NLRP3/cryopyrin is necessary for IL-\(\beta\) release in response to hyaluronan, an endogenous trigger of inflammation in response to injury

Kenshi Yamasaki‡, Jun Muto‡, Kristen R. Taylor‡, Anna L. Cogen‡, David Audish‡, John Bertin#, Ethan P. Grant#, Anthony J. Coyle#, Amirhossein Misaghi*, Hal M. Hoffman*†, Richard L. Gallo‡.

From the ‡Division of Dermatology, University of California, San Diego and VA San Diego Health Care System, San Diego, California 92161, USA, *Department of Pediatrics, University of California San Diego, California 92161, USA, †Ludwig Institute of Cancer Research, California 92161, USA, #Formerly at Millennium Pharmaceuticals, Cambridge, Massachusetts 02139, USA.

Running title: HA activation of inflammasome through CD44.

Address correspondence to: Richard L. Gallo, MC 9111B, 3350 La Jolla Village Drive, San Diego, California 92161, Tel: (858) 642-3504, Fax: (858) 642-2435, E-mail: rgallo@ucsd.edu

Inflammation under sterile conditions is a key event in autoimmunity and following trauma. Hyaluronan, a glycosaminoglycan released from the extracellular matrix after injury, acts as an endogenous signal of trauma and can trigger chemokine release in injured tissue. Here we investigated if NLRP3/cryopyrin, a component of the inflammasome, participates in the inflammatory response to injury or the cytokine response to hyaluronan. Mice with a targeted deletion in cryopyrin showed a normal increase in Cxcl2 in response to sterile injuries but had decreased inflammation and release of IL-\(\beta\). Similarly, addition of hyaluronan to macrophages derived from cryopyrin-deficient mice increased release of Cxcl2 but did not increase IL-\(\beta\) release. To define the mechanism of hyaluronan mediated activation of cryopyrin, elements of the hyaluronan recognition process were studied in detail. IL-\(\beta\) release was inhibited in peritoneal macrophages derived from CD44-deficient mice, in a MH-S macrophage cell line treated with antibodies to CD44, or by inhibitors of lysosome function. The requirement for CD44 binding and hyaluronan internalization could be bypassed by intracellular administration of hyaluronan oligosaccharides (10 – 18 mer) in LPS-primed macrophages. Therefore, the action of CD44 and subsequent hyaluronan catabolism trigger the intracellular cryopyrin ~ IL-\(\beta\) pathway. These findings support the hypothesis that hyaluronan works through IL-\(\beta\) and the cryopyrin system to signal sterile inflammation.

Inflammation, as defined by changes in vascular permeability and leukocyte recruitment, is an essential step for the control of microbial invasion. Specific microbial products trigger this process through a diverse array of innate immune pattern recognition receptors. However, an inflammatory response independent of infection is also an important process for maintenance of biological homeostasis. For example, normal wound healing requires a controlled inflammatory response to enable the recruitment of monocytes and the release of growth factors required for repair. This response can occur in the absence of microbial stimuli. Furthermore, inflammation and the release of proinflammatory mediators is also associated with many diseases such as rheumatoid arthritis and Crohn’s disease (1). These diseases are not well understood in terms of their triggers, but rather are described by the subsequent release of proinflammatory mediators. Identification of the triggers of sterile inflammation represents an important goal with immediate diagnostic and therapeutic significance.

Recent work has begun to elucidate pathways of inflammation that occur in the absence of microbial stimuli. Stress signals such as heat-shock proteins, intracellular components of necrotic cells not normally seen by immune cells, and components of the extracellular matrix have all been implicated as endogenous triggers of injury (2-4). Among this group is the glycosaminoglycan hyaluronan (HA)¹, an
important structural component of the extracellular matrix that is also a common component of bacterial surfaces. HA is synthesized at the cell surface and typically exists as a high molecular weight polymer greater than 10^6 Da and composed of repeating disaccharide units of N-acetylglucosamine and glucuronic acid (5,6). Unlike other glycosaminoglycans such as heparan sulfate or chondroitin sulfates that encode specific activity by use of a diverse disaccharide sequence, HA is not sulfated or epimerized and only changes in HA size, concentration, and location affect function.

We have previously developed murine models of sterile injury to identify the innate elements that recognize and mediate sterile inflammation (7). Our results demonstrated that a) the initiation of a sterile intrinsic inflammatory process is dependent on TLR4 activation, b) sterile injury induces HA accumulation at the injured site, and c) sterile intrinsic inflammation resembles signaling events that are activated by HA. Furthermore, we have defined a novel alternative recognition complex for HA that involves TLR4, MD-2 and CD44 (7). Taken together with other work associating HA and innate pattern recognition (4,8-10), these observations have provided new insight into mechanisms responsible for sterile inflammation.

Recently, the NLR (Nucleotide-binding domain and Leucine rich Repeat containing) family has been extensively analyzed as a group of intracellular pattern recognition receptors (11). NLRs have a leucine rich repeat (LRR) that recognize pathogen-associated molecular patterns (PAMPs) including bacterial cell wall components and viral nucleic acids. NOD2 and NLRP3/cryopyrin are two of the best characterized NLRs. NOD2 recognizes the bacterial peptidoglycan-derived molecule muramyl dipeptide (MDP) and activates the NF-kB pathway to induce inflammatory responses (12). Mutations of the NOD2 gene were identified in individuals with familial cold autoinflammatory syndrome (FCAS), Muckle-Wells syndrome (MWS), and neonatal onset multisystem inflammatory disease (NOMID) (18-20). These individuals have recurrent or chronic inflammatory symptoms, including fever, arthritis, and a urticaria-like eruption characterized by neutrophilic infiltration. In FCAS, symptoms can be elicited by cold provocation by a mechanism that appears to be mediated through the skin (15,21).

