The Role of Extracellular Matrix Fragments in Osteoarthritis

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Abstract

Osteoarthritis (OA) is the most common musculoskeletal disorder worldwide. Despite this, there are currently no disease modifying treatments available for OA, restricting treatments to managing pain or total joint replacement. Therefore, it is critical to understanding the complex pathophysiology of OA to generate less severe interventions. OA initiation is a complex process, thought to compose of a biomechanical stressor, which releases fragments of the joint extracellular matrix (ECM) into the synovial cavity. This subsequently allows these ECM fragments to serve as danger-associated molecular patterns (DAMPs), which upon binding to various receptors can release pro-inflammatory and catabolic mediators. The pro-inflammatory molecules can further inflammation in the synovium, and the catabolic factors can lead to degeneration of articular cartilage, a hallmark of OA progression. This literature review summarizes the role of ECM fragments in the OA inflammatory response and assessed their potential as therapeutic targets.

Layman Summary

For humans, bones are critical for supporting the body and helping us moving, making them critical for everyday activities such as walking. Where bones meet one another, they are covered in a tissue called 'cartilage' and surrounded by a small amount of fluid that together allow the bones to move without friction. Over time or due to an injury, the cartilage can wear down. When the cartilage breaks down, this causes a significant amount of pain and results in a chronic condition called 'osteoarthritis'. Osteoarthritis typically occurs in the hands, hips and knees and is becoming more prevalent with today's aging population. When a person has osteoarthritis, they usually have pain in their joints, stiffness, swelling, and a decreased ability to move.

Typically, osteoarthritis was considered a "wear-and-tear" disease. This means that it was thought that due to joint injury or overuse, age, and/or obesity, individuals with osteoarthritis were developing degradation of cartilage. However, in recent years, it has become clear that this idea of how osteoarthritis occurs may not be so simple. It appears that inflammation is also involved in the progression of osteoarthritis. Inflammation is a physical response to certain molecules where your immune system fights off outside invaders, damage or infection. For example, when a virus enters the body, the immune system recruits different types of cells to fight the virus and make sure that it cannot infect more of the body. In osteoarthritis, however, the 'invading' factors are thought to come from our own cartilage. However, it is important to realize that the cause(s) of osteoarthritis are currently heavily debated, and it appears likely that both a mechanical cause and inflammation are involved in the disease.

Currently, it is thought that due to some type of trigger, parts of the cartilage break off and enter the fluid that enables the bones to move smoothly. This trigger could be mechanical, such as when you put a lot of strain on your joints through, for instance, physical activity. When these parts of the cartilage enter the fluid that lubricates the joints, they can cause inflammation. This is what is thought leads to the painful symptoms of osteoarthritis. Currently, there are no treatments to stop or reverse osteoarthritis, so the treatments that do exist focus on treating the painful symptoms. To do so, painkillers are used, and doctors also recommend lifestyle changes including increased exercise or a change in diet, depending on the person's lifestyle. If the osteoarthritis has progressed to the point that the patients cannot cope with the pain anymore, a joint replacement surgery is performed.

Since there are no treatments that currently stop or revert the progression of osteoarthritis, it is important that we focus on different causes of the disease. In doing so, hopefully we can find new targets for drugs that will be able to stop osteoarthritis from progressing, instead of only treating the symptoms. Therefore, this review focused on the role of inflammation in osteoarthritis. More specifically, it focuses on what is currently known about the fragments that break off from the cartilage and how they cause or maintain inflammation. It is suggested that it is unclear how the fragments initially break off and cause inflammation, but that some play a role in making the inflammation worse whereas some may play a role in starting the inflammatory response. Based on these findings, different therapeutic targets were identified, some of which are currently in pre-clinical studies. These involve targeting those fragments causing inflammation directly or stopping them from being generated at all. We still have much to learn about the processes by which the fragments cause inflammation, and this is one of the first steps necessary to develop new therapies that will help halt osteoarthritis instead of temporarily treating the symptoms.

