboratory as previously described (8,36).

Cells and viruses. THP-1 [TIB-202D; American Type Culture Collection (ATCC), Manassas, VA, USA] were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA) (culture medium), 100 μM non-essential amino acids, 100 U/ml penicillin and 100 μg/ml streptomycin, and were incubated at 37˚C with 5% CO_2. THP-1 cells were differentiated with 1 μM PMA for 12 h, and subsequently incubated with heat-inactivated HTNV, HTNV at indicated multiplicity of infection (MOI) or mock (medium only) for 1 h before the supernatant was removed and replaced with culture medium. PMA-THP-1 were cultured for 6 h to 3 days before use.

HTNV 76-118 was propagated in the sucking mice brain. All the animal experiments were approved by the Fourth Military Medical University Medical Ethics Committee (Xi'an, China) (approval no. XJYYLL-2012508) (37). Viral titer was determined by $TCID_{50}$ on Vero E6 cells (Vero C1008; ATCC CRL-1586), and was converted to plague-forming units using the Reed-Muench method. The virus was heat inactivated at 56˚C for 30 min. Inactivation was confirmed when infection of Vero E6 cells for 3 days yielded no detectable viral nucleoprotein.

Immunofluorescence assay. For the detection of immunofluorescence, human umbilical vein endothelial cells (HUVEC) and Vero E6 cells were directly seeded in coverslips in a 24-well plate, and when adherent and 70% confluent were infected with HTNV (MOI=1) for 90 min. The THP-1 cells were differentiated with PMA for 12 h in a 24-well plate supplement with coverslips. Seventy-two hours post-infection, cells were washed three times with Dulbecco's phosphate-buffered saline (DPBS; HyClone) and fixed with 4% paraformaldehyde for 15 min at room temperature. Cells were subsequently permeabilized with 0.5% Triton X-100 (Sigma-Aldrich) for 10 min and washed again with DPBS. HTNV NP present in cytosol was stained by mAb fluorescein isothiocyanate-1A8 specific for HTNV nucleoprotein and Hoechst 33258 (100 ng/ml; Beyotime) was used to stain the cell nuclei. The cells were observed using a BX60 fluorescence microscope (Olympus, Tokyo, Japan).

Quantitative polymerase chain reaction (qPCR). The specific oligonucleotide primers were: Human *GAPDH* forward, 5'-ACC CAC TCC TCC ACC TTT G-3'; and reverse, 5'-ATC TTG TGC TCT TGC TGG G-3'; *NLRP3* forward, 5'-CTT CCT TTC CAG TTT GCT GC-3'; and reverse, 5'-TCT CGC AGT CCA CTT CCT TT-3'; human *IL-1β* forward, 5'-CAG CCA ATC TTC ATT GCT CA-3'; and reverse, 5'-TCG GAG ATT CGT AGC TGG AT-3'; caspase-1 forward, 5'-GGA CTC TCA GCA GCT CCT CAG GCA-3'; and reverse, 5'-GCA AAG CTT GAC ATT CCC TTC TGA GCC-3'; and *ASC* forward, 5'-CCT ACG GCG CCG AGC TCA C-3'; and reverse, 5'-CTC CAG AGC CCT GGT GCG T-3'; which were synthesized at Sangon Biotech (Shanghai, China). For qPCR, total RNA was isolated using RNAiso and converted to cDNA immediately using the PrimeScript™ RT Master mix (Perfect Real-Time) (Takara, Dalian, China) following the manufacturer's instructions and stored at -20˚C until use. Each cDNA was amplified with the previously listed primers and SYBR Premix Ex Taq™ II (Tli RNaseH Plus) (Takara) for 40 cycles, and results were analyzed using the Mx3005P System Software (ABI Stratagene, La Jolla, CA, USA).

Caspase-1 activity assays. The activity of caspase-1 was determined based on the ability of caspase-1 to change

acetyl-Tyr-Val-Ala-Asp p-nitroanilide (Ac-YVAD-pNA) into the yellow formazan product p-nitroaniline (pNA) using a Caspase-1 Activity kit (Beyotime). Cell lysates were centrifuged at 13,000 x g for 10 min, and the protein concentrations were determined by the Bradford protein assay. Cellular extracts (30 μg of protein) were incubated in a 96-well microtiter plate with 20 ng of Ac-DEVD-pNA overnight at 37˚C. The absorbance values of pNA at 405 nm, optical density $(OD)_{405}$, were measured using a 96-well plate reader (BioTek, Santa Barbara, CA, USA). An increase in the OD_{405} indicated activation of caspase-1.

RNA interference. Small hairpin (sh) RNA lentivirus against human *NLRP3* (target, 5'-GGA GAG ACC TTT ATG AGA AAG-3'), human *ASC* (target, 5'-GCA AGA TGC GGA AGC TCT TCA-3'), human caspase-1 (target, 5'-GCA CAC GTC TTG CTC TCA TTA-3') and scrambled sequence (5'-GTT CTC CGA ACG TGT CAC GT-3') were synthesized at GenePharma (Shanghai, China). Undifferentiated THP-1 cells were infected with shRNA lentivirus (MOI=100) and selected over three passages in the presence of puromycin (Sangon, Shanghai, China). Specifically, THP-1 cells stably expressing indicated shRNAs were selected with 1 μg/ml puromycin for 2 weeks, followed by 0.5 μg/ml puromycin for an additional 2 weeks, with the medium changed every 3 days.

