Fragmented hyaluronan has no alarmin function assessed in arthritis synovial fibroblast and chondrocyte cultures

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Abstract

Hyaluronan (HA) is a large polymer and an important component of the extracellular matrix. During homeostasis, high molecular mass HA is the predominant form, but upon inflammation, degradation products of HA accumulate. These HA fragments (HA-fs) have been reported to possess pro-inflammatory activities and thus act as alarmins, notifying immune cells of danger via TLR4 and CD44. HA is found in large quantities in synovial joint fluid. In order to reveal a potential role of HA-fs in arthritis pathogenesis, the *in vitro* effects of HA of various molecular masses (from 1680 kDa to oligo-saccharide HA) on synovial fibroblasts and chondrocytes from rheumatoid arthritis patients, and on peripheral blood mononuclear cells from healthy donors, were investigated. TLR4 and CD44 surface expression was confirmed by immunocytochemistry, and cell activation was determined based on cytokine and chemokine production. While the cell types investigated expressed TLR4 and CD44, no increased release of IL-1ß, IL-6, IL-8, IL-10, IL-12 or TNF-α was detected after HA stimulation. Similarly, HA did not enhance activation after priming cells with low doses of LPS or by forming complexes with LPS. Hence, this study does not support the common view of HA-fs being pro-inflammatory mediators and it is not likely that HA-fs generated during arthritis contribute to disease pathogenesis.

Introduction

Hyaluronan (HA) is a large glycosaminoglycan that is mainly synthesized and secreted by stromal cells. Following secretion, HA either remains associated with the cell or becomes a component of the extracellular matrix, where it plays a major role in maintaining tissue integrity and homeostasis. HA is found in almost every tissue and significant amounts are located in synovial joint fluid, cartilage and the skin.[1] At homeostasis, HA predominantly exists in its native high-molecular mass (MM) form ($>1 \times 10^3$ kDa), and its continuous synthesis is balanced by cellular uptake and by degradation mediated by HA-specific enzymes: hyaluronidases.[2] The normal process of HA turnover is primarily upheld by nearby tissue macrophages. HA is internalized through the CD44 receptor into endosomes and subsequently lysosomes, where it is first degraded into oligosaccharides and then to its individual sugar components by hyaluronidases.[3,4] Via drainage, HA can also enter the lymphatics, and become degraded in the liver.[5] During inflammation when homeostasis is lost, increased HA synthesis is accompanied by a decrease in MM, possibly due to altered activity rates of both HA synthases and hyaluronidases.[6,7] The generated HA fragments (HA-fs) are often smaller than

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500 kDa in size. Such low-MM HA may also be generated as a result of physical damage. In mouse models of inflammation, HA-fs have been shown to accumulate extracellularly to an extent that correlates with the inflammatory intensity.[6–8] Reduction of HA-fs by blocking hyaluronidases has been reported to reduce TNF-α-induced TLR4 and CD44 expression and thereby reduce the production of pro-inflammatory cytokines.[9] How and where the degradation of HA occurs during inflammation is not clear, but it may result from the release of hyaluronidases from damaged or necrotic cells, or breakdown by reactive oxygen species released from infiltrating immune cells.[10,11]

HA-fs, including oligosaccharide HA (oligo-HA), have been reported to possess pro-inflammatory activities and thus act as an alarmin, an endogenous molecule that notifyies immune cells of danger and initiates an inflammatory response. However, the literature is conflicting in many aspects. In the 1990s, the first articles were published reporting that HA-fs have the capacity to induce pro-inflammatory chemokines, cytokines and NF-κB-mediated inducible nitric oxide synthase (iNOS) in mouse macrophages.[12–17] At first it was thought that CD44 alone facilitated the observed response mediated by HA-fs, but in several subsequent studies, HA-fs of MM between 135–200 kDa were proposed to bind TLR2 and/or TLR4, and thereby induce inflammatory activities in mouse macrophages and dendritic cells (DCs).[18–20] In two other studies, increased release of IL-1ß, TNF-α and IL-12 by mouse DCs after stimulation with oligo-HA (4-, 6- and 8-mers) was observed to occur independently of CD44, TLR2 and RHAMM, with TLR4 being the sole receptor being involved.[21,22] Interestingly, HA-fs with MM between 80–200 kDa, which in past studies were reported to stimulate macrophages, had no pro-inflammatory effect on DCs in one of these studies.[21] Next, a heterocomplex of the receptors TLR4, CD44 and MD-2 was suggested to mediate the pro-inflammatory effect of an oligo-HA (of undefined size) in both mouse and human cell lines.[23] The same group also proposed a model for NLRP3 inflammasome activation stimulated by HA-fs via a complex involving both TLR4 and CD44, with IL-1ß release as a downstream result.[24] An increase of IL-1ß and TNF-α mRNA expression was also observed in chondrocytes from healthy mice upon stimulation with HA-fs of 50 kDa, a response that is dependent on CD44.[25] Although most of the work regarding the role of HA-fs as alarmins has been performed using murine cells, it has also been reported that human macrophages, monocytes, fibroblasts, chondrocytes and alveolar epithelial cells are activated upon stimulation with HA-fs with MMs ≤ 500 kDa.[26–29] In addition to their endogenous alarmin functions, alarmins may enhance the response to other inflammatory mediators by complex formation. This has been demonstrated for the alarmin high mobility group box 1 protein (HMGB1), which can form enhancing complexes with LPS, IL-1β, CXCL12 and other inflammatory mediators.[30,31] Whether HA-fs exert their alarmin functions by forming complexes is presently unknown.

