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CRITICAL ROLE FOR CRYOPYRIN/NALP3 IN ACTIVATION OF CASPASE-1 IN RESPONSE TO VIRAL INFECTION AND DOUBLE-STRANDED RNA*

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Running Title: Activation of Cryopyrin Inflammasome by viruses

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Viral infection induces the production of IL-1β and IL-18 in macrophages through the activation of caspase-1, but the mechanism by which host cells sense viruses to induce caspase-1 activation is unknown. In this report we identify a signalling pathway leading to caspase-1 activation that is induced by double-stranded ribonucleic acid (dsRNA) and viral infection that is mediated by Cryopyrin/Nalp3. Stimulation of macrophages with dsRNA, viral RNA or its analog poly(I:C) induced the secretion of IL-1β and IL-18 in a cryopyrin-dependent manner. Consistently, caspase-1 activation triggered by poly(I:C), dsRNA and viral RNA was abrogated in macrophages lacking cryopyrin or the adaptor ASC, but proceeded normally in macrophages deficient in TLR3 or TLR7. We also show that infection with Sendai and Influenza viruses activate the cryopyrin inflammasome. Finally, cryopyrin was required for IL-1β production in response to poly(I:C) *in vivo*. These results identify a mechanism mediated by cryopyrin and ASC that links dsRNA and viral infection to caspase- 1 activation resulting in IL-1β and IL-18 production.

Innate immunity is the initial line of host defence against microbial pathogens, including viral infection. The early recognition of viruses by the host initiates signalling pathways leading to the induction of anti-viral responses, including the secretion of type I interferons (IFN-α and IFN-β) and proinflammatory cytokines (1-3). Recognition of pathogens including viruses by the host immune system relies on the detection of conserved molecular structures that are shared by large pathogens (pathogen-associated molecular patterns, PAMPs). In the case of viruses, genomic DNA and single stranded (ss) RNA or double-stranded (ds) RNA produced during viral replication are sensed by host cells to induce anti-viral responses (3). Viral DNA, ssRNA and dsRNA are recognized by a subfamily of Toll-like receptors (TLR3, 7, 8 and 9) in endosomes after the endocytosis of viral particles in mammalian cells (4-9). Upon activation, these TLRs recruits the adaptor proteins MyD88 or TRIF to activate the IkB kinase (IKK) complex and the IKK-related kinases, TBK1 and IKKε (1,10). Viral dsRNA is recognized by TLR3 and the two cytosolic RNA helicases, the retinoic acid inducible gene I (RIG-I) and MDA5, that induces activation of IKK and TBK1/IKKε (4,11,12). Activation of the IKK complex that includes the catalytic subunits IKKα and IKKβ as well as the regulatory subunit IKKγ/NEMO mediates NF-κB activation

whereas that of TBK1 and IKKε induces phosphorylation and activation of IRF3 or IRF7 (10,13,14). The nuclear translocation of NF-κB and IRF3/IRF7 mediates the transcriptional activation of interferon and cytokine genes that limit viral replication and promote adaptive immune responses (15-17).

Signalling pathways other than NF-κB or IFN that are activated upon viral recognition and mediate anti-viral responses are poorly understood. Previous studies have shown that infection of macrophages with certain viruses including Influenza A and Sendai virus induce IL-1β and IL-18 secretion (18,19), but the mechanism by which host cells sense viruses to induce caspase-1 activation is unknown. Both IL-1β and IL-18 are synthesized as inactive cytoplasmic precursors that are processed into biologically active mature forms in response to various proinflammatory stimuli including viruses by caspase-1, a cysteine protease (18,20,21). Caspase-1 is synthesized as an inactive zymogen that becomes activated by cleavage at aspartic residues to generate an enzymatically active heterodimer composed of a 10 kDa and a 20 kDa chain (20). Recent studies have implicated members of the NOD-like receptor (NLR) family of proteins (also called NOD-LRR or CATERPILLER) in the regulation of caspase-1 activation in response to microbial pathogens (22,23). The NLR family is composed of 23 cytosolic proteins including Nod1, Nod2, Cryopyrin/Nalp3 and Ipaf. The structure of NLRs include an amino-terminal effector binding region that consists of protein-protein interaction domains such as CARD (caspase-recruitment domains) or pyrin, a central nucleotide binding oligomerization domain that acts to oligomerize these proteins, and carboxyl-terminal leucine-rich repeats (LRRs) that are required to detect specific PAMPs (22). Cryopyrin forms an endogenous multi-protein complex containing ASC and caspase-1 dubbed "the inflammasome" which promotes caspase activation and processing of pro-IL-1β (24,25). Notably, missense mutations in the CIAS1 gene that encodes Cryopyrin cause three autoinflammatory disorders characterized by deregulated production of IL-1β (26). Cryopyrin senses bacterial RNA, synthetic anti-viral purine analogs and monosodium urate or calcium pyrophosphate dehydrate crystals (27,28). In addition, other results indicate that Cryopyrin regulates caspase-1 activation in response to factors that induce intracellular K+ efflux, such as certain toxins and high concentrations of extracellular ATP (29,30).

The genome of poxviruses encode Pyrin-containing proteins that interact with components of the inflammasome and inhibit caspase-1 activation and the processing of IL-1β and IL-18 induced by diverse stimuli (31). These results suggest that certain viruses target components of caspase-1 activation pathways to circumvent host anti-viral responses. However, the signalling pathways that link viral infection to caspase-1 activation and IL-1β/IL-18 are unknown. Here we show that viral dsRNA and its analog poly(I:C) as well as viral infection activate caspase-1 through cryopyrin, resulting in the production of active IL-1β and IL-18. The signalling pathway stimulated by dsRNA and cryopyrin was independent of TLR3 or TLR7. These results identify a novel TLR-independent signalling pathway that is mediated by cryopyrin and ASC and leads to the secretion of pro-inflammatory cytokines in response to viral infection.

EXPERIMENTAL PROCEDURES

Mice - Cryopyrin, ASC, and TLR7 KO mice have been described (27,32,33). TLR3 KO mice (4) were obtained from Jackson Laboratory.

Macrophages - Bone marrow was prepared from leg bones of 5-20 weeks old mice. Legs were dissected and the bone marrow flushed out. Bone marrow cells were cultured with IMDM media supplemented with 30% L929 supernatant containing macrophage stimulating factor, glutamine, sodium pyruvate, 10% heat-inactivated fetal bovine serum (FBS; Gibco BRL), 50 μg/ml penicillin and 50 μg/ml streptomycin at 37°C, 5% CO2 for 5 days (Bone-marrow differentiation media; BMDM). Bone-marrow macrophages (BMMs) were then harvested

with rubber scrapers and seeded. Peritoneal macrophages were elicited to the peritoneum of mice and isolated 4 days after the injection of 4% thioglycolate broth. After 1 day, non-adherent cells were removed, and the remaining macrophages were incubated in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% heat-inactivated FBS, 50 μg/ml penicillin, 50 μg/ml streptomycin.

In vitro RNA transcription, viruses and viral RNA - The dsRNA for hsp90 and LacZ were made according to the protocol (34). To prepare flock house virus (FHV) dsRNA, the plasmids pFHV2[0,0] and p2VHF[2,0] (35) were used to generate viral (+) and (-) ssRNAs, respectively. Plasmids were linearized with RsrII, *in vitro* transcripts were synthesized using T7 polymerase and an Ambion Megascript kit per the manufacturer's instructions, and RNA was purified by phenol-chloroform extraction and ethanol precipitation and quantitated by spectrophotometry. Equal amounts of (+) and (-) RNA transcripts were mixed in RNase-free water, heated to 75°C for 30 min, and allowed to cool gradually at room temperature to form dsRNA. Viral RNA integrity and formation of dsRNA were assessed by non-denaturing agarose gel electrophoresis. Murine Sendai virus (strain Cantell) was purchased from American Type Culture Collection and Influenza A/Puerto Rico/8/34 virus (H1N1) was a gift from James R. Becker, Jr (University of Michigan). Rotavirus (strain SA11-4F) dsRNA was isolated by phenol-chloroform extraction from virus grown in monkey kidney (MA014) cells and purified by CsCl centrifugation (36).

