Hyaluronan (HA) fragments as initiators or enhancers of inflammation in arthritis patients

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MASTER THESIS PROJECT, KTH
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1. Abstract

Degraded fragments of Hyaluronan (HA) accumulate at sites of inflammation. Whether these fragments are proinflammatory is debated and not known. Many studies suggest that HA is proinflammatory, but some claim that the proinflammatory effects could be caused by endotoxin contamination. Elevated levels of HA fragments are observed in arthritis patients, making it relevant to study in the context of this disease. The joint-specific cells, chondrocytes and synovial fibroblasts, were in this study stimulated with HA of four different molecular weights followed by quantification of key proinflammatory cytokines (IL-6, IL-8 and IL-1β) with ELISA to establish if HA acts proinflammatory in these cells. PBMCs from healthy controls was also used in the stimulation experiments. HA was investigated as an (i) initiator or (ii) enhancer of inflammation. In this study, HA did not increase the release of IL-6, IL-8 or IL-1β in synovial fibroblasts or chondrocytes. HA did not increase the release of IL-6 or IL-1β in PBMCs. The results of this study suggest that HA is not proinflammatory to cells involved in the pathogenesis of arthritis. They also support the claim that HA is not an alarmin. The proinflammatory effects following HA stimulation may have been caused by endotoxin contamination.
2. Sammanfattning

Förhöjda nivåer av fragmenterat Hyaluronan (HA) existerar vid platsen för inflammation. Huruvida dessa fragment är proinflammatoriska är ett debatterat ämne som ännu inte är fullt utrett. Flertalet artiklar som försökt reda ut detta hävdar att HA är proinflammatoriskt, medan vissa forskare föreslår att den proinflammatoriska effekten kan vara orsakad av endotoxinkontaminering. Förhöjda nivåer av HA fragment har påvisats hos artritpatienter, vilket gör det relevant att studera dessa i patienter med artrit. I denna studie stimulerades ledpsecifika celler, kondrocyter och synoviala fibroblaster, med HA av fyra olika molekylvikter. Därefter följde mätning av centrala proinflammatoriska cytokiner (IL-6, IL-8 och IL-1β) med ELISA i cellmediet för att fastställa om HA verkar proinflammatoriskt i dessa celler. PBMCs från friska kontroller stimulerades också i experimenten. Det undersöktes i denna studie om HA kan (i) initiera eller (ii) förstärka inflammation. HA inducerade inte frisläppning av IL-6, IL-8 eller IL-1β i synoviala fibroblaster eller kondrocyter. HA inducerade inte frisläppning av IL-6 i PBMCs. Resultaten av denna studie föreslår att HA inte verkar proinflammatoriskt i celltyperna som medverkar i sjukdomsförloppet i artrit. Resultaten stödjer även påståendet att HA inte är ettalarmin, samt att proinflammatoriska effekter av HA stimulering kan ha orsakats av endotoxinkontaminering.
3. Introduction

3.1. Rheumatoid arthritis
Rheumatoid arthritis (RA) is a chronic inflammatory and destructive disease, posing not only high personal costs for the affected individual in terms of pain and disability, but also high socioeconomic costs. Being one of the most prevalent chronic inflammatory diseases,\(^1\) it has a relatively constant incidence (as of 2002) of 0.5% – 1% in many populations.\(^2\) The disease primarily affects the joints, although it often develops to include extra-articular manifestations as well, for example rheumatoid nodules (firm lumps under the skin often localised near the arthritic joints\(^3\)), pulmonary involvement, cardiovascular, psychological and skeletal comorbidities.\(^4\) Characteristics of RA include synovial inflammation and swelling of the joints, and eventually the disease causes damage and break-down of cartilage and bone.\(^4\)

RA patients generally experience swollen and fragile joints, stiffness of joints in the morning and have abnormally high levels of C-reactive protein or erythrocyte sedimentation rate. These features are however not exclusive to RA, but are also observed in other types of arthritis such as reactive arthritis, infectious arthritis, and Lyme disease. Most commonly, patients who seek care for their symptoms are first diagnosed with undifferentiated arthritis, i.e. arthritis that cannot at that time be characterized as a specific disease.\(^5\) These patients are subsequently followed and can later formally be diagnosed with RA if symptoms remain for more than 6 weeks and the patient meets the diagnostic criteria for RA, as described in the American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis,\(^6\) or later by the 2010 Rheumatoid Arthritis Classification Criteria.\(^7\)

There are today several different treatments available for RA, with the majority aiming at reversing the inflammation and alleviating the symptoms. Nonetheless, the disease is chronic and cannot with current treatments be cured.\(^8\) Thus, an unmet need in treating RA patients in the clinic is continuously being dealt with, and currently there is a lot of research taking place to establish the cause of disease in order to prevent it and treat it at an earlier stage.

3.2. Juvenile Idiopathic arthritis
Juvenile idiopathic arthritis (JIA) is a broad umbrella term referring to all the chronic inflammatory arthritides with onset in children and adolescents up until the age 16 years and lasting for longer than 6 weeks.\(^9\) JIA is the most prevalent chronic rheumatic condition in
children and a common cause of disability. When patients are diagnosed with JIA, it is often a diagnosis of exclusion, due to the lack of distinctive characteristics of the disease. The broad term JIA is further divided into seven disease categories based on the clinical and laboratory features observed during the first 6 months after the patient has been diagnosed with the disease. These different categories are: (i) polyarthritis RF-positive, (ii) polyarthritis RF-negative, (iii) oligoarthritis (persistent and extended), (iv) psoriac arthritis, (v) enthesitis-related arthritis, (vi) systemic onset JIA, and (vii) undifferentiated arthritis. The seven sub-types of JIA are briefly described in table 1.

### Table 1: Brief descriptions of JIA subtypes

<table>
<thead>
<tr>
<th>Sub-type of JIA</th>
<th>Arthritis pattern</th>
<th>Additional symptoms that may occur</th>
<th>Autoantibodies</th>
<th>Predisposition</th>
</tr>
</thead>
<tbody>
<tr>
<td>(i) and (ii) Polyalthritis (RF+ or RF-)</td>
<td>≥ 5 joints affected, both small and big, usually symmetrical</td>
<td>Iridocyclitis</td>
<td>20% RF positive</td>
<td>Girls</td>
</tr>
<tr>
<td>(iii) Oligoarthritis (persistent or extended)</td>
<td>&lt; 5 of the big joints affected, usually asymmetrical</td>
<td>20-30% with chronic uveitis</td>
<td>70-80% ANA positive</td>
<td>Girls, early onset &lt;6 years</td>
</tr>
<tr>
<td>(iv) Psoriatic arthritis</td>
<td>Variable</td>
<td>Psoriasis, dactylitis, nail pitting</td>
<td>75% ANA positive</td>
<td>No gender bias</td>
</tr>
<tr>
<td>(v) Enthesitis-related arthritis</td>
<td>Hips and back</td>
<td>Inflammation of tendons or ligaments insert</td>
<td>No RF or ANA</td>
<td>Boys &gt; 6 years</td>
</tr>
<tr>
<td>(vi) Systemic onset JIA (soJIA)</td>
<td>Often affects several joints, usually symmetrical. May not be present at onset.</td>
<td>Fever, rash, systemic organ involvement (liver, kidney, lymph nodes, lungs, heart)</td>
<td>No RF or ANA</td>
<td>No gender bias</td>
</tr>
<tr>
<td>(vii) Undifferentiated</td>
<td>Patients do not fit into any, or fit into several of the JIA sub-types above.</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

RF = rheumatoid factor, ANA = antinuclear antibodies.