In the last decade, the role of HA-fs (including oligo-HAs) as alarmins has been questioned and concerns about possible endotoxin contamination in the HA preparations used in earlier studies have been raised.[32,33] In two different reports, HA and HA-fs with 11–1000 kDa MMs neither activated NFκB nor induced increases of iNOS, NO or TNF-α production in mouse macrophages[32,34] or splenocytes.[32] In studies where endotoxin levels were reduced to minimal levels (0.03 endotoxin units (EU)/mg), HA-fs failed to increase NO production in murine and human tissue macrophages.[35,36] Likewise, in another recent study, endotoxin-free HA-fs, including oligo-HAs, did not upregulate co-stimulatory molecules and did not induce IL-1ß, TNF-α or IL-12 production in mouse macrophages and DCs.[33] In contrast to the majority of studies in this field, the more recent reports suggest that the accumulation of HA-fs in inflamed tissue might be a consequence, rather than a driver, of inflammation.

Increased levels of circulating HA have been reported in several human inflammatory diseases, including rheumatoid arthritis (RA).[37–40] RA is a chronic autoimmune disease of unknown cause, which is associated with an inflamed synovium and a progressive destruction of bone and cartilage. Normal synovial fluid contains large amounts of high-MM HA, which has important lubricating function for the joints. However, in the inflamed synovium of RA patients, the activity of hyaluronidases is augmented, which suggests a higher presence of HA-fs as compared to healthy joints.[41] Apart from immune cells infiltrating the arthritic joint, resident tissue cells such as fibroblasts and chondrocytes are considered important for the perpetuation of the initiated inflammation. Synovial fibroblasts actively communicate with the immune cells infiltrating the synovial compartment through the release of pro-inflammatory cytokines.[42] Chondrocytes populating the cartilage have been suggested to secrete cytokines and enzymes, which escalate the degradation process in the RA joint.[43] Even though HA-fs are reported to be potent alarmins and are allegedly present in the RA joint, little is known about the potentially activating or enhancing effect of HA-fs in the dysregulated inflammatory response that characteristic of RA. In this in vitro study, we investigated whether HA-fs (including oligo-HAs) could stimulate or enhance pro-inflammatory cytokine and chemokine production in synovial fibroblasts and chondrocytes derived from patients with RA, and in PBMCs from healthy donors. None of the patient-derived synovial cell types have been used before in a similar experimental setting.

Materials and methods

HA panel

Pharmaceutical-grade high-MM HA ~ 1680 kDa (HA15M-1) and HA-fs of various MMs (~ 234 kDa (HA200K), ~ 28 kDa (HA20K) and 4–10 kDa (HA5K)) were purchased from Lifecore Biomedical (Chaska, MN, US), and are referred to as 1.5 M, 200 K, 20 K and 5 K, respectively. The HA forms used originated from bacteria and were isolated after bacterial fermentation. The oligo-HA (HYA-OLIGO6EF) with a MM of approximately ~ 1.2 kDa was purchased from Amsbio (Abingdon, UK) and is referred to as 6-mer. The 6-mer was prepared by enzymatic digestion of HA of animal origin using hyaluronidase Type V from Sheep testes and subsequently purified. All HA samples were certified to have low endotoxin levels (< 0.01 EU/mg for Lifecore HA and < 1 EU/mg for Amsbio oligo-HA).

Cell isolation and culture conditions

Synovial fibroblasts were isolated from RA patients ($n = 3$) undergoing knee replacement surgery at Karolinska University Hospital and cultured in complete media consisting of high-Glc DMEM, 10% heat-inactivated FCS, 100 µg/ml penicillin–streptomycin and 2 mM L-glutamine from Sigma-Aldrich (Saint Louis, MO, US) in standard tissue culture (TC) flasks (Sarstedt, Nümbrecht, Germany) in a TC incubator at 37°C with 5% CO_2. Chondrocytes were isolated from RA patients ($n = 3$) undergoing knee replacement surgery at Karolinska University Hospital. Chondrocytes were cultured in complete media with DMEM-F12 (Sigma-Aldrich) in standard TC flasks (Sarstedt) in a TC incubator at 37°C with 5% CO_2. PBMCs were purified from blood samples from healthy adult donors ($n = 3$) using standard Ficoll-Paque (Ficoll-Paque Plus, GE Healthcare, Uppsala, Sweden) separation (www.miltenyibiotec.com). Washed cells were resuspended in Roswell Park Memorial Institute-1640 media supplemented with 10% heat-inactivated FCS, 100 µg/ml Penicillin–Streptomycin and 2 mM L-glutamine (Sigma-Aldrich). All procedures were approved by the Institutional Ethical Committee (Solna, Stockholm, Sweden, ethical number 2009/1262-31/3) and are in compliance with all ethical standards and patients' written consent, in accordance with the Declaration of Helsinki.

Cell stimulation

Prior to stimulation experiments, cells were harvested at 80–90% confluency using trypsin (Sigma-Aldrich). Cells were used between passages 3–5 for synovial fibroblasts and passages 1–3 for chondrocytes. Synovial fibroblasts, chondrocytes and PBMCs were seeded in 96-well cell culture plates (Sarstedt) at a concentration of 6000 cells/well (synovial fibroblasts and chondrocytes) or 100,000 cells/well (PBMCs). All cells were left to adhere overnight (ON, 18 h) in a TC incubator at 37°C with 5% CO_2 prior to stimulation. HAs of different MMs were used in a concentration of 100 µg/ml, except for 6-mer HA, which was used at 10 µg/ml.

Experiments to test the capacity of HA-fs as inducers of inflammation. Cells were stimulated with 10 ng/ml LPS (from *Escherichia coli* strain 055:B5, cell culture-tested, γ-irradiated and purchased from Sigma-Aldrich) or HAs of different MMs, and incubated in a TC incubator at 37°C with 5% CO_2 for 21 h.

Experiments to test the capacity of HA-fs to act as the first or second signal in inflammasome activation. To measure IL-1ß secretion, two signals are required: one to up-regulate the expression of pro-IL-1β and a second signal to activate the inflammasome assembly necessary for the cleavage of pro-IL-1β to IL-1β. To orchestrate this two-step process, cells were incubated with either LPS or HAs of different MMs for 4 h as the first step, followed by the addition of 5 mM ATP (Sigma-Aldrich) or HA as the second step for 1 h.