1. Introduction

Osteoarthritis (OA) is a chronic joint disease with rising prevalence due to an aging and increasingly obese population. It is clinically characterized by joint instability, stiffness, and varying degrees of impaired mobility.[1–3] OA is the most common musculoskeletal disorder with a complex pathophysiology affecting all joint tissues.[4,5] Diagnosis of OA can be performed via imaging studies, which demonstrate changes in the bone such as osteophyte formation and joint space narrowing.[6] OA is characterized by a degradation of articular cartilage, damage to ligaments and menisci, as well as inflammation of the synovial membrane found lining the joint cavity, resulting in a complex pathophysiology.[7,8] Current treatment options focus on alleviating symptoms rather than treating the origins of the disease, despite the increasing incidence of OA.[9] As there are currently no disease modifying drugs (DMOADs), treatment plans are often restricted to life-style changes, treating painful symptoms with non-steroidal anti-inflammatory drugs (NSAIDs) and other analgesics. The current end-point for OA is joint replacement surgery.[9]

Traditionally, OA was regarded to be a "wear-and-tear" disease where continuous mechanical loading of the joints or traumatic injury to the joints was thought to cause a deterioration in articular cartilage.[8,10] While biomechanical factors are known to progress osteoarthritis through the degradation of articular cartilage, it has become clear in recent years that the inflammatory response also plays a role in osteoarthritis progression. This, in turn, mediates changes in extracellular matrix (ECM) composition, which is critical to maintaining a healthy joint environment.[8] A novel hypothesis of OA pathogenesis postulates that biomechanical injury or insult in the joint triggers the release of cartilage fragments into the synovial cavity.[4] Upon release of cartilage ECM fragments into the synovial cavity due to abnormal mechanical loading or injury in the joint, inflammatory mediators, namely cytokines and chemokines, are produced and released into the synovial fluid (SF).[8,11,12]

This perspective has gained increasing traction in recent decades, and ECM fragments are thought to play a role in the low-grade inflammation observed in OA progression. Despite this there is currently no overview of the contribution of each ECM fragment to the inflammatory response in OA. Therefore, this review focuses on the mechanisms by which ECM-derived DAMPs enact their biological effect(s), specifically in the context of inflammation. Through this, the potential for therapeutic approaches with regards to the ECM DAMPs will be addressed.

2. Structure of the Articular Cartilage and Synovium

2.1. Cartilage

OA is characterized by a degradation of articular cartilage, leading to a loss of joint space.[7] Human articular cartilage is approximately of specialized connective tissue 2 to 4 millimetres in thickness that covers the ends of the long bone in synovial joints.[5,13] It enables a low-friction articulation and allows the subchondral bone to withstand significant mechanical loading.[5,13] Simply stated, cartilage consists of chondrocytes embedded in the ECM. The different components function synergistically to maintain proper matrix hydration and allow for the mechanical properties of the cartilage.[14]

The general ECM structure in articular cartilage can be described as two distinct phases: a solid and liquid phase.[15] The liquid phase of the ECM, is composed of water (~80% of the wet weight) and electrolytes, including calcium, potassium, sodium and chlorine.[13,15] The solid phase is a three-dimensional network structured by collagen fibrils and proteoglycans.[5,15] Approximately 90-95% of the collagen found in articular cartilage is type II collagen.[14] Proteoglycans exist as either monomers or aggregates within the ECM.[14] Aggrecans are the most abundant proteoglycans found in the articular cartilage. They have a core protein backbone to which glycosaminoglycans (GAGs), chondroitin sulfate (CS) and keratan sulfate (KS) are covalently bound.[5,14] The aggrecans are non-covalently bound to another long GAG chain and hyaluronic acid (HA) via specialized link proteins.[5] The positively charged electrolytes and water are attracted to the negatively charged GAG chains of the aggrecans in the solid phase, which allows for adequate hydration.[13] The components of the solid network are submerged in the liquid phase. Together these phases work to withstand compression and shear resistance, allowing for adequate biomechanical functioning, supported by the liquid phase.[5,14,15]

