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 whether NLRP3/cryopyrin, a component of the inflammasome, participates in the inflammatory response to injury or the cytokine response to cryopyrin-deficient mice increased release of Cxcl2 but did not increase IL-1β release. To define the mechanism of hyaluronan-mediated activation of cryopyrin, elements of the hyaluronan recognition process were studied in detail. IL-1β release was inhibited in peritoneal macrophages derived from CD44-deficient mice, in an 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 lipopolysaccharide-primed macrophages. Therefore, the action of CD44 and subsequent hyaluronan catabolism trigger the intracellular cryopyrin→IL-1β pathway. These findings support the hypothesis that hyaluronan works through IL-1β 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 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), 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 mass 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 stimulate inflammation.
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 that recognizes pathogen-associated molecular patterns including bacterial cell wall components and viral nucleic acids. NOD2 and NLR family, pyrin containing 3 (NLRP3)/cryopyrin are two of the best characterized NLRs. NOD2 recognizes the bacterial peptidoglycan-derived molecule muramyl dipeptide and activates the NF-κB pathway to induce inflammatory responses (12). Mutations of the NOD2 gene were identified in individuals with chronic inflammatory disorders such as Crohn disease (13, 14) and Blau syndrome (15). Mouse knockin mutants of NOD2, which have the same mutation in NOD2 as human patients with Crohn disease, showed elevated proinflammatory cytokines following muramyl dipeptide challenge or dextran sodium sulfate-induced bowel inflammation (16). NLRP3, also known as cryopyrin, CIAS1, NALP3, PYPAF1, forms an “inflammasome” with ASC (apoptosis-associated speck-like protein containing a CARD) and caspase-1 to convert pro-IL-1β to active IL-1β (17). Mutations in NLRP3 were identified in individuals with familial cold autoinflammatory syndrome (FCAS), Muckle-Wells syndrome, and neonatal onset multisystem inflammatory disease (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). Because 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 that 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 the American Type Culture Collection (ATCC, catalogue number CRL-2019). Cells were maintained in RPMI 1640 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. HA preparations were free of DNA and protein contamination as preparations showed no absorbance at 260 and 280 nm. In addition, to ensure purity, 1-ml HA batches were boiled for 1 h and 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. Hylan™, rooster comb HA, was purchased from Fidia Farmaceutici (Abano Terme, Italy). LPS (from *Escherichia coli* K12 D31n4 (RE), catalogue number 302) was obtained from LIST Biologics, Inc. (Campbell, CA). Amirolide hydrochloride hydrate, bafilomycin A1, and chloroquine diphosphate were obtained from Sigma. Rat anti-mouse CD44 monoclonal antibodies KM114 and IM7 were obtained from BD Biosciences and Santa Cruz Biotechnology, 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 Life Sciences, Lowell, MA). For experiments, media were removed from cells and replaced with media containing either HA or LPS at the indicated concentration. 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 were replaced with media containing HA and inhibitors. Cells were allowed to incubate for 18 h, and 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). 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 RPMI 1640 media. Collected cells were counted and plated in RPMI 1640 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 × 10⁵ 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 the designated concentrations of HA, and supernatants and RNA were collected as described previously.

**Models of Skin Sterile Inflammation**—Cryopyrin-deficient mouse (*Nlrp3*−/−) were generated as described previously (22) and backcrossed 10 generations to a C57/B6 background. *Tlr4*−/− (C3H/HeJ), *Tlr4*+/+ controls, CD44-deficient mouse (*Cd44*tm1H9262, *Cd44*−/−), and *Cd44*+/+ controls (B6129SF2/J) were all purchased from The Jackson Laboratory. For bead injection, 0.5 mg of 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 of Cytodex bead slurry were 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 buffer (50 mm HEPES, 150 mm NaCl, 0.05% SDS, 0.25% deoxycholate, 0.5% Nonidet P-40, pH 7.4) with protease inhibitor mixture (Complete™ EDTA-free; Roche Diagnostics) and were beaten with 2.4-mm zirconia beads by a mini-beadbeater apparatus (Bio-Spec 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 the University of California San Diego 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 (Bio-Rad) as described by the manufacturer’s protocol. TaqMan gene expression assays (Applied Biosystems) were used to analyze expression of Il1b (assay ID: Mm00432282-m1) and Cxcl2 (assay ID: Mm00436450-m1) as described by the manufacturer’s instructions (76). 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 controls).