The difference between JIA and RA is complicated and not completely established. Similarities include that both RA and most JIA subtypes are autoimmune arthritis conditions characterized by joint destruction, clinical and pathological features are shared, and both diseases are genetically complex (see section 3.4.). One difference between RA and JIA include the clinical phenotype of the diseases, where JIA is divided into seven different categories, and RA represents a more homogenous disease, commonly divided into the two subgroups seronegative and seropositive patients. Some JIA patients receive disease remission, unlike RA patients, and other JIA patients are later diagnosed with RA. Polyarthritis RF-positive is the JIA subtype which is most phenotypically similar to RA in adults and is considered to be
representative of childhood RA. JIA patients who are positive for both RF and ACPA (see section 3.3.) phenotypically resemble adult patients with RA.

Considering the lack of understanding of why and how these arthritic diseases evolve, the amount of suffering they pose to the affected individuals and costs to society, research aiming at a deeper understanding of the underlying causes of disease is required to develop successful treatments. It is hoped that focus on finding biomarkers will enable more indications of subtypes within RA and JIA that will improve the prognosis, and enable the use of personalized and targeted medicine.  

3.3. Autoimmunity
RA and the majority of JIA subtypes are autoimmune diseases, meaning that the immune system of the patient erroneously reacts to and attacks “self-tissue”. Autoimmune diseases are a diverse group with over 80 chronic disorders, affecting over 4% of the western population. These diseases are characterized by the production of antibodies with reactivity towards self-antigens, so called autoantibodies. Autoantibodies have shown to be important biomarkers of autoimmune diseases. Autoimmune disorders are also characterized by auto-reactive T-cells, i.e. T-cells which have escaped the immunological tolerance mechanisms which purpose is to eliminate or inactivate T-cells containing auto-reactivity. Furthermore, autoimmune diseases extending beyond arthritides, such as type 1 diabetes and multiple sclerosis, are strongly associated with the genetic region human leukocyte antigen (HLA), which is encoding the human major histocompatibility complex (MHC) region. The HLA region is currently considered to be one of the most important regions in the human genome with regards to autoimmunity, as it encodes many genes that are key in immune responses. Specific components encoded by the HLA class II region has a role in non-self-antigen presentation to T-helper cells and has been specifically associated with RA, implicating this pathway’s significance in the disease development.

In 1939, Erik Waaler discovered the first human autoantibody rheumatoid factor (RF) in sera from an RA patient. Since then, several other autoantibodies such as anti-citrullinated protein antibodies (ACPAs) reacting to citrullinated proteins and peptides (where arginine has been converted to the α-amino acid citrulline as a result of a post-translational modification), and antinuclear antibodies (ANAs) with reactivity towards several nuclear macromolecules have been detected in RA and JIA patients. The presence of autoantibodies against post-
translationally modified proteins, and the presence of RF in RA and JIA patients is clinically referred to as seropositivity, and has been associated with more severe symptoms and damage to the joints in RA. If these autoantibodies are not present, the patients are referred to as seronegative.

The increase in joint damage observed in seropositive patients has been suggested to arise from the fact that ACPAs form immune complexes with antigens containing citrulline, and further that these complexes binds RF, leading to complement activation. It has previously been shown that ACPAs are able to bind citrullinated residues on several self-proteins such as type II collagen, fibronectin, fibrinogen, histones, vimentin and α-enolase. The presence of ACPAs in the blood circulation has been detected several years before clinical onset of the disease, referred to as pre-rheumatoid arthritis. It would be valuable if this could be detected and therapy started earlier, since early diagnosis in best case can result in disease remission and has the potential to minimize damage to bone and cartilage.

3.4 Etiology
JIA and RA are complex diseases, which refers to that they are caused by a combination of genetic, epigenetic and environmental factors, as opposed to Mendelian diseases which are caused by one disease-causing gene. More than 100 different genetic loci, both within and outside the HLA region, have been associated with a risk of developing RA through genome-wide association studies. Regarding JIA, the progress in finding susceptibility genes has not been as rapid, and a handful of non-HLA genes have to date been identified. The heritability in RA is implied from descriptive epidemiology, although compared to some other autoimmune diseases, the genetic influence of RA appears to be smaller. Nevertheless, a positive family history is associated with a higher risk of developing the disease and studies have shown that 50-60% of RA occurrence in twins is due to shared genetic effects, and heritability is approximated to 50% for seropositive patients and 20% for seronegative patients. Having a first-degree relative (sibling, child or parent) with RA increases the risk of developing RA with a two-to five-fold, making it one of the strongest risk factors for RA. There are also environmental triggers described to be associated with the disease, including for example smoking.

Epigenetics, a term coined in 1942 by C.H. Waddington, which in short words means the changes that occur in phenotype without changes in the genotype can also influence the
disease pathogenesis in RA. It has for example been shown that changes in DNA methylation (an epigenetic modification) occur in immune cells of RA patients.35

3.5. Inflammation
RA pathogenesis is characterized by an inflammation in the synovial membrane which occurs upon infiltration of leukocytes into the synovial compartment. This leads to swelling of the joint and synovial membrane (synovitis).4 Migration of cells into the synovial compartment is made possible by endothelial activation of synovial microvessels, which increases their expression of various adhesion molecules and chemokines.4 Microenvironmental changes in combination with architectural reorganization of the synovium, as well as fibroblast activation, lead to the formation of an inflamed synovial tissue in RA,4 where the cell composition of the synovium typically includes both innate immune cells (monocytes, mast cells, dendritic cells) and adaptive immune cells (T cells and B cells).1

3.5.1. Cells of the synovium
Besides the infiltrating immune cells, tissue cells of the synovium and cartilage are important contributors to the development of RA. Two of these cell types, synovial fibroblasts and chondrocytes, are in focus in this thesis. Rheumatoid arthritis synovial fibroblasts (RASFs) are believed to mediate the majority of joint destruction pathways observed in RA. Activated RASFs are engaged in the induction and continuation of the disease. When these RASFs have become activated (which is believed to occur early in RA) they start to produce and secrete proteins (for example cytokines, see below). The cytokines are important for the interactions with immune cells responsible for the progressive destruction of cartilage and bone which is observed in RA joints.36

Chondrocytes are the cells that populate the articular cartilage, which has important functions in the joints in providing a low-friction, articulating interface between the opposing bone surfaces. At normal conditions, the chondrocytes maintain a stable equilibrium of synthesis and degradation of matrix components such as collagen and proteoglycans, like aggrecan, -attached to hyaluronan. In RA, the cartilage matrix components become digested, and it is believed that chondrocytes participate in degrading their own matrix. It has also been suggested that chondrocytes can themselves secrete proinflammatory cytokines which increase tissue catabolism and suppress the anabolic repair processes. This disequilibrium is thought to contribute to the fast degradation of cartilage matrix components observed in RA joints.37
3.5.2. Cytokines

Cytokines are small proteins released from cells, which are important for the interactions and communication between cells. Cytokines can have an effect on the cells that release them, nearby cells or distant cells, and are often produced in cascades, whereby one cytokine stimulates its target cell to produce other types of cytokines. Proinflammatory cytokines have a crucial role in the induction and progression of RA and other inflammatory disorders, and the production of them by synovial cells is central to the pathogenesis of the disease. Specifically which cytokines that are expressed may change over time in the disease development, but some important ones are discussed below.