Microbial ligands and antibodies- Pam2CGDPKHPKSF (FSL-1), Pam3CSK4, poly(I:C), and CpG oligonucleotide (5'-TCCATGACGTTCCTGACGTT-3') were purchased from Invivogen. poly(A), poly(C), poly(G), and poly(U) single strand (ss) RNA and poly(I)·poly(C) dsRNA were from Sigma. Purified total RNA from *E. coli* was purchased from Ambion. *Bacillus anthracis* PA and LF were obtained from List Biologicals Laboratories and were used at 1 μg/ml concentration. Aliquots of RNA samples were incubated with RNases (Ambion) as suggested by the manufacturer. Rabbit anti-mouse caspase-1 was a generous gift of P. Vandenabeele (Ghent University, Ghent, Belgium). The antibodies for mouse Iκ-Bα, phospho-Iκ-Bα, p38, phospho-p38, ERK, phospho-ERK were from Cell Signalling.

Immunoblotting - Cells were washed twice with PBS and scraped in lysis buffer solution (150 mM NaCl, 10 mM Tris [tris(hydroxymethyl)aminomethane; pH 7.4], 5 mM EDTA [ethylenediaminetetraacetic acid], 1 mM EGTA [ethylene glycol tetraacetic acid], 0.1% Nonidet P40 [NP40]) supplemented with 1x protease inhibitor mixture (Roche Applied Science). For analysis of caspase-1 activation, macrophages were cultured with stimuli for 1-3 h and then with medium containing 5 mM ATP (Sigma) for 30 min. Extracts were prepared from cells and culture supernatants by adding lysis buffer containing 1% NP-40 supplemented with complete protease inhibitor cocktail (Roche, Mannheim, Germany) and 2 mM dithiotheitol. Samples were clarified, denaturated with SDS buffer and boiled for 5 min, separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were immunoblotted with primary antibodies and proteins detected with appropriate secondary anti-rabbit antibody conjugated to horseradish peroxidase followed by enhanced chemiluminescence.

Measurements of cytokines - Macrophages were stimulated with various microbial and synthetic ligands for 24 h and the supernatants were analyzed for IL-1β, IL-18, TNFα and IL-6 secretion. The following ligand concentrations were used: FLS-1, Pam3CSK4 were used at 1 μg/ml and poly(I:C) at 2.5 μg/ml. Mouse cytokines were measured in culture supernatants by enzyme-linked immunoabsorbent assay (ELISA) kits (R and D Systems, Minneapolis, MN).

RESULTS

Cryopyrin is required for IL-1β and IL-18 secretion in response to the dsRNA analog poly(I:C) - To assess a role for cryopyrin in immune responses induced by viruses, macrophages from wild-type (WT) and cryopyrin deficient mice were stimulated with polyinosinic-polycytidylic acid (poly(I:C), a synthetic dsRNA analog that mimics viral infection, and a synthetic diacylated lipopeptide (FSL-1, TLR2 agonist) or *E. coli* RNA as controls. Incubation of wild-type macrophages with poly(I:C) induced IL-1β, but this response was abolished in cryopyrin-null macrophages (Fig. 1*A* and 1*B*). Consistent with previous results, production of IL-1β in response to *E. coli* RNA, but not the FSL-1, was deficient in cryopyrin-null macrophages (Fig. 1*A*). Additionally, cryopyrin was required for IL-18 induced by poly(I:C), but dispensable for IFNα production (Fig. 1*C* and 1*D*). These results indicate that cryopyrin is specifically required for IL-1β/IL-18 secretion induced by poly(I:C).

Cryopyrin-dependent IL-1β secretion by poly(I:C) is independent of NF-κB and MAPK activation - Stimulation of macrophages with poly(I:C) induces the secretion of several pro-inflammatory cytokines (4). We found that cryopyrin was dispensable for the production of TNFα and IL-6 induced by poly(I:C) (Fig. 2*A* and 2*B*). In contrast, production of TNFα and IL-6 required TLR3 (Fig. 2*C* and 2*D*), as previously reported (4). The induction of IL-1β secretion is thought to involve the upregulation of pro-IL-1β through transcriptional mechanisms via NF-κB and then a second stimulus that leads to the activation of caspase-1, processing of pro-IL-1β and release of mature IL-1β (37,38). Stimulation with poly(I:C) induced comparable levels of NF-κB, ERK and p38 activation in WT and cryopyrin-/- macrophages (Fig. 2*E*). By contrast, the activation of NF-κB and MAPKs was abolished in TLR3-deficient macrophages (Fig. 2*F*). These results demonstrate that cryopyrin-mediated IL-1β secretion induced by poly(I:C) is independent of NF-κB and MAPK as well as TLR3.

Cryopyrin but not Nod2/TLR3/TLR7 is essential for activation of Caspase-1 in response to poly(I:C) - Proteolytic activation of procaspase-1 is a critical step in the induction of IL-1β secretion (39,40). Importantly, processing of pro-caspase-1 was induced rapidly (by 1h) and in a dose-dependent manner after stimulation of WT macrophages with poly(I:C), as determined by the detection of the mature 20 kDa subunit of caspase-1 (Fig. 3*A* and 3*B*). Such activation of caspase-1 was abrogated in macrophages lacking cryopyrin (Fig. 3*A* and 3*B*) or ASC (Fig. 3*C*), an adaptor that links cryopyrin to caspase-1 (25,41). In contrast, activation of caspase-1 induced by poly(I:C) was unimpaired in Nod2- and TLR3- or TLR7-deficient macrophages (Fig. 3*D-F*). These results demonstrate that cryopyrin is essential for caspase-1 processing in response to poly(I:C). Furthermore, TLR3 is required for NF-κB and MAPK activation, but dispensable for caspase-1 activation.

Cryopyrin-dependent IL-1β and IL-18 secretion and caspase-1 activation by poly(I:C) requires dsRNA structure - We next tested several ssRNA and dsDNA compounds to determine the structural requirements for IL-1β and IL-18 secretion and caspase-1 activation induced by poly(I:C). Importantly, the synthetic ssRNA analogues (polycytidylic acid (poly(C), polyuridylic acid (poly (U) or polyinosinic (poly(I)) neither induce IL-1β, IL-18 secretion (Fig. 4*A*-4*B*) nor caspase-1 activation (Fig. 4*C*). Moreover, the dsDNA analogues, polydeoxyinosinic–deoxycytidylic acid (poly(dI:dC) and polydeoxyinosinic–deoxcytidylic acid (poly(dG:dC), did not have any effect on IL-1β secretion or caspase-1 activation (Fig. 4*A*-*C*). To further verify these results, we treated poly(I:C) with a panel of RNases that cleave ssRNA, dsRNA or both. Digestion of poly(I:C) with RNase A and RNase T1 that are specific for ssRNA had little or no effect on caspase-1 activation induced by poly(I:C) (Fig. 4*D*). In contrast, treatment of poly(I:C) with RNase V1 that cleaves dsRNA or with benzonase that digests both ss and dsRNA abolished its ability to induce processing of caspase-1 (Fig. 4*D*).

Together these results indicate that the dsRNA structure of poly(I:C) is essential for cryopyrin-dependent induction of IL-1β secretion and caspase-1 activation in macrophages.

Viral and non-viral dsRNA produced in vitro induce cryopyrin-dependent caspase-1 activation – Poly(I:C) does not represent all viral and non viral dsRNA. To assess if *in vitro* transcribed dsRNAs induce caspase-1 activation, we produced ~ 700-bp dsRNA fragments from the 5' coding region of the lacZ and Drosophila Hsp83 genes using *in vitro* transcription system (34,35). In addition, complementary plus-sense (+) and minus-sense (-) ssRNAs of flock house virus (FHV) were produced by in vitro transcription and annealed to form viral dsRNA. Stimulation of macrophages with FHV dsRNA as well as non-viral dsRNAs induced caspase-1 activation as effectively as poly(I:C) that was used as a control (Fig. 5*A*). As expected, neither the (+) ssRNA nor the (–) ssRNA used in preparing FHV dsRNA was able to induce processing of Caspase-1 (Fig. 5*B*). As it was observed with poly(I:C), digestion of FHV dsRNA with RNases that digest dsRNA, but not ssRNA, abolished their ability to induce caspase-1 activation (Fig. 5*C-D*). Additionally, treatment of dsRNAs with *E. coli* RNase III, an endoribonuclease that cleaves dsRNA into 10-18 bp dsRNA fragments had no effect on their ability to induce caspase-1 activation (Fig. 5*E*).