Since disorders associated with mutations in NLRP3 are examples of inflammation under sterile conditions, and HA has been shown to be a trigger of sterile inflammation, we sought to further understand the mechanism of the response to HA by examining the role of cryopyrin during injury and after exposure to HA. Our results show cryopyrin and IL-1β are integral to sterile inflammation and the response to HA. These observations provide new insight into the function of HA as a ‘danger signal’ of injury.

**EXPERIMENTAL PROCEDURES**

**Cells, media and reagents** — The mouse alveolar macrophage cell line MH-S was purchased from American Type Culture Collection (ATCC, catalog CRL-2019). Cells were maintained in RPMI1640 media supplemented with L-glutamine, 10% heat-inactivated fetal calf serum (FCS), penicillin/streptomycin (100 units/ml and 50 mg/ml, respectively) and 50 µM 2-mercaptoethanol.

Human umbilical cord hyaluronan was purchased from Sigma-Aldrich (St. Louis, MO). HA preparations were free of DNA and protein contamination as preparations showed no absorbance at 260 nm and 280 nm. In addition, to ensure purity, 1 ml HA batches were boiled for one hour then run on two successive endotoxin-removal columns (Associates of Cape Cod Inc., East Falmouth, MA) to remove potential endotoxin contamination. Short HA oligosaccharides (4 – 18 mer, 0.777 – 3.5 kDa) were a generous gift from Dr. Paul L. DeAngelis at the University of Oklahoma. Hyalgan™, rooster comb HA, was purchased from Fidia.
Farmaceutici, Italy. LPS (from *E. coli* K12 D31m4 (RE), catalogue #302) was obtained from LISP Biologics, Inc. (Campbell, CA). Amirolide hydrochloride hydrate, bafilomycin A1, and chloroquine diphosphosphate were obtained from Sigma. Rat anti-mouse CD44 monoclonal antibodies KM114 and IM7 were obtained from BD Biosciences and Santa Cruz, respectively.

**In vitro cell stimulation and sample collection (MH-S Cells)** — MH-S cells were grown to confluence in a 96-well flat bottom plate (Corning Incorporated Life Sciences, Lowell, MA). For experiments, media was removed from cells and replaced with media containing either HA or LPS at the indicated concentrations. All stimulations were done in low serum media (1% FCS). In assays with chemical inhibitors and antibodies, cells were incubated with inhibitors or antibodies for 30 min and then media replaced with media containing HA and inhibitors. Cells were allowed to incubate for 18 h, media were then collected and spun at 1,000 g for 10 min at 4 °C to remove any debris. Cell media were stored at –20 °C until analysis. RNA was extracted from adherent cells after supernatant collection using TRIzol reagent (Invitrogen, Carlsbad, CA). RNA was stored at –80 °C.

**Macrophage collection and stimulation in vitro** — Mice were injected intraperitoneally with 3 ml of 5% thioglycolate. After 3 days, macrophages were collected through intraperitoneal lavage of 10 ml of RPMI1640 media. Collected cells were counted, and plated in RPMI1640 media supplemented with L-glutamine, 10% FCS, penicillin/streptomycin (100 units/ml and 50 mg/ml, respectively) and 50 μM 2-mercaptoethanol at a density of 5×10^5 cells/well in 96-well plates. Cells were allowed to recover for 24 h. After recovering, cells were stimulated for 18 h at 37 °C with designated concentration of HA, and supernatants and RNA were collected as previously described.

**Models of skin sterile inflammation** — Cryopyrin-deficient mouse (Nlrp3−/−) were generated as previously described (22) and backcrossed 10 generations to a C57/B6 background. Tlr4−/− (C3H/HeJ), Tlr4+/+ controls, CD44-deficient mouse (Cd44^−/−, Cd44^+/+), and Cd44^+/+ controls (B6129SF2/J) were all purchased from Jackson Laboratories. For bead injection, 0.5 mg Cytodex™ microcarrier beads (Sigma-Aldrich) were placed in 25 ml sterile endotoxin-free PBS, maintained in sterile conditions, and autoclaved. Mice were anesthetized with isoflurane and fur was plucked from the back area and wiped with an ethanol pad. Two hundred fifty μl Cytodex™ bead slurry was injected subcutaneously into the backspace using a sterile 25-gauge needle and sterile 1 ml syringe, creating a large bubble in the skin. For the liquid nitrogen sterile inflammation model, a CRY-AC liquid nitrogen dispenser with a 1 mm nozzle (Brymill Cryogenic Systems, Ellington, CT) was used to administer a steady liquid nitrogen 1 cm diameter circle to the mouse back for 15 s. This provided a frozen injury that was kept frozen for a full 60 s. After 48 h, mice were euthanized by over-anesthesia with halothane and an 8-mm punch biopsy was used to isolate either injured or non-injured skin regions on the backs of the mice. The skin sections were placed in tube with 500 ml 1× radioimmune precipitation assay (RIPA) buffer (50 mM HEPES, 150 mM NaCl, 0.05% SDS, 0.25% deoxycholate, 0.5% NP-40, pH 7.4) with protease inhibitor mixture (Complete™ EDTA-free; Roche, Indianapolis, IN), and were beaten with 2.4 mm Zirconia beads by a mini-beadbeater apparatus (BioSpec Products, Inc., Bartlesville, OK) for 50 s on full speed. Extracts were then sonicated for 5 min in ice-cold water and spun down at 12,000 g for 10 min at 4 °C. Supernatant was removed and kept at –20 °C until analysis. Animal procedures were approved by UCSD and the Veterans Affairs San Diego Healthcare System subcommittee on animal studies.

