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Role of the Leucine-rich Repeat (LRR) Domain of Cryopyrin/ NALP3 in Monosodium Urate Crystal-induced Inflammation

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Abstract

Objectives—The mechanism by which MSU crystals intracellularly activate the Cryopyrin inflammasome is unknown. Here, we used a mouse molecular genetics-based approach to test if the LRR domain of Cryopyrin is required for MSU crystal-induced inflammation.

Methods—Cryopyrin knockout lacZ ($\text{Cryo}^{-Z/-Z}$) and LRR deletion lacZ ($\text{Cryo}^{\Delta\text{LRR } Z/\Delta\text{LRR } Z}$) mice were generated using BAC-based targeting vectors, which allow for large genomic deletions. Bone marrow-derived macrophages (BMDMs) from $\text{Cryo}^{\Delta\text{LRR } Z/\Delta\text{LRR } Z}$, $\text{Cryo}^{-Z/-Z}$, and their congenic wildtype (WT) mice were challenged with endotoxin-free MSU crystals under serum-free conditions. Phagocytosis and cytokine expression were assessed by flow cytometry and ELISA analysis. MSU crystals also were injected into mouse synovial-like subcutaneous air pouches. The *in vivo* inflammatory responses were examined.

Results—Release of IL-1 β , but not CXCL1 and TNF α , was impaired in $\text{Cryo}^{\Delta\text{LRR } Z/\Delta\text{LRR } Z}$ and $\text{Cryo}^{-Z/-Z}$ BMDMs compared to WT BMDMs in response to not only MSU crystals but also other known stimuli that activate the cryopyrin inflammasome. In addition, comparable percentage of MSU crystals taken up by each type of BMDMs was observed. Moreover, total leukocytes infiltrated in the air pouch and IL-1 β production were attenuated in $\text{Cryo}^{-Z/-Z}$ and $\text{Cryo}^{\Delta\text{LRR } Z/\Delta\text{LRR } Z}$ mice at 6 h post-injection of MSU crystals compared to WT mice.

Conclusions—MSU crystal-induced inflammatory responses were comparably attenuated both *in vitro* and *in vivo* in $\text{Cryo}^{\Delta\text{LRR } Z/\Delta\text{LRR } Z}$ and $\text{Cryo}^{-Z/-Z}$ mice. Hence, the LRR domain of Cryopyrin plays a role in mediating MSU crystal-induced inflammation in this model.

Introduction

In gout, the deposition of monosodium urate (MSU) crystals in articular joints and bursal tissues can be asymptomatic or be associated with the pathogenesis of acute, episodic, self-limiting joint inflammation (1–3). The interaction of MSU crystals with resident cells such as synovial lining cells and macrophages in the joint is believed to be the primary trigger for the acute neutrophil ingress that drives episodes of gouty arthritis (4). Cells encountering MSU crystals express a broad array of inflammatory mediators that drive and amplify acute

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gouty inflammation, including arachidonate metabolites, the cytokines IL-1 β , TNF α , CXCL1 (GRO α), CXCL8 (IL-8) (5–9), and the calgranulins S100A8 and S100A9 (10).