Cryopyrin/ASC-dependent activation of caspase-1 by naturally produced viral dsRNA - We next tested the ability of genomic dsRNA purified from rotavirus grown in monkey kidney cells to induce caspase-1 activation. Incubation of macrophages with naturally produced rotavirus dsRNA induced caspase-1 activation in WT, but not cryopyrin-deficient cells (Fig. 6*A*). Similarly, cryopyrin was required for the activation of caspase-1 induced by viral dsRNA purified from plant cells infected with brome mosaic virus as well as by *in-vitro* produced brome mosaic viral dsRNA. (Fig. 6*B*). In contrast, caspase-1 activation triggered by the lethal toxin of *Bacillus anthracis*, consisting of the protective antigen (PA) and lethal factor (LF) that depends on Nalp1b (42), proceeded normally in the absence of cryopyrin (Fig. 6*A*). Consistent with the results obtained with poly(I:C), activation of caspase-1 induced by rotavirus dsRNA required ASC but not TLR3, or TLR7 (Fig. 6*D-F*). Furthermore, treatment of naturally produced rotavirus and dsRNA with RNase V1, but not RNase III, abolished their ability to induce caspase-1 activation (Fig. 6*G*).

Infection of macrophages with Sendai and Influenza viruses induce Cryopyrin-dependent caspase-1 activation - We next tested the ability of Sendai and Influenza A virus, two viruses known to stimulate IL-1β secretion in human macrophages (18,21), to induce caspase-1 activation in WT and cryopyrin-deficient macrophages. Macrophages were infected with Sendai or Influenza A virus at low multiplicity of infection (MOI) and cell extracts were examined for pro-caspase-1 processing by immunoblotting. The analysis revealed that both viruses induced caspase-1 activation in WT but not cryopyrin-deficient macrophages (Fig. 7*A*). As it was observed with viral dsRNA, activation of caspase-1 triggered by viral infection was independent of TLR3 and TLR7 (Fig. 7*B*).

Cryopyrin is required for production of IL-1β and IL-18 after administration of Poly(I:C) in vivo - We next examined whether cryopyrin plays a role in the production of pro-inflammatory cytokines in the animal. Intraperitoneal administration of poly(I:C) induced the production of IL-1β, IL-18, TNF-α and IL-6 in the serum of wild-type mice. Similarly the bacterial lipopeptide Pam3CSK4 (TLR2 agonist) also induced the production of IL-1β, TNF-α and IL-6 in the serum of wild-type mice. (Fig. 8). In contrast, the serum levels of IL-1β, IL-18 and to lesser extend IL-6 were greatly reduced while those of TNFα were unimpaired in cryopyrin KO mice after injection with poly(I:C) (Fig. 8*A*, 8*B*, 8*C,* 8*D,* 8*G* and Supplemental Fig. S1). The reduced levels of IL-6 detected in the serum of cryopyrin-null mice after stimulation with poly(I:C) is presumably due

to the induction of IL-6 by IL-1β in vivo (43). Notably, the production of pro-inflammatory cytokines including IL-1β was unimpaired in cryopyrin-deficient mice after intraperitoneal administration of Pam3CSK4, demonstrating the specific role of cryopyrin in regulating poly(I:C) responses *in vivo.*

DISCUSSION

Infection of monocytes and macrophages with a variety of viruses is known to induce the secretion of IL-1β and IL-18 through the activation of caspase-1, but the molecular mechanisms involved have remained largely unknown. These studies demonstrate that cryopyrin plays an essential role in the secretion of IL-1β and IL-18 by sensing viral dsRNA and inducing the activation of caspase-1. Viral dsRNA also triggers the production of type I IFNs, TNFα and IL-6 through TLR3 and the RIG-1stimulation (4,11), but these immune responses were independent of cryopyrin. The latter responses are mediated through the activation of the transcriptional factors IRF3/IRF7 and NF-κB (16,44). Thus, dsDNA induces at least three distinct defense signaling pathways in host cells to limit viral infection.

IL-1β and IL-18 are induced by a variety of microbial stimuli through the activation of caspase-1. IL-1β is considered to be a master cytokine in that mediates several innate and adaptive immune responses directly or through the induction of other cytokines such as IL-6 (43). In addition, IL-1β is a potent pyrogen that is involved in the development of fever in response to pathogen infection (45). However, there is little evidence for a role of IL-1β in host defense against viral infection. In contrast, IL-18, a cytokine that stimulate NK cells and CD8+ T cells and is potently synergistic with IL-12 for this function (46), is known to play an important role in viral clearance (47,48). Furthermore, administration of IL-18 can protect the host against infection with herpes simplex and vaccinia virus (49,50). A role for IL-18 in viral infection is also supported by the finding that the genome of several poxviruses viruses encode proteins that bind and inactivate IL-18 (51).

Functional analyses of RNA compounds and enzymatic studies revealed that dsRNA, but not ssRNA, activate caspase-1 through cryopyrin. The ability of cryopyrin to discriminate between dsRNA and ssRNA provides a mechanism to sense viral RNA and to avoid harmful activation of the cryopyrin inflammasome by endogenous RNA. Treatment of 700 bp dsRNA fragments with RNase III, an endoribonuclease that cleaves both synthetic and natural dsRNA into small duplex products averaging 10-18 bp in length (52), had no effect on its ability to induce caspase-1 activation. It is known that mammalian RNase III enzymes such as Dicer process dsRNA into 21-24 nt dsRNAs that can induce degradation of homologous mRNAs and specific gene silencing (53). It is also known that certain siRNAs can induce IFN responses and toxic effects in mammalian cells (54). Our results suggest both long viral and endogenous dsRNA fragments or their shorter dsRNA products generated by RNase III enzymes might lead to cryopyrin activation and IL-1β/IL-18 secretion.

There are several mechanisms by which dsRNA could induce the activation of caspase-1 through cryopyrin. Viral dsRNA is produced during viral infection in the cytosol of host cells and could be sensed directly by cryopyrin. There is evidence that several NLR proteins including Nod1, Nod2, Ipaf, and cryopyrin can recognize microbial structures (22,23). However, there is no evidence that the microbial products physically interact with the LRRs of these NLR proteins (55). The sensing of dsRNA by cryopyrin is most likely to be indirect as it been proposed for the recognition of microbial components by plant NLR homologs (55,56). Cryopyrin has been shown to form a large multi-protein complex containing ASC that promotes the activation of caspase-1 in cell-free systems (25). Thus, a possible model is that the LRRs of cryopyrin sense dsRNA produced during viral infection, which induces the oligomerization of cryopyrin and recruitment of caspase-1 via ASC and possibly other factors involved in caspase-1 activation.

In summary, our results have shown a novel role for cryopyrin in activation of

inflammasome by dsRNA/viral RNA and by viral infection. These findings provide important insight into the role of NLR proteins in the host response to dsRNA. The possibility that certain siRNAs might activate the cryopyrin inflammasome warrant further investigation as such siRNAs are being explored for their potential therapeutic use (57,58).

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FOOTNOTES

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[1]The abbreviations used are: BM, bone marrow; CARD caspase-recruitment domain; KO, knock-out; NLR, NOD-like receptor; MOI, multiplicity of infection; NOD, nucleotide-binding, oligomerization domain; TLR, Toll-like receptor; LRR, leucine-rich repeat.

FIGURE LEGENDS

Fig. 1. Cryopyrin is required for IL-1β and IL-18 secretion in response to poly(I:C). *A-C*, Macrophages from WT (filled bars) or cryopyrin$^{-/-}$ mice (open bars) were stimulated with the indicated stimuli for 24 h and cell-free supernatants were analyzed by ELISA for production of IL-1β□□□□□□□. *D*, Macrophages described above were stimulated with the indicated

concentrations of poly(I:C) for 24 h and cell-free supernatants were analyzed by ELISA for production of IFNα. The data are means ± S.D. of triplicates.