Tumor necrosis factor (TNF)-α in combination with interleukin-1β (IL-1β) play a crucial role in RA as they up-regulate each other’s expression in a positive feed-back loop which is responsible for most of the indicative symptoms. TNF-α and IL-1β also induce proliferation of activated fibroblasts which produce proteolytic enzymes causing break-down of cartilage, and activate cells which in turn demineralize bone. It is believed that two distinct signals are required in order to release mature IL-1β from cells. These two signals are referred to as the priming step and the activation step, where priming often is induced by bacterial toxins such as Lipopolysaccharide (LPS), leading to de novo synthesis of inactive, pro-IL-1β. The second signal can for example be induced by exogenous ATP activation. This step leads to assembly and activation of the inflammasome, a multiprotein complex important for the innate immune response, responsible for activating caspase-1. Caspase-1 is required to cleave pro-IL-1β into its mature and active form, which subsequently gets released by the cells.

The expression of IL-6 in synovial fibroblasts is induced by IL-1β in combination with IL-17. IL-6 is a multifunctional, proinflammatory cytokine that is highly expressed in many different inflammatory diseases, including RA and JIA. The activity of IL-6 contributes to both the local and systemic symptoms of RA, and also induce the acute-phase response, which in turn activates complement, induces other proinflammatory cytokines and stimulates neutrophil chemotaxis. It is suggested that IL-6 is capable of mediating both proinflammatory effects and antiinflammatory effects and it exists at elevated levels in both synovial fluid and serum of patients with RA. Another proinflammatory cytokine which has an important role in joint diseases is IL-8, which has also been found in high concentrations in synovial fluids of patients with arthritis. A relationship between these three important cytokines IL-6, IL-8 and IL-1β
has been found in RA patients, where IL-6 and IL-8 was also correlated with the level of inflammation of the synovial fluid. 43

3.6.Treatments
The main different types of treatments used for treating RA and JIA are: (i) non-steroidal anti-inflammatory drugs (NSAIDs), which have anti-inflammatory and pain-relieving properties although little effect on the underlying causes of disease and cartilage erosion;44 (ii) pain-killers, (iii) disease-modifying antirheumatic drugs (DMARDs), which can slow the progression of RA, reduce cartilage erosion, and is currently recommended to use in the early phases of RA when the disease is progressing rapidly, (iv) Cortisone treatment, for decreasing the inflammation, and (v) biological agents, such as antibodies.

Most commonly, RA patients are first given a long-term treatment in the form of a DMARD, such as Methotrexate, in combination with pain-killers and a drug aiming at decreasing the inflammation. Cortisone tablets or cortisone injections can be prescribed meanwhile the DMARD starts to have effect. If Methotrexate does not give full effect, it is combined with other DMARDs such as Sulphasalazine. If the combination therapy does not have effect, biological agents are generally prescribed. For JIA patients, Methotrexate is also the most commonly used DMARD, due to its effectiveness at acceptable toxicity levels.10

NSAIDs have been the mainstay treatment form for all subtypes of JIA but should not be used as a monotherapy for more than two months if arthritis persists. Only some types of these drugs, such as naproxen and ibuprofen, are approved for use in children. Other common treatments in children with JIA include intra-articular corticosteroid injections.10

Biological agents include anti-TNF-α drugs, and represents an important drug class for both RA and JIA patients.44 There are currently five different treatments for blocking TNF-α, and other cytokines which can be blocked with biological agents include IL-6 and IL-1β. The number of biological agents for treating RA and JIA are believed to increase within the near future due to the extensive research taking place in this field.45

To conclude, current treatments for RA and JIA mainly aim at reversing inflammation and sooth the symptoms. By decreasing the inflammation, the damage can be prevented and physical function can be improved. The treatment of these diseases also include regular assessment of
how far the disease has progressed to adapt and optimize the therapeutic approach. If the disease is not treated adequately, manifestations other than in the joints can arise, for example vasculitis or interstitial lung disease. Furthermore, medical treatments are generally combined with physical and psychological therapy. 

3.7. Alarmins
Alarmins are endogenous molecules belonging to the damage-associated molecular pattern (DAMP) group of proteins. Following an infection or tissue damage, alarmins are released from cells into the extracellular space, where they act as signals of danger. The release of alarmins can occur by both active and passive processes. The active processes take place in activated (immune) cells, while the passive release for example takes place during cell death. Alarmins are important for the immune system in triggering inflammation and tissue repair, and some alarmins common in chronic inflammatory joint diseases include High Mobility Group Box 1 (HMGB1), S100 proteins and IL-33. Alarmins are currently seen as potential markers of the destructive state of joints, as well as therapeutic targets, making them highly relevant to study in diseases resulting in joint damage.

In normal conditions, alarmins have important intracellular functions, but upon activation, after transportation out into the extra-cellular milieu, they act as inflammatory mediators. The excreted alarmins can recruit immune cells of the antigen-presenting type, for example dendritic cells (DCs). Alarmins bind to pattern recognition receptors (PRRs) on immune cells, such as the toll-like receptors (TLRs) TLR-2 and TLR-4, as well as the Receptor for Advanced Glycosylation of End products (RAGE). Upon binding, specific signalling pathways are activated in the immune cells. The stimulated immune cells will subsequently initiate both the innate and adaptive immune response, for example by releasing cytokines, and this ultimately leads to inflammation and/or tissue regeneration. High extracellular levels of alarmins, such as HMGB1, have been detected in synovial tissue biopsies from RA patients and plays an important role in the pathogenesis of arthritis.