The naked MSU crystal has a negatively charged, highly reactive surface that nonspecifically binds at least 25 different serum proteins (11) and also binds plasma membrane proteins including certain integrins (12,13). MSU crystal binding of C5, and C5 catalysis on the crystal surface (14), promotes C5b-C9 membrane attack complex assembly that contributes to both intra-articular CXCL8 expression and acute neutrophilic inflammation in experimental MSU crystal-induced knee arthritis (15). A number of studies have demonstrated the importance of innate immunity in acute gouty inflammation. MSU crystals can functionally engage the canonical signaling pathway from TLR2 to NF- κ B activation mediated by the shared TLR and IL-1 receptor adaptor protein MyD88 (16). TLR2 and TLR4 each mediate macrophage uptake of the MSU crystal *in vitro* and MSU-crystal-induced inflammation *in vivo* (17). In addition, MyD88 plays a major role in macrophage uptake of the MSU crystal and is essential for MSU crystal-induced inflammation *in vivo* (17). Moreover, direct engagement of CD14 which is a shared TLR2 and TLR4 adaptor molecule, is a major determinant of the inflammatory potential of the MSU crystals (18). Furthermore, the cytoplasmic Cryopyrin (also known as NALP3, *NLRP3*) inflammasome complex, which is principally expressed in phagocytes, is pivotal for acute MSU crystal-induced inflammation (19). MSU crystals appear to be among the stimuli that trigger aggregation and activation of the cryopyrin inflammasome through pyrin-pyrin domain interactions of Cryopyrin and of the adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC). Activation of the inflammasome complex results in the recruitment and proteolytic cleavage of caspase-1 (19). In this context, MSU crystal-induced caspase-1 activation, and subsequent cleavage, maturation and release of IL-1 β are markedly decreased in macrophages from mice deficient in Cryopyrin, ASC or caspase-1 *in vitro* (19). Moreover, MSU crystal-induced peritoneal neutrophil influx is blunted in Cryopyrin, ASC and caspase-1 deficient mice (19,20).

Similar to TLR domain structure, Cryopyrin has an LRR domain at its C-terminus which is also proposed to be a ligand sensing motif (21). In this model, cryopyrin is normally present in the cytoplasm in an inactive form, but becomes active when the LRR domain is engaged by an agonist. This is thought to be due to the conformational rearrangement of this molecule which exposes the oligomerization domain (NBS/NACHT) and subsequently the effector domain (pyrin binding domain) (21). In this study, we investigated if the LRR domain of Cryopyrin is required for MSU crystal-induced inflammation using a novel recombinant mouse with the cryopyrin LRR domain deleted and fused to the reporter lacZ (*Cryo Δ LRR Z/ Δ LRR Z*). Macrophages from these mice stimulated *in vitro* do not induce caspase-1 activation and IL-1 β release. The *in vivo* inflammatory response in subcutaneous air pouches in these mice is significantly reduced. In addition, the IL-1 β release in *Cryo Δ LRR Z/ Δ LRR Z* macrophages *in vitro* in response to several other known Cryopyrin activators is also decreased markedly.

MATERIALS AND METHODS

Reagents

All chemical reagents were obtained from Sigma-Aldrich (St. Louis, MO), unless otherwise indicated. Triclinic MSU crystals were prepared under pyrogen-free conditions, using uric acid pretreated for 2 hours at 200°C prior to crystallization (17). The crystals were suspended at 25 mg/ml in sterile, endotoxin-free PBS, and verified to be free of detectable LPS contamination (<0.025 Endotoxin Units/ml) by the limulus amebocyte lysate assay (BioWhittaker, Inc., Walkersville, MD). Peptidoglycan and R837 were obtained from InVivogen (San Diego, CA), and bacterial RNA was from Ambion (Austin, TX).