Fig. 2. Cryopyrin-dependent IL-ıβ secretion by poly(I:C) is independent of NF-κB and MAPK activation. *A-B*, Macrophages from WT and Cryopyrin$^{-/-}$ mice or *C-D*, WT and TLR3$^{-/-}$ mice were stimulated with indicated concentrations of poly(I:C) for 24 h and cell-free supernatants were analyzed by ELISA for production of TNFα and IL-6. *E,* Macrophages from WT and cryopyrin$^{-/-}$ mice or *F*, WT and TLR3$^{-/-}$ mice were stimulated with poly(I:C) for indicated times. Cell extracts were immunoblotted with antibodies that detect total and phosphorylated (activated) forms of the indicated proteins. The data are means ± S.D. of triplicates and are representation of at least two independent experiments.

Fig. 3. Cryopyrin and ASC but not Nod2/TLR3/TLR7 are essential for activation of caspase-1 in response to poly(I:C). *A-F*, Macrophages from WT and mutant mice were stimulated with 5 μg/ml poly(I:C) or with the indicated concentrations for the indicated times and then cells were pulsed transiently with ATP for 30 min. Cell extracts were immunoblotted with a caspase-1 antibody. Arrows denote procaspase-1 and its processed p20 subunit. Results are representative of three separate experiments.

Fig. 4. Cryopyrin-dependent IL-1β and IL-18 secretion and caspase-1 activation by poly(I:C) requires dsRNA structure. *A-B*, Macrophages from WT (filled bars) or cryopyrin$^{-/-}$ mice (open bars) were stimulated with the indicated stimuli for 24 h and cell-free supernatants were analyzed by ELISA for production of IL-1β and IL-18. The data are means ± S.D. of triplicates. *C-D*, Macrophages from WT mice were stimulated with indicated stimuli for 3 h and then cells were pulsed transiently with ATP for 30 min. Cell extracts were immunoblotted with a caspase-1 antibody. Arrows denote procaspase-1 and its processed p20 subunit. Results are representative of three separate experiments.

Fig. 5. Endogenous and viral dsRNA produced *in vitro* induce cryopyrin-dependent caspase-1 activation. *A-B*, Macrophages from WT and cryopyrin$^{-/-}$ mice were stimulated with *in vitro* produced viral RNA, non-viral dsRNA from LacZ and Hsp83 gene, plus-sence (+) and minus-sence (–) ssRNAs and poly(I:C) for 3 h and then cells were pulsed transiently with ATP for 30 min. Cell extracts were immunoblotted with a caspase-1 antibody. Arrows denote procaspase-1 and its processed p20 subunit. *C-E*, Macrophages from WT mice were stimulated for 3 h with dsRNA preparations digested with indicated RNases and then pulsed transiently with ATP for 30 min. Cell extracts were immunoblotted with a caspase-1 antibody. Results are representative of three separate experiments.

Fig. 6. Cryopyrin/ASC-dependent activation of caspase-1 by naturally produced viral dsRNA. *A-F*, Macrophages from WT and indicated mutant mice were stimulated with *A-B,* indicated stimuli or *C-F,* purified dsRNA from rotavirus-infected cells. *G*, Macropages were stimulated with indicated RNA preparations digested with RNaseV1 and RNase III. All the above treated cells were stimulated for 3 h and then pulsed transiently with ATP for 30 min. Cell extracts were immunoblotted with a caspase-1 antibody. Results are representative of three independent experiments.

Fig. 7. Infection of macrophages with Sendai and Influenza A viruses induce cryopyrin-dependent caspase-1 activation. *A*, Macrophages from WT and the indicated mutant mice were stimulated with Sendai and Influenza A viruses at the indicated MOI or with *B*, Sendai virus for indicated time and then cells were pulsed transiently with ATP for 30 min. Cell extracts were immunoblotted with a caspase-1 antibody. Arrows denote procaspase-1 and its processed p20 subunit. Results are representative of at least two separate experiments.

Fig. 8. Cryopyrin is required for IL-1β production in response to poly(I:C) *in vivo*. *A-G*, Groups of WT mice and cryopyrin$^{-/-}$ mice (KO) (n=4) were injected with poly(I:C) (200 μg) or *D-F*, with Pam3CSK4 (200 μg) i. p. and *A*, *D*, the serum levels of IL-1β, *B, E,* IL-6, *C, F,* TNFα or *G,* IL-18 were determined by ELISA at the indicated times. The serum of mice that were not injected did not contain any detectable levels of cytokines. Error bars represent standard deviation of values obtained from 4 mice. An independent experiment is shown as Supplemental Fig. S1.

Supplementary Fig. S1. Cryopyrin is required for IL-1β production in response to poly(I:C) *in vivo*. *A-C*, Groups of WT mice and cryopyrin$^{-/-}$ mice (KO) (n=4) were injected with poly(I:C) (200 μg) i. p. and *A*, the serum levels of IL-1β, *B,* IL-6 or *C,* TNFα were determined by ELISA at the indicated times. The serum of mice that were not injected did not contain any detectable levels of cytokines. Error bars represent standard deviation of values obtained from 4 mice.

Figure 1

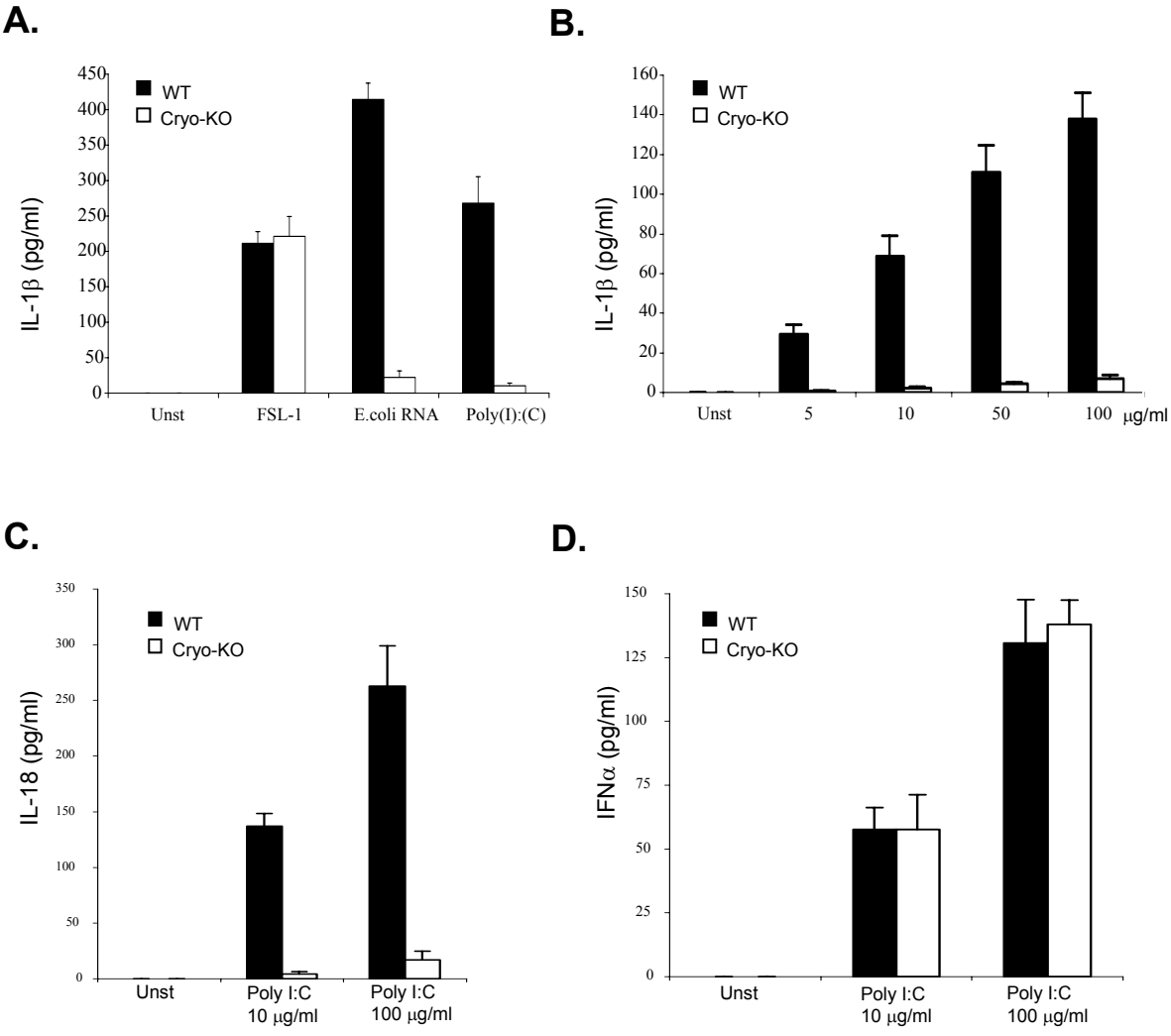

A.
B.
C.
D.
WT
Cryo-KO
IL-1β (pg/ml)
450
400
350
300
250
200
150
100
50
0
Unst
FSL-1
E.coli RNA
Poly(I):(C)
160
140
120
100
80
60
40
20
0
5
10
50
100 μg/ml
IL-18 (pg/ml)
350
300
250
200
150
100
50
0
Poly I:C
10 μg/ml
Poly I:C
100 μg/ml
IFNα (pg/ml)
150
125
100
75
50
25
0