3.7.1. HMGB1: a well-studied alarmin
HMGB1 is an abundant protein with important roles both intra- and extra-cellularly. The protein is normally present in the cell nucleus where it associates with linear DNA in a non-sequence dependent way, and binding preferentially occur to bent or distorted DNA. HMGB1 has an architectural role in several DNA-dependent processes which occur in the nucleus,
including transcription, replication, recombination, and repair, and it also aids binding of transcription factors to DNA.\textsuperscript{51}

The role of HMGB1 as a mediator of inflammation was originally defined by Wang et al., in 1999 in endotoxin-stimulated mice,\textsuperscript{52} and since then, the protein has become extensively studied for its critical role in sterile inflammation and autoimmunity, where RA has been at the core of the research.\textsuperscript{50} Evidence of the important role of HMGB1 in RA pathogenesis include the fact that it is released at the site of joint inflammation, and injection of HMGB1 in a healthy joint causes the development of arthritis.\textsuperscript{53} Expression of abnormal, extranuclear HMGB1 has been detected in both synovial tissue\textsuperscript{54} and serum\textsuperscript{55} of RA patients. It has been suggested that HMGB1 triggers joint inflammation by activating macrophages and inducing production of IL-1β,\textsuperscript{56} and it has been described as a novel biomarker in RA pathogenesis.\textsuperscript{57} It has further been shown that HMGB1 in complex with LPS can increase the production of proinflammatory cytokines (TNF-α, IL-6 and IL-8) compared to LPS alone, in synovial fibroblasts isolated from RA patients.\textsuperscript{49}

\subsection*{3.8. Hyaluronan}
Hyaluronan (HA) is a non-sulphated polysaccharide consisting of alternating D-glucuronic acid and D-N-acetylglucosamine units bound through β-1,4 and β-1,3 glycosidic bonds.\textsuperscript{58} Originally being isolated from the vitreous humour in 1934,\textsuperscript{59} it was later recognized to constitute a major part of the extracellular matrix (ECM)\textsuperscript{60} where it exerts the majority of its important functions. In vertebrates, HA is found in all tissues and fluids, but is especially abundant in humans in the umbilical cord, synovial fluid, the dermis skin layer and the vitreous body of the eye.\textsuperscript{61} HA is not, in contrast to other glycosaminoglycans, synthesized in the ER/Golgi compartment, but instead synthesized directly at the plasma membrane by membrane-bound glycosyltransferases, after which it protrudes into the extracellular space.\textsuperscript{62}

The biological functions that has been attributed to HA are widespread and includes space filling, lubrication of joints, tissue repair, water homeostasis\textsuperscript{61} and regulation of cells.\textsuperscript{60} HA can reach remarkable molecular masses, which explains some of its functions in the ECM. HA is constantly produced and degraded in a natural process by endogenous enzymes (hyaluronidases) and has a very high turnover rate, with almost one third of the total HA in our bodies being replaced by newly-formed HA every 24 hours.\textsuperscript{63} Due to this high turnover rate,
which is important for maintaining tissue homeostasis, all different molecular weights of HA exists at all times in our bodies.

Except for being degraded naturally by hyaluronidases, HA can also be fragmented in other ways, by for example reactive oxygen species (ROS) and hypohalous acids (present in trauma) and bacterial hyaluronidases which are all substances generated in inflammatory responses.\(^{58}\)

Due to some important properties that HA possesses such as high viscoelasticity, biocompatibility and non-immunogenicity, it is widely used in the biomedical and pharmaceutical industries, and HA is a common ingredient in face creams and other cosmetic products. Furthermore, HA has been studied as a drug delivery system through many different routes (e.g. ophthalmic and dermal routes).\(^{64}\) Furthermore, intra-articular injections of HA is used to as a treatment for knee osteoarthritis, where it has been suggested to decrease the symptoms.\(^{65}\)

The role of HA in inflammation is a debated and controversial subject, which is far from completely understood. It has been suggested that HA can possess both pro- and anti-inflammatory properties, which is dependent on its molecular weight (size). Following tissue injury, HA and its degraded fragments accumulate,\(^{63}\) and low molecular mass (LMW) HA fragments are generated during inflammation. It is however still unclear whether HA fragments also induce inflammation, or if they are present only as a result of inflammation.\(^{66}\) It is also known that at sites of inflammation, high molecular weight (HMW) HA can become depolymerized to LMW fragments by ROS or other enzymes.\(^{67}\)

The majority of authors and articles regarding HA’s role in inflammation suggest that HA (and specifically LMW fragments) is proinflammatory and can act as an alarmin in immune cells (as reviewed in Lee-Sayer et al., 2015).\(^{68}\) Some of the results of these studies are summarized in table 2.
Table 2: Studies claiming that HA is proinflammatory.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>HA molecular weight</th>
<th>HA source</th>
<th>Effect</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse alveolar macrophages</td>
<td>200 kDa</td>
<td>Human umbilical cord</td>
<td>Induced expression and production of murine metalloelastase</td>
<td>Horton et al., 1999.69</td>
</tr>
<tr>
<td>Human monocyte-derived dendritic cells</td>
<td>4-mers and 6-mers</td>
<td>HA for clinical applications, endotoxin content &lt;0.1 ng/ml.</td>
<td>Increased production of IL-12, IL-1β and TNF-α.</td>
<td>Termeer et al., 2000.70</td>
</tr>
<tr>
<td>Human peripheral blood mononuclear cells (PBMCs)</td>
<td>100-150 kDa</td>
<td>Pig skin</td>
<td>Induced IL-6 and Monocyte Chemoattractant protein 1</td>
<td>Yamawaki et al., 2008.71</td>
</tr>
<tr>
<td>Bronchiolar epithelial cells</td>
<td>370 kDa</td>
<td>Commercially purchased</td>
<td>Induced IL-8 production</td>
<td>Mascarenhas et al., 2004.72</td>
</tr>
<tr>
<td>Mouse chondrocytes</td>
<td>50 kDa</td>
<td>Purchased HA sodium salt.</td>
<td>Increased production of MMP-3 and iNOS</td>
<td>Campo et al., 2009.73</td>
</tr>
<tr>
<td>Mouse synovial fibroblasts</td>
<td>4-mers</td>
<td>Purchased HA sodium salt.</td>
<td>High production of TNF-α and IL-1β</td>
<td>Campo et al., 2013.74</td>
</tr>
<tr>
<td>Mouse synovial fibroblasts</td>
<td>6-mers</td>
<td>Purchased HA sodium salt.</td>
<td>Upregulation of IL-18 and IL-33</td>
<td>Campo et al., 2012.75</td>
</tr>
</tbody>
</table>

MMP-3: matrix metallopeptidase-3, iNOS: inducible nitric oxide synthase.

There are however some more recent studies that contradict the statement that HA acts as an alarmin and instead suggests that HA and fragments thereof does not alone induce cytokine production, and is not proinflammatory. Some of these studies are summarized in table 3.
Table 3: Studies claiming that HA is not proinflammatory.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>HA molecular weight</th>
<th>HA source</th>
<th>Effect</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human lung fibroblasts as a model for RASFs</td>
<td>900 kDa</td>
<td>Commercially bought</td>
<td>Supressed LPS-induced MMP-1 expression</td>
<td>Hirabara et al., 2013.</td>
</tr>
<tr>
<td>Mouse dendritic cells and macrophages</td>
<td>1680 kDa, 234 kDa, 28 kDa, &lt;10 kDa</td>
<td>Pharmaceutical grade HA</td>
<td>Pharmaceutical grade HA did not stimulate TNF-α or IL-1β production</td>
<td>Dong et al., 2016.</td>
</tr>
<tr>
<td>Mouse macrophages</td>
<td>11 kDa, 52 kDa, 87 kDa, 250 kDa, 970 kDa</td>
<td>Pharmacological grade HA</td>
<td>No stimulation of production of TNF-α or NFκB</td>
<td>Krejcová et al., 2009.</td>
</tr>
<tr>
<td>Rodent air pouch model</td>
<td>5-150 kDa</td>
<td>Commercially purchased</td>
<td>No stimulation of production of TNF-α or IL-1β</td>
<td>Huang et al., 2014.</td>
</tr>
<tr>
<td>Mouse mesangial cells</td>
<td>&lt;1500 Da and 1500-3000 Da</td>
<td>Commercially purchased</td>
<td>No stimulation of production of IL-6</td>
<td>Ebid et al., 2013.</td>
</tr>
</tbody>
</table>

MMP-1: matrix metalloproteinase 1, NFκB: nuclear factor kappa-light-chain-enhancer of activated B cells.