Experiments to test the capacity of HA-fs to enhance inflammation. Cells were first primed with a low concentration of LPS (0.5 ng/ml for synovial fibroblasts and chondrocytes, and 10 pg/ml for PBMCs) for 2 h, followed by stimulation with HAs for 19 h (total stimulation time 21 h). The priming concentration of LPS was determined by a titration study prior to the experiment and cells were considered primed when a low, but still detectable, release of IL-6 was recorded in the cell culture media after 21 h, as measured by ELISA DuoSet (R&D systems, Minneapolis, MN, US). After stimulation, cells were spun down ($300 \times g$ for 5 min at 20°C), and supernatants collected and analysed either directly or stored at -20°C until quantification of cytokines and chemokines.

Immunocytochemistry

Cells were seeded at a density of approximately 6000 cells/well (synovial fibroblasts and chondrocytes) and 200,000 cells/well (PBMCs) on eight-chamber polystyrene vessel TC-treated glass slides (Falcon, Corning Inc., NY, US) or Poly-D-Lysine cellware culture slides (Discovery Labware, Corning Inc., MA, USA) and incubated ON at 37°C with 5% CO_2. Slides were washed twice with PBS, fixed with 2–4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) (Histolab Products, Askim, Sweden) for 10 min at 4°C in darkness, before being washed twice with PBS (cold PBS for PBMCs) and stored at 4°C in PBS until staining. Slides

were blocked with 5% FCS (Sigma-Aldrich) for 10 min at room temperature (RT, 21°C) and washed in PBS (3 × 5 min).

For TLR4 staining, slides were blocked with 5% normal donkey serum (Sigma-Aldrich) for 1 h at RT and washed again in PBS (3 × 5 min), before being incubated ON at 4°C with 1 µg/ml anti-TLR4 (8694 from Santa Cruz Biotechnology Inc., Santa Cruz, CA, US) or an irrelevant IgG Ab from goat serum (Sigma-Aldrich). Slides were washed again in PBS (3 × 5 min) and incubated with Alexa Fluor 488-conjugated donkey anti-goat (A11058, Molecular Probes Inc., Eugene, OR, US) at a 1:1000 dilution for 1 h at RT.

For CD44 staining, slides were blocked with 5% normal goat serum (DAKO, Glostrup, Denmark) for 1 h at RT. PBMCs were additionally blocked with Background Buster (Innovex Biosciences, Richmond, CA, US) at RT for 30 min. The slides were washed in PBS (3 × 5 min) before being incubated at 4°C ON with 1 µg/ml anti-CD44 (ab6124 from Abcam, Cambridge, MA, US) or an irrelevant mouse anti-IgG2a Ab (DAKO). After washing in PBS (3 × 5 min), slides were incubated with biotinylated goat anti-mouse IgG2a (Invitrogen, Frederick, CA, US) at a 1:800 dilution for 1 h at RT, washed (3 × 5 min) in PBS and incubated for 30 min at RT with streptavidin-conjugated Alexa Fluor 594 (Invitrogen), diluted 1:500.

After incubation with the secondary Abs, all slides (for TLR4 and CD44 staining) were washed again in PBS (3 × 5 min) followed by one wash in 70% ethanol and slides were left to dry. Slides were mounted in ProLong Gold antifade reagent with 4′,6-diamidino-2-phenylindole (Life Technologies, Eugene, OR, US) and analysed in a Leica DM RXA2 microscope (Leica Microsystems, Wetzlar, Germany).

Quantification of cytokines and chemokines

Cytokine bead array (CBA, B&D Biosciences, Pharmingen, San Diego, CA, US) was used for the detection of TNF-α, IL-1ß, IL-6, IL-8 and IL-10, analysed according to the manufacturer's instructions. IL-1ß, IL-6 and IL-8 were additionally quantified with ELISA DuoSet (R&D systems). Raw data was analysed using the Flow Cytometric Analysis Program (FCAP) Array Software (BD Biosciences, San Jose, CA, US) for CBA and SoftMax Pro 6.2.1 (Molecular Devices, Sunnyvale, CA, US) for ELISA.

Statistical analysis

All graphs were made using GraphPad Prism version 7.03. *P*-values were calculated by using one-way ANOVA with Dunnett's multiple comparison post-test. A *P*-value ≤ 0.05 was considered statistically significant.

Results

HA-fs do not induce an inflammatory response in synovial fibroblasts, chondrocytes or PBMCs

Synovial fibroblasts and chondrocytes from RA patients and PBMCs derived from healthy blood donors were incubated with HAs of different MMs or LPS. To estimate the stimulatory properties of HA-fs, the secretion of key inflammatory cytokines and chemokines – IL-1ß, IL-6, IL-8, IL-10 and TNF-α – was measured in cell culture media after 21 h of incubation using CBA and ELISA.

LPS, but not HAs, induced significant secretion of IL-6 and IL-8 in synovial fibroblasts and of IL-6 in chondrocytes from RA patients as compared to non-stimulated cells (Figure 1a and b). Similarly, LPS, but not Has, upregulated IL-6, IL-8, TNF-α and IL-10 in PBMCs from healthy donors (Figure 1(c)). CBA was run for the first individual for all cell types, but as the synovial fibroblasts and chondrocytes did not release TNF-α and IL-10 (Supplementary Figure 1(a) and (b)), these cell types were analysed using IL-6 and IL-8 ELISA. No stimulus induced IL-1ß secretion in any cell type. A higher concentration of HA (200 µg/ml) or increased incubation time (72 h) did not induce a response to HA in any of the investigated cell types (data not shown). To make sure that no analytes were degraded during freeze–thawing cycles, cell culture media from one individual representing each cell type was determined by CBA before and after freezing. No analytes were lost after one freeze–thawing cycle (data not shown).