Figure 2

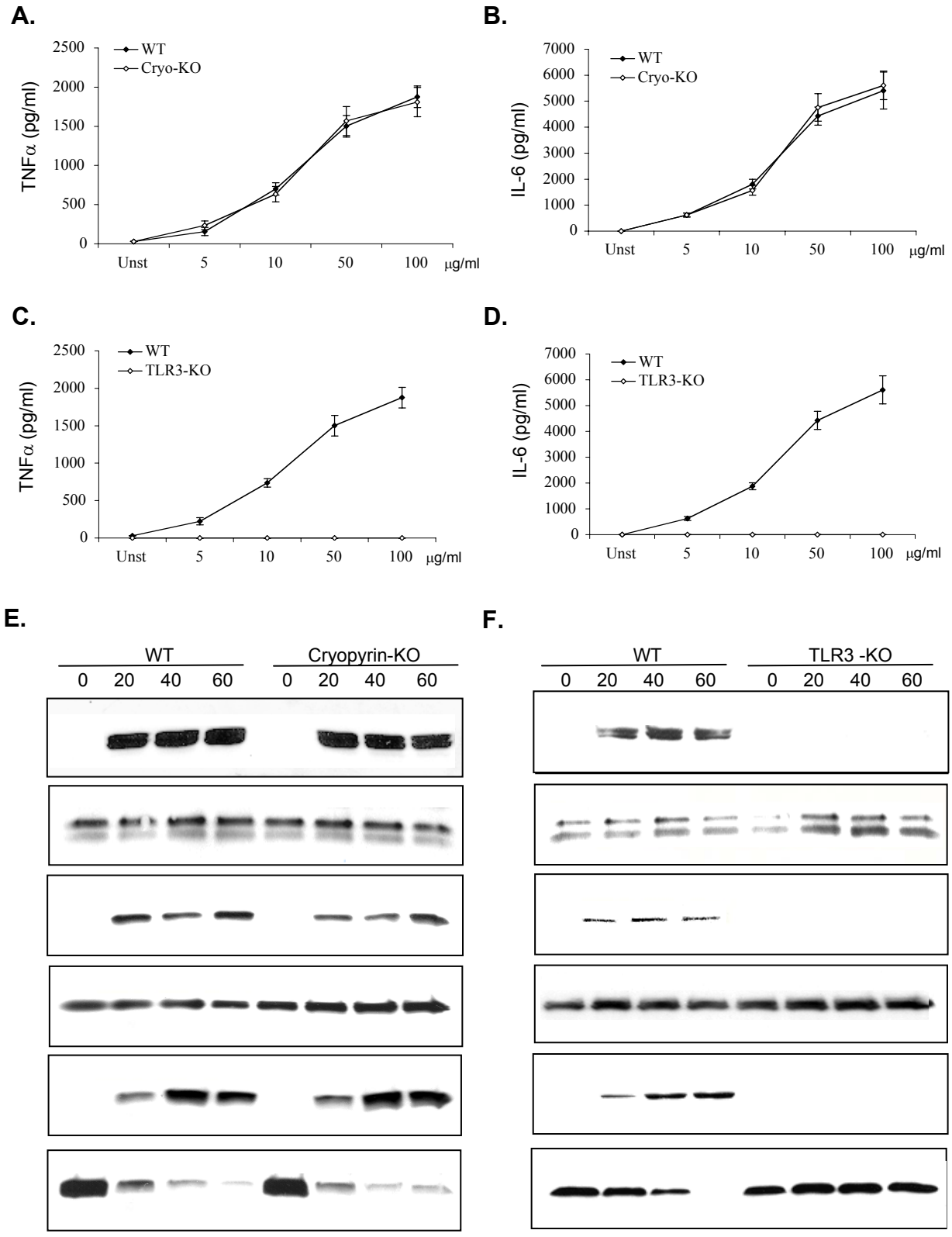

A.
TNFα (pg/ml)
2500
2000
1500
1000
500
0
WT
Cryo-KO
Unst
5
10
50
100
μg/ml
B.
IL-6 (pg/ml)
7000
6000
5000
4000
3000
2000
1000
0
WT
Cryo-KO
Unst
5
10
50
100
μg/ml
C.
TNFα (pg/ml)
2500
2000
1500
1000
500
0
WT
TLR3-KO
Unst
5
10
50
100
μg/ml
D.
IL-6 (pg/ml)
7000
6000
5000
4000
3000
2000
1000
0
WT
TLR3-KO
Unst
5
10
50
100
μg/ml
E.
WT
Cryopyrin-KO
0
20
40
60
0
20
40
60
F.
WT
TLR3 -KO
0
20
40
60
0
20
40
60
(min)
P-ERK
ERK
P-p38
p38
P-IκBα
IκBα