Some authors of the studies in table 3 hypothesize that endotoxins and other unrelated proteins in the HA preparations used may be the cause for an observed proinflammatory effect when cells are stimulated with the HA. HA samples derived from e.g. the umbilical cord are known to contain protein and nucleic acids, and HA contamination can also be introduced when preparing or handling the samples. It is important to establish whether the proinflammatory effects observed in the studies summarized in table 2 were due to contamination of the HA samples or caused by the HA. Endotoxins are cumbersome to separate from HA, but since concentrations as low as 10 pg/ml of endotoxin LPS can have proinflammatory effects in immune cells, it is important to ensure that HA preparations are endotoxin-free.

Furthermore, the literature summarized in table 2 is also conflicting regarding which receptors HA is signalling through, what the proinflammatory outcome is, and exactly what sizes of HA that are proinflammatory.

It has been shown in several studies that RA patients have elevated levels of HA in the circulation. One study demonstrated that RA patients had, in average, a seven-fold higher level
of HA in plasma than the age-matched, non-arthritic control group. These studies also suggest that the amount of HA elevation may reflect the level of synovial involvement\textsuperscript{81} and inflammation\textsuperscript{82} in arthritis patients. Other studies focusing on serum levels of HA in JIA patients have concluded that measuring serum HA levels could be a useful tool for diagnosing some sub-types of the disease, such as polyarticular\textsuperscript{83} and systemic onset\textsuperscript{84} JIA.

HA’s signalling route has been suggested to go through TLR-4\textsuperscript{85} which is a receptor that has previously been stated to be highly expressed in synovial fibroblasts.\textsuperscript{86} Other proposed receptors include CD44\textsuperscript{67} and TLR-2.\textsuperscript{87} Since HA is naturally found at a high abundance in synovial fluid\textsuperscript{61} and its fragmentation is related to inflammation\textsuperscript{66}, it is of high interest to study in inflammatory joint diseases such as RA and JIA, in cells present at the site of inflammation (synovial fibroblasts and chondrocytes).

3.9. Aim
The aim of this master thesis project is to investigate the role of hyaluronan (HA) as an alarmin, and its capacity to induce inflammation in the context of arthritis. To investigate this, the inflammatory responses of degraded HA of different molecular weights, and the well-known alarmin HMGB1, was studied in joint tissue cells. Furthermore, levels of HA and HMGB1 were quantified in synovial fluid and plasma samples from patients suffering from JIA.
4. Materials and Methods

4.1. Cell cultures
Synovial fibroblasts and chondrocytes were isolated from RA patients undergoing knee replacement surgery at the Karolinska University Hospital. Cells were cultured in standard tissue culture (TC)-flasks (Sarstedt). Synovial fibroblasts were cultured in complete media consisting of high glucose Dulbecco’s Modified Eagle’s Medium (DMEM, Sigma-Aldrich), 10% heat-inactivated Fetal Bovine Serum (FBS, Sigma-Aldrich), 100 µg/ml Penicillin-Streptomycin (Sigma-Aldrich) and 2 mM L-glutamine (Sigma-Aldrich). Chondrocytes were cultured in complete media with DMEM-F12 (Sigma-Aldrich) instead of DMEM. Cells were grown in a tissue culture incubator at 37 °C and 5% CO₂ and were routinely split by Trypsinization (Sigma-Aldrich) at 70-80% confluency. All synovial fibroblasts used were between passages 3 to 8 and chondrocytes were between passages 1 to 4.

4.2. Immunofluorescence staining
8000 cells/well were seeded on an 8-chamber culture slide and cultured overnight at 37 °C and 5% CO₂. Supernatant was discarded and the slide was washed with PBS two times and fixed with 4% Paraformaldehyde (PFA) in phosphate buffer 0.1 M, pH 7.4 (Histolab products) for 10 minutes (min) on ice. The fixative was discarded and the slide was washed with PBS two times and stored in 4 °C in PBS.

Slides were blocked with 5% Fetal Calf Serum (FCS, Sigma-Aldrich) for 10 min at room temperature (RT) and washed in PBS 3 x 5 min. Slides were blocked with Normal Donkey Serum (NDS) for one hour at RT and washed with PBS 3 x 5 min. Primary antibody was added to the slide and incubation overnight at 4 °C followed. The primary antibody used was: TLR-4: 8694 (Santa Cruz Biotechnology) and as a negative control an irrelevant goat IgG antibody (Sigma-Aldrich). All primary antibodies were used at a concentration of 1 µg/ml. Slides were washed 3 x 5 min in PBS and incubated with secondary antibody (Alexa 594, donkey anti-goat, A11058, lot 440197, Molecular probes) for 1 hour at RT. Slides were washed for 3 x 5 min in PBS and Phalloidin (Thermo Scientific) diluted in 1% BSA was added to the slide and incubated for 20 min at RT. Slides were washed 3 x 5 min in PBS and washed in 70% Ethanol and left to dry. Slides were mounted in ProLong Gold antifade reagent with DAPI (Molecular probes by Life Technologies) and analysed in an immunofluorescent microscope using the Leica system.
4.3. Patient samples

Plasma and synovial fluid samples from the Juvenile Arthritis BioBank Astrid Lindgrens Children Hospital (JABBA) were used in this thesis to measure levels of HA, HMGB1 and cytokines IL-6 and IL-8.

4.4. Isolation of PBMCs

PBMCs from healthy volunteers were purified using standard Ficoll-Paque (Ficoll-Paque Plus, GE Healthcare) separation with PBS as buffer. The cells were re-suspended and seeded in RPMI-1640 media (Sigma-Aldrich) supplemented with 10% heat-inactivated FBS (Sigma-Aldrich), 100 µg/ml Penicillin-Streptomycin (Sigma-Aldrich) and 2 mM L-glutamine (Sigma-Aldrich) in 96-well round-bottom plates (Sarstedt) with 300,000 cells/well and total volume 180 µl/well.

4.5. Stimulations

Chondrocytes and synovial fibroblasts were harvested at 80-90% confluency and seeded in 96-well flat-bottom plates (Sarstedt) in complete media at a cell concentration of 6000 cells/well and a volume of 180 µl/well. Cells were cultured overnight in a tissue culture incubator at 37ºC and 5% CO₂ prior to all experiments. Cells were stimulated with HA of four different molecular weights, with or without LPS priming prior to HA stimulation. Details about the different stimulations are described in table 4. The total volume after adding the stimulant was 200 µl/well.