**ELISA** — Mouse IL-1β and Cxcl2/MIP-2 in mouse skin and cultured media of MH-S cells and macrophages were measured by IL-1β and MIP-2 ELISA Duo set (R&D Systems) according to the manufacturer’s instructions.

**Quantitative RT-PCR** — Real-time PCR was used to determine the induction of IL-1β and Cxcl2 mRNA following sterile injury and HA stimulation. cDNA was synthesized from RNA by the iScript cDNA Synthesis Kit (BioRad) as described by the manufacture’s protocol. TaqMan Gene Expression Assays (Applied Biosystems)
were used to analyze expression of *Il1b* (assay ID: Mm00434228-m1) and *Cxcl2* (assay ID: Mm00436450-m1) as described by the manufacturer instruction (the user bulletin #2 by Applied Biosystems). *Gapdh* mRNA was used as an internal control to validate RNA for each sample. *Il1b* and *Cxcl2* mRNA were calculated as relative expression to *Gapdh* mRNA, and all data are presented as normalized data against each control (mean of non-treated skin or non-stimulated cells).

**Endosome/Lysosome acidification** — Endosome/lysosome acidification was monitored by LysoSensor™ Green DND-189 (Invitrogen), which is a pH-sensitive dye and fluoresces green in acidic compartments (pKa = 5.2). MH-S cells and macrophages were cultured in 8-well chamber slides (Nunc International) for 24 h. One µM of LysoSensor™ was added to culture media for 30 min. After washing with PBS once, cells were treated with 25 µg/ml HA for designated time at 37°C. Media were removed, and cells were fixed with Prolong anti-fade reagent (Invitrogen) and a cover slip. Fluorescence was observed using an Olympus BX41 fluorescent microscope (Scientific Instrument Company, Temecula, California).

**RNA interference** — Hyaluronidase specific siRNAs and mock siRNA (siGLO) were obtained from Dharmacon, Inc (Chicago, IL). Transfection of siRNAs were performed with siLentFect™ transfection reagent (BioRad). MH-S cells were cultured for 48 h, and replaced with 5% FCS containing media. siRNAs (final concentration at 10 nM) with transfection agent were added every 24 h up to 72 h (total of three transfection). In the case of both Hyal1 and Hyal2 siRNA treatment, 5 nM each of siRNA (total 10 nM in final concentration) was used for transfection. Then the medium was replaced with 100 µl of low serum media (1% FCS) containing HA. Culture media was collected 18 h after HA stimuli for cytokine ELISA.

**Fluorescein-labeling of Hyaluronan** — HA was labeled with Fluorescein-amine, isomer 1 (Sigma-Aldrich) as previously described (23). HA (50 mg) was dissolved in 40 ml water for 12 hr. The hyaluronan solution was then diluted in 20 ml of dimethyl sulphoxide (DMSO). The HA solution was reacted with fluorescein amine (25 mg), acetaldehyde (25 µl) and cyclohexyl isocyanide (25 µl) dissolved in a 0.5 ml of DMSO at 22 °C for 5 h with continuous stirring. The product was precipitated in 240 ml of ethanol saturated with sodium chloride. The precipitate was allowed to sediment, and the supernatant was decanted. The precipitation was repeated until the product was free from non-bound fluorescein. This fluorescein-labeling method conjugates fluorescein in carboxyl hydroxy group of N-acetyl-D-glucosamine (GlcNAc) of hyaluronan. Thus, hyaluronidase and chondroitinase digestion does not interfere the fluorescein conjugated to GlcNAc. The fluorescein-labeled HA (fl-HA) was dialyzed against distilled water with 1 kDa cutoff filter for further experiments and in some cases further defined by dialysis first through 30 kDa dialysis membrane and then collected in the retentate of 10 kDa dialysis (Microcon™, Millipore).

**HA internalization** — MH-S cells and peritoneal macrophages from wt and *Cd44−/−* mice were cultured in 8-well chamber slides (Nunc International) for 24 h. Fluorescein-labeled HA (fl-HA) was added to culture media for indicated time. After washing with PBS thrice, cells were fixed with ProLong™ Gold antifade reagent with DAPI (Invitrogen) and a cover slip. Fluorescence was observed using an Olympus BX41 fluorescent microscope (Scientific Instrument Company, Temecula, California) or a Zeiss LSM 510 confocal microscope (Carl Zeiss, Inc.).