Enzyme-linked immunosorbent assay (ELISA) and immunoblot. The supernatant and cell lysate of PMA-differentiated THP-1 cells were collected at the given time points between 6 h and 3 days post-infection, as indicated. The concentration of the IL-1β level in the cell culture supernatant was determined by ELISA according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). Samples were tested in triplicate and data were analyzed using GraphPad software version 5.0 (GraphPad Software, San Diego, CA, USA). The presence of pro-caspase-1 and pro-IL-1β in cell lysate was measured by immunoblot as described previously (38), and the bio-active form of IL-1β and caspase-1 in the supernatant were detected with antibodies that target IL-1β and caspase-1.

ATP assay. THP-1 culture supernatant was collected at the indicated time points post-infection. The concentration of ATP was determined immediately using the ATP assay kit (Beyotime) according to the manufacturer's instructions.

ROS release assay. The assay is based on the incorporation of 2',7'-dichlorofluorescein diacetate into the cell. THP-1 cells were infected with HTNV for 1 day, or were treated with 100 μM H_2O_2 as a positive control or with cold DPBS as a negative control for 10 min, stained with 10 μM H2DCFDA (Sigma-Aldrich), and subsequently ROS release was evaluated by Cytomics FC 500 (Beckman-Coulter, Fullerton, CA, USA).

Statistical analyses. All the data are expressed as mean ± standard deviation. The statistical significance of the obtained data was analyzed using a two-tailed unpaired t-test in GraphPad Prism 5. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Induction and secretion of IL-1β by HTNV in THP-1 cells. The human monocytic cell line, THP-1, is widely used in inflammasome research (19), and it has previously been established that Hantavirus can infect THP-1 cells (39,40). Vero E6, HUVEC and PMA-differentiated THP-1 were infected with HTNV at an MOI of 1, and intracellular nucleoprotein was detected at 3 days post-infection by immunofluorescent staining. The fraction of THP-1 cells infected with HTNV was lower than the fraction of Vero E6 and HUVEC cells (Fig. 1A), consistent with previously published data (40). As the level of secreted IL-1β indicates activation of inflammasome, ELISA was used to assess the concentration of IL-1β in cell supernatants. Significantly high levels of IL-1β (95.6±27.0 pg/ml) secretion from PMA-differentiated THP-1 were detected 24 h after HTNV infection compared to the control group (16.2±5.0 pg/ml) ($P<0.05$) (Fig. 1B). The concentration of IL-1β corresponded with the MOI of HTNV (Fig. 1B), and the level of IL-1β in the supernatant peaked at 24 h post-infection (Fig. 1D). PMA-differentiated THP-1 cells incubated with heat-inactivated HTNV also secreted increased levels of IL-1β (33.2±9.0 pg/ml), however, this was less than that of the PMA-differentiated THP-1 cells incubated with infectious HTNV and there was no statistical significance compared to the mock group (Fig. 1B).

Secretion of IL-1β requires two independent processes. First is the expression of pro-IL-1β and, second is the inflammasome cleavage of pro-IL-1β into IL-1β (19). LPS is routinely employed *in vitro* to initiate the expression of pro-IL-1β (41). To examine whether LPS-enhanced pro-IL-1β expression further increased HTNV-induced IL-1β secretion, PMA-differentiated THP-1 cells were infected in the presence or absence of LPS. The addition of LPS increased the level of IL-1β secreted by HTNV-infected PMA-differentiated THP-1 cells 24 h post-infection, as illustrated in Fig. 1C; 87.3±14.8 pg/ml for MOI=0.1 and 142.8±19.7 pg/ml for MOI=2 compared to 47.8±1.6 pg/ml for MOI=0.1 and 119.7±33.8 pg/ml for MOI=2. There was no statistical significance between these two groups, indicating that HTNV was likely inducing pro-IL-1β expression independently.

HTNV activates caspase-1 and pro-IL-1β in THP-1 cells. As HTNV induced IL-1β secretion in the absence of LPS, it is likely that HTNV induced pro-IL-1β expression in THP-1 cells. To evaluate this hypothesis, the total protein from HTNV-infected THP-1 cells was isolated and the level of pro-IL-1β and pro-caspase-1 protein was assessed by immunoblot. Increased levels of pro-IL-1β and pro-caspase-1 were detected in cells incubated with either HTNV or LPS and ATP (Fig. 2A), which is consistent with our previous results (Fig. 1C). Bioactive caspase-1 is required to cleave pro-IL-1β into IL-1β. In order to investigate whether caspase-1 was activated during HTNV infection, the culture supernatant of HTNV-infected THP-1 was ultra-filtered and an increased concentration of secreted caspase-1 was detected post-infection (Fig. 2B). In addition, the activity of caspase-1 was detected in THP-1 cell lysates, and the activation of caspase-1 was significantly increased 12 h post-infection (1.137±0.064) compared to the mock group (0.15±0.026) ($P<0.0001$) (Fig. 2C). These results indicate