Mice

Mice were generated by Regeneron Pharmaceuticals using the VelociGene approach as described (22). This approach has been useful for studying *in situ* expression of targeted proteins particularly when specific antisera are unavailable. A targeting vector was constructed that included an in-frame reporter lacZ gene cloned next to an out-of-frame neomycin resistance gene flanked by loxP sites and driven by a promoter that allowed for positive selection in both bacterial and mammalian cells. The LacZ-Neo cassette was ligated to double-stranded oligonucleotides and used for the generation of bacterial artificial chromosome-based targeting vectors lacking the cryopyrin LRR domain (Cryo^{ΔLRR Z/ΔLRR Z}) or deficient in cryopyrin (Cryo^{-Z/-Z}) as shown in figure 1B. These constructs were microinjected into embryonic stem cells derived from the 129/Sv C57BL/6 F1 background to allow for proper recombination. Correctly targeted embryonic stem cells carrying the targeting construct were injected into BALB/c blastocysts, which were then implanted in CD1 pseudopregnant foster mothers. Male chimeras were bred with C57BL/6 to screen for germline-transmitted offspring. Mice bearing the targeted allele were screened by PCR. To confirm gene expression of the “truncated” cryopyrin (ΔLRR), RNA isolated from bone marrow (Trizol) of these mice was subjected to RT-PCR (ABI Taqman) using the following exonic primer pairs, 5'-CGAGAAAGGCTGTATCCCAG and 5'-GCTAGGATGGTTTTCCCGAT (Exons 1–3), 5'-CACGTGGTTTCCTCCTTTTG and 5'-TGGTGAAGGAGGGCTTGATA (Exons 3–9), 5'-CACGTGGTTTCCTCCTTTTG and 5'-TTGACTGTAGCGGCTGATGTTG (Exon 3 to lacZ), 5'-GGTAAACTGGCTCGGATTAGGG and 5'-TTGACTGTAGCGGCTGATGTTG (lacZ to lacZ), and 5'-GGTCTTACTCCTTGAGGCCATGT and 5'-GACCCCTTCATTGACCTCAACTACA (GAPDH). Protein expression of the “truncated” cryopyrin (ΔLRR) was also confirmed by Western blot analysis on bone marrow derived macrophages of these mice using antibodies to either β-gal (Invitrogen, CA) or Cryopyrin N14 (Santa Cruz, CA).

Isolation and Culture of Murine Macrophages

All animal experiments were done humanely under institutionally approved protocols. Background matched wild type (WT), Cryo^{-Z/-Z} and Cryo^{ΔLRR Z/ΔLRR Z} mice were backcrossed at least 4 generations on a C57BL/6 background and were maintained under specific pathogen-free conditions and genotyped by PCR. Bone marrow derived macrophages (BMDMs) were prepared from 8–10 weeks old homozygous Cryo^{-Z/-Z} and Cryo^{ΔLRR Z/ΔLRR Z} as well as congenic WT control mice.

Western blot and Immunoprecipitation (IP)

BMDMs were lysed with the buffer containing 50mM Tris pH7.8, 50mM NaCl, 0.1% NP40, 5mM EDTA, 10% glycerol, 1mM PMSF, and protease inhibitors (Roche) on ice and passed through a 22g needle 10 times. For the IP experiment, cell lysates were incubated with anti-Cryopyrin N14 Peptide Antibody (Santa Cruz) at 4°C overnight and Protein G Sepharose Fast Flow (Sigma) was added for 2 hours at 4°C. Beads were spun down and washed three times with the same buffer. The washed beads or cell lysates were separated on 4–15% SDS-PAGE gels and then transferred to PVDF membranes. The membranes were then used for immunoblot analyses with antibodies indicated. Same amount of conditioned media was subjected to SDS/PAGE and Western blot analyses on caspase-1 and IL-1β expression using the antibodies to caspase-1 (BioVision) and IL-1β (BioVision).

Assays of phagocytosis and cytokine production

BMDMs of individual genotypes were treated with MSU crystals (0.5 mg/ml) for 2 h at 37°C and then were washed 3 times with cold PBS containing 5 mM EDTA, and harvested

in the same buffer. The proportion of the macrophages taking up MSU crystals was assessed by flow cytometry analysis based on increased side-scatter (23).

We evaluated generation of IL-1 β and CXCL1 by DuoSet ELISA (R&D Systems, Inc. Minneapolis, MN) following the manufacturer's protocol by testing conditioned media collected from mouse BMDMs (5×10^5 /well) stimulated with MSU crystals (0.5 mg/ml) for 24 h.