**HA stimulation by lipofection** — DOTAP Liposomal Transfection Reagent was obtained from Roche and used according to manufacturer instruction. Briefly, 2.5 µg of HA oligosaccharides were incubated with 3 µl of DOTAP for 30 min in a final volume of 15 µl serum free media. After incubation, 100 µl of low serum media (1% FCS) was added. MH-S cells were stimulated with 0.2 µg/ml of LPS in low serum media (1% FCS) for 3 h prior to HA oligosaccharides stimulation. After LPS-priming, media were replaced with the HA oligosaccharides and DOTAP-containing media. Culture media was collected 18 h after treatment for cytokine ELISA.
**Statistical Analysis** — Results are expressed as the mean ± SEM and are representative of at least three separate experiments. One-way ANOVA was used to determine significance, which was analyzed by GraphPad Prism 4 (GraphPad Software, Inc.), unless otherwise stated. Two-way ANOVA was used to determine significance in the experiment of dose-dependent IL-1β and Cxcl2 secretion by HA.

**RESULTS**

**Sterile injury induces inflammation through Cryopyrin and IL-1β.** Tissue injury results in an inflammatory response. Models of skin injury that lack exposure to microbes, such as freezing or disruption of the matrix by subcutaneous sterile bead injection, induce the release of fragments of hyaluronan (HA) that in turn stimulates a TLR4 dependent increase in CXCL2 chemokines (7). To further examine other pathways that may be involved in the tissue response to injury, we examined the response of cryopyrin-deficient mice (Nlrp3–/–) to sterile injury in two model systems. Following sterile injury by either liquid nitrogen freezing or dermal disruption through injection of sterile beads, cryopyrin null mice showed less skin erythema than control animals (Figure 1a). Because cryopyrin could potentially influence this phenotype through its action on IL-1β release (22,24,25), we measured the abundance of IL-1β in the lesional skin of these animals and observed there was no increase in IL-1β in cryopyrin null mice as compared to controls, whereas similar increases in Cxcl2/MIP-2 were observed in both mice (Figure 1b, c). This selective lack in IL-1β increase but not Cxcl2/MIP-2 in cryopyrin null mice contrasts with Tlr4–/– mice that have much less IL-1β (Figure 1d) and, as previously reported, Cxcl2/MIP-2 mRNA in both wt and cryopyrin null mice though the magnitude of this response was slightly lower in cryopyrin null mice (Figure 2e, f). In contrast to the cryopyrin null mice, IL-1β release and transcription were suppressed in macrophages derived from Tlr4-deficient mice (Figure 2g, h). These data again show that HA induces the release of Cxcl2/MIP-2 and IL-1β by different mechanisms, and that transcriptional control was not dependent on cryopyrin but on TLR4.

**CD44 is required for HA-dependent IL-1β release.** CD44 binds HA and associates with TLR4 for HA-dependent Cxcl2/MIP-2 induction (7). Therefore, to further understand the mechanism of IL-1β release in response to HA, the role of CD44 was studied. CD44-deficient macrophages showed significantly less IL-1β release following HA stimulation (Figure 3a, P < 0.001). A similar result was observed in the MH-S macrophage cell line pretreated with anti-CD44 antibodies (Figure 3b, P < 0.01), demonstrating that CD44 is involved in release of both IL-1β and Cxcl2/MIP-2.

**Involvement of the lysosome and hyaluronidase in IL-1β release by HA.** Following binding to CD44, this cell surface receptor can act to facilitate endocytosis to the lysosome (27-30). Lysosomal function is thought to play a role in the intracellular processing and degradation of HA (31), an event that may be important to the response to HA. HA internalization in MH-S cells could be directly visualized by uptake of fluorescein-labeled HA (fl-
HA) (Figure 4a). The uptake of HA increased in a time-dependent manner up to 24 h, and much less fl-HA uptake was observed in macrophages from \textit{Cd44}−/− mice (Figure 4b). To examine if these events are important to the IL-1β response, MH-S cells exposed to HA were examined with Lysosensor™, a marker that senses aciditrophic compartments, such as lysosomes and endosomes (pKa = 5.2). Aciditrophic compartments were detected within 2 h of exposure to HA (Figure 3c). HA also enabled detection of acidic intracellular compartments in peritoneal macrophages from wt mice, but much less in CD44-deficient macrophages (Figure 3d).

Next, cells were treated with pharmacological inhibitors of lysosome function. These agents included NH$_4$Cl, which inhibits lysosome activation and disrupts hyaluronidase activity (27,29), Bafilomycin A1, a specific inhibitor of vacuolar type H$^+$–ATPase (V-ATPase) that inhibits acidification of lysosomes/endosomes and maturation of lysosomal proteases including cathepsins (32,33), and chloroquine, a lysosomotropic agent (27,29,34). Each of these inhibitors of lysosome activation significantly suppressed IL-1β release by HA (Figure 3e). In contrast, use of amiloride to inhibit the plasma membrane Na$^+$–H$^+$ exchanger (NHE1) and inhibit cytoplasmic acidification (31) did not suppress IL-1β release by HA, confirming specific involvement of lysosome function in IL-1β release by HA (Figure 3e). HA increased IL-1β mRNA transcription more than 20-fold in the presence of these inhibitors, suggesting inhibition of lysosome function does not block induction of transcription (Figure 3f). In addition, as lysosomal function can influence hyaluronidase activity and the subsequent processing of HA after binding to CD44, we employed an RNA interference technique to test the involvement of hyaluronidase in IL-1β release. Treatment with siRNA for Hyal 1 and Hyal2 suppressed Hyal gene expression to less than 20% of untreated cells (Figure 3h, i). The combination of Hyal1 and Hyal2 siRNA significantly suppressed HA-dependent IL-1β release, and Hyal1 siRNA or Hyal2 siRNA alone was not enough to suppress IL-1β release (Figure 3g). Neither of these siRNAs affected IL-1β transcription (data not shown). The results suggest that Hyal1 and Hyal2 are involved in the post-transcriptional events leading to IL-1β release by HA. The lack of an effect of single knock down of Hyal1 or Hyal2 also suggests that there is redundancy between Hyal1 and Hyal2 function on the process. These data show that CD44 is involved in HA-dependent endocytosis and that lysosomal function and hyaluronidase activity is essential to IL-1β release.