The articular chondrocytes are responsible for maintaining ECM homeostasis via regulation of their catabolic and anabolic activities.[2,16] The ECM is synthesized by chondrocytes, implying a symbiotic relationship between chondrocytes and the ECM, whereby the chondrocytes are responsible for matrix synthesis and turnover, which directly mediates chondrocyte function.[14] Articular cartilage has a highly ordered structure dependent on i) the distance from the joint surface and ii) the relation to the distance of the chondrocytes.[14] There are four zones in the articular cartilage: the superficial, middle, deep, and calcified zones.[13] Each of these zones has a distinct composition of chondrocyte phenotype and morphology, as well as ECM structure (Figure 1).[5,13]

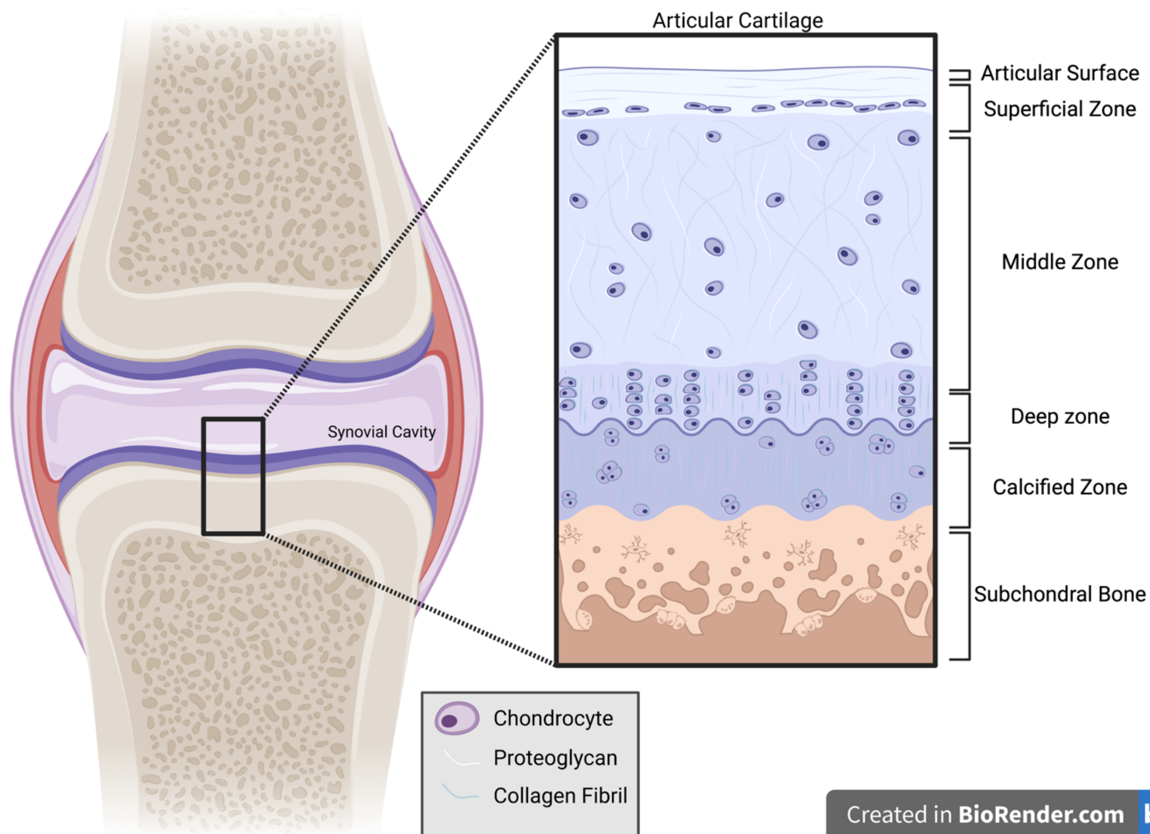

Figure 1. Articular Cartilage Structure. There are various zones in the articular cartilage, each with their own distinct composition and ECM organization. The superficial zone is the thinnest zone, comprised of thin collagen fibrils aligned in parallel to the joint surface with elongated, inactive chondrocytes. The middle zone is thicker, which spherical chondrocytes and larger, non-parallel collagen fibrils. The deep zone contains spheroidal cells in a columnar orientation with the collagen fibrils oriented in parallel perpendicular to the joint surface. Lastly, the zone of calcified cartilage contains collagen fibrils directly inserted into the calcified cartilage, providing the mechanical transition from cartilage to bone, anchoring the tissues together.[14]