Studies of synovial-like subcutaneous air pouches

Subcutaneous pouches were generated by the injection of sterile-filtered air to generate an accessible space that developed a synovium-like membrane within 7 days, as described (17). In brief, anaesthetized 8–10 week-old WT, Cryo^{-Z/-Z} and Cryo Δ LRR Z/ Δ LRR Z mice were injected with 5 ml of sterile air into the subcutaneous tissue of the back, followed by a second injection of 3 ml of sterile air into the pouch 3 days later. MSU crystals (3 mg), in 1 ml of sterile, endotoxin-free PBS, were injected into the pouch 7 days after the first injection of air. Mice were sacrificed and pouch fluids were harvested at specific time points by injecting 5 ml of PBS containing 5 mM EDTA, and cells infiltrating into the air pouch were counted manually using a hemocytometer. Smears of cells from the air pouches on the slides were prepared by centrifugation of 10^5 of cells in cytofunnels (ThermoShandon, Pittsburgh, PA) in a Cytospin 4 centrifuge (ThermoShandon, Pittsburgh, PA) at 110 g for 2 min. Leukocyte population counts were measured via Wright-Giemsa staining of cytospin slides. IL-1 β was determined by ELISA, as above, in supernatants of air pouch exudates.

Statistical analyses

Data are presented as the mean $\pm$ SD. Statistical analyses were performed using the two-tailed Student's *t* test.

Results

Generation and Characterization of Cryo Δ LRR Z/ Δ LRR Z mice

To investigate whether the cryopyrin LRR domain plays a role in inflammatory response, we generated Cryopyrin LRR deletion mutant mice (Cryo Δ LRR Z/ Δ LRR Z), as well as Cryopyrin knockout (Cryo^{-Z/-Z}) mice (Figure 1A–C) as described in Methods. To examine the expression of the recombinant gene, we first isolated RNA from bone marrow from these mice, performed reverse transcription, followed by PCR using primers derived from exonic sequence coding for various domains. We confirmed that the “truncated” cryopyrin LRR mutant was expressed at the RNA level (Figure 1D). Next, we isolated cell lysates from BMDMs of wild type (WT), Cryo Δ LRR Z/ Δ LRR Z and Cryo^{-Z/-Z} mice and measured protein expression by Western blot using an antibody to β -gal. Expression of the “truncated” cryopyrin (Δ LRR)fused to β -gal and expression of β -gal alone in cryopyrin knockout in which the entire gene is replaced by β -gal was observed (Figure 1E). Immunoprecipitation analyses with a cryopyrin specific antibody also confirmed expression of the “truncated” cryopyrin (Δ LRR)fused to β -gal in Cryo Δ LRR Z/ Δ LRR Z mice (data not shown), as well as expression of cryopyrin in WT mice (data not shown). These mice were viable and fertile, pups were born at the expected Mendelian ratio, and there was no apparent phenotype.

Attenuation of MSU crystal-induced inflammatory responses in Cryo^{-Z/-Z} mice

First, BMDMs were generated from Cryo^{-Z/-Z} and the congenic WT mice. As with our previous studies (17,18), to avoid potential masking effects of both serum protein opsonization of the crystals (1) and of crystal-induced complement activation (14,15), BMDMs were treated with endotoxin-free MSU crystals at the concentration of 0.5 mg/ml under entirely serum-free conditions. At 24 h, MSU crystals induced release of IL-1 β and

CXCL1 (Figure 2A and 2B) in WT BMDMs. However, release of IL-1 β (Figure 3A), but not of CXCL1 (Figure 2B) and TNF α (data not shown) was blunted in Cryo^{-Z/-Z} BMDMs in response to MSU crystals. Notably, there is no impairment in uptake of MSU crystals in Cryo^{-Z/-Z} BMDMs after 2 hours stimulation at 37°C (Figure 2C), suggesting that cryopyrin is not involved in phagocytosis of MSU crystals. *In vivo* studies using the air pouch model revealed that MSU crystal-induced leukocyte infiltration peaked at 6 hours post-injection of MSU crystals, and was self-limiting by 24 hours post-injection of MSU crystals in the air pouch of WT mice (Figure 2D). In contrast, total number of leukocytes infiltrated in the air pouch of Cryo^{-Z/-Z} mice was markedly suppressed at 6 hours post-injection (Figure 2D). This result was in consistent with previous observation in the peritonitis model (20).