**Intracellular HA induces IL-1β release from LPS-primed macrophages.** The size of HA is crucial in its biological function (35), and processing to small HA is dependent on hyaluronidase activity on both the cell surface and intracellularly. HA fragments in the 200 – 500 kDa range induce inflammatory cytokines, however, smaller HA fragments less than 100 kDa have been reported to show different effects on host cells (36-41). Therefore, we next tested if smaller extracellular HA fragments induce IL-1β release. The activity of short HA oligosaccarides (4 – 18 mer, 0.777 – 3.5 kDa) and isolated large HA, Hyalgan™ (size from 500 to 730 kDa), were compared to the purified HA preparations used previously that contained HA at wide range of sizes up to 500 kDa (42). The size-purified preparations were used at thrice the concentration used for the mixed reagent (100 µM, 77.7 µg/ml of 4 – 18 mer oligosaccharide and 350 µg/ml of 18 mer oligosaccharide). Neither preparation induced IL-1β release, although the 18-mer oligosaccharides did induce Cxcl2/MIP-2 (Figure 5a, b). Hyalgan™, up to 50 µg/ml, induced neither IL-1β nor Cxcl2/MIP-2 mRNA and protein in the media (data not shown). Furthermore, digested HA less than 10 kDa was minimally internalized in MH-S (Supplementary Figure 2a), a finding consistent with the limited ability of small HA fragments to induce IL-1β.

Given our previous results that indicated that intracellular uptake and processing of HA is necessary for IL-1β response, and prior reports that HA, when digested by hyaluronidases intracellularly, results in a smaller size (less than 20 kDa) (29,31,43), we next tested if small HA oligosaccharides could induce IL-1β release if introduced to the intracellular space. HA oligosaccharides were added intracellularly using the cationic transfectant DOTAP™. MH-S cells were treated with LPS at low concentration to induce transcription of IL-1β mRNA without inducing the release of IL-1β protein. When
introduced to the intracellular space, small HA oligosaccharides significantly increased IL-1β release from LPS-treated cells (Figure 5c). Introduction of small HA oligosaccharides to intracellular space did not influence the appearance of intracellular aciditrophic compartments as detected by Lysosensor (Supplementary Figure 2b). This amount of HA oligosaccharides did not induce IL-1β release when added only to the extracellular space (DOTAP–) (Figure 5c). In contrast to IL-1β release, the small HA oligosaccharides when present in the extracellular space could induce Cxcl2/MIP-2 in the presence of low concentrations of LPS (Figure 5d).

**DISCUSSION**

The results presented here show that inflammation in vivo following sterile injury is partially dependent on the presence of functional cryopyrin, and associated with the release of IL-1β. We also show here in model cell culture systems that the response to HA mimics observations in mice, and that the macrophage IL-1β response is also dependent on cryopyrin. Furthermore, this response involves CD44, the lysosome, and HA metabolism by hyaluronidases. When combined with prior studies of the function of the inflammasome (7,11) these observations suggest a possible model whereby CD44 serves to trigger and direct the cellular response to HA (Figure 6). Deposition of HA after sterile injury can be a ‘danger signal’ leading to the induction of IL-1β and other cytokines, including Cxcl2/MIP-2. The response to HA may modify the response to microbial stimuli such as those detected by the leucine rich repeats (LRR) of cryopyrin (44-46). Taken together, our observations suggest the HA recognition response through cryopyrin is integral to sterile inflammation.

Numerous physiological changes such as injury, shock (47,48), heat (49), and cold (7) increase HA concentration locally and systematically. HA fragment sizes are altered in these conditions, and altered sizes of HA show different effects on host cells. HA fragments ranging from 200 – 250 kDa induce inflammatory cytokines (36-41). HA oligosaccharides from 6 – 20-mers have angiogenic properties through endothelial cell proliferation and migration (50-53). Here, we have shown that intracellular and extracellular HA oligosaccharides ranging from 10 – 18-mers have different effects on IL-1β release and Cxcl2 chemokine release. Small intracellular HA oligosaccharides, but not extracellular HA oligosaccharides, induced IL-1β release in the presence of TLR4 activation. On the other hand, Cxcl2 chemokines such as MIP-2 could be induced by extracellular HA. Mixed preparations of HA could activate both IL-1β and Cxcl2/MIP-2 when applied extracellularly, and this response involved the action of both TLR4 and CD44, although further studies are required to see if other HA receptors such as RHAMM (54) and LYVE1 (55) are also involved in this process. Our data suggest that extracellular high molecular weight HA binds to CD44, is internalized and degraded within the cell, and that these HA fragments are capable of interacting with cryopyrin and inducing IL-1β (31,43). Thus, altered sizes of intracellular and extracellular HA stimulate host cells differently.