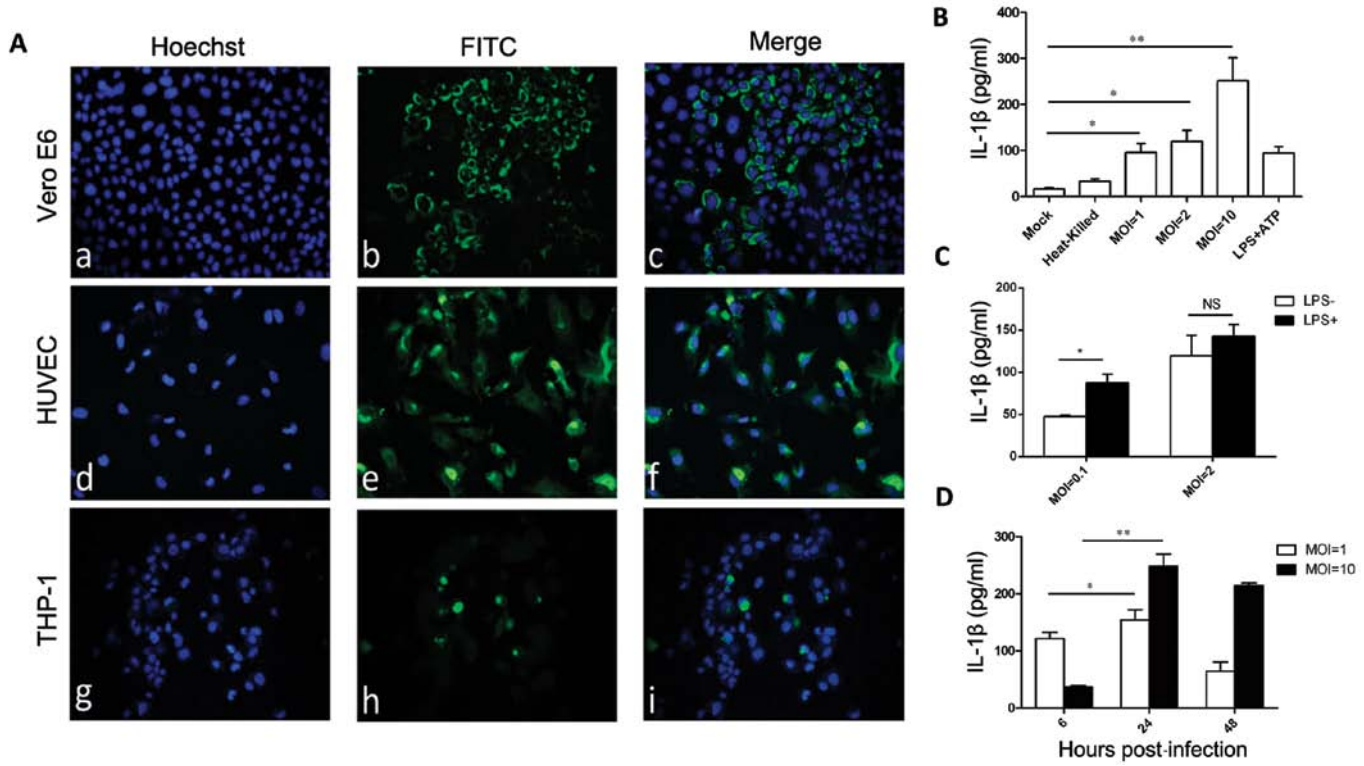

Figure 1. Hantaan virus (HTNV) induces the production of interleukin-1β (IL-1β) in THP-1 cells. (A) Immunofluorescent staining of HTNV nucleoprotein in infected Vero E6, human umbilical vein endothelial cells (HUVEC) and phorbol 12-myristate-13-acetate (PMA) differentiated-THP-1. (A-C) Vero E6, (D-F) HUVEC and (G-I) THP-1 were infected with HTNV and were fixed 72 h post-infection. (A, D and G) Nuclei were stained with Hoechst; and (B, E and H) HTNV nucleoprotein was detected with fluorescein isothiocyanate (FITC)-1A8. Data are representative of three independent experiments. (B) Enzyme-linked immunosorbent assay (ELISA) detection of IL-1β in the supernatant of HTNV-infected THP-1. PMA-differentiated THP-1 cells were incubated with infectious HTNV at a multiplicity of infection (MOI) of 1, 2 or 10, or heat-inactivated HTNV, lipopolysaccharide (LPS) + adenosine triphosphate (ATP) at 24 h post-infection. (C) ELISA detection of IL-1β in the supernatant of HTNV-infected THP-1. PMA-differentiated THP-1 cells were incubated with HTNV at an MOI of 2 or 0.1 in the presence or absence of PMA, and 24 h post-infection the IL-1β level in the supernatant was measured by ELISA. (D) ELISA detection of IL-1β in the supernatant of HTNV-infected THP-1. PMA-differentiated THP-1 cells were incubated with HTNV at an MOI of 1 or 10, and the level of IL-1β in the supernatant was measured by ELISA at the indicated times post-infection. (B-D) Data are representative of three independent experiments each performed in triplicate (errors bars represent standard error of the mean). *P<0.05, **P<0.01; NS, not significant.

that HTNV-infected THP-1 generated the bioactive form of caspase-1.

Incubation with the potent proteasome inhibitor MG132 significantly reduced the concentration of IL-1β in the culture supernatant of HTNV-infected cells, 88.1±4.8 pg/ml compared to 21.6±1.6 pg/ml (P=0.047) (Fig. 2D), indicating that the proteasome may participate in the process of HTNV IL-1β induction. To further investigate the role of caspase-1 in HTNV-induced IL-1β secretion, cells were incubated with the specific inhibitors of caspase-1, Z-VAD-FMK and Boc-D-CMK, in advance of HTNV infection. Z-VAD-FMK and Boc-D-CMK significantly reduced the level of IL-1β secretion (P<0.05) (Fig. 2E), indicating that caspase-1 participated in HTNV-induced IL-1β secretion.

mRNA level of ASC and caspase-1 are elevated in the HTNV-infected THP-1. To address the mechanism by which IL-1β secretion was triggered, the total cellular RNA was extracted from HTNV-infected THP-1 cells. Using qPCR, the mRNA levels of *ASC* and caspase-1, key molecules forming the inflammasome, were measured (Fig. 3). The level of caspase-1 mRNA was increased by ~6-fold in HTNV-infected cells 24 h post-infection (Fig. 3B). The level of *ASC* mRNA was increased by ~5-fold 6 h post-infection, and reached an increase of 10-fold 24 h post-infection (Fig. 3A). Thus, these results indicate that HTNV-infected THP-1 induce expression of *ASC* and caspase-1, which participate in the induction of IL-1β secretion.