2.2. The Synovium and Synovial Fluid

The synovium is a well-vascularized and innervated membrane composed of two thin layers, the intima and the sub-intima, where the former is adjacent to the joint and the latter is what joins the fibrous capsule of the joint.[17,18] The outer layer, the sub-intima is composed of type I collagen and microvascular blood supply, and is essentially acellular.[18] The intima, on the other hand, is next to the joint cavity and approximately 20-40 um thick and composed of two types of specialized cells: macrophage-like cells and synovial fibroblasts.[17,18] The macrophage-like synoviocytes ensure elimination of excess material and pathogens from the joint, and are also responsible for the secretion of cytokines and chemokines which maintain inflammation and cartilage degeneration. The synovial fibroblasts have two primary functions: i) producing hyaluronic acid (HA) and ii) serving as a barrier to keep synovial fluid (SF) in the joint.[17] The synovial membrane secretes SF to lubricate the joint and contributes to its biomechanical properties, maintain transport of oxygen and nutrients to the cartilage, and determines which molecules can enter the joint space.[17]

2.2.1. Inflammation in the Joint

There is also cellular cross-talk between the macrophage-like synoviocytes, fibroblast synoviocytes and articular chondrocytes.[4] Activated synovial fibroblasts secrete cytokines, growth factors, and matrix metalloproteinases (MMPs), which contributes to macrophage-like synoviocyte activation and the activation of catabolic pathways in chondrocytes. Similarly, activated macrophages secrete pro-inflammatory mediators that activate synovial fibroblasts and chondrocytes. Through this, they promote

the degradation of ECM components. These ECM products then activate the synovial fibroblasts and macrophages, which produce pro-inflammatory and catabolic mediators.[4,18] This results in a subsequent increase in production of proteolytic enzymes such as MMPs and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTs), which not only lead to degradation of the articular cartilage, but cleavage of the ECM. This allows the fragments that are cleaved off to function as DAMPs and further amplify synovial inflammation and the inflammatory response and further cartilage degradation.[18]

2.3. ECM Components as DAMPs

DAMPs are endogenous molecules which can either be released from the ECM or necrotic cells and cause inflammation *in vivo*.[11] DAMPs bind to pattern recognition receptors (PRRs), which includes Toll-like receptors (TLRs) and NOD-like receptors (NLRs).[8,11] PRRs are expressed in immune cells, articular chondrocytes, and synovial fibroblasts.[19–22] The DAMPs can then bind to their respective receptors and initiate signalling pathways, increasing production of inflammatory mediators such as interleukin(IL)-1β, tumour necrosis factor (TNF)-α, and IL-6, amongst others. This can activate various downstream transcription factors, which regulate inflammatory response.[11,23]

In OA progression, ECM-derived DAMPs have been observed to interact primarily with three receptor families: TLRs, integrin receptors, and NLRs (Figure 2). Humans have ten functional TLRs, which are constitutively expressed on immune cells. TLR1-7 and TLR9 have been observed in the synovial membrane in OA patients.[24,25] Specifically TLR2 and TLR4 have been implicated in OA, as their expression has been increased in cartilage lesions as well as the synovial membrane.[11] There are two major signalling pathways induced by TLR4 activation: the myeloid differentiation factor-88 (MyD88)-dependent and MyD88-independent pathways. The MyD88-dependent pathway involves receptor polymerization inducing an interaction of IL-1 receptor-associated kinases (IRAKs), which autophosphorylate and activate tumour necrosis factor receptor-associated factor 6 (TRAF6).[26–28] The recruitment of these adaptor proteins allows for activation of downstream transcription factors, such as NF-κB activation via the three mitogen activated kinase (MAPK) pathways: i) extracellular signal-regulated kinase (ERK)1/2 ii) c-Jun N-terminal kinase (JNK)1/2 ii) and p38.[29] ERK1/2 then activates c-Fos and JNK1/2 activates c-Jun, which both lead to nuclear transcription of activator protein 1 (AP1). P38 activates CREB, which leads to nuclear transcription of cyclic adenosine monophosphate (cAMP)-response element binding protein (CREB). Lastly, TRAF6 can also lead to nuclear factor κB (NF-κB) activation, which can produce pro-inflammatory mediators such as IL-1, TNF-α, and IL-6/-8. Additionally, NF-κB activation can induce cartilage catabolism by inducing MMP-1, -3, and -13 and ADAMTS-4, -5 production, thereby inhibiting cartilage anabolism (Figure 2).[29] ECM-derived DAMPs may also interact with integrin receptors. Upon ligands binding to integrin receptor α5β1, IL-4 is produced which binds to the IL-4 receptor. This results in a subsequent activation of protein kinase C Δ (PKCΔ) and phospholipase C, which leads to the activation of the same three MAPK pathways as TLR2/-4 activation, thereby leading to NF-κB activation and catabolism (Figure 2).[29]