Impaired MSU crystal-induced inflammatory responses in Cryo ^{Δ LRR Z/ Δ LRR Z} mice

Similar *in vitro* and *in vivo* studies as shown in Figure 2 were carried out in Cryo ^{Δ LRR Z/ Δ LRR Z} mice. As seen in Figure 3A and 3B, MSU crystal-induced release of IL-1 β , but not CXCL1 was blunted in BMDMs of Cryo ^{Δ LRR Z/ Δ LRR Z} mice *in vitro*, which is comparable to that in BMDMs of Cryo^{-Z/-Z} mice. *In vivo* air pouch model experiments demonstrated that MSU crystal-induced leukocyte infiltration observed in WT mice at 6 hours post-injection was markedly decreased in the Cryo ^{Δ LRR Z/ Δ LRR Z} mice (Figure 3C). In addition, there was a significant decrease in IL-1 β release in the air pouch of Cryo ^{Δ LRR Z/ Δ LRR Z} mice compared with that in the air pouch of WT mice (Figure 3D). These data suggest that Cryopyrin LRR domain is required for mediating MSU crystal-induced inflammatory responses.

Attenuation of Caspase-1 activation and IL-1 β cleavage in Cryo ^{Δ LRR Z/ Δ LRR Z} BMDMs in response to MSU crystals *in vitro*

Next, we examined caspase-1 activation in BMDMs of Cryo ^{Δ LRR Z/ Δ LRR Z} mice in response to MSU crystals *in vitro*. As depicted in the top panel of Figure 4, MSU crystal-induced caspase-1 activation observed in WT BMDMs was diminished in BMDMs of not only Cryo^{-Z/-Z} but also Cryo ^{Δ LRR Z/ Δ LRR Z} mice. Similarly, IL-1 β release was repressed in BMDMs of Cryo^{-Z/-Z} mice and Cryo ^{Δ LRR Z/ Δ LRR Z} mice in response to MSU crystals, compared with that in WT BMDMs (bottom panel of Figure 4). This suggests that the LRR domain of Cryopyrin is needed for MSU crystal-induced caspase-1 activation and IL-1 β release in BMDMs.

Impaired IL-1 β release in Cryo ^{Δ LRR Z/ Δ LRR Z} BMDMs in response to several other known Cryopyrin activators *in vitro*

To determine if Cryopyrin LRR domain is generally required for mediating IL-1 β release in response to inflammatory stimuli, we examined IL-1 β release in BMDMs of Cryo ^{Δ LRR Z/ Δ LRR Z} mice and compared with that in WT and Cryo^{-Z/-Z} BMDMs in response to several stimuli known to activate cryopyrin such as peptidoglycan (PGN), bacterial RNA, R837 and crude LPS (cLPS). As seen in Figure 5A, IL-1 β release was induced by all of these stimuli in WT BMDMs, but was reduced significantly in BMDMs of Cryo ^{Δ LRR Z/ Δ LRR Z} mice, which is comparable to that in BMDMs of Cryo^{-Z/-Z} mice. In contrast, there was no significant difference in CXCL1 release in all types of BMDMs in response to all the stimuli (Figure 5B). These data suggest that Cryopyrin LRR domain is essential for mediating IL-1 β release in response to inflammatory stimuli known to activate the Cryopyrin inflammasome.

Discussion

In this paper, we demonstrated that the novel Cryo ^{Δ LRR Z/ Δ LRR Z} mice have decreased MSU crystal-induced caspase-1 activation and IL-1 β release in BMDMs *in vitro* and leukocyte

infiltration in the air pouch model *in vivo*. In addition, we showed that the Cryo^{ΔLRR Z/ΔLRR Z} have decreased IL-1β release in BMDMs in response to several other known Cryopyrin activators *in vitro*.