A model of HA engagement to CD44 and eventual digestion by hyaluronidases has been proposed as a mechanism of HA degradation (31,43). Hyaluronidase-1, -2, and -3 (Hyal-1, -2, and -3) are somatically expressed. Hyal-1 is a lysosomal enzyme, which is active in acidic conditions (56,57). Hyal-2 is also an acid-active enzyme, which is linked to the plasma membrane by glycosylphosphatidylinositol (58-61). The extracellular high molecular weight HA is cleaved by Hyal-2 to 20-kDa fragments, followed by internalization to deliver the HA fragments to lysosomes through endosomes. In the acidified lysosomes, Hyal-1 degrades 20-kDa HA fragments to even smaller fragments that are predominantly tetrasaccharides (43). However, the current results from Hyal1 and Hyal2 siRNA experiments suggest there is also redundancy between Hyal1 and Hyal2 function in HA metabolism. The lack of an effect seen with functional suppression of only one Hyaluronidase might be explained by compensation by the other Hyaluronidase. Supporting this are preliminary observations showing that both Hyal1 and Hyal2 can both localize at the cell membrane and in cytosol although under normal conditions Hyal1 expresses predominantly in the cytosol and Hyal2 localize mostly at the cell membrane. Furthermore, Hyal1 can be detected extracellularly in serum.
Therefore, it is reasonable to conclude that Hyal1 can localize at the cell membrane and possibly compensate for loss of Hyal2. Further studies are required to better define this catabolism model, but our results clearly show that intracellular HA catabolism and sensing of HA oligosaccharides by cryopyrin is a trigger of IL-1β release and inflammation.

NLR molecules contain a LRR domain and are predicated to function as pattern recognition receptors (11), much like the membrane associated TLRs (62,63). Because of their intracellular localization NLRs may sense digested molecules of pathogen-related structures, including the peptidoglycan-derived molecules meso-diaminopimelic acid and muramyl dipeptide that have been shown to stimulate NOD1 and NOD2, respectively (12,64,65). Now, we show that HA induces release of IL-1β in a cryopyrin-dependent manner. This requires both IL-1β mRNA transcription through TLR4 signaling and cryopyrin/inflammasome activation by endocytosed and digested HA (Figure 6). CD44 is a key factor in this mechanism for both mRNA transcription and cryopyrin/inflammasome activation, because 1) HA-CD44 can bind to and activate TLR4 signaling (7) and 2) CD44 is endocytic receptor for HA catabolism (43). An intermediate size of HA is required for efficient HA endocytosis though molecular structure analysis showed 8–10 mer HA oligosaccharides are minimum for CD44 binding (66). Blocking of HA endocytosis and suppression of hyaluronidase Hyal1 and Hyal2 reduced HA-dependent IL-1β release, also suggesting HA catabolism is necessary for the activation of intracellular NLRP3/inflammasome. Furthermore, a requirement of intracellular HA oligosaccharides was shown by DOTAP-mediated intracellular administration of HA oligos in LPS-primed cells. It is not currently clear why extracellular HA oligos, which are supposed to be of sufficient length for CD44 binding, cannot induce IL-1β mRNA transcription, and why smaller digested HA is not efficiently endocytosed. The HA-size might affect the nature of the receptor complex, such as CD44-TLR2/TLR4, and the recruitment of adaptor molecules required for endocytosis. Further evaluation of hyaluronidase function in HA endocytosis is required to address this issue in the future, but our data show for the first time that HA catabolism and intracellular HA oligosaccharides are required to activate the cryopyrin/inflammasome process.

IL-1β processing by caspase-1 takes place in secretory lysosomes with phospholipase involvement (67). HA stimulation might also activate an alternative IL-1β release mechanism. The secretory lysosome model is only one of the models of IL-1β secretion (68), and others are accumulation of IL-1β in microdomains of the membrane (69), directly passing through the membrane (70), and in a multivesicular body of an exosome (71). These secretory models are could be also co-activated in IL-1β release by HA, and remain for future evaluation.

HA is not only produced by the host and a signal of tissue injury. HA is also expressed on the extracellular surface of microbes. Group A Streptococcus (GAS) produces HA on its capsule, possibly to mimic a mammalian host. Phagocytic cells, including macrophages, phagocytose GAS and digest them. Thus, once phagocytosed and degraded, bacterial HA may also stimulate cryopyrin and induce IL-1β release. Recent reports have shown that the extracellular or intracellular localization of the pathogen distinguishes its capacity to stimulate host cells. For example, intracellular flagellin directly stimulates Ipaf, one of the intracellular caspase activators, independent of TLR5 that is an extracellular receptor for flagellin (72-74). Our findings showed mixed extracellular large molecular weight HA or intracellular small HA oligosaccharides, but not extracellular small HA oligosaccharides, induced IL-1β release. These suggest that localization of HA along with its size dictate function.