For IL-1β to be secreted from cells, two signals are required: one to upregulate the expression of pro-IL-1β and another to activate caspase-1 via the assembly of inflammasomes, which cleave pro-IL-1β into its mature and active form. The positive control in our experimental system was represented by cells first stimulated with the TLR4 agonist LPS for 4 h (signal 1) followed by the addition of ATP for 1 h (signal 2). This combination induced a significant release of IL-1β from PBMCs, but not from synovial fibroblasts or chondrocytes (Figure 2). The ability of HAs of different MMs to replace either the first or second signals in this system was investigated. Again, none of the different HA sizes could increase pro-inflammatory activities in PBMCs, or replace LPS or ATP as the first or second signals in this system. Incubation with only LPS, but not with HAs of any MM, induced small but non-significant amounts of IL-1β in PBMCs from healthy donors (Figure 2). Thus, in contrast to previous studies reporting increased inflammatory activities in both immune and non-immune cells after stimulation with HA-fs, we could not detect any such pro-inflammatory effect on synovial fibroblasts or chondrocytes from RA patients or PBMCs from healthy donors.
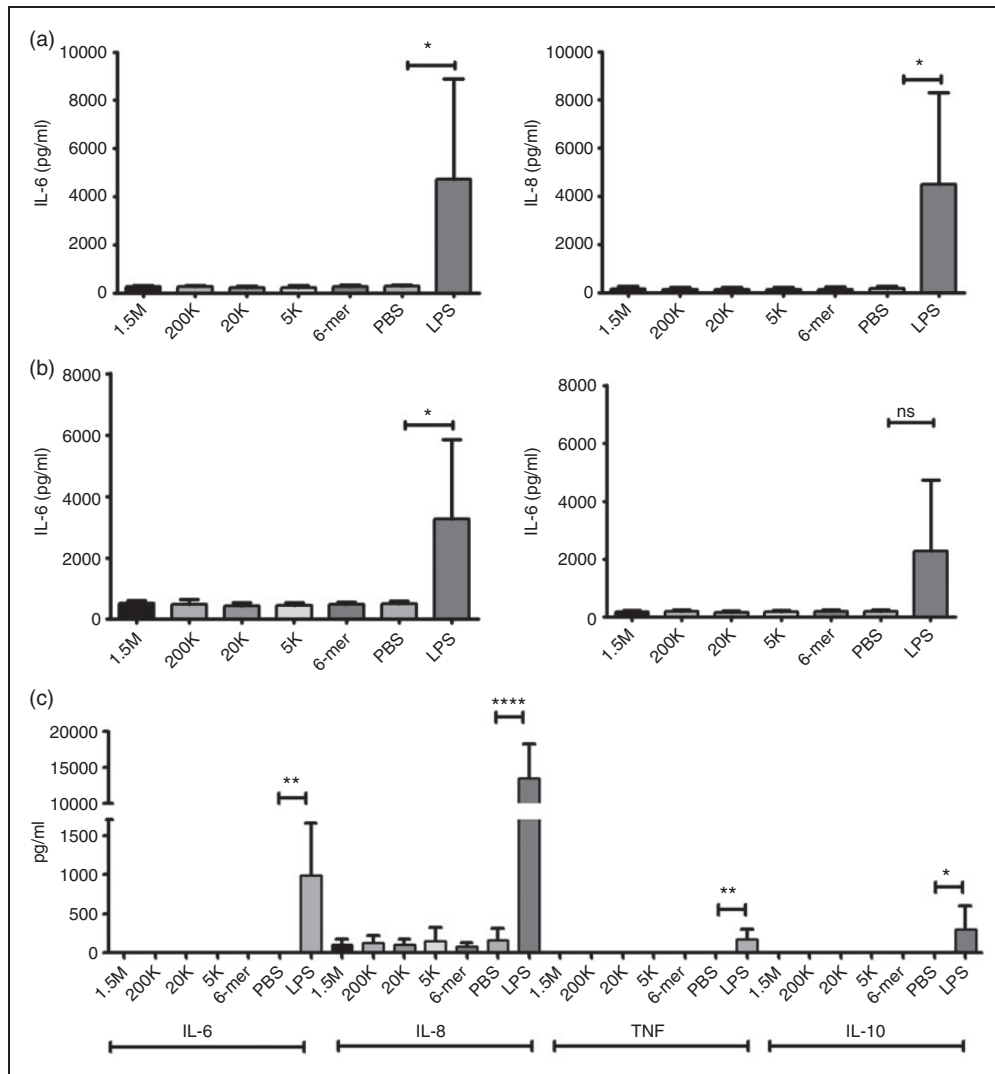
Figure 1. HA fragments do not function as inducers of inflammatory cytokine production in synovial fibroblasts, chondrocytes or PBMCs. Cells from three individuals per cell type were incubated with 10 ng/ml LPS or 100 μg/ml HA (10 μg/ml for 6-mer HA) of different MMs for 21 h. LPS, but not HA, significantly increased the release of the pro-inflammatory cytokines IL-6 and IL-8 from synovial fibroblasts (a) and chondrocytes (b) from RA patients and PBMCs from healthy donors (c). PBMCs, but not synovial fibroblasts or chondrocytes, also released TNF-α and IL-10 upon LPS stimulation, but not after stimulation with HA (c). HA samples were: 1.5 M (~1680 kDa), 200 K (~234 kDa), 20 K (~28 kDa), 5 K (4-10 kDa) and 6-mer (~1.2 kDa). Cytokines and chemokines were measured in cell culture media by CBA and ELISA, and P-values were calculated using Dunnett's multiple comparison post-test. $*(P < 0.05)$, $**(P < 0.01)$ and $***(P < 0.001)$.