When cells were stimulated with HA alone, the role of HA as an initiator of inflammation was investigated. When LPS-primed cells were stimulated with HA, the potentially enhancing proinflammatory effect of HA was tested. Cells in the LPS-priming experiments were first stimulated with LPS for 48 hours, followed by HA stimulation for 24 hours.

The concentration of LPS used for the priming experiments was determined by a LPS titration in each cell type where a concentration of LPS which gave a low, but still detectable response in the proinflammatory cytokine release (IL-6) was observed, to make sure that cells were indeed “primed” and started releasing proinflammatory cytokines.

When IL-1β was used as a read-out, the positive control was represented by cells stimulated first with LPS for 4 hours, followed by ATP stimulation for 1 hour, as IL-1β release is dependent on two signals, see section 3.5.2. In this experiment, the capacity of HA to replace LPS or ATP in this two-hit model was tested. Details about the stimulation experiments are shown in table 4.
When stimulations were stopped, cells were spun down (300G, 5 min, 20 °C) and supernatant was collected and saved in -20 °C for ELISA experiments.

**Table 4: Details about the stimulation experiments.** LPS (10 ng/ml) was used as a positive control, except for in the IL-1β experiment were LPS as a first hit (10 ng/ml) and ATP (5 mM) as a second hit was used as a positive control. PBS (at time 0h) was used as a negative control in all experiments.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Cell types</th>
<th>Stimulant</th>
<th>Stimulation time</th>
<th>Cytokines measured</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA alone*</td>
<td>Synovial fibroblasts, chondrocytes, PBMCs***</td>
<td>HA*</td>
<td>21 h</td>
<td>IL-6, IL-8</td>
</tr>
<tr>
<td>HMGB1 alone**</td>
<td>Chondrocytes, PBMCs</td>
<td>HMGB1***</td>
<td>21 h</td>
<td>IL-6</td>
</tr>
<tr>
<td>HA* after priming cells with LPS</td>
<td>Synovial fibroblasts, chondrocytes</td>
<td>HA* after priming with LPS (0,5 ng/ml)</td>
<td>72 h (48 h + 24 h)</td>
<td>IL-6, IL-8</td>
</tr>
<tr>
<td>HA* as the 1st or 2nd hit to induce IL-1β production</td>
<td>Synovial fibroblasts, chondrocytes</td>
<td>1st hit: HA* at 0 h 2nd hit: ATP**** at 4 h or 1st hit: LPS (10 ng/ml) at 0 h 2nd hit: HA* at 4 h</td>
<td>5 h (4h + 1h)</td>
<td>IL-1β</td>
</tr>
</tbody>
</table>

*HA was used in a concentration of 100 µg/ml at various molecular weights (MW): 1-1.8 MDa (high MW), 150-300 kDa (medium MW), 21-40 kDa (low MW) and HA <10kDa.

**HMGB1 was used in a concentration of 5 µg/ml

***Only IL-6 was used as a read-out for PBMCs

****ATP was used in a concentration of 5 mM.

### 4.6. ELISA

To quantify the levels of cytokines present in the patient samples and cell supernatants, Enzyme-Linked Immunosorbent Assay (ELISA) was used. ELISA is a technique that is based on an immunoassay principle and uses an enzyme as the reporter label.\(^{89}\) In sandwich ELISA, used in this thesis, an antibody pair (one capture antibody and one detection antibody) is used to quantify a specific analyte, for example a cytokine, of interest.\(^{89}\)

ELISA (DuoSet, R&D systems) was run according to manufacturer’s instructions in duplicates to quantify the concentration of IL-6, IL-8 and IL-1β released from cells to the extracellular
milieu (supernatant). A DuoSet was also used (R&D systems) to measure the concentration of HA in plasma and synovial fluid (SF) samples from the JABBA cohort. Before the SF samples could be analysed in the HA ELISA, SF was clarified of cells and debris by centrifugation at 3000G, 4 °C, 30 min. An HMGB1 ELISA kit was purchased from IBL International and run according to the manufactures’ instructions. ELISA endpoint protocol was used, and absorbance was measured at 450 nm with subtraction of readings at 570 nm to correct for optical imperfections in the plate. A standard curve was created by reducing the data by generating a four-parameter logistic (4-PL) curve fit as described by the manufacturer. The data was analysed using the program SoftMax Pro 6.2.1 (Molecular Devices).

4.7. Luminex
Luminex was used to quantify the levels of IL-6 and IL-8 in JIA patients. It is a multiplex bead array system which is more suited for high-throughput analyses than ELISA. Luminex utilizes beads in suspension to capture the ligands, and a dual laser system for detection as opposed to an enzyme in ELISA. The beads (microspheres) are dyed with known proportions of red and near-infrared fluorophores, defining a “spectral address” of the bead. This system enables that up to 100 different detection reactions can occur simultaneously.

In this thesis, the Luminex kit Human Cytokine/Chemokine Magnetic Bead Panel (for quantification of IL-6 and IL-8) and were purchased from Merck, and run in the core facility at CMM according to the manufacturer’s instructions.

4.8. Reagents
Pharmaceutical grade Hyaluronan of four different molecular weights (1-1.8 MDa (High MW), 150-300 kDa (Medium MW), 21-40 kDa (Low MW) and HA <10 kDa were purchased from Lifecore Biomedical. Phosphate buffered Saline (PBS) was purchased from Sigma-Aldrich. Disulfide-HMGB1 (0, 61 mg/ml) was a kind gift from Kevin J. Tracey, the Feinstein Institute for Medical Research. Adenosine 5’-triphosphate (ATP) disodium salt hydrate, product number: A2383 was purchased from Sigma-Aldrich and diluted in distilled water to a concentration of 200 mM which was stored in -20 °C until usage. Lipopolysaccharide (LPS) from Escherichia coli (055:B5) cell culture tested, γ-irradiated with lot number 037K4068 was purchased from Sigma-Aldrich.
4.9. *Statistical analysis*

All graphs were made using GraphPad Prism version 7.03. The number of experiments (n) for one cell type, one stimulation type and one read-out is displayed in the figure legends. P-values were calculated by comparing all samples to the control sample (PBS) using a one-way ANOVA with Dunnett’s multiple comparison post-test. A p-value ≤ 0.05 was considered statistically significant. When n=1 the error bars derive from duplicate samples in the ELISA protocol.
5. Results

5.1. Staining

To establish that cells used in the stimulation experiments could interact with the stimulants used, (LPS, HA and HMGB1) the expression of TLR-4, which is the main receptor for LPS and HMGB1 interaction, and also suggested to bind HA, was examined. Synovial fibroblasts have previously been reported to express TLR-4,49 and PBMCs contain monocytes, which have also been reported to express TLR-4.92 To establish that chondrocytes express TLR-4, they were stained with a TLR-4 antibody, which showed that this receptor is expressed on the chondrocytes. The staining is shown in figure 1.

5.1.1. Chondrocytes

Figure 1. Cellular staining of TLR-4 in chondrocytes. (A) shows a negative control, were cells are stained with an unspecific IgG antibody. (B) shows the TLR-4 expression (red). Green colour shows the actin, blue colour shows the nuclei.