NLRs are another DAMP receptor family, where the best characterized receptor is NLRP3, which is highly expressed in macrophages, chondrocytes, synoviocytes, and osteoblasts. Upon activation by ligand binding, NLRP3 oligomerizes, enabling it to interact with its adaptor proteins, C-terminal caspase recruitment domain (ASC) and Cardinal. This then creates a complex which can recruit procaspase-1. After activation, it forms a multimeric structure called the 'inflammasome', thereby inducing production of pro-inflammatory cytokines IL-1β and IL-18.[27,30] This subsequently induces the secretion of pro-inflammatory cytokines in the OA SF (Figure 2).[11,31,32] Another NLR is NOD2, which activates the three MAPK pathways, thereby eliciting the same downstream effects as TLR2/4 and integrin receptor α5β1 (Figure 2).

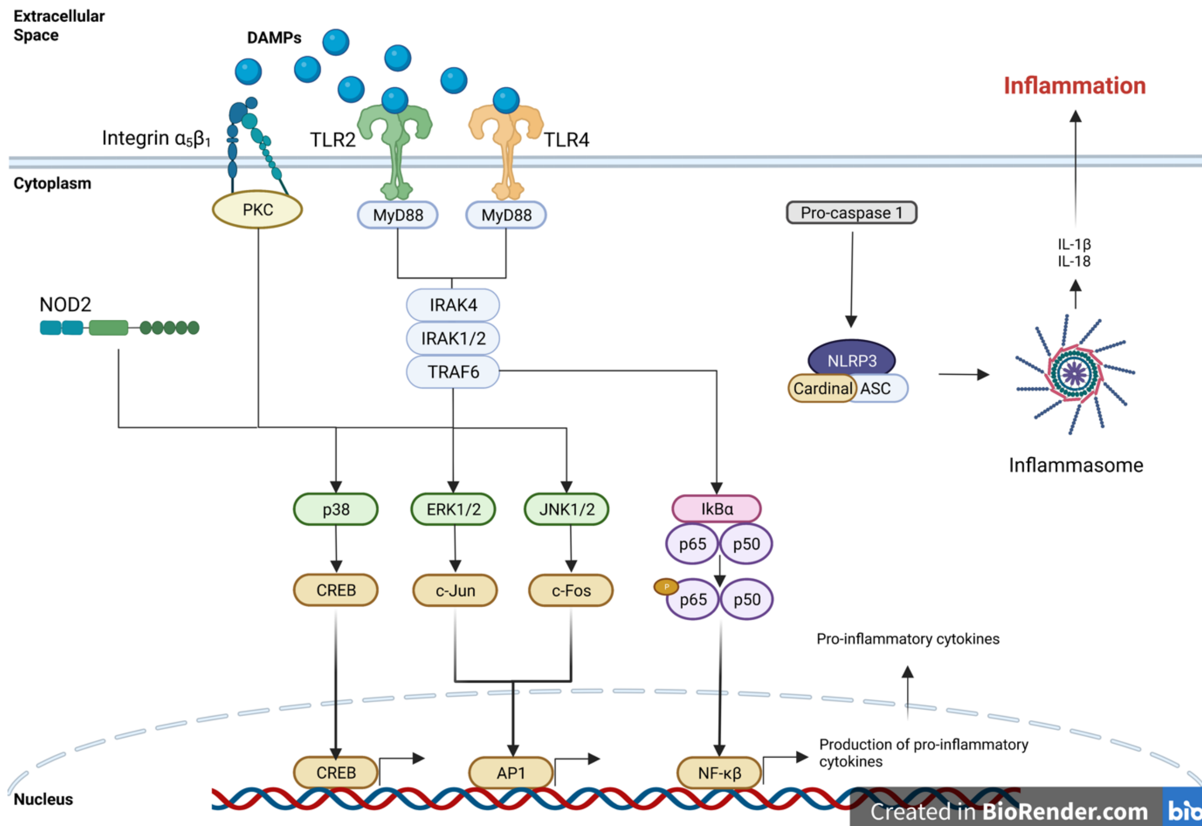

Figure 2. Signalling pathways involved in ECM-derived DAMP signalling. Figure demonstrates activation of the TLR2/4, integrin α5β1, NOD2, and NLRP3 receptors. TLR2/4 are activated by DAMP binding on the cell surface, and upon binding can signal via MyD88-dependent signalling, as shown in the figure. This leads to downstream activation of the three MAPK pathways, p38, ERK1/2, and JNK1/2, which are able to lead to the production of pro-inflammatory cytokines via CREB and AP1 through downstream activation. The downstream effects of integrin α5β1 activation are the same as those described for TLR2/4 activation. Additionally, upon TLR2/4 activation, TRAF6 can also activate NF-κB, thereby also generating production of pro-inflammatory cytokines. Activation of NOD2 also activates the 3 MAPK pathways, which results in the production of pro-inflammatory cytokines. Lastly, upon ligand binding, NLRP3 can recruit its adaptor proteins, Cardinal and ASC, leading to oligomerization of the compound and leading to the inflammasome, which can also generate an inflammatory response by production of IL-1β and IL-18. *Abbreviations: DAMPs: Danger-associated molecular patterns ; MAPK: mitogen-activated protein kinase: TLR2: Toll-like receptor 2; TLR4: Toll-like receptor 