The Cryopyrin inflammasome is activated by a number of pathogen-associated patterns (PAMPs) including bacterial muramyl dipeptide (MDP), a degradation product of the bacterial cell wall component PGN, the microbial toxins, RNA of bacterial and viral origin, and cytosolic microbial and host DNA (24–27), as well as danger-associated molecular patterns (DAMPs) such as ATP, imidazoquinoline, MSU crystals, asbestos and silica (25,28–33). It is still not clear how Cryopyrin senses these diverse activators to trigger inflammasome complex formation that leads to caspase-1 activation and IL-1β release. One putative mechanism is that each of the activators directly or indirectly interacts with the LRR domain of Cryopyrin, leading to a conformational change in Cryopyrin and subsequently to inflammasome assembly. MDP was recently demonstrated to directly bind to recombinant NALP1, and MDP interaction with the LRR region of NALP1 is essential for caspase-1 activation mediated by the reconstituted NALP1 inflammasome (34). This data indicate that NALPs could directly interact with their activators. Although we do not yet know if MSU crystals activation occurs via direct interaction with the LRR domain of Cryopyrin, our study suggest a role for the LRR domain of Cryopyrin in MSU crystal-induced inflammatory responses.

Interestingly, recent findings showed that potassium efflux, lowering intracellular potassium levels, is a common requirement for Cryopyrin inflammasome activation triggered by all known activators including MSU crystals (33,35,36). In addition to potassium efflux, reactive oxygen species (ROS) production is a necessary step in activation of the Cryopyrin inflammasome (32,33). It is unlikely that each of the activators is “specifically” recognized by Cryopyrin. Tschopp’s group proposed that these activators could induce a cellular stress situation that in all cases result in modification of one or more membrane-associated proteins, which then trigger a signaling cascade leading to activation of Cryopyrin (32). Since we observed that IL-1β release in BMDMs of Cryo^{ΔLRR Z/ΔLRR Z} mice was impaired in response to several known activators of Cryopyrin *in vitro*, it is possible that these activators may activate protein(s) which directly interact(s) with the Cryopyrin LRR domain.

The chaperone heat shock protein 90 (Hsp90) and the co-chaperone-like, ubiquitin ligase-associated protein SGT1 have been shown to bind the LRR domain of Cryopyrin, which are essential for the function of Cryopyrin inflammasome (37). They maintain Cryopyrin in an inactive but signaling-competent state, and disassociate from Cryopyrin once activating signals are detected, therefore allowing conformational change of Cryopyrin that enables the interaction of Cryopyrin with other components such as ASC and pro-caspase-1. In the absence of Hsp90, Cryopyrin becomes unstable, and is degraded by the proteasome (37). Thus, if Cryopyrin is missing the LRR domain, the Cryopyrin inflammasome will lose function since Hsp90 and SGT1 cannot interact with Cryopyrin. In addition, Cryopyrin without LRR domain may be unstable, and therefore unable to form an inflammasome complex. This may explain why there was impaired IL-1β release in BMDMs of Cryo^{ΔLRR Z/ΔLRR Z} mice in response to MSU crystals and several other known activators of Cryopyrin *in vitro*. At present, we are not sure if this result is due to instability of the truncated Cryopyrin.

The LRR domain of human Cryopyrin has been shown to be alternatively spliced in the 3’ region of the gene, resulting in a large number of Cryopyrin forms of differing lengths and LRR composition (38). One of the most common alternative splice forms expressed in human leukocytes, lacks most of the LRR domain, which is similar to the