5.2. Levels of HA, HMGB1, IL-6 and IL-8 in JIA patients.

To investigate the presence of HA, HMGB1 and proinflammatory cytokines (IL-6 and IL-8) in arthritis patients, these analytes were quantified in JIA patient samples (plasma and synovial fluid (SF)) from the JABBA cohort. HA, HMGB1, IL-6 and IL-8 were all detectable in both plasma and SF samples, with higher levels observed in the SF samples. These results are shown in figure 2. HMGB1 and HA samples were measured using ELISA and IL-6 and IL-8 were measured using Luminex.
Figure 2. HA, HMGB1, IL-6 and IL-8 are all detectable in JIA patients. Levels of (A) HA, (B) HMGB1, (C) IL-6 and (D) IL-8 are detectable with ELISA/Luminex in JIA patients in the circulation (plasma) and synovial fluid (SF) samples. The graphs show the amount of each analyte in the patient samples, with the mean of all the samples indicated in each graph.
5.3. Stimulations

5.3.1. IL-6 and IL-8

5.3.1.1 Synovial fibroblasts

Synovial fibroblasts were stimulated with HA of four different molecular weights for 21 hours to establish if HA could work as an inducer of inflammation in these cells. Proinflammatory cytokines IL-6 and IL-8 were measured in the cell supernatants using ELISA. HA of the various molecular weights did not significantly increase the release of IL-6 or IL-8 (see figure 3A and 3B). To test whether HA could work as an enhancer of inflammation, cells were primed with LPS for 48 hours, followed by HA stimulation for 24 hours. These experiments showed that HA could not enhance the release of IL-6 or IL-8 compared to LPS alone. (see figure 3C and 3D).

![Figure 3](image)

**Figure 3.** LPS but not HA of different molecular weights significantly increase synovial fibroblasts' release of proinflammatory cytokines. Experiments to test the role of HA as an inducer (A), (B) or enhancer (C), (D) of inflammation in synovial fibroblasts show that none of the HA samples of various molecular weights had a significant effect on (A), (C) IL-6 or (B), (D) IL-8 release. The values are here shown as means with standard deviations. For experiment (A): n=7, (B): n=5 (except for LPS (10 ng/ml) where n=2, (C): n=3 (except for LPS (0.5 ng/ml) where n=2, (D): n=2 (except for LPS (10 ng/ml) where n=1.
5.3.1.2 Chondrocytes

Chondrocytes were stimulated with HA of four different molecular weights for 21 hours to establish if HA could work as an inducer of inflammation in these cells. Proinflammatory cytokines IL-6 and IL-8 were measured in the cell supernatants using ELISA. Chondrocytes were also stimulated with the well-known alarmin HMGB1, followed by measurement of IL-6 release. HA of the various molecular weights did not significantly increase the release of IL-6 or IL-8 (see figure 4A and 4B). HMGB1 showed elevated release of IL-6 but the effects were not statistically significant (figure 4A). To test whether HA could work as an enhancer of inflammation, cells were primed with LPS for 48 hours, followed by HA stimulation for 24 hours. These experiments showed that HA could not enhance the release of IL-6 or IL-8 compared to LPS alone. (see figure 4C and 4D).

Figure 4. LPS but not HA of different molecular weights increase chondrocytes’ release of proinflammatory cytokines. Experiments to test the role of HA as an inducer (A), (B) or enhancer (C), (D) of inflammation in chondrocytes show that none of the HA samples of various molecular weights had a significant effect on (A), (C) IL-6 or (B), (D) IL-8 release. HMGB1 did increase, but not significantly, the release of IL-6 in chondrocytes (A). The values are here shown as means with standard deviation. For experiment (A): n=5, (except for HMGB1 (5µg/ml where n=2), (B): n=3 (except for LPS (10 ng/ml) where n=2, (C): n=2, (D): n=2 (except for LPS (10 ng/ml) where n=1.
5.3.1.3 PBMCs
To investigate the effect of HA on immune cells, PBMCs from healthy controls were stimulated with HA of four different molecular weights to establish if HA could work as an inducer of inflammation in these cells. PBMCs were also stimulated with the well-known alarmin HMGB1. The proinflammatory cytokine IL-6 was measured in the cell supernatants using ELISA. HA of the various molecular weights did not significantly increase the release of IL-6 in the PBMCs. HMGB1 did significantly increase the release of IL-6 in these cells. These results are shown in figure 5.

Figure 5. LPS and HMGB1, but not HA of different molecular weights, significantly increase PBMCs’ release of IL-6. Experiments to test the role of HA as an inducer of inflammation in PBMCs indicate that none of the HA samples of various molecular weights had a significant effect on IL-6 release. HMGB1 significantly increased the IL-6 release in PBMCs. The values are here shown as means with standard deviation. N=6 except for LPS (10 ng/ml) where n=5 and HMGB1 (5 µg/ml) where n=2.
5.3.2. **IL-1β**

It was finally investigated if HA of four different molecular weights could replace either the first or second hit in the two-step model of IL-1β release. These experiments were performed in PBMCs, synovial fibroblasts and chondrocytes. IL-1β release was increased by LPS, but not HA in PBMCs. IL-1β was not produced at all in synovial fibroblasts or chondrocytes. The results are shown in figure 6.

**Figure 6. IL-1β release was increased by LPS but not HA stimulation.** Experiments to test the role of HA samples of various molecular weights as the first or second hit required for IL-1β release indicate that HA has no effect on IL-1β production. (A) shows that the combination LPS and ATP, but not LPS and HA or HA and ATP increase the release of IL-1β in PBMCs. IL-1β was not released at all in (B) synovial fibroblasts or (C) chondrocytes. The values are here shown as means with standard deviations. In (A): n=3, (B): and (C): n=1 and the error bars derive from the duplicates in ELISA.
In conclusion, the results (figure 3-6) show that in these experiments, LPS but not pharmaceutical grade HA of four different molecular weights had proinflammatory effects on synovial fibroblasts, chondrocytes and PBMCs. In addition, HMGB1 had a proinflammatory effect in PBMCs and a suggested effect in chondrocytes. The stimulations were performed in three different RA patients in synovial fibroblasts, one RA patient in chondrocytes and 7 healthy volunteers (PBMCs).
6. Discussion

6.1. Summary of the project

It has been shown that RA patients have elevated (up to a seven-fold higher) levels of HA in the circulation, and it has even been suggested that the amount of HA elevation can reflect the level of synovial inflammation, and is thus important to study in arthritis. HA is also naturally found at a high abundance in synovial fluid, where inflammation in arthritis occurs. HA’s role as an initiator or enhancer of inflammation is a debated and controversial subject which has not yet been resolved. It is important to establish if and how HA fragments contribute to the inflammatory response observed in arthritis.

It has in this study been investigated whether pharmaceutical grade HA of the four different molecular weights 1-1.8 MDa, 150-300 kDa, 21-40 kDa and HA <10 kDa can work as an initiator or enhancer of inflammation in the cell types synovial fibroblasts, chondrocytes and PBMCs. This has been performed by quantifying the proinflammatory cytokines IL-6, IL-8 and IL-1β in supernatants from stimulated and non-stimulated cells. The study thus re-evaluates HA’s potential role as an alarmin in human cells. HA’s potential to induce inflammation and work as an alarmin has been reported in several studies and by several different authors.