4 ; NOD2: Nucleotide-binding oligomerization domain-containing protein 2 ; NLRP3 : NOD-like receptor family pyrin domain containing 3 ; ASC: C-terminal caspase recruitment domain ; PKC: Protein kinase C; MyD88: Myeloid differentiation primary response 88 ; IRAK4: Interleukin-1 receptor-associated kinase 4 ; IRAK1/2: Interleukin-1 receptor-associated kinase 1/2 ; TRAF6: Tumour necrosis factor receptor-associated factor 6 ; ERK1/2: Extracellular signal-regulated kinase 1/2 ; c-Jun N-terminal kinase 1/2 ; CREB: cyclic adenosine monophosphate element binding protein; AP1: Activator protein 1; Iκ-Bα: Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; P: phosphate; NF-κB: Nuclear factor-κB, IL-1β: Interleukin 1-beta: IL-18: Interleukin-18*

The DAMPs observed in OA can be classified as intracellular or extracellular.[11,27] Intracellular DAMPs include immunogenic molecules that are released due to the breakdown of necrotic and apoptotic cells, and include calcium-binding S100-proteins, high mobility group box-1 (HMBG1), uric acid and heat shock proteins (HSPs), to name a few.[8,11] Extracellular DAMPs are ECM components such as glycoproteins, proteoglycans and glycosaminoglycans.[11] ECM-derived DAMPs originate from cleavage of ECM components during tissue injury by proteases such as matrix metalloproteinases (MMPs) or aggrecanases such as ADAMTS-4 and -5.[11,33] These proteases are able to cleave a large number of ECM molecules, such as fibronectin (FN), low-molecular weight (LWH-)HA, tenascin-C (TN-C), members of the small leucine-rich protein (SLRP) family, aggrecans, collagens, and cartilage oligomeric matrix protein (COMP).[11] The cleavage of the ECM components then exposes cryptic epitopes, thereby allowing recognition of ligands, where these ECM degradation products can engage with PRRs and initiate the pro-inflammatory response after release into the SF. ECM-derived DAMPs can therefore act as autonomous triggers of the inflammatory response by interacting with the specific

PRRs, and can amplify or maintain the inflammatory response, depending on which signalling pathway(s) are involved.