Figure 3

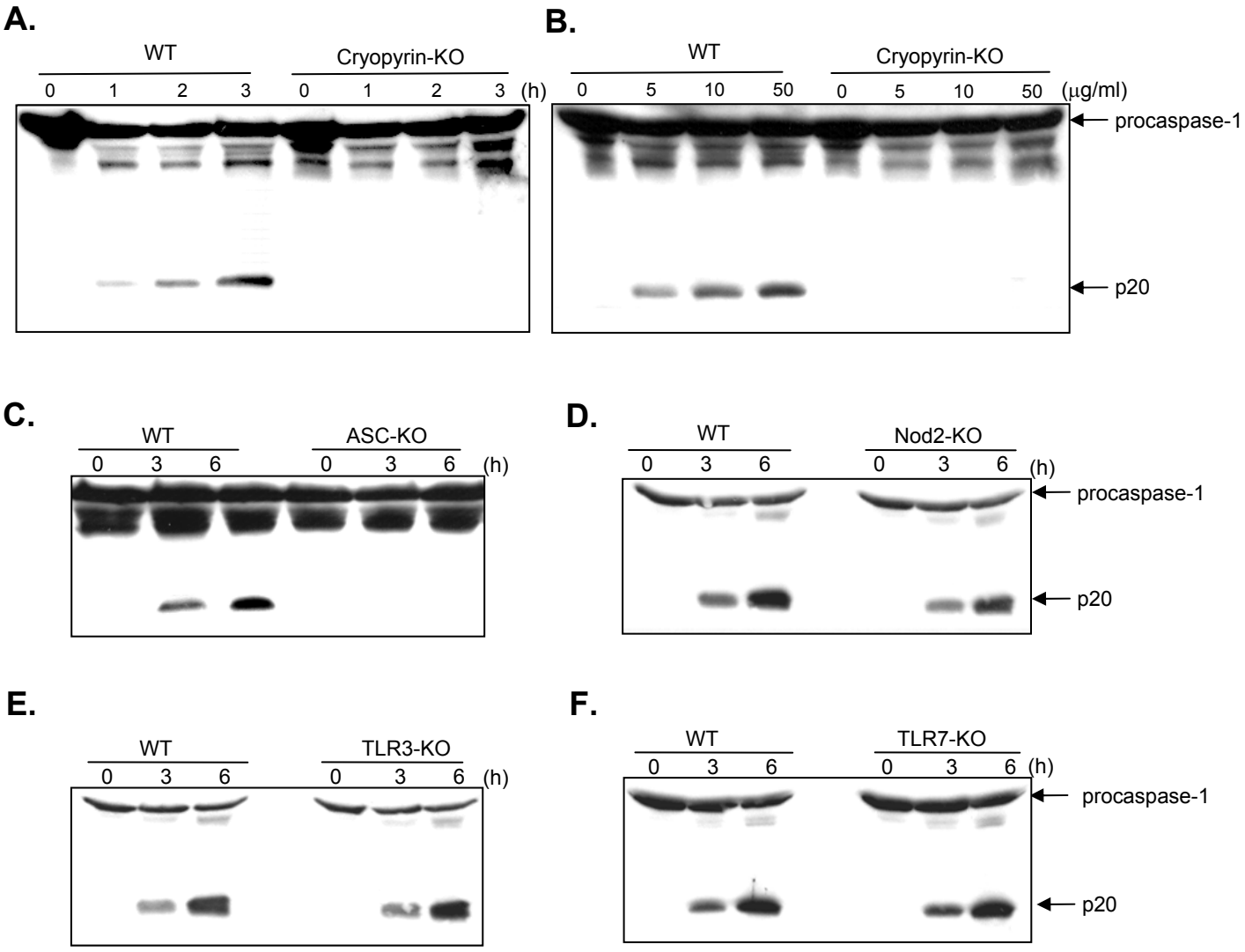

A.
WT
Cryopyrin-KO
0 1 2 3 0 1 2 3 (h)
B.
WT
Cryopyrin-KO
0 5 10 50 0 5 10 50 (μg/ml)
procaspase-1
p20
C.
WT
ASC-KO
0 3 6 0 3 6 (h)
D.
WT
Nod2-KO
0 3 6 0 3 6 (h)
procaspase-1
p20
E.
WT
TLR3-KO
0 3 6 0 3 6 (h)
F.
WT
TLR7-KO
0 3 6 0 3 6 (h)
procaspase-1
p20

Figure 4

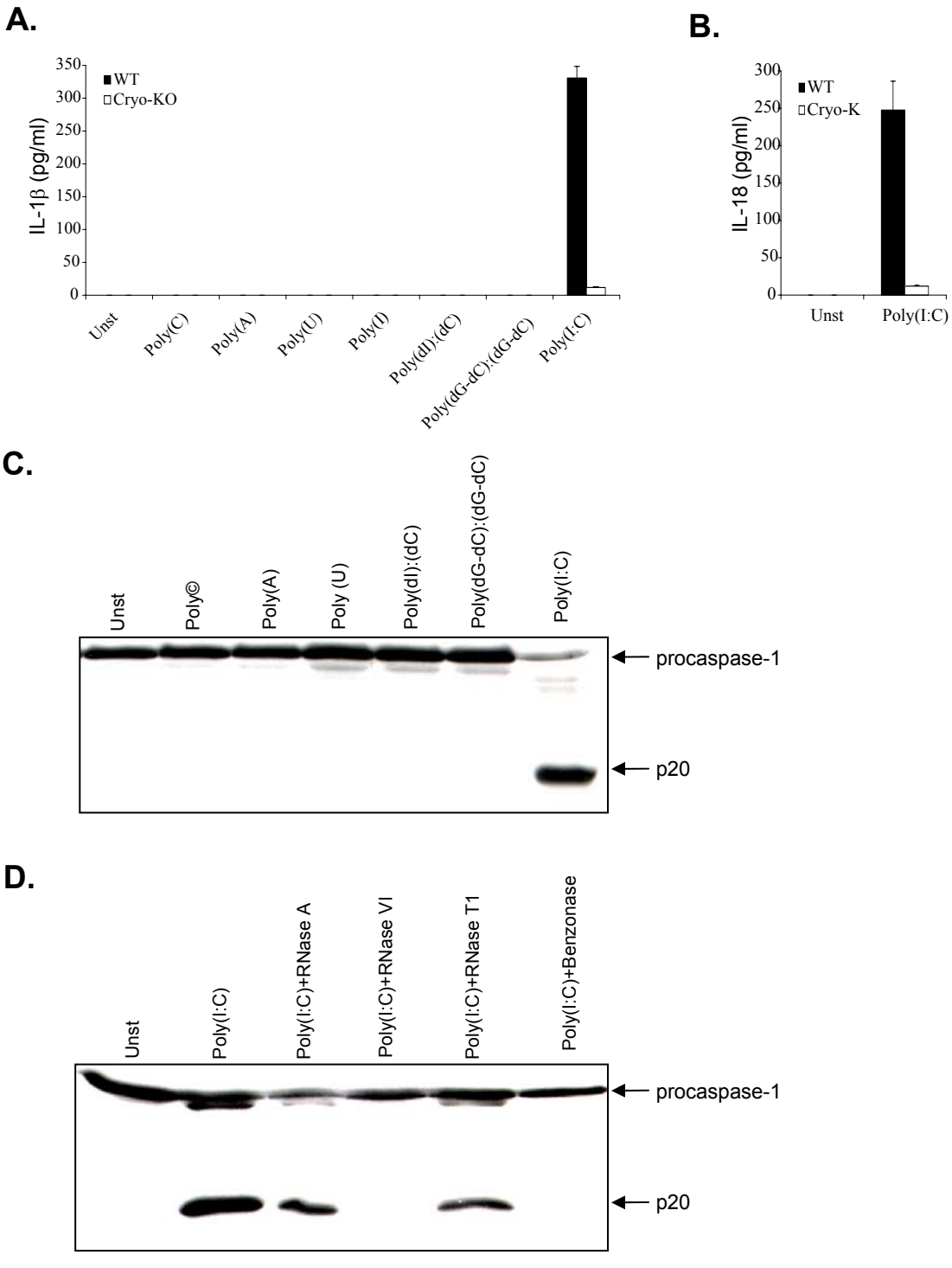

A.
WT
Cryo-KO
IL-1β (pg/ml)
350
300
250
200
150
100
50
0
Unst
Poly(C)
Poly(A)
Poly(U)
Poly(I)
Poly(dI):(dC)
Poly(dG-dC):(dG-dC)
Poly(I:C)
B.
WT
Cryo-K
IL-18 (pg/ml)
300
250
200
150
100
50
0
Unst
Poly(I:C)
C.
Unst
Poly©
Poly(A)
Poly (U)
Poly(dI):(dC)
Poly(dG-dC):(dG-dC)
Poly(I:C)
procaspase-1
p20
D.
Unst
Poly(I:C)
Poly(I:C)+RNase A
Poly(I:C)+RNase VI
Poly(I:C)+RNase T1
Poly(I:C)+Benzonase
procaspase-1
p20