HA-fs do not enhance the inflammatory response in LPS-primed synovial fibroblasts, chondrocytes or PBMCs

The ability of HA-fs to enhance an inflammatory response was investigated by stimulating LPS-primed cells with HAs of different MMs. The priming concentration was determined by titration of LPS and the concentration, which induced a low but still detectable amount of IL-6 in the different cell types, was chosen (data not shown). Cells were stimulated for 2 h with the priming concentrations of LPS followed by incubation with HAs of different MMs for 19 h (in total 21 h). Low concentration of LPS induced detectable amounts of

both IL-6 and IL-8 in all cell types, but the addition of HA did not enhance this secretion (Figure 3). Again, synovial fibroblasts and chondrocytes did not release IL-10 or TNF-α (Supplementary Figure 1(c) and (d)), and PBMCs released very low levels of these cytokines after stimulation with the priming concentration of LPS (Figure 3(c)). To test the ability of HA-fs to form complexes with other ligands and modulate their inflammatory effect, HAs of different MMs were co-incubated overnight with the priming concentration of LPS before being added to synovial fibroblasts from one RA patient and incubated for 21 h. Again, in this experiment, HA-fs appeared to be immunologically inert and mixtures had no effect on the release of
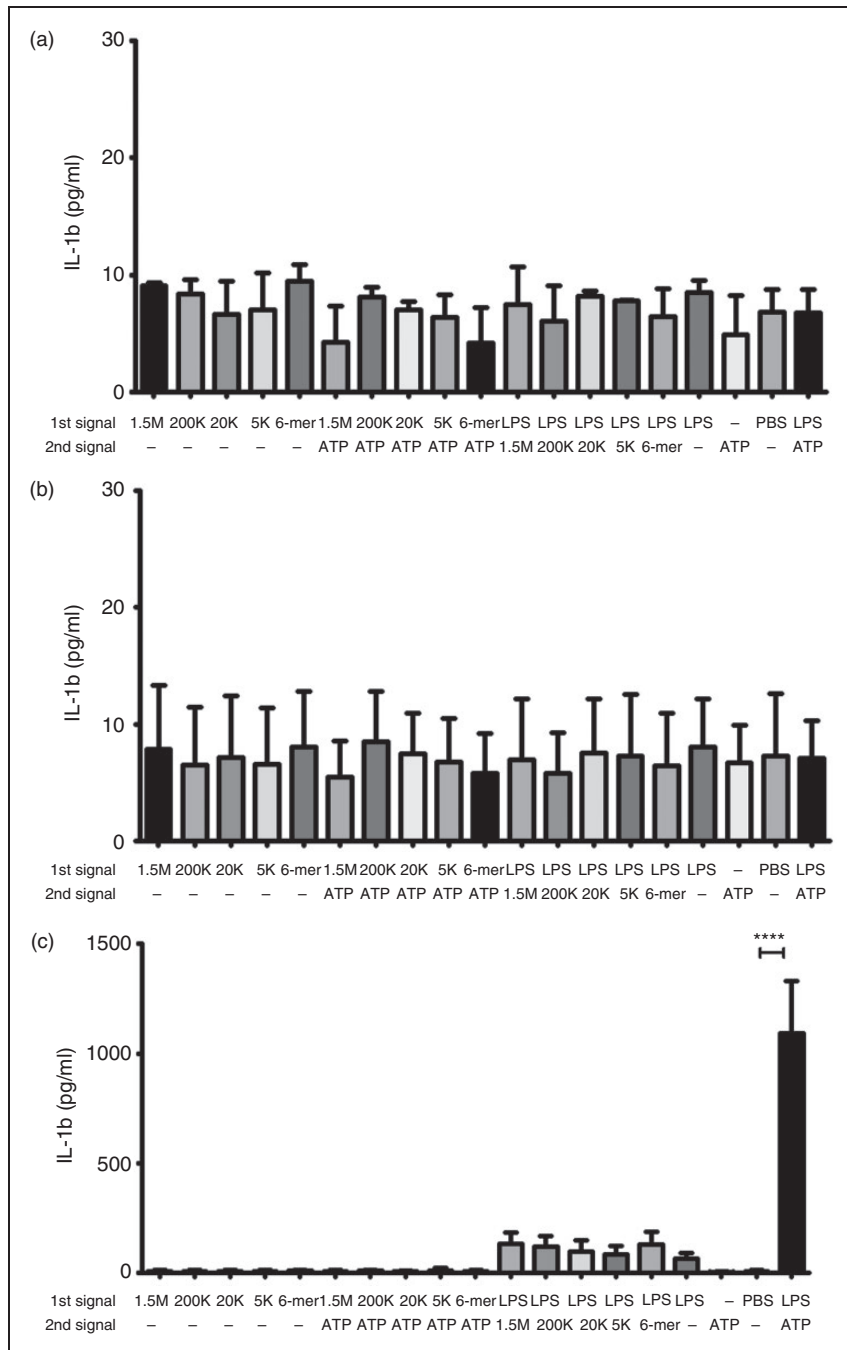
Figure 2. HA fragments cannot serve as first or second signals in inflammasome activation for IL-1β release. Cells from three individuals per cell type were incubated with 10 ng/ml LPS or 100 μg/ml HA (10 μg/ml for 6-mer HA) of different MMs for 4 h, followed by the addition of 5 mM ATP or HAs of the different MMs for 1 h. Synovial fibroblasts and chondrocytes did not release IL-1β upon stimulation with any of the combinations ((a) and (b)). In PBMCs, the only combination that significantly increased the release of IL-1β was LPS followed by ATP. HA samples of different MMs were: 1.5 M (∼1680 kDa), 200 K (∼234 kDa), 20 K (∼28 kDa), 5 K (4-10 kDa) and 6-mer (∼1.2 kDa). Cytokines and chemokines were measured in cell culture media by ELISA and P-values were calculated using Dunnett's multiple comparison post-test. *(P < 0.05), **(P < 0.01) and ***(P < 0.001).

pro-inflammatory cytokines and chemokines (Supplementary Figure 2). We concluded that HA-fs do not have enhancing properties when added to already activated RA synovial fibroblasts and chondrocytes or PBMCs from healthy donors.