We again show here that HA can be a danger signal in initiating an inflammatory reaction. CD44, at least partially, is involved in this induction of cytokines in sterile injury. Our model focuses on the situation of acute skin inflammation, in which preexisting HA can be degraded and can induce inflammation. However, in another model of tissue inflammation, bleomycin-induced inflammation in lung, HA has been implicated in more chronic inflammation. In contrast to acute injury where HA degradation only takes place initially, a chronic inflammatory process may set for further deposition of extracellular matrix molecules including HA (75).
Here, mice lacking CD44 fail to clear HA, and show prolonged and massive deposition of HA in such a bleomycin model. This prolonged deposition of HA might activate other HA receptors such as RHAMM that promotes inflammatory cell migration (54) and LYVE1 that induces lymphatic vessel migration (55). Therefore, further comparison of our acute sterile injury model and the bleomycin model might reveal altered functions and mechanisms of HA in different phases of inflammation. Further studies are required to examine the distinct functions of HA and CD44 in acute and chronic inflammation.

The mutations observed in individuals with FCAS, MWS, and NOMID are thought to be gain-of-function mutations. The urticaria-like dermatoses observed in FCAS are induced by cold provocation, although the mediators that sense cold and provoke this inflammatory reaction are not well known. Our cold sterile injury model induces HA deposition at the injured site and HA induces IL-1β and Cxcl2/MIP-2 (7). This IL-1β release by HA is dependent on cryopyrin. Therefore, HA may be a mediator of cold-induced inflammation in individuals with FCAS, in which mutated cryopyrin is activated by stimuli that are not sensed by non-mutant cryopyrin. These findings could help to understand the mechanisms involved in FCAS and other cold-induced inflammatory disorders.

Taken together, our data show that HA is involved in systems that sense the tissue environment. HA associates with CD44 and stimulates pattern recognition receptors on the cell surface such as TLR4, and within the cell such as cryopyrin. Mechanisms of HA catabolism, such as endocytosis and hyaluronidase activity, are necessary for stimulation of intracellular cryopyrin and inflammasome processing to release IL-1β. Cxcl2 chemokine response to HA is dependent on different events. Therefore, the regulation of HA catabolism and complex recognition of HA oligosaccharides may be key variables that modify the host inflammatory response during injury and during a variety of autoimmune disorders.

REFERENCES


**FOOTNOTES**

This work was supported by the grant from NIH P01-HL057345 to R.L.G. The abbreviations used are:

HA, hyaluronan; LPS, lipopolysaccharide; GAG, glycosaminoglycan; TLR, Toll-like receptor; NLRP3, NLR (nucleotide-binding domain and leucine rich repeat containing) family, pyrin containing 3; IL-1β, interleukin 1 beta; MIP-2, macrophage inflammatory protein-2; FCAS, familial cold autoinflammatory syndrome; MWS, Muckle-Wells syndrome; CINCA, Chronic infantile neurological cutaneous and articular; V-ATPase, vacuolar type H+-ATPase; NHE1, Na+-H+ exchanger 1; LRR, leucine-rich repeat; PAMP, pathogen-associated molecule pattern; GAS, group A *streptococcus*, PBS, phosphate-buffered saline; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl) ethyl]glycine; TBS, Tris-buffered saline; Mβ-CD, Methyl-β-cyclodextrin; RNAi, RNA interference; siRNA, small interference RNA: fl-HA, fluorescein-labeled hyaluronan.

**FIGURE LEGENDS**

**Figure 1: Sterile injury induced less IL-1β in cryopyrin null mouse.**

(a): Cryopyrin null (*Nlrp3–/–*) and Wt mouse were injured by liquid nitrogen or sterile bead-injection as described in methods and skin erythema monitored. Representative images of the skin surface 48 h after injury from three independent experiments are shown.

(b, c): Injured skin of Cryopyrin null (ko) and wt mouse was excised, and IL-1β (b) and Cxcl2/MIP-2 (c) measured by ELISA. Bars indicate mean of each group. **: \( P < 0.01 \).

(d): TLR4 null (ko) and wt mouse were injured by liquid nitrogen or sterile bead-injection. Skin lesions were excised and IL-1β measured by ELISA. Bars indicate mean of each group. *: \( P < 0.05 \), **: \( P < 0.01 \).

**Figure 2: IL-1β release by HA depends on cryopyrin.**

(a, b): Peritoneal macrophages from cryopyrin null (*Nlrp3–/–*) and Wt control mice were treated with HA (25 µg/ml) or LPS (25 µg/ml) for 18 h, and IL-1β (a) and Cxcl2/MIP-2 (b) in cultured media measured by ELISA. Mean and SEM are shown. Wt: gray bars, cryopyrin null (*Nlrp3–/–*): black bars. Statistical analyses were done to compare *Nlrp3–/–* and Wt in each stimulus. \(**: \ P < 0.001\).

(c – f): Peritoneal macrophages from cryopyrin null (*Nlrp3–/–*) and Wt control mice were treated with various concentrations of HA for 18 h. IL-1β (c) and Cxcl2/MIP-2 (d) release in cultured media was measured by ELISA. mRNA abundance for *Il1b* (e) and *Cxcl2* (f) was measured 18 h after exposure to various doses of HA by quantitative RT-PCR. Quantitative RT-PCR data are shown as relative expression.
compared to control untreated Wt peritoneal macrophages. Statistical analyses were done to compare Nlrp3^{−/−} and Wt in each concentration. *: P < 0.05, **: P < 0.01.