Effect of NLRP3, ASC and caspase-1 shRNA on IL-1β secretion. As the NLRP3 inflammasome is activated by numerous viruses (17,19,24,25,26,42-52), we suspect that NLRP3 may participate in HTNV induction of IL-1β. To evaluate the role of NLRP3 in the induction of IL-1β following HTNV infection, an shRNA lentivirus was used to knockdown the expression of *NLRP3*. In comparison to the non-targeting control shRNA (NC), the cells in which *NLRP3* was knocked down secreted less IL-1β (Fig. 4). As a control, the level of IL-1β induced by knockdown of *ASC* and caspase-1 was also compared to further examine the role of *NLRP3* in HTNV infection and this determined that, as expected, the cells in which *ASC* and caspase-1 were knocked down also generated less IL-1β (Fig. 4). These results indicate that the assembly of the NLRP3 inflammasome complex is responsible for the increased secretion of IL-1β in HTNV-infected THP-1 cells.

HTNV activation of NLRP3 correlates with ROS release but not the extracellular ATP level. ATP is regarded as a damage signal that can induce the formation of inflammasome (19,53).

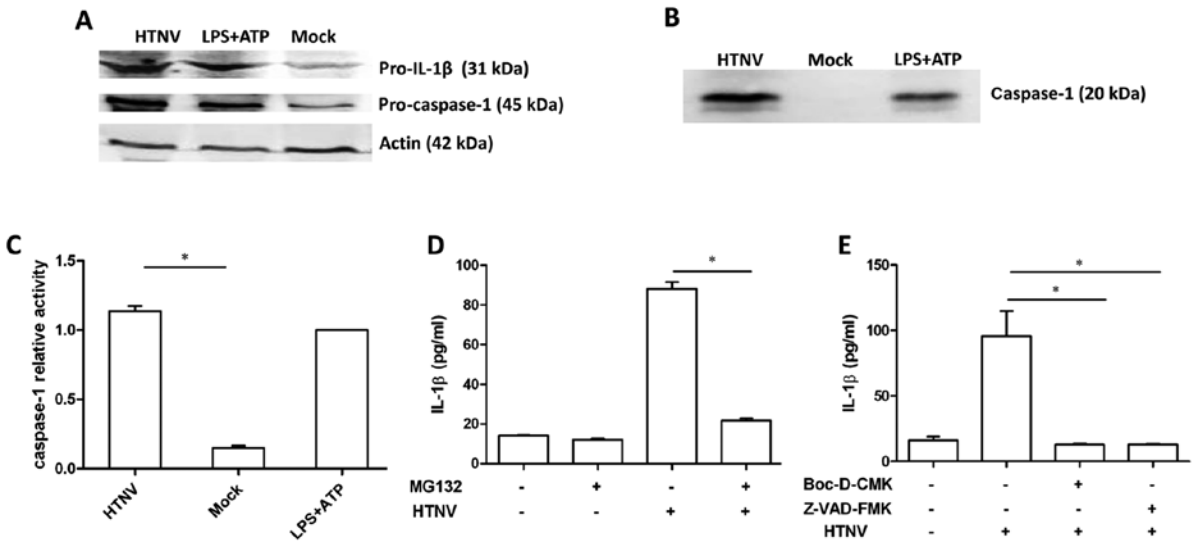

Figure 2. Hantaan virus (HTNV) activates pro-interleukin-1β (IL-1β) and caspase-1 expression and activation. (A) Immunoblot detection of pro-IL-1β and pro-caspase-1 in THP-1 cell lysates following HTNV infection [multiplicity of infection (MOI)=1]. (B) Immunoblot detection of bioactive caspase-1 in the supernatant following HTNV infection (MOI=1) of THP-1. (C) The activity of caspase-1 was determined following HTNV infection of THP-1. Cells were incubated with lipopolysaccharide (LPS) and adenosine triphosphate (ATP), HTNV (MOI=1) or mock-infected with culture media only, and 24 h post-infection, cell lysates were centrifuged and incubated with Ac-DEVD-pNA. The OD_{405} values of pNA were measured and represent the activation of caspase-1. (D) Enzyme-linked immunosorbent assay (ELISA) detection of IL-1β in the supernatant of HTNV-infected THP-1 cells. Cells were pretreated in the presence or absence of 10 μM MG132, and subsequently HTNV at an MOI of 1. Twenty-four hours post-infection, the IL-1β level in the supernatant was measured by ELISA. (E) ELISA detection of IL-1β in the supernatant of HTNV-infected THP-1 cells. Cells were pretreated in the presence or absence of 50 μM Boc-D-CMK and 0.05 μM Z-VAD-CMK, and subsequently HTNV at an MOI of 1. Twenty-four hours post-infection the IL-1β level in the supernatant was measured by ELISA. Data are representative of three independent experiments each performed in triplicate (errors bars represent standard error of the mean). *P<0.05.

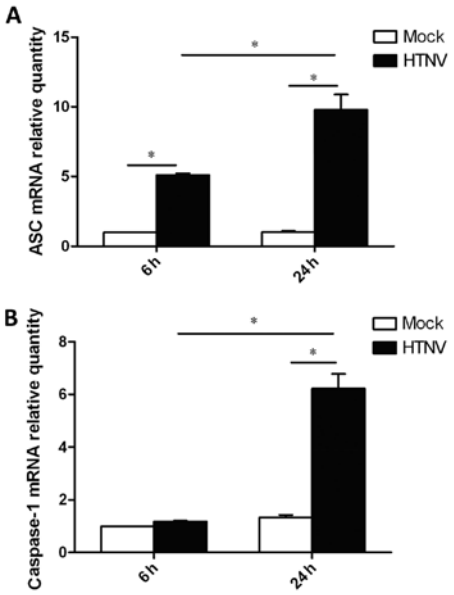

Figure 3. Hantaan virus (HTNV) induces the increase of mRNA level of *ASC* and caspase-1 in THP-1 cells. Cells were exposed to HTNV at a multiplicity of infection (MOI) of 10 at the indicated times post-infection and analyzed by quantitative polymerase chain reaction. Results are expressed as fold induction of transcripts in HTNV-infected THP-1 relative to those in mock-infected cells. Data are representative of three independent experiments each performed in triplicate (errors bars represent standard error of the mean). *P<0.05.