Synovial fibroblasts, chondrocytes and PBMCs express CD44 and TLR4

The main receptors reported to facilitate pro-inflammatory activities by HA-fs are CD44 and TLR4. To
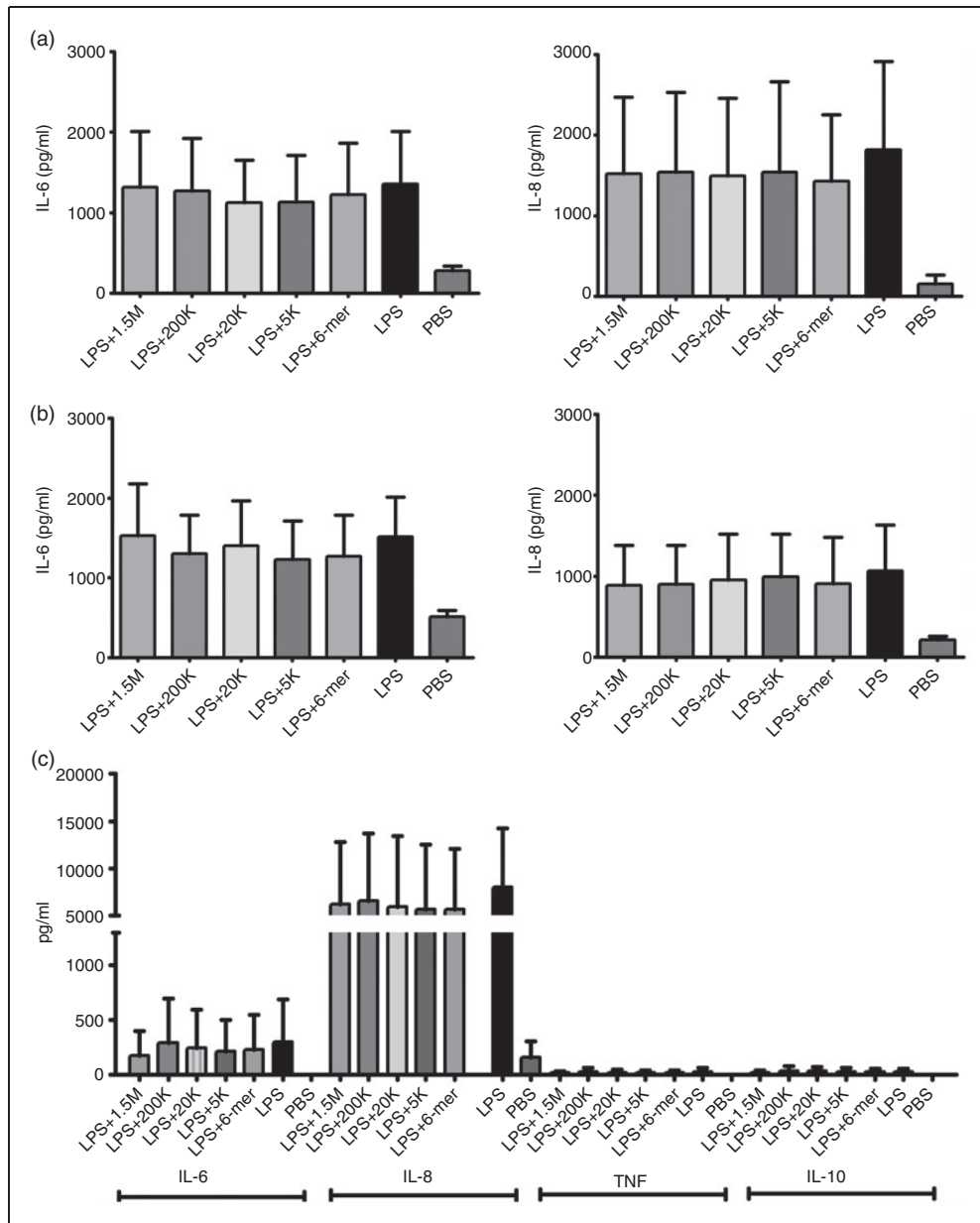
Figure 3. HA fragments do not function as enhancers of inflammatory cytokine production in LPS-primed synovial fibroblasts, chondrocytes or PBMCs. Cells from three individuals per cell type were primed with low levels of LPS (0.5 ng/ml in synovial fibroblasts and chondrocytes, and 10 pg/ml in PBMCs) for 2 h, followed by the addition of 100 µg/ml HAs (10 µg/ml for 6-mer HA) of different MMs for 19 h. Low levels of LPS alone induced the release of IL-6 and IL-8 from synovial fibroblasts (a) and chondrocytes (b) from RA patients and PBMCs from healthy controls (c). This effect was not enhanced by the addition of HAs of any MM. HA samples of different MMs were: 1.5 M (∼1680 kDa), 200 K (∼234 kDa), 20 K (∼28 kDa), 5 K (4-10 kDa) and 6-mer (∼1.2 kDa). Cytokines and chemokines were measured in cell culture media by CBA and ELISA, and P-values were calculated using Dunnett's multiple comparison post-test. *(P < 0.05), **(P < 0.01) and ***(P < 0.001).

exclude the possibility that the unresponsiveness to stimulation by HA-fs (including oligo-HA) seen in this study is a result an absence or low expression of the receptors on the cell surfaces, their expression was determined by immunofluorescent staining. Non-permeabilized synovial fibroblasts, chondrocytes and PBMCs were stained for the presence of surface TLR4 and CD44 using specific Abs. To control for unspecific staining, cells were also stained with irrelevant Abs as negative controls. Strong expression of both TLR4 and CD44 was observed in all three cell types (Figure 4). Accordingly, an absence of the main receptors suggested to be responsible for pro-inflammatory signalling of HA-fs could not explain the unresponsiveness of cells to HA-fs observed in this study.