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 the designated time at 37 °C. Media were removed, and cells were fixed with ProLong Gold antifade reagent (Invitrogen) and a coverslip. Fluorescence was observed using an Olympus BX41 fluorescent microscope (Scientific Instrument Co.,) or a Zeiss LSM 510 confocal microscope (Carl Zeiss, Inc.).

HA Stimulation by Lipofection—DOTAP™ liposomal transfection reagent was obtained from Roche Applied Science and used according to the manufacturer’s instructions. Briefly, 2.5 μg of HA oligosaccharides were incubated with 3 μl of DOTAP for 30 min in a final volume of 15 μl of serum free media. After incubation, 100 μl of low serum media (1% FCS) were added. MH-S cells were stimulated with 0.2 μg/ml LPS in low serum media (1% FCS) for 3 h prior to HA oligosaccharide stimulation. After LPS-priming, media were replaced with the HA oligosaccharides and DOTAP-containing media. Culture media were collected 18 h after treatment for cytokine ELISA.

Statistical Analysis—Results are expressed as the mean ± S.E. and are representative of at least three separate experiments. One-way analysis of variance was used to determine significance, which was analyzed by GraphPad Prism 4 (GraphPad Software, Inc.), unless otherwise stated. Two-way analysis of variance 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 HA that in
HA Induces IL-1β Secretion through Cryopyrin—Because the release of fragments of HA from the extracellular matrix has previously been associated with the sterile inflammatory response (7, 8, 10, 26), we next examined whether HA can directly induce IL-1β release. Peritoneal macrophages of WT mice, but not cryopyrin null mice (Nlrp3−/−), showed a large increase in IL-1β release (Fig. 2a). The presence of cryopyrin did not affect the release of Cxcl2/MIP-2 in response to HA or LPS (Fig. 2b). HA-dependent IL-1β release showed dose dependence, and 10–25 μg/ml HA induced the highest release of IL-1β (Fig. 2c). Diminished IL-1β induction by HA digested with hyaluronidase or chondroitinase ABC confirmed HA as the active molecule (supplemental Fig. 1, a and b). These doses of HA also induced Cxcl2/MIP-2, but lack of cryopyrin did not affect Cxcl2/MIP-2 induction (Fig. 2d). Interestingly, HA induced an increase in IL-1β and Cxcl2/MIP-2 mRNA in both WT and cryopyrin null mice, although the magnitude of this response was slightly lower in cryopyrin null mice (Fig. 2, e and f). In contrast to the cryopyrin null mice, IL-1β release and transcription were suppressed in macrophages derived from Tlr4-deficient mice (Fig. 2, g and 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 (Fig. 3a, p < 0.001). A similar result was observed in the MH-S macrophage cell line pretreated with anti-CD44 antibodies (Fig. 3b, p < 0.01), demonstrating that CD44 is involved in the 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 fl-HA (Fig. 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 Cd44−/− mice (Fig. 4b). To examine whether these events are important to the IL-1β response, MH-S cells exposed to HA were examined with lysensor, a marker that senses acidotrophic compartments, such as lysosomes and endosomes (pKaw = 5.2). Acidotrophic compartments were detected within 2 h of exposure to HA (Fig. 3c). HA also enabled detection of acidic intracellular compartments in peritoneal macrophages from WT mice but much less in CD44-deficient macrophages (Fig. 3d).

Next, cells were treated with pharmacological inhibitors of lysosomal function. These agents included NH4Cl, 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 (Fig. 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, con-
HA Activation of Inflammasome through CD44

Figure 2. IL-1β release by HA depends on cryopyrin. a and b, peritoneal macrophages from cryopyrin null (Nlpr3<sup>−/−</sup>) 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 were measured by ELISA. Mean and S.E. are shown. Gray bars, WT; black bars, cryopyrin null (Nlpr3<sup>−/−</sup>). Statistical analyses were done to compare Nlpr3<sup>−/−</sup> and WT in each stimulus. ***: p < 0.001. c–f, peritoneal macrophages from cryopyrin null (Nlpr3<sup>−/−</sup>) 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 as compared with control untreated WT peritoneal macrophages. Statistical analyses were done to compare Nlpr3<sup>−/−</sup> and WT in each concentration. *: p < 0.05, ***: p < 0.001. g, h, peritoneal macrophages from Tlr4 null (Tlr4<sup>−/−</sup>) and WT mice were treated with HA (25 μg/ml) for 18 h, and IL-1β in cultured media (g) and the level of Il1b mRNA (h) were measured. mRNA was measured by quantitative RT-PCR and shown as relative expression over the control (WT indicates non-treated). Mean and S.E. are shown on the graphs. **: p < 0.01, ***: p < 0.001.