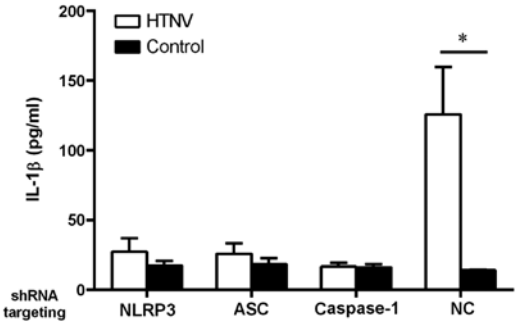

Figure 4. Knockdown of *NLRP3*, *ASC* and caspase-1 reduces the level of interleukin-1β (IL-1β) induced by Hantaan virus (HTNV) in THP-1 cells. Cells were infected with small hairpin RNA-Lentivirus targeting different genes associated with inflammasome, and were subsequently incubated with HTNV at a multiplicity of infection (MOI) of 1. Twenty-four hours post-infection, the IL-1β level in the supernatant was measured by enzyme-linked immunosorbent assay (ELISA). Data are representative of three independent experiments each performed in triplicate (errors bars represent standard error of the mean) *P<0.05. NC, non-specific control.

We speculated that extracellular ATP may be involved in HTNV-induced IL-1β secretion. The supernatant from HTNV-infected THP-1 cells was collected at different time points, but no change was found in the level of ATP correlating with HTNV infection (data not shown). However, increased levels of ROS were detected in HTNV-infected THP-1 cells (Fig. 5A). To further investigate the role of ROS in inflammasome formation during HTNV infection, THP-1 cells were treated with the ROS inhibitors apocynin and ammonium PDTC, prior to HTNV infection. The level of IL-1β in the supernatant of HTNV-infected cells was decreased by ROS inhibitors (Fig. 5B); 153.9±36.7 pg/ml compared to 13.5±2.5 pg/ml for apocynin (P<0.001) and 29.0±2.8 pg/ml for PDTC (P<0.001). Thus, during HTNV infection, ROS induced

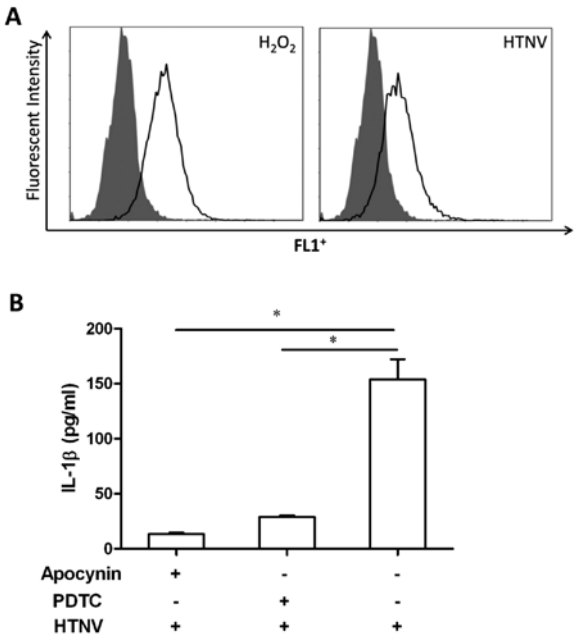

Figure 5. Level of reactive oxygen species (ROS) is unregulated in HTNV-infected THP-1 cells. (A) Flow cytometry analysis of intracellular ROS. THP-1 cells were infected with HTNV [multiplicity of infection (MOI)=1] for 30 h, subsequently stained with H2DCFDA, and fluorescence was detected by flow cytometry. As a positive control, cells were treated with 0.1 μM H_2O_2 for 30 min. Results are presented relative to the fluorescence-activated cell sorting mean fluorescence intensity in the control cells. (B) Enzyme-linked immunosorbent assay (ELISA) detection of the level of interleukin-1β (IL-1β) in HTNV-infected THP-1 supernatants. Cells were pretreated in the presence or absence of 100 μM pyrrolidine dithiocarbamate (PDTC) and 100 μM apocynin, and subsequently treated with HTNV at an MOI of 1. Twenty-four hours post-infection the level of IL-1β in supernatant was measured by ELISA. Data are representative of three independent experiments each performed in triplicate (errors bars represent standard error of the mean). $^*P<0.05$.

by HTNV contributed to NLRP3 inflammasome formation in THP-1 cells.

Discussion

Secreted cytokines produce an inflammatory microenvironment aiding the elimination of pathogens; however, excessive cytokine secretion can cause damage to host tissues and produce a 'cytokine storm', which is speculated to be the main cause of mortality resulting from SARS-CoV and pandemic influenza A infection (54-57). Induction of pro-inflammatory molecules may play an important role in the pathogenesis of HFRS (13-15); however, little is known regarding the mechanism by which hantavirus elicits an inflammatory processes. As an important cytokine in the inflammation and angiogenesis, IL-1β is critical for induction of fever and an inflammatory microenvironment inhibiting pathogen invasion (16,58,59). However, IL-1β is also considered as a detrimental factor in certain cases, inducing hyperpermeability of the endothelium. IL-1β has been found to induce endothelial hyperpermeability during dengue hemorrhagic fever (17,24,25). *In vivo* and *in vitro* studies have reported that the level of circulating IL-1β was elevated in HFRS patients (15) and was induced in hantavirus-infected cells (39,40). The present study reports that secretion of IL-1β from HTNV-infected THP-1 cells requires the assembly of the NLRP3 inflammasome and is ROS-signal dependent.