Discussion

In this study, we evaluated the inflammatory properties of HA-fs (∼234, ∼28 and 4–10 kDa and 6-mer) in synovial fibroblasts and chondrocytes derived from patients with RA and in PBMCs from healthy donors. While the function of HA-fs as alarmins has been intensely studied, a consensus regarding their properties and mechanisms of action remains to be reached. This is mainly due to the many conflicting reports regarding which MM of HA is stimulatory, which receptors are important in conveying this signal, which cell types can be activated and what is the exact outcome. Recent reports have questioned whether HA-fs have pro-inflammatory properties at all. Here, we investigated the responsiveness of synovial fibroblasts and chondrocytes from RA patients to HA-fs. Both synovial fibroblasts and chondrocytes play an important role in the chronic inflammation seen in RA as they can both receive and secrete inflammatory signals such as cytokines. HA metabolism is altered during RA with higher degree of HA-fs formed. High HA-fs levels have also been correlated with higher inflammatory activity. Thus, it is plausible that both synovial fibroblasts and chondrocytes are exposed to HA-fs

and that such exposure might contribute to their activated states in RA. However, how and if they respond to HA-fs has not been studied before. In this study, we investigated not only the directly activating properties of HA-fs, but also the capacity of HA-fs to enhance rather than induce inflammation, a function previously described for alarmins.

First, we incubated cells with HAs of different MMs and measured the release of pro-inflammatory cytokines and chemokines in cell culture media to define the immunoactivating properties of HA-fs. None of the investigated HA forms increased the secretion of cytokines or chemokines by any of the cell types studied. This finding stands in contrast to most studies conducted to date, which have demonstrated a capacity of HA-fs to stimulate inflammation in both mouse and human cells. For example, 4.3 kDa HA-fs have been recorded to induce CD44-mediated release of IL-6 and IL-8 in human dermal fibroblasts,[26] and 200 kDa HA, as well as oligo-HA (4-, 6- and 8-mers), were described in two separate studies as potent stimulators of IL-8 production in human endothelial and epithelial cells.[18,28] Oligo-HAs (4–14-mers) have also been reported to stimulate the production of IL-1ß, TNF-α and IL-12 in human blood-derived DCs.[21] In our study,
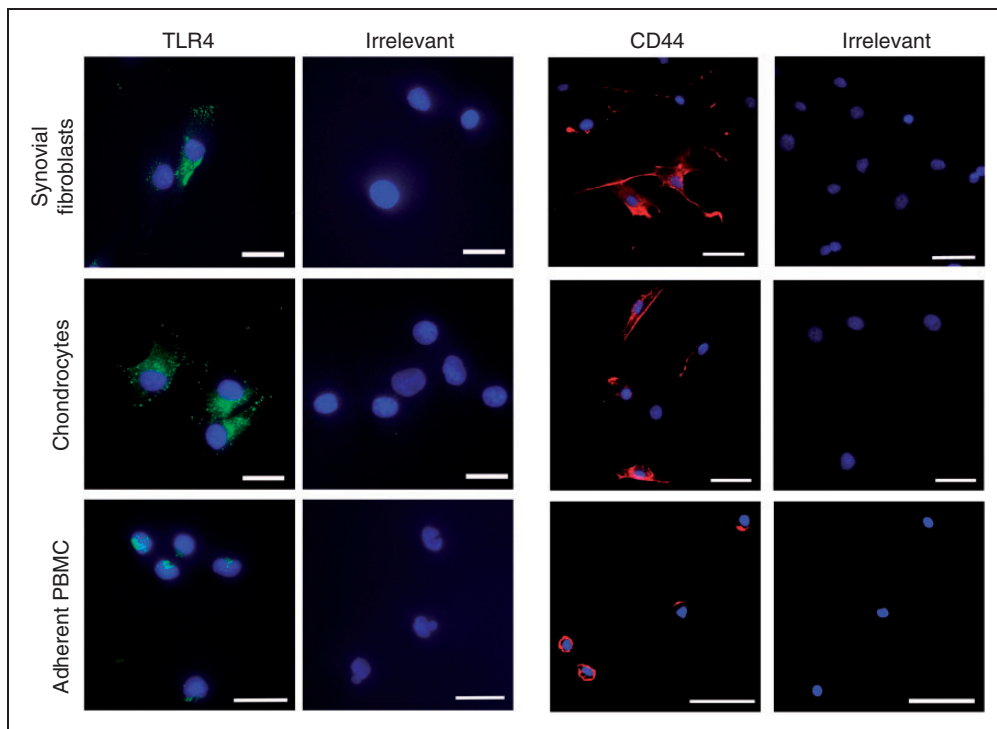
Figure 4. TLR4 and CD44 are expressed on synovial fibroblasts, chondrocytes and adherent PBMCs. Cells from one individual per cell type were seeded in chamber slides without exogenous stimulation, and immunocytochemical staining was used to determine the expression of TLR4 (green, Alexa Fluor 488) and CD44 (red, Alexa Fluor 594). Nuclei were counterstained with DAPI (blue). Synovial fibroblasts, chondrocytes and adherent PBMCs all expressed TLR4 and CD44. Left-side of all pictures show Ab staining and right-side control staining with irrelevant Ab (64 × magnification, scale bar is 50 μm).

the corresponding MMs of HAs reported to be pro-inflammatory were represented by 200 K HA-fs, 5 K HA-fs and a HA 6-mer.