Firming specific involvement of lysosome function in IL-1β release by HA (Fig. 3e). HA increased IL-1β mRNA transcription more than 20-fold in the presence of these inhibitors, suggesting that inhibition of lysosome function does not block induction of transcription (Fig. 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 Hyal1 and Hyal2 suppressed Hyal gene expression to less than 20% of untreated cells (Fig. 3, h and 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 (Fig. 3g). Neither of these siRNAs affected IL-1β mRNA 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 Macrophage—The size of HA is crucial in its biological function (35), and processing to small HA is dependent on hyaluronidase activity both on 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 whether smaller extracellular HA fragments induce IL-1β release. The activity of short HA oligosaccharides (4–18-mer, 0.777–3.5 kDa) and isolated large HA, Hyalgan (size from 500 to 730 kDa) were compared with the purified HA preparations used previously that contained HA at a 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 4-mer oligosaccharide and 350 μg/ml 18-mer oligosaccharide). Neither preparation induced IL-1β release, although the 18-mer oligosaccharides did induce Cxcl2/MIP-2 (Fig. 5, a and 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 (supplemental Fig. 2a), a finding consistent with the limited ability of small HA fragments to induce IL-1β.

Given our results that indicated that intracellular uptake and processing of HA are 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 whether small HA oligosaccharides could induce IL-1β release if introduced to the intracellular space. HA oligosaccharides were added intracellularly using the cationic transfection DOTAP. MH-S cells were treated with LPS at a low concentration to induce transcription of IL-18 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 (Fig. 5c). Introduction of small HA oligosaccharides to intracellular space did not influence the appearance of intracellular acidotroph compartments as detected by LysoSensor (supplemental Fig. 2b). This amount of HA oligosaccharides did not induce IL-1β.
Then the cells were treated with **: lysosome inhibitors NH4Cl (20 mM), bafilomycin A1 (100 nM), chloroquine, and IL1b mRNA (**: 0.001). 18 h after the addition of HA (25 \( \mu g/ml \) ) in the presence of antibodies. IL-1 \( \beta \) stimulation. Acidic intracellular compartments, which are indicated by green fluorescence, were observed at 1 and 2 h after HA addition. Scale bar, 50 \( \mu m \).

CD44 affects IL-1 \( \beta \) release in response to HA. *: peritoneal macrophages from CD44-deficient mice and WT mice were treated with HA (25 \( \mu g/ml \) ) for 18 h, and IL-1 \( \beta \) in cultured media was measured by ELISA. Mean \pm S.E. 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 \( \mu g/ml \) ) each for 30 min. MH-S cells were then exposed to 25 \( \mu g/ml \) HA for 18 h in the presence of antibodies. IL-1 \( \beta \) in cultured media was measured by ELISA. **: \( p < 0.01 \), ***: \( p < 0.001 \). c: MH-S cells were treated with 1 \( \mu M \) of LysoSensor (Green DND-189, Invitrogen) for 30 min and washed once with PBS. After the addition of HA (25 \( \mu g/ml \)) fluorescence was monitored at the time points indicated by the digits in the upper right corner. Fluorescence was observed at 1 and 2 h after HA addition. Scale bar, 50 \( \mu m \).

d: peritoneal macrophages from CD44-deficient mice and WT mice were treated with 1 \( \mu M \) of LysoSensor for 30 min and washed once with PBS. After two hours after the addition of HA (25 \( \mu g/ml \)), fluorescence was monitored. Scale bar, 50 \( \mu m \).

e: peritoneal macrophages from CD44-deficient mice and WT mice were treated with 1 \( \mu M \) of LysoSensor for 30 min and washed once with PBS. After two hours after the addition of HA (25 \( \mu g/ml \)), fluorescence was monitored. Scale bar, 50 \( \mu m \).

HA Activation of Inflammasome through CD44

**FIGURE 3.** a: peritoneal macrophages from CD44-deficient mice and WT mice were treated with HA (25 \( \mu g/ml \) ) for 24 h. Scale bar, 50 \( \mu m \). b: peritoneal macrophages from WT and CD44 \( ^{-/-} \) mice were treated with HA for the indicated times. Numbers indicate the percentage of fluorescein-positive cells (percentage of fl-HA-positive cells/4,6-diamidino-2-phenylindole (DAPI)). Scale bar, 50 \( \mu m \).