Not only pathogens, but also aluminum, asbestos, silica crystals and monosodium urate crystals were identified as 'danger' signals inducing formation of the inflammasome (30,60). The present study identified that HTNV efficiently induces the expression of pro-IL-1β in THP-1 cells, and the secretion of mature IL-1β. Previously, TLR3 and RIG-I have been shown to sense hantavirus and induce expression of type I interferon (61,62), and the NLRP3 inflammasome can be induced by viral RNA via activation of TLR3 and RIG-I (51,63). HTNV induced less IL-1β in the presence of the ROS and NF-κB inhibitor PDTC in the present study. PDTC likely inhibited the translation of pro-IL-1β and inflammasome formation. A live virus induces more mature IL-1β secretion compared to a heat-inactivated virus, suggesting that HTNV-induction of IL-1β secretion requires cytosolic viral replication.

The outcome of the inflammasome signal is generation of bioactive caspase-1 and subsequent cleavage of pro-IL-1β (18,20). The mRNA level of *ASC* and caspase-1 were increased 24 h post-infection in the present study, indicating that HTNV may alter the transcriptional level inflammasome components, as suggested by previous findings (46,64). A significant increase was also identified in the level of ASC and caspase proteins in HTNV-infected THP-1, and increased caspase-1 activity. By knocking down the level of caspase-1 in THP-1, the secretion of IL-1β was reduced, consistent with the established mechanism by which RNA viruses activate inflammasome in primary macrophages or THP-1 cells (18,46,65-68).

As opposed to the AIM2 and IFI16 inflammasomes, which can sense DNA (69-72), the molecular partners of NLRP3 remain unknown (19,42). Previous studies have indicated that certain pathogens can directly activate the inflammasome. For example, the M2 ion channel protein of influenza A has been reported to activate the NLRP3 inflammasome (23). Dengue virus interacts with CLEC5A to form the NLRP3 inflammasome (25). The component of the hantavirus HTNV that is responsible for induction of the NLRP3 inflammasome requires further investigation, but the present results support the role of ROS in the induction of the NLRP3 inflammasome complex.

In conclusion, the present study indicates that the NLRP3 inflammasome complex formation and the subsequent induction of bioactive caspase-1 and the cleavage of pro-IL-1β are responsible for HTNV-induced IL-1β secretion in THP-1 cells. These results provide an important insight into the role of the NLRP3 inflammasome in the pathogenesis of HFRS, and may highlight potential strategies for the treatment of HFRS.