As a comparison to RA synovial fibroblasts and chondrocytes, we used PBMCs from healthy donors, since both synovial fibroblasts and chondrocytes from healthy individuals are rare and difficult to obtain. None of the HA-fs used in our study had an effect on the production of inflammatory cytokines and chemokines (IL-1ß, IL-6, IL-8, IL-10 and TNF-α) in PBMCs. The lack of response to stimulation with HA-fs of these specialized immune cells is notable and stands in contrast to a study of human monocytes, in which cells released IL-6 and IL-8 upon stimulation with HAs specified as 'fragments of signalling size'.[29] The lack of response to HA-fs by any of the investigated cell types in this study could not be explained by cells lacking the suggested signalling receptors TLR4 and CD44. Synovial fibroblasts and chondrocytes from RA patients, as well as adherent PBMCs (monocytes) from healthy donors, all express these cell surface receptors, as verified by immunocytochemistry. Also, all cell types responded to small amounts of LPS, which is the prototype TLR4 ligand. Similar to LPS, HA-fs are reported to signal through TLR4.

During homeostatic non-inflammatory conditions, most immune cells (except alveolar macrophages) do not bind HA.[44] CD44 expression, and thereby the ability to bind HA, increases when cells are exposed to an inflammatory stimulus. Human monocytes increase their binding to HA after stimulation with LPS or TNF-α.[44] Therefore, we tested the ability of HAs of different MMs to stimulate already activated cells and thereby enhance the release of cytokines and chemokines. In addition, we investigated if HA-fs can form complexes with LPS and thereby augment inflammation. Such a scenario has been described for the well-studied alarmin HMGB1, which can form complexes with immunostimulating molecules including IL-1ß, CXCL12, DNA and LPS, and enhance innate immune responses.[30,31] An enhancing stimulatory effect could not be recorded from HA-fs in this study in either of these two experimental settings. Taken together, we conclude that HAs of the MMs used in this study do not by themselves activate, nor do they enhance, a pro-inflammatory response in synovial fibroblasts and chondrocytes derived from RA patients or PBMCs from healthy donors.

Our results contradict previous reports suggesting that HA-fs (including oligo-HA) are recognized as alarmins by various cells expressing CD44 and TLR4. There are different possible explanations for these conflicting results, including deviations in experimental setup or choice of read-out. Immunoactivation can be measured in multiple ways, such as the changed expression of cell surface costimulatory molecules, or the maturation and migration of immune cells. The cytokines and chemokines investigated in this study are classic inflammatory markers and were chosen to resemble previous studies. Our general experimental settings were also comparable to previous studies, including incubation time and the concentrations of HAs used in the attempts to stimulate cells. The MMs of HA-fs used (∼234, ∼28 and 4-10 kDa and 6-mer) have also been reported previously to induce inflammation.[13,14,18–22,24,26,28] We also investigated time and concentration dependence by stimulation with higher concentrations of HA-fs (up to 200 µg/ml) and longer incubation times (up to 72 h). In addition, we investigated a longer LPS priming step (48 h before adding HA-fs) without observing an activation of the cells in terms of cytokine or chemokine release in any of the settings. We argue that our experiments are comparable with previously reported studies in which HA-fs have been suggested to possess alarmin functions and that we are unlikely to have excluded the right signaling fragment size of HA.

In the majority of studies in which HA-fs have been defined to be pro-inflammatory, HA was purified from tissues such as human umbilical cord and rooster comb. This results in HA preparations containing a wide range of different MMs. In our study, and in the other recent studies indicating that HA-fs are immunologically inert, HAs of more specific MMs have been used. HA preparations are now available that are generated from enzymatic synthesis or purified after bacterial fermentation to yield defined MMs. Furthermore, HAs and HA-fs are available from several commercial suppliers. As the source of HA-fs in earlier studies is different from ours, this could be another possible reason for the differing results. However, bacterial HA synthases are not known to generate HA molecules that are any different from their mammalian counterparts, so this explanation is also unlikely.[45] The pharmaceutical-grade HA purified from bacteria has endotoxin levels below 0.01 EU/mg according to the manufacturer. In some of the studies where HA-fs were reported to have pro-inflammatory properties, the potential problem with contamination has been addressed. A standard method to remove endotoxin from HA is by treatment with the neutralizing agent polymyxin B. This method was also used in a study where HA originating from human umbilical cord induced various pro-inflammatory activities in mouse innate immune cells. Interestingly, this method was demonstrated to be insufficient to remove all endotoxins. Further analysis demonstrated that residual TLR ligands were still present in the HA preparation, which still induced inflammation.[33] Hence, this study suggests that other studies using polymyxin B to ensure HA purity may have reported results caused by remaining endotoxin contamination. In another study of HA-fs and their stimulatory effect on monocytes, DNA contaminated HA and was demonstrated to be responsible for the induced pro-inflammatory

response in monocytes.[46] It is worth noting that the direct binding of HA-fs to TLR4 has yet to be established and that contaminants from HA production (such as LPS) can give similar results.

In conclusion, we report that endotoxin-free HA-fs of various MMs were unable to induce or enhance pro-inflammatory cytokine release from synovial fibroblasts and chondrocytes from RA patients or from PBMCs from healthy donors, despite their proven expression of the HA receptors CD44 and TLR4. Our results imply that it is unlikely that HA-fs contribute to the inflammatory processes that are ongoing in arthritic joints, although other cell types in the synovial compartment have to be tested in order to completely exclude this possibility. This study also supports a scenario where the increased levels of HA-fs reported during the inflammatory response are a consequence, rather than the cause of, inflammation. To our knowledge, the ability of HA-fs to induce or enhance pro-inflammatory activities in joint-derived cells from RA patients has not been investigated previously; thus, our study contributes important information on the role of HA-fs as alarmins and in arthritis pathogenesis. Furthermore, HA is a molecule widely that is used for multiple medical device applications, as well as in the cosmetic industry, hence it is important to fully understand its effect on physiological processes, an on inflammation in particular.

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