Figure 3: CD44 affects IL-1β release in response to HA.

(a): Peritoneal macrophages from CD44-deficient mice and Wt mice were treated with HA (25 µg/ml) for 18 h, and IL-1β in cultured media were measured by ELISA. Mean ± SEM are shown.

(b): MH-S macrophage cell line was pretreated with rat anti-mouse CD44 monoclonal antibodies KM114 and IM7, or control rat IgG (10 µg/ml each) for 30 min. MH-S cells were then exposed to 25 µg/ml of HA for 18 h in the presence of antibodies. IL-1β in cultured media were measured by ELISA. **: P < 0.01, ***: P < 0.001.

(c): MH-S cells were treated with 1 µM of Lysosensor™ (Green DND-189, Invitrogen) for 30 min and washed once with PBS. After addition of HA (25 µg/ml), fluorescence was monitored at time points indicated by digits in upper right corner that denote hours after HA stimulation. Acidic intracellular compartments, which are indicated by green fluorescence, was observed at 1 and 2 h after HA addition. Scale bar: 50 µm.

(d): Peritoneal macrophages from CD44-deficient mice and Wt mice were treated with 1 µM of Lysosensor™ for 30 min and washed once with PBS. Two hour after addition of HA (25 µg/ml) fluorescence were monitored. Scale bar: 50 µm.

(e, f): MH-S cells were pre-treated with indicated agents or DMSO (0.1%) as vehicle control for 30 min. Then MH-S cells were treated with 25 µg/ml of HA for 18 h in the presence of agents, and IL-1β in cultured media (e) and Il1b mRNA (f) were measured. Mean and SEM are shown. The agents used were; lysosome inhibitors NH₄Cl (20 mM), bafilomycin A1 (100 nM), chloroquine (100 µM), and Na⁺–H⁺ exchanger inhibitor amiloride (500 µM). Statistical analyses were done to compare with each vehicle treated samples. *: P < 0.05, **: P < 0.01, ***: P < 0.001.

(g): MH-S cells were treated with siRNAs for 72 h. Then the cells were treated with 25 µg/ml of HA for 18 h in the presence of agents, and IL-1β in cultured media were measured. Mean and SEM are shown. The agents used were; none: no pre-treatment, T.R.: transfection reagent alone, Mock: mock transfected, Hyal1: Hyal1 siRNA-transfected, Hyal2: Hyal2 siRNA-transfected, Hyal1/2: both Hyal1 and Hyal2 siRNA-transfected. *: P < 0.05.

(h, i): MH-S cells were treated with siRNAs for 72 h, and Hyal1 (h) and Hyal2 (i) mRNA expression were examined.

Figure 4: CD44 is involved in HA endocytosis.

(a): MH-S cells were treated with fluorescein-labeled HA (fl-HA, 25 µg/ml) for 24 h. Scale bar: 50 µm.

(b): Peritoneal macrophages from wt and Cd44^{−/−} mice were treated with fl-HA for indicated time. Numbers indicate percentage of fluorescein-positive cells (% of fl-HA positive cells / DAPI). Scale bar: 50 µm.

Figure 5: Intracellular HA oligosaccharides induced IL-1β release from LPS-primed macrophages.

(a-d): MH-S cells were stimulated with HA oligosaccharides (100 µM) or HA (25 µg/ml) for 18 h, and IL-1β (a) and Cxcl2/MIP-2 (b) in cultured media were measured. mRNA abundance for Il1b (c) and Cxcl2 (d) at 18 h was measured by quantitative RT-PCR. Quantitative RT-PCR data are shown as relative expression compared to untreated control (none). Mean and SEM are shown on the graphs. Statistical analyses were done to compare with non-treated samples. **: P < 0.01.
(e-h): MH-S cells were pre-treated with or without LPS (0.2 µg/ml) for 3 h. Then media was replaced, 2.5 µg of HA oligosaccharides were added to the media or permitted access to the intracellular space by membrane transfection with DOTAP™. IL-1β (e) and Cxcl2/MIP-2 (f) in cultured media was measured after 18 h stimulation. mRNA abundance for Il1b (c) and Cxcl2 (d) at 18 h are shown as relative expression compared to untreated control (none). Mean and SEM are shown on the graphs. Statistical analyses were done to compare with each vehicle treated samples. *: P < 0.05, **: P < 0.01, ***: P < 0.001.

Figure 6: A model of HA catabolism and inflammasome activation.

HA binding to CD44 activates p38 MAPK and NF-κB through TLR4 to increase IL-1β and Cxcl2 mRNA transcription. Endocytosis of CD44 and hyaluronidase activity hydrolyses HA to small oligosaccharide fragments. Small HA oligosacchrides activate the inflammasome through NLRP3/cryopyrin to convert pro-IL-1β to active IL-1β and release IL-1β.
Figure 1: Yamasaki K., et al
Figure 2. Yamasaki K., et al.
Figure 4: Yamasaki K. et al
Figure 6: Yamasaki K., et al.
NLRP3/cryopyrin is necessary for IL-1β release in response to hyaluronan, an endogenous trigger of inflammation in response to injury

J. Biol. Chem. published online March 3, 2009

Access the most updated version of this article at doi: 10.1074/jbc.M806084200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2009/03/04/M806084200.DC1

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/early/2009/03/03/jbc.M806084200.citation.full.html#ref-list-1