**FIGURE 4.** CD44 is involved in HA endocytosis. a: MH-S cells were treated with fl-HA (25 \( \mu g/ml \) ) for 24 h. Scale bar, 50 \( \mu m \). b: peritoneal macrophages from WT and CD44 \( ^{-/-} \) mice were treated with HA for the indicated times. Numbers indicate the percentage of fluorescein-positive cells (percentage of fl-HA-positive cells/4,6-diamidino-2-phenylindole (DAPI)). Scale bar, 50 \( \mu m \).

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-1\( \beta \). We also show here in model cell culture systems that the response to HA mimics observations in mice and that the macrophage IL-1\( \beta \) 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 (Fig. 6). Deposition of HA after sterile injury can be a danger signal leading to the induction of IL-1\( \beta \) 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 of cryopyrin (44–46). Taken together, our observations suggest release when added only to the extracellular space (Fig. 5c, DOTP-\( ^{-} \)). In contrast to IL-1\( \beta \) release, the small HA oligosaccharides when present in the extracellular space could induce Cxcl2/MIP-2 in the presence of low concentrations of LPS (Fig. 5d).

**FIGURE 3.** a: peritoneal macrophages from CD44-deficient mice and WT mice were treated with HA (25 \( \mu g/ml \) ) for 18 h, and IL-1\( \beta \) in cultured media was measured by ELISA. Mean \pm S.E. 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 \( \mu g/ml \) ) each for 30 min. MH-S cells were then exposed to 25 \( \mu g/ml \) HA for 18 h in the presence of antibodies. IL-1\( \beta \) in cultured media was measured by ELISA. **: \( p < 0.01 \), ***: \( p < 0.001 \). c: MH-S cells were treated with 1 \( \mu M \) of LysoSensor (Green DND-189, Invitrogen) for 30 min and washed once with PBS. After the addition of HA (25 \( \mu g/ml \)) fluorescence was monitored at the time points indicated by the digits in the upper right corner. Fluorescence was observed at 1 and 2 h after HA addition. Scale bar, 50 \( \mu m \).

d: peritoneal macrophages from CD44-deficient mice and WT mice were treated with 1 \( \mu M \) of LysoSensor for 30 min and washed once with PBS. Two hours after the addition of HA (25 \( \mu g/ml \)) fluorescence was monitored. Scale bar, 50 \( \mu m \).

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**FIGURE 4.** a: MH-S cells were treated with fl-HA (25 \( \mu g/ml \) ) for 24 h. Scale bar, 50 \( \mu m \). b: peritoneal macrophages from WT and CD44 \( ^{-/-} \) mice were treated with HA for the indicated times. Numbers indicate the percentage of fluorescein-positive cells (percentage of fl-HA-positive cells/4,6-diamidino-2-phenylindole (DAPI)). Scale bar, 50 \( \mu m \).

**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-1\( \beta \). We also show here in model cell culture systems that the response to HA mimics observations in mice and that the macrophage IL-1\( \beta \) 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 (Fig. 6). Deposition of HA after sterile injury can be a danger signal leading to the induction of IL-1\( \beta \) 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 of cryopyrin (44–46). Taken together, our observations suggest release when added only to the extracellular space (Fig. 5c, DOTP-\( ^{-} \)). In contrast to IL-1\( \beta \) release, the small HA oligosaccharides when present in the extracellular space could induce Cxcl2/MIP-2 in the presence of low concentrations of LPS (Fig. 5d).
that 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 to 250 kDa induce inflammatory cytokines (36–41). HA oligosaccharides from 6- to 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- to 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 whether other HA receptors such as RHAMM (receptor for hyaluronan-mediated motility) (54) and LYVE1 (lymphatic vessel endothelial hyaluronan receptor 1) (55) are also involved in this process. Our data suggest that extracellular high molecular weight HA binds to CD44, that it 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 mass 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 that 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 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 localizes 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 leucine-rich repeat 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 peptido-glycan-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 the 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 (Fig. 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 an endocytic receptor for HA catabolism (43). An intermediate size of HA is required for efficient HA endocytosis, although molecular structure analysis showed that 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 that 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 oligosaccharides in LPS-primed cells. It is not currently clear why extracellular HA oligosaccharides, 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 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 produces HA on its capsule, possibly to mimic a mammalian host. Phagocytic cells, including macrophages, phagocyte Group A Streptococcus 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 that 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 in 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, which promotes inflammatory cell migration (54), and LYVE1, which 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, Muckle-Wells syndrome, and neonatal onset multisystem inflammatory disease 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.

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