3. ECM-derived DAMPs and their Contributions to Inflammation in OA

3.1. Fibronectin & Fibronectin Fragments

Fibronectin (FN) is an 230- to 270-kDa adhesive glycoprotein responsible for regulation of various cellular processes including adhesion, motility, growth, differentiation, and opsonization.[34–36] Native FN exists as a dimer, where each monomer comprises three homologous repeating units: 12 type I, 2 type II, and 15-17 type III domains.[36,37] These globular domains are connected by flexible, protease-sensitive regions.[38] There are two forms of FN, which are classified based on their solubility: the plasma FN, which is water soluble and produced primarily in liver hepatocytes and travels through the plasma, and the cellular FN, which is water insoluble and found in tissues.[34,36,38] The cellular FN is locally secreted by fibroblasts, chondrocytes, and synovial cells and is found on cell surfaces in the ECM, including the synovial membrane, cartilage, and SF.[34,36,39]

Upon inflammation, an increase in FN-degrading proteases is observed, leading to cleavage of FN from the ECM and thereby generate FN-Fragments (FN-Fs), which are the main candidates suspected of maintaining cartilage degradation and synovial inflammation.[34] The cleaving of FN results in different sizes of FN-Fs. Thus far, 29-, 30-, 45-, 120-, and 200-kDa FN-Fs have been found in OA cartilage and SF, where each has a different binding affinity.[34] Cleavage of FN into FN-Fs is mediated primarily by MMP-1, MMP-3 and MMP-13, and to a lesser extent, ADAMTS-4 and ADAMTS-5.[40] There are three regions where splicing can generate fragment isoforms: the extra domain A (EDA or EIIIA), the extra domain B (EDB or EIIIB) and the variable length (V or IIICS) domain.[36,38] The EDA and EDB domains are not present in plasma FN, but are in cellular plasma and in disease.[38,41] The EDB+, EDB-, EDA-, and V+ variants are present in the cartilage, meniscus, and synovial membrane of OA patients. In contrast, the EDA+ variant was rarely observed.[42] The effects of the different FN domains have been eloquently described elsewhere.[36]

FN-Fs act as DAMPs where they act in a positive feedback loop that leads to an increased expression of pro-inflammatory cytokines, nitric oxide (NO), other inflammatory molecules, proteinases, and pro-catabolic mediators.[34] FN-Fs are known enact differential biological effects compared to native FN.[34,38,43] Native FN was found to not impact cartilage degradation, in contrast to the injection of a FN-F causing a 70% loss of cartilage proteoglycans via increased MMP release.[44] The differeing functions between native FN and FN-Fs can likely be attributed to fragmentation exposing cryptic binding sites that are blocked by the secondary or tertiary structure in native FN, and/or that domains of the molecules may lose functions they had prior to proteolysis.[38]

FN-Fs can interact with TLR2/4 and integrin receptor $\alpha5\beta1$, where upon binding to the receptor, PKCΔ is activated, leading to the activation of ERK1/2, JNK1/2, and p38.[29] After stimulation using IL-1β and three FN-Fs (29-, 45-, and 70-kDa), TLR2 expression was upregulated through an IL-1β-dependent mechanism mediated by MyD88 and NF-κB translocation.[45] While many sizes of FN-Fs have been observed, this review will focus primarily on the 29-kDa FN-F, as it is best characterized in the available literature.

The 29-kDa FN-F is the most potent activator of chondrocytes and stimulator of pro-inflammatory mediators. This is mediated via various pathways, which occurs primarily through binding of TLR2.[46] Upon addition of a recombinant 29-kDa FN-F, Gemba et al. found that this induces activation of MAPK and production of IL-1β, which is known to serve as an activator of inflammatory mediators and regulate inflammation in an autocrine loop.[46] MAPK was found to further determine activation of ERK, c-Jun NH(2) terminal kinase and p38 MAPK. Furthermore, expression of iNOS mRNA was observed 6 hours post-stimulation, and NO production was increased 48 hours post-stimulation, indicating FN-

Fs may contribute to oxidative stress and ROS involvement and promote further inflammation, although this was not elucidated in this study.[46]