Cryo^{ΔLRR Z/ΔLRR Z} studied here in mice. It is still unclear whether these alternatively spliced forms are expressed at the protein level or if these forms have any unique function. It is also unclear if Cryopyrin alternative splicing occurs in mice. One model proposes that the LRR domain serves an inhibitory role in its native state by preventing inflammasome oligomerization. *In vitro* evidence suggests that expression of a truncated Cryopyrin results in a constitutively active Cryopyrin inflammasome (39,40), which could have a similar effect as the gain of function mutations observed in patients with Cryopyrinopathies. However, interpretation of these *in vitro* models may be limited. This is due to that three different components of inflammasome (Cryopyrin, ASC, caspase 1) and pro-IL-1β have to be simultaneously introduced into a cell line (which is normally a non-myeloid cell line such as 293 cells) via transfection. Efficiency of expression of all four components may be inconsistent from cell to cell with some cells expressing only some of the components due to the difficulty of simultaneous transfection of four different cDNA constructs. In addition, the expression level of each component is artificial, and may not truly reflect the endogenous level of each component in myeloid cells. These limitations prompted us to study the absence of the LRR domain in an *in vivo* mouse model.

In contrast, the Cryo^{ΔLRR Z/ΔLRR Z} mice are phenotypically normal, similar to the Cryo^{-Z/-Z} mice, and our *in vitro* mouse studies do not support the previous data observed in transfected human cell lines. Our data is more consistent with the LRR domain having a functional role in PAMP or DAMP sensing, a structural role in cryopyrin protein stability, or contributory role in inflammasome protein-protein interactions. The primary limitation of this mouse model is the construct design, which included a LacZ fusion in place of the LRR domain. This approach was chosen in order to confirm expression of the protein, and study tissue distribution of the ΔLRR form of cryopyrin. However, LacZ is a relatively large protein and evidence suggests that oligomerization of multiple cryopyrin monomers, adaptor proteins, and chaperone proteins is crucial to inflammasome function (24,37,40). Therefore, it is possible that the LacZ fusion product in the Cryo^{ΔLRR Z/ΔLRR Z} mice interferes with inflammasome oligomerization resulting in the observed null phenotype.

The role of LRR domain of Cryopyrin in MSU crystal-induced inflammatory responses suggested by our studies in Cryo^{ΔLRR Z/ΔLRR Z} mice makes it a potential drug target for gouty inflammation. Because the LRR domain of Cryopyrin has a potential to directly or indirectly engage with a vast array of structurally unrelated PAMPs or DAMPs to activate the Cryopyrin inflammasome leading to innate immune inflammatory responses, targeting at the LRR domain of Cryopyrin has a huge drug potential for host defense.

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Figure 1.

Schematic illustration of constructs used for generation of Cryopyrin LRR deletion mutant mice. A. Cryopyrin/NLRP3 gene with the deleted region (LRR domain). 50–200 bp homology boxes (uHB/dHB) upstream and downstream of the deleted region are PCR amplified. B. The homology boxes are ligated to the lacZ/Neo cassette and transformed into recombination proficient *E. coli* harboring a BAC containing the Cryopyrin/NLRP3 locus. The lacZ construct recombines with the BAC to make a targeting BAC, which is linearized and electroporated into ES cells where it recombines with a native NLRP3 allele. C. The Cryo^{ΔLRR Z} and Cryo^{-Z/-Z} alleles. D. Confirmation of Cryopyrin LRR deletion (Cryo^{ΔLRR Z/ΔLRR Z}) and Cryopyrin knockout (Cryo^{-Z/-Z}) RNA expression and E. protein expression. PCR was performed on cDNA from WT, Cryo^{ΔLRR Z/ΔLRR Z}, and Cryo^{-Z/-Z} BMDMs using exonic primer pairs for Exons 1–3, Exons 3–9, Exon 3 to lacZ, lacZ to lacZ, and GAPDH as described in the Methods. E. Confirmation of Cryopyrin LRR deletion (Cryo^{ΔLRR Z/ΔLRR Z}) protein expression from BMDM cell lysates on Western blot using an antibody to β-galactosidase (β-gal). The band size (molecular weight) is consistent with fusion protein consisting of truncated cryopyrin protein and β-gal.

Figure 2.