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References

1. Vaheri A, Strandin T, Hepojoki J, *et al*: Uncovering the mysteries of hantavirus infections. Nat Rev Microbiol 11: 539-550, 2013.
2. Mir MA: Hantaviruses. Clin Lab Med 30: 67-91, 2010.
3. Mustonen J, Mäkelä S, Outinen T, *et al*: The pathogenesis of nephropathia epidemica: new knowledge and unanswered questions. Antiviral Res 100: 589-604, 2013.
4. Macneil A, Nichol ST and Spiropoulou CF: Hantavirus pulmonary syndrome. Virus Res 162: 138-147, 2011.
5. Jonsson CB, Figueiredo LT and Vapalahti O: A global perspective on hantavirus ecology, epidemiology, and disease. Clin Microbiol Rev 23: 412-441, 2010.
6. Watson DC, Sargianou M, Papa A, Chra P, Starakis I and Panos G: Epidemiology of Hantavirus infections in humans: a comprehensive, global overview. Crit Rev Microbiol 40: 261-272, 2014.
7. Yi J, Xu Z, Zhuang R, *et al*: Hantaan virus RNA load in patients having hemorrhagic fever with renal syndrome: correlation with disease severity. J Infect Dis 207: 1457-1461, 2013.
8. Yu L, Bai W, Wu X, *et al*: A recombinant pseudotyped lentivirus expressing the envelope glycoprotein of Hantaan virus induced protective immunity in mice. Virol J 10: 301, 2013.
9. Linderholm M and Elgh F: Clinical characteristics of hantavirus infections on the Eurasian continent. Curr Top Microbiol Immunol 256: 135-151, 2001.
10. Gupta S, Braun M, Tischler ND, *et al*: Hantavirus-infection confers resistance to cytotoxic lymphocyte-mediated apoptosis. PLoS Pathog 9: e1003272, 2013.
11. Krautkrämer E, Grouls S, Stein N, Reiser J and Zeier M: Pathogenic old world hantaviruses infect renal glomerular and tubular cells and induce disassembling of cell-to-cell contacts. J Virol 85: 9811-9823, 2011.
12. Rang A: Modulation of innate immune responses by hantaviruses. Crit Rev Immunol 30: 515-527, 2010.
13. Li M, Ji Y, Dong Y, Zhou Y, Ren H and Xie M: The detection of vascular endothelial growth factor in serum of patients with hemorrhagic fever with renal syndrome. Inflammation 36: 962-967, 2013.
14. Saksida A, Wraber B and Avšič-Županc T: Serum levels of inflammatory and regulatory cytokines in patients with hemorrhagic fever with renal syndrome. BMC Infect Dis 11: 142, 2011.
15. Wang L, Li XL, Dai Y, Qiu ZF and Li TS: Change of plasma pro-inflammatory cytokines levels in patients with hemorrhagic fever with renal syndrome. Zhongguo Yi Xue Ke Xue Yuan Xue Bao 30: 607-609, 2008 (In Chinese).
16. Zhu W, London NR, Gibson CC, *et al*: Interleukin receptor activates a MYD88-ARNO-ARF6 cascade to disrupt vascular stability. Nature 492: 252-255, 2012.
17. Hottz ED, Lopes JF, Freitas C, *et al*: Platelets mediate increased endothelium permeability in dengue through NLRP3-inflammasome activation. Blood 122: 3405-3414, 2013.
18. Gram AM, Frenkel J and Ressing ME: Inflammasomes and viruses: cellular defence versus viral offence. J Gen Virol 93: 2063-2075, 2012.
19. Bauernfeind F, Ablasser A, Bartok E, *et al*: Inflammasomes: current understanding and open questions. Cell Mol Life Sci 68: 765-783, 2011.
20. Rathinam VA, Vanaja SK and Fitzgerald KA: Regulation of inflammasome signaling. Nat Immunol 13: 333-342, 2012.
21. Kanneganti TD: Central roles of NLRs and inflammasomes in viral infection. Nat Rev Immunol 10: 688-698, 2010.
22. Ito M, Yanagi Y and Ichinohe T: Encephalomyocarditis virus viroporin 2B activates NLRP3 inflammasome. PLoS Pathog 8: e1002857, 2012.
23. Ichinohe T, Pang IK and Iwasaki A: Influenza virus activates inflammasomes via its intracellular M2 ion channel. Nat Immunol 11: 404-410, 2010.
24. Tan TY and Chu JJ: Dengue virus-infected human monocytes trigger late activation of caspase-1, which mediates pro-inflammatory IL-1β secretion and pyroptosis. J Gen Virol 94: 2215-2220, 2013.
25. Wu MF, Chen ST, Yang AH, *et al*: CLEC5A is critical for dengue virus-induced inflammasome activation in human macrophages. Blood 121: 95-106, 2013.
26. Dowling JK and O'Neill LA: Biochemical regulation of the inflammasome. Crit Rev Biochem Mol Biol 47: 424-443, 2012.
27. Lupfer C and Kanneganti TD: The expanding role of NLRs in antiviral immunity. Immunol Rev 255: 13-24, 2013.
28. Proell M, Gerlic M, Mace PD, Reed JC and Riedl SJ: The CARD plays a critical role in ASC foci formation and inflammasome signaling. Biochem J 449: 613-621, 2013.
29. Kanneganti TD, Lamkanfi M, Kim YG, *et al*: Pannexin-1-mediated recognition of bacterial molecules activates the cryopyrin inflammasome independent of Toll-like receptor signaling. Immunity 26: 433-443, 2007.
30. Hornung V, Bauernfeind F, Halle A, *et al*: Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. Nat Immunol 9: 847-856, 2008.
31. Cruz CM, Rinna A, Forman HJ, Ventura AL, Persechini PM and Ojcius DM: ATP activates a reactive oxygen species-dependent oxidative stress response and secretion of proinflammatory cytokines in macrophages. J Biol Chem 282: 2871-2879, 2007.
32. Cassel SL, Eisenbarth SC, Iyer SS, *et al*: The Nalp3 inflammasome is essential for the development of silicosis. Proc Natl Acad Sci USA 105: 9035-9040, 2008.
33. Koshiba T, Bashiruddin N and Kawabata S: Mitochondria and antiviral innate immunity. Int J Biochem Mol Biol 2: 257-262, 2011.
34. Nakahira K, Haspel JA, Rathinam VA, *et al*: Autophagy proteins regulate innate immune responses by inhibiting the release of mitochondrial DNA mediated by the NALP3 inflammasome. Nat Immunol 12: 222-230, 2011.
35. Zhou R, Yazdi AS, Menu P and Tschopp J: A role for mitochondria in NLRP3 inflammasome activation. Nature 469: 221-225, 2011.
36. Xu Z, Wei L, Wang L, Wang H and Jiang S: The in vitro and in vivo protective activity of monoclonal antibodies directed against Hantaan virus: potential application for immunotherapy and passive immunization. Biochem Biophys Res Commun 298: 552-558, 2002.
37. Cheng L, Yu L, Wu X, *et al*: Induction of specific humoral and cellular immune responses in a mouse model following gene fusion of HSP70C and Hantaan virus Gn and S0.7 in an adenoviral vector. PLoS One 9: e88183, 2014.
38. Deng JX, Nie XJ, Lei YF, *et al*: The highly conserved 5' untranslated region as an effective target towards the inhibition of Enterovirus 71 replication by unmodified and appropriate 2'-modified siRNAs. J Biomed Sci 19: 73, 2012.
39. Markotic A, Hensley L, Daddario K, Spik K, Anderson K and Schmaljohn C: Pathogenic hantaviruses elicit different immunoreactions in THP-1 cells and primary monocytes and induce differentiation of human monocytes to dendritic-like cells. Coll Antropol 31: 1159-1167, 2007.
40. Shin OS, Yanagihara R and Song JW: Distinct innate immune responses in human macrophages and endothelial cells infected with shrew-borne hantaviruses. Virology 434: 43-49, 2012.
41. Davis BK, Wen H and Ting JP: The inflammasome NLRs in immunity, inflammation, and associated diseases. Annu Rev Immunol 29: 707-735, 2011.
42. Leavy O: Inflammasome: Turning on and off NLRP3. Nat Rev Immunol 13: 1, 2013.
43. Bird L: Innate immunity: Linking mitochondria and microbes to inflammasomes. Nat Rev Immunol 12: 229, 2012.
44. Henao-Mejia J, Elinav E, Strowig T and Flavell RA: Inflammasomes: far beyond inflammation. Nat Immunol 13: 321-324, 2012.
45. Leemans JC, Cassel SL and Sutterwala FS: Sensing damage by the NLRP3 inflammasome. Immunol Rev 243: 152-162, 2011.
46. Burdette D, Haskett A, Presser L, McRae S, Iqbal J and Waris G: Hepatitis C virus activates interleukin-1β via caspase-1-inflammasome complex. J Gen Virol 93: 235-246, 2012.
47. Barlan AU, Griffin TM, McGuire KA and Wiethoff CM: Adenovirus membrane penetration activates the NLRP3 inflammasome. J Virol 85: 146-155, 2011.
48. Nour AM, Reichelt M, Ku CC, Ho MY, Heineman TC and Arvin AM: Varicella-zoster virus infection triggers formation of an interleukin-1β (IL-1β)-processing inflammasome complex. J Biol Chem 286: 17921-17933, 2011.
49. Rajan JV, Rodriguez D, Miao EA and Aderem A: The NLRP3 inflammasome detects encephalomyocarditis virus and vesicular stomatitis virus infection. J Virol 85: 4167-4172, 2011.
50. Komune N, Ichinohe T, Ito M and Yanagi Y: Measles virus V protein inhibits NLRP3 inflammasome-mediated interleukin-1β secretion. J Virol 85: 13019-13026, 2011.
51. Poeck H, Bscheider M, Gross O, *et al*: Recognition of RNA virus by RIG-I results in activation of CARD9 and inflammasome signaling for interleukin 1 beta production. Nat Immunol 11: 63-69, 2010.