Figure 5

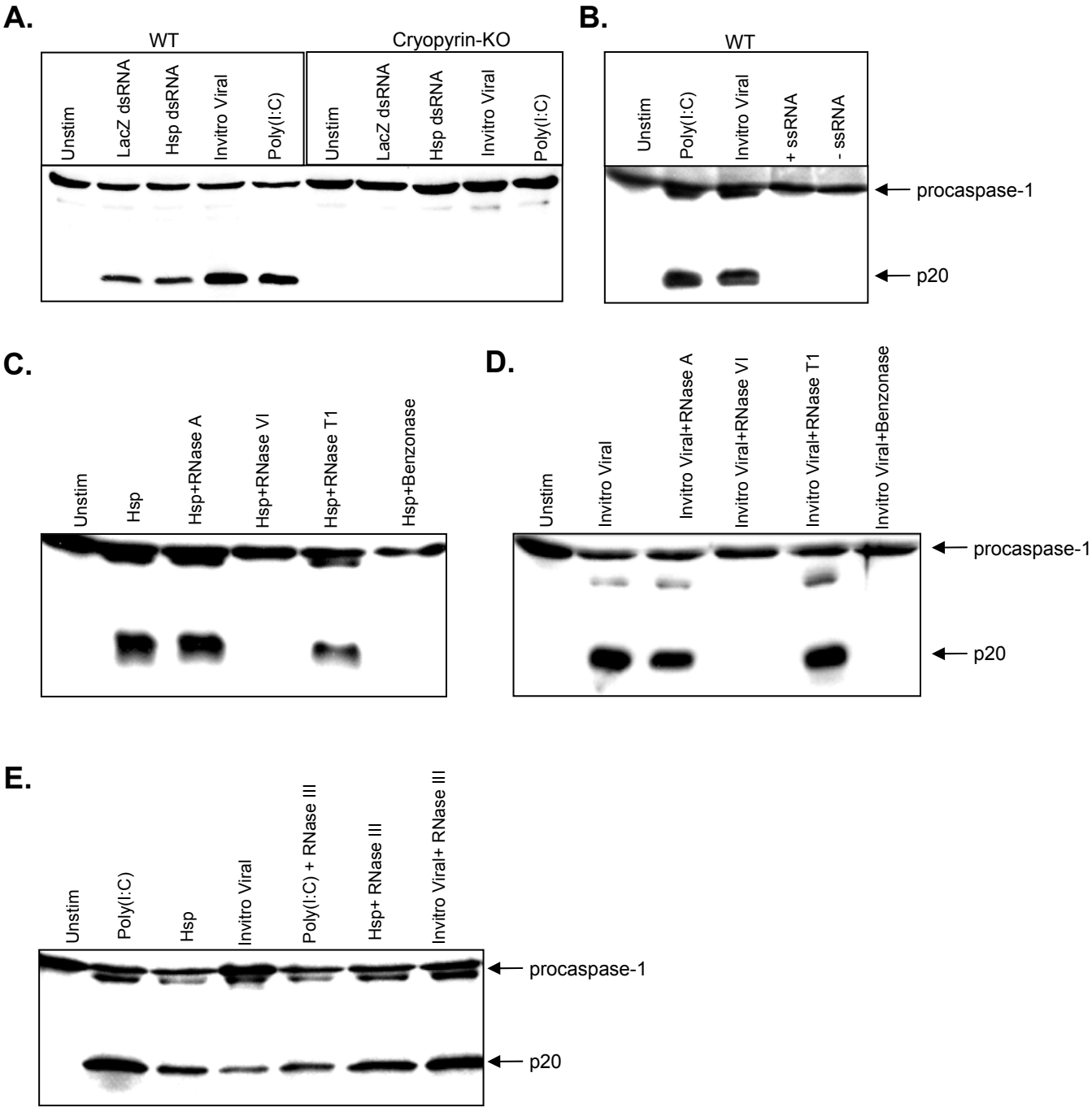

A.
WT
Cryopyrin-KO
Unstim
LacZ dsRNA
Hsp dsRNA
Invitro Viral
Poly(I:C)
B.
WT
Unstim
Poly(I:C)
Invitro Viral
+ ssRNA
- ssRNA
procaspase-1
p20
C.
Unstim
Hsp
Hsp+RNase A
Hsp+RNase VI
Hsp+RNase T1
Hsp+Benzonase
D.
Unstim
Invitro Viral
Invitro Viral+RNase A
Invitro Viral+RNase VI
Invitro Viral+RNase T1
Invitro Viral+Benzonase
procaspase-1
p20
E.
Unstim
Poly(I:C)
Hsp
Invitro Viral
Poly(I:C) + RNase III
Hsp+ RNase III
Invitro Viral+ RNase III
procaspase-1
p20

Figure 6

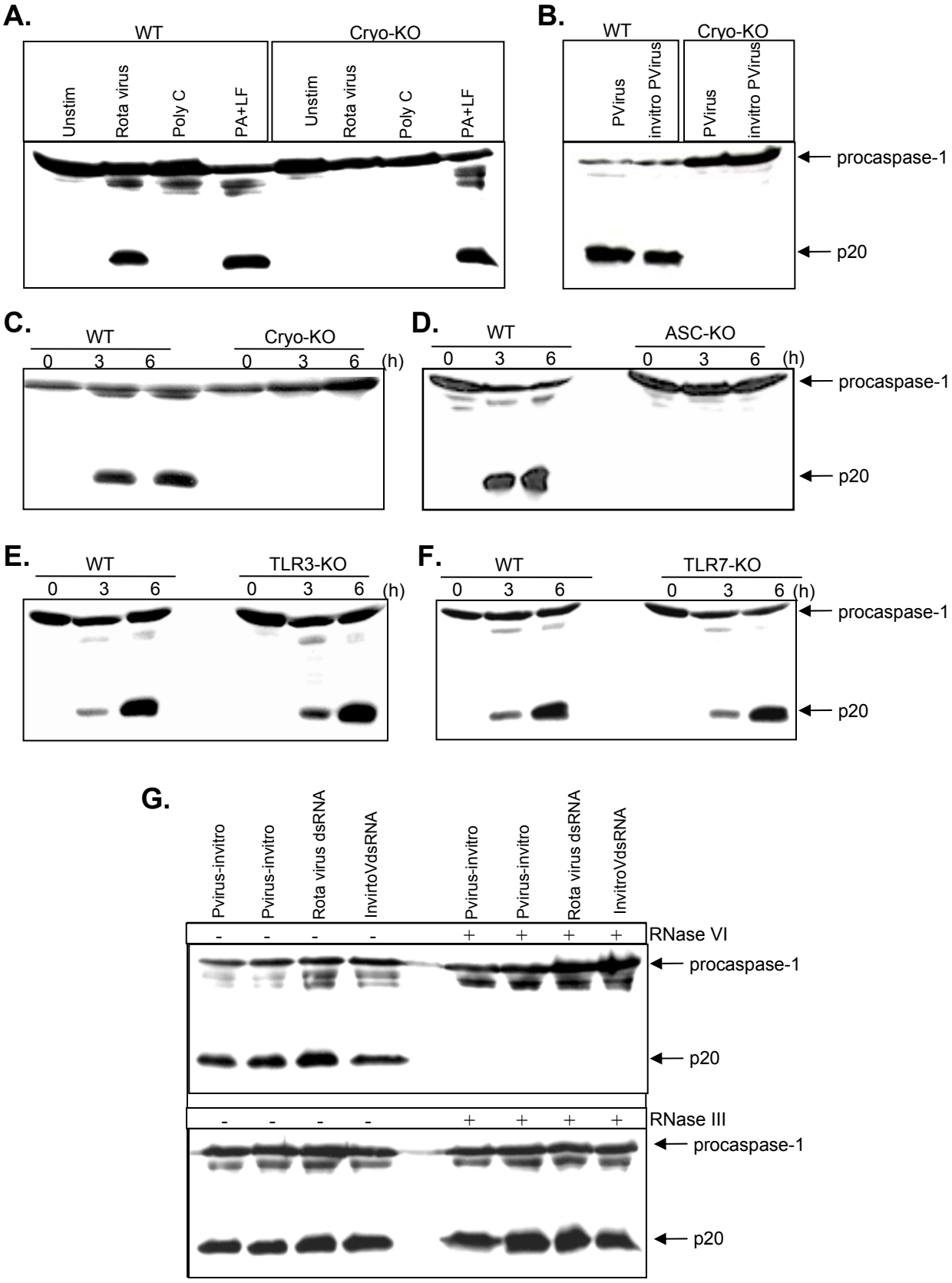
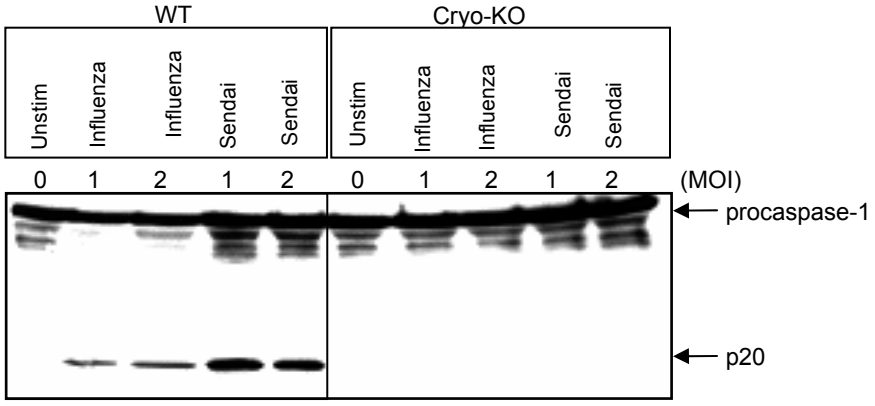
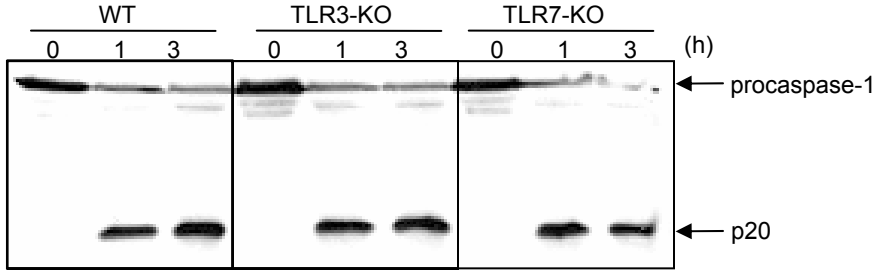