This study shows that pharmaceutical grade HA of these four molecular weights do not have proinflammatory effects in synovial fibroblasts, chondrocytes or PBMCs under the conditions used in these experiments. It can further be concluded that HA did not enhance the release of proinflammatory cytokines in the cells when they were primed with the endotoxin LPS prior to HA stimulation. Neither was HA able to induce IL-1β release by replacing either LPS or ATP in the two-step model of IL-1β release.

The conclusion that HA does not, under these circumstances, work as an alarmin in the cells investigated, is important, and conflicting with the majority of other studies in the field, which suggest that HA (<500 kDa) can work as an alarmin in immune cells, including PBMCs.71 In this study, three of four HA sizes (MMW, LMW and <10 kDa HA) used to stimulate cells were within the size range which have been reported to be proinflammatory.
6.2. Cells investigated

Both synovial fibroblasts and chondrocytes are important in the RA pathogenesis, and are known to be active in the degradation process that occurs in the RA joints. PBMCs are immune cells and exists in the circulation. Other cell types which could be investigated to gain a better understanding of the role that HA fragments have in the RA joints include the infiltrating leukocytes, resident macrophages, or cells from the bone compartment, which could all contribute to disease progression.

It should be noted that the synovial fibroblasts and chondrocytes used in this study have derived from individuals diagnosed with RA, and what potential effect this has on the cells is not known. These cells may be activated compared to cells from a healthy joint, and thus react less or more to inflammatory molecules. It should however also be noted that this study investigated HA fragments’ effect in arthritis, making it relevant to study cells derived from arthritis patients. Furthermore, when cells are isolated from their natural milieu (in this case an inflammatory milieu) and cultured in a lab through several passages, they are not phenotypically the same as they were in the joint, and the in vitro conditions are different compared to the in vivo setting.

To compare the arthritis-derived cells with cells derived from healthy controls, these cell types would need to be isolated from healthy individuals, something which is not ethically feasible. This is due to the fact that the cells are isolated during knee replacement surgery, which is a procedure that a healthy volunteer normally would not do. Nevertheless, alternative models such as dermal fibroblasts, and other types of cartilage cells could potentially be used for this purpose. The PBMCs were isolated from healthy controls, and undoubtedly it would also be of interest to compare these results with patients diagnosed with arthritis. Unfortunately, this was not feasible in this project, but is something that could be investigated in the future.

It should further be noted regarding the stimulation experiments with PBMCs, that there is a lot of variation between different people (a large individual variation). For example, the relative proportion of different cells in PBMCs can rapidly change following e.g. an inflammatory response, something that could affect cytokine release. To receive higher statistical power from these experiments, more healthy controls and experiments would be required. The time frame for this project did not allow for more experiments to be performed, so therefore this is also a suggestion for further studies.
6.3. HMGB1

The results from this study also show that HMGB1 works as a potent alarmin by significantly increasing the release of the proinflammatory cytokine IL-6 in PBMCs from healthy controls. In chondrocytes, the release of IL-6 was increased, but not to a statistically significant level. However, in one of the two stimulations with HMGB1 and IL-6 as a read-out, IL-6 levels was significantly increased compared to the control sample (PBS), suggesting that HMGB1 is a potent alarmin in these cells. In the graph, both experiments were included, which causes the low value for IL-6 release after HMGB1 stimulation. The reason for the lower release of IL-6 in the second stimulation was probably due to repeated thawing and freezing of the HMGB1 sample. It is well-known that HMGB1 is sensitive to oxidization and might have lost its proinflammatory properties. This experiment must be repeated to draw any conclusions about HMGB1’s ability to induce proinflammatory cytokines in chondrocytes.

6.4. Endotoxin contamination of HA samples

It is interesting to discuss why so many studies show different results regarding HA’s capacity to induce inflammation. One theory which has been put forward is that many of these studies have used HA from sources which might contain endotoxins and unrelated proteins, which may be the reason for its proinflammatory effects. In one study, it was for example shown that polymyxin B treatment does not fully remove endotoxins from HA samples and that more purification steps are required.66

It can further be observed that it is only more recent papers which suggest that proinflammatory effects have been due to endotoxin contaminations. The sources from which HA samples are derived varies a lot (see table 2 and 3). Before, it was common to isolate HA from rooster combs, while today streptococcal fermentation is the most common way to produce HA.94 It would be interesting to compare HA and the endotoxin levels from different sources to see what effect this has on the proinflammatory outcome.

6.5. Suggestion for future studies

To investigate the effect of endotoxin contamination of HA samples, an endotoxin (e.g. LPS) could be titrated in to a pure HA sample to determine the minimal response concentration, and compared to levels seen in different HA preparations. Read-outs for this experiment could be the same as the ones used in this study. Furthermore, the possibility of complex formation between HA and LPS needs to be investigated. To confirm that complexes are formed, gel
electrophoresis could be used. In order to conclude that an observed proinflammatory effect is not caused by endotoxins, all HA being used in stimulation experiments should be endotoxin-tested before stimulation to establish that it is not the endotoxins which causes the proinflammatory effect.

It would also be interesting to study additional molecular weights of HA since it is known that the function that HA exerts in the body will depend on its molecular weight. For example, a HA size with even lower molecular weight, so called ultra-low molecular weight HA would be interesting to study, since it has proposed proinflammatory effects. The concentration of HA used in the stimulations could also be varied and titrated to induce inflammation. The concentration used in this project, 100 µg/ml, has been used in several other studies with similar aims, and makes the results more comparable, but not necessarily optimized. Furthermore, the kinetics of HA stimulation could be studied as well as adding more read-outs of proinflammatory activity in the cells. Examples of additional read-outs include TNF-α, MMP-1 and measuring RNA expression of inflammatory genes. Read-outs IL-6 and IL-8 were chosen in this study because synovial fibroblasts are known to produce them, and IL-1β was chosen due to its central role in RA pathogenesis.

6.6. Conclusion

To conclude, this project has shown that cells present at the site of inflammation in RA, chondrocytes and synovial fibroblasts (shown in literature⁴⁹) should be able to bind HA due to their expression of TLR-4. It also concludes that HA, HMGB1 and proinflammatory cytokines IL-6 and IL-8 are present at the site of inflammation in JIA patients. Finally, HA of the four molecular weights investigated do not work as an initiator or enhancer of inflammation in chondrocytes and synovial fibroblasts from arthritis patients, or in PBMCs from healthy controls.

Considering the extensive usage of HA in for example the biomedical and pharmaceutical industries, it is important to determine the inflammatory effect of HA. Since HA is frequently used as a treatment in osteoarthritis patients by intra-articular injections, it is necessary to establish that HA does not contribute to the already existing inflammation in the arthritic joint. It is also important to establish the role of alarmins in general in the abnormal inflammation that occur in the arthritic joints to develop more effective treatments.
7. Acknowledgements

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