52. Kaushik DK, Gupta M, Kumawat KL and Basu A: NLRP3 inflammasome: key mediator of neuroinflammation in murine Japanese encephalitis. PLoS One 7: e32270, 2012.
53. Schroder K and Tschopp J: The inflammasomes. Cell 140: 821-832, 2010.
54. Lau SK, Lau CC, Chan KH, *et al*: Delayed induction of proinflammatory cytokines and suppression of innate antiviral response by the novel Middle East respiratory syndrome coronavirus: implications for pathogenesis and treatment. J Gen Virol 94: 2679-2690, 2013.
55. Tisoncik JR, Korth MJ, Simmons CP, Farrar J, Martin TR and Katze MG: Into the eye of the cytokine storm. Microbiol Mol Biol Rev 76: 16-32, 2012.
56. Cheng XW, Lu J, Wu CL, *et al*: Three fatal cases of pandemic 2009 influenza A virus infection in Shenzhen are associated with cytokine storm. Respir Physiol Neurobiol 175: 185-187, 2011.
57. Huang KJ, Su IJ, Theron M, *et al*: An interferon-gamma-related cytokine storm in SARS patients. J Med Virol 75: 185-194, 2005.
58. Hussain SP and Harris CC: Inflammation and cancer: an ancient link with novel potentials. Int J Cancer 121: 2373-2380, 2007.
59. Sims JE and Smith DE: The IL-1 family: regulators of immunity. Nat Rev Immunol 10: 89-102, 2010.
60. Dostert C, Petrilli V, Van Bruggen R, Steele C, Mossman BT and Tschopp J: Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica. Science 320: 674-677, 2008.
61. Handke W, Oelschlegel R, Franke R, Kruger DH and Rang A: Hantaan virus triggers TLR3-dependent innate immune responses. J Immunol 182: 2849-2858, 2009.
62. Lee MH, Lalwani P, Raftery MJ, *et al*: RNA helicase retinoic acid-inducible gene I as a sensor of Hantaan virus replication. J Gen Virol 92: 2191-2200, 2011.
63. Guillot L, Le Goffic R, Bloch S, *et al*: Involvement of toll-like receptor 3 in the immune response of lung epithelial cells to double-stranded RNA and influenza A virus. J Biol Chem 280: 5571-5580, 2005.
64. Kummer JA, Broekhuizen R, Everett H, *et al*: Inflammasome components NALP 1 and 3 show distinct but separate expression profiles in human tissues suggesting a site-specific role in the inflammatory response. J Histochem Cytochem 55: 443-452, 2007.
65. Allen IC, Scull MA, Moore CB, *et al*: The NLRP3 inflammasome mediates in vivo innate immunity to influenza A virus through recognition of viral RNA. Immunity 30: 556-565, 2009.
66. Ichinohe T, Lee HK, Ogura Y, Flavell R and Iwasaki A: Inflammasome recognition of influenza virus is essential for adaptive immune responses. J Exp Med 206: 79-87, 2009.
67. Kanneganti TD, Body-Malapel M, Amer A, *et al*: Critical role for Cryopyrin/Nalp3 in activation of caspase-1 in response to viral infection and double-stranded RNA. J Biol Chem 281: 36560-36568, 2006.
68. Thomas PG, Dash P, Aldridge JR Jr, *et al*: The intracellular sensor NLRP3 mediates key innate and healing responses to influenza A virus via the regulation of caspase-1. Immunity 30: 566-575, 2009.
69. Kerur N, Veettil MV, Sharma-Walia N, *et al*: IFI16 acts as a nuclear pathogen sensor to induce the inflammasome in response to Kaposi sarcoma-associated herpesvirus infection. Cell Host Microbe 9: 363-375, 2011.
70. Unterholzner L, Keating SE, Baran M, *et al*: IFI16 is an innate immune sensor for intracellular DNA. Nat Immunol 11: 997-1004, 2010.
71. Rathinam VA, Jiang Z, Waggoner SN, *et al*: The AIM2 inflammasome is essential for host defense against cytosolic bacteria and DNA viruses. Nat Immunol 11: 395-402, 2010.
72. Hornung V, Ablasser A, Charrel-Dennis M, *et al*: AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC. Nature 458: 514-518, 2009.