A.
WT
Cryo-KO
Unstim
Rota virus
Poly C
PA+LF
Unstim
Rota virus
Poly C
PA+LF
B.
WT
Cryo-KO
PVirus
invitro PVirus
PVirus
invitro PVirus
procaspase-1
p20
C.
WT
Cryo-KO
0 3 6 0 3 6 (h)
D.
WT
ASC-KO
0 3 6 0 3 6 (h)
procaspase-1
p20
E.
WT
TLR3-KO
0 3 6 0 3 6 (h)
F.
WT
TLR7-KO
0 3 6 0 3 6 (h)
procaspase-1
p20
G.
Pvirus-invitro
Pvirus-invitro
Rota virus dsRNA
InvirtoVdsRNA
Pvirus-invitro
Pvirus-invitro
Rota virus dsRNA
InvitroVdsRNA
- - - - + + + +
RNase VI
procaspase-1
p20
- - - - + + + +
RNase III
procaspase-1
p20

Figure 7

A.


WT
Cryo-KO
Unstim
Influenza
Influenza
Sendai
Sendai
Unstim
Influenza
Influenza
Sendai
Sendai
0 1 2 1 2 0 1 2 1 2
(MOI)
procaspase-1
p20

B.


WT
TLR3-KO
TLR7-KO
0 1 3 0 1 3 0 1 3
(h)
procaspase-1
p20

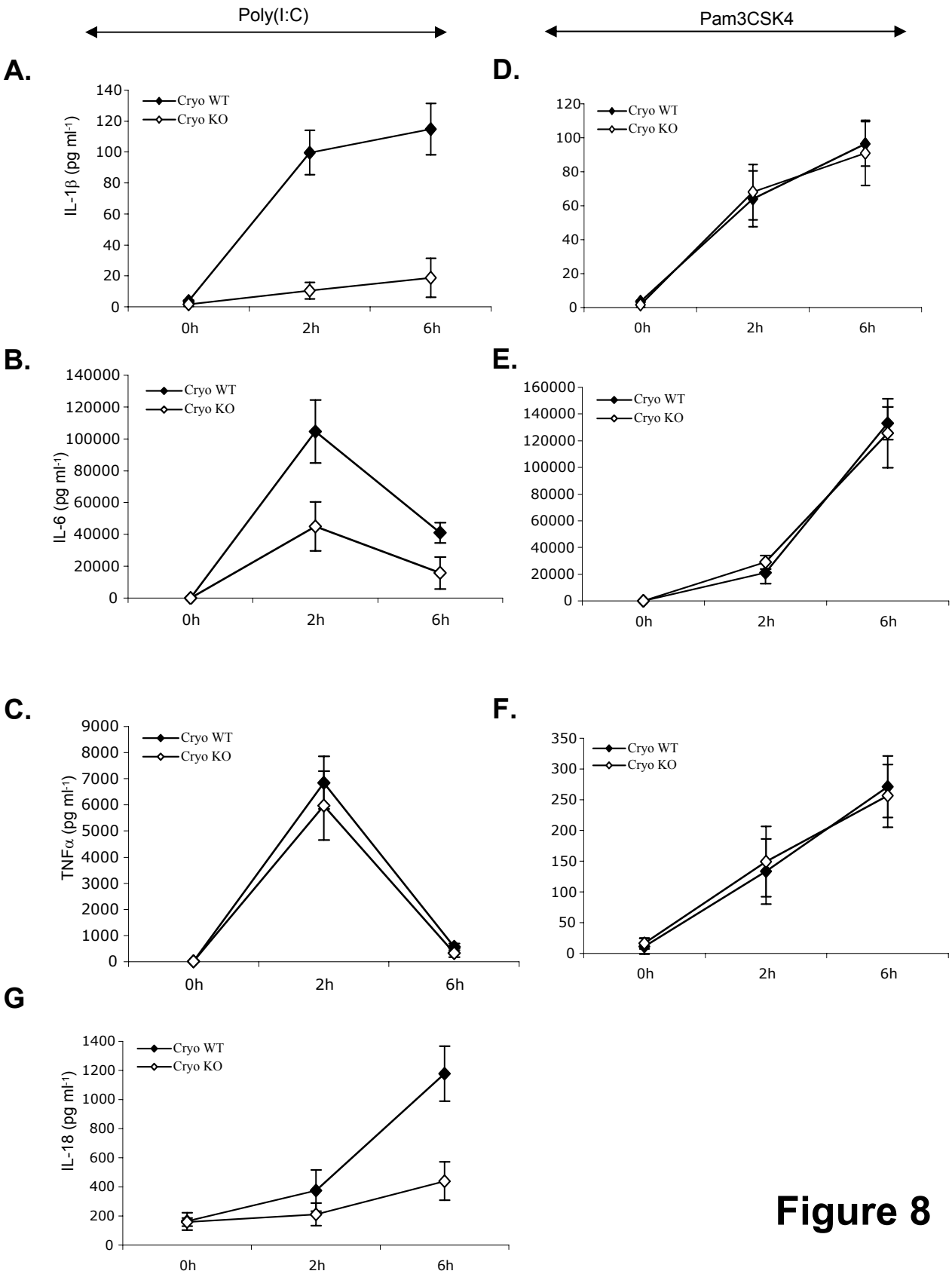
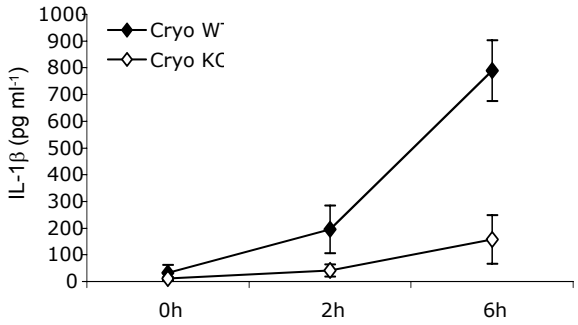
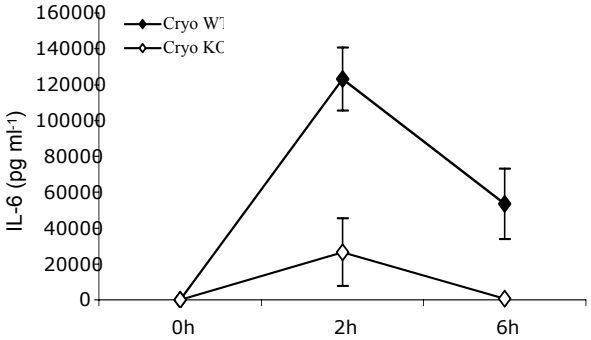

Poly(I:C)
Pam3CSK4
A.
D.
B.
E.
C.
F.
G
Cryo WT
Cryo KO
IL-1β (pg ml-1)
IL-6 (pg ml-1)
TNFα (pg ml-1)
IL-18 (pg ml-1)
0h
2h
6h

Figure 8

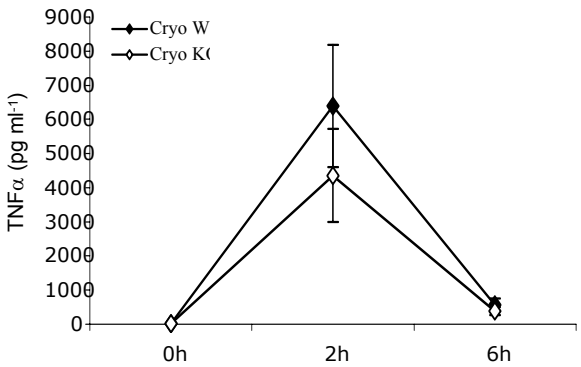
A.



B.



C.



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Supplementary Figure 1