Effects of Cryopyrin deficiency on inflammatory responses to MSU crystals. BMDMs prepared from WT and Cryo^{-2/-2} mice were incubated with MSU crystals (0.5 mg/ml) for 18 h (A, B) and 2 h (C) under serum-free conditions, as described in the Methods. Conditioned media were assayed for cytokines IL-1 β (A) and CXCL1 (B) by ELISA, as described in the Methods. The percentages of BMDMs taking up the MSU crystals were estimated by flow cytometry based on increase in the side scatter profile (C). Subcutaneous air pouches with a synovium-like lining were created in mice via injections of sterile air, as described in the Methods. At 7 days after the first injection of air, a 1 ml suspension of 3 mg MSU crystals in PBS was injected into the air pouches. Mice were euthanized at the times indicated, and the air pouch exudates were harvested by washing with 5 ml of PBS containing 5 mM EDTA. The leukocyte counts were measured at each time point using a hemocytometer as described in the Methods, in the exudates of air pouches of WT and Cryo^{-2/-2} mice after injection with MSU crystals (3 mg) (D). Data shown in panel A, B and C are representative of 3 different experiments, using cells from ≥ 3 different mice of each genotype. * $p < 0.001$ relative to WT cells. Data shown in D are mean $\pm$ SD of 9 WT and 9 Cryo^{-2/-2} mice. # $p < 0.05$ relative to WT mice.

Figure 3.

Impaired inflammatory responses to MSU crystals in Cryo Δ LRR Z/ Δ LRR Z mice. BMDMs prepared from Cryo Δ LRR Z/ Δ LRR Z and the WT control mice were incubated with MSU crystals (0.5 mg/ml) for 18 h (A, B) under serum-free conditions, as described in the Methods. Conditioned media were assayed for cytokines IL-1 β (A) and CXCL1 (B) by ELISA, as described in the Methods. Subcutaneous air pouches with a synovium-like lining were created in mice via injections of sterile air, as described in the Methods. At 7 days after the first injection of air, a 1 ml suspension of 3 mg MSU crystals in PBS was injected into the air pouches. Mice were euthanized at the times indicated, and the air pouch exudates were harvested by washing with 5 ml of PBS containing 5 mM EDTA. The leukocyte counts were measured at each time point using a hemocytometer as described in the Methods, in the exudates of air pouches of WT and Cryo Δ LRR Z/ Δ LRR Z mice after injection with MSU crystals (3 mg) (C). IL-1 β production was measured by ELISA from the supernatants of air pouch exudates after cells were removed by sedimentation (D). Data shown in panel A, B and D are representative of 3 different experiments from ≥ 3 different mice of each genotype. * $p < 0.01$ relative to WT. Data shown in C are mean $\pm$ SD of 10 WT and 9 Cryo Δ LRR Z/ Δ LRR Z mice. # $p < 0.05$ relative to WT mice.

Figure 4.

Impaired caspase-1 activation and IL-1 β cleavage in response to MSU crystals in BMDMs of Cryo ^{Δ LRR Z/ Δ LRR Z} and Cryo^{-Z/-Z} mice *in vitro*. BMDMs prepared from Cryo ^{Δ LRR Z/ Δ LRR Z}, Cryo^{-Z/-Z} and the WT control mice were incubated with MSU crystals (0.5 mg/ml) for 18 h under serum-free conditions, as described in the Methods. Conditioned media were subjected to Western blot analysis with antibodies to caspase-1 and IL-1 β .

Figure 5. Impaired IL-1 β release in response to several other stimuli known to activate cryopyrin inflammasome in Cryo Δ LRR Z/ Δ LRR Z BMDMs *in vitro*. BMDMs prepared from Cryo Δ LRR Z/ Δ LRR Z, Cryo $^{-Z/-Z}$ and the WT control mice were incubated with PGN (2 μ g/ml), bacterial RNA (1 μ g/ml), R837 (5 μ g/ml) and cLPS (1 μ g/ml) for 18 h under serum-free conditions. Conditioned media were assayed for IL-1 β release by ELISA. *, #, **, ## p<0.05 relative to WT.