Hwang et al. determined involvement of the cyclic guanosine monophosphate (GMP)-adenosine monophosphate (AMP) synthase (cGAS) and its adaptor protein stimulator of interferon gene (STING) pathway.[47] Increased levels of the cGAS/STING pathway were found in OA cartilage, and this resulted in the increased expression of pro-inflammatory cytokines mediated by the cGAS/STING/TANK-binding kinase 1 (TBK1)/IRF3 pathway, which was in turn regulated by TLR2 and NOD2.[47] Therefore, the cGAS/STING pathway may have a role in DAMP-induced damage in cartilage by maintaining TLR2 signalling and thereby inflammation.

The 29-kDa FN-Fs were also found to induce NOD2 activation in human articular chondrocytes.[48] Through NOD2 silencing with siRNAs, it was found that NOD2 is involved in 29-kDa FN-F-induced TLR2 expression. NOD2 and TLR2 were both found to modulate expression of receptor-interacting protein 2 (RIP2) and MyD88, and increase the interaction of NOD2, RIP2, and transforming growth factor β-activated kinase 1 (TAK1), which is critical for TLR2-mediated signalling. This led to the upregulation of pro-inflammatory cytokines and catabolic enzymes.[48] A later study also reported involvement of the transcription factor tonicity-responsive enhancer-binding protein (TonEBP) pathway. TonEBP responds to changes in osmolality and modulates immune responses independent of osmolality. It was found that TonEBP expression was significantly higher in human OA cartilage compared to healthy cartilage, and upon stimulation with 29-kDa FN-Fs, this increased expression of MMP-1, -3, and -13.[49] Therefore, it could be postulated that this may result in further FN fragmentation.

Although less potent than the 29-kDa FN-F, other sizes of FN-Fs have also been implicated in the progression of OA. After stimulating synovial fibroblasts and human articular chondrocytes with 30-kDa FN-F, protein expression levels of TLR2, nitric oxide synthase 2 (NOS2), cyclooxygenase 2 (COX2), prostaglandin-endoperoxide synthase (PTGS2), and MMP-13 were increased.[50] Additionally, gene expression levels of TLR2 were also observed to be increased.[50] The 45-kDa FN-F was found to induce MMP-13 and -3 synthesis in cultured chondrocytes or cartilage explants, thereby promoting a pro-catabolic cartilage phenotype in the chondrocytes independent of IL-1.[51] Furthermore, in human articular cartilage explant models, 40-kDa FN-Fs stimulated production of MMP-1, -2, -9 and -13 through the CD44 receptor.[52] In rheumatoid synovial fibroblasts, 40-kDa FN-Fs demonstrated increased MMP production via MAPK activation.[37]

As previously stated, FN-Fs also signal via the integrin receptor α5β1. It was determined that upon stimulation with FN-Fs, MMP-1 and -3 expression was increased in rabbit synovial fibroblasts upon binding to the α5β1 receptor.[43] Forsyth et al. determined that MAPK signalling mediated MMP-13 release in human articular chondrocytes stimulated with a 120-kDa FN-F, and that autocrine production of IL-1 contributes to additional MMP-13 production.[53] A follow-up study found that MMP-13 gene upregulation induced by FN-F binding in chondrocytes can be blocked through addition of insulin-like growth factor (IGF-1) and osteogenic protein-1 (OP-1) in a dose-dependent manner. It was found that the decreased MMP-13 protein expression is due to the down-regulation of pro-inflammatory molecule production stimulated by NF-κB.[54] Lastly, it was also determined that upon stimulation using a 110-kDa FN-F in human articular chondrocytes, there was a >2-fold increase in IL-6, IL-8, monocyte chemoattractant protein (MCP-1) mediated by α5β1 binding.[55]

Upon *in vitro* stimulation of articular cartilage explants, a 1 nM concentration of the 29-kDa FN-F, induced an increase in proteoglycan release, whereas concentrations of 0.1-1 uM suppress proteoglycan synthesis and enhances release of MMPs, temporarily.[56] The 1 nM concentration also led to the release of anabolic mediators such as IGF-1, and TGF-β.[56] At increased concentrations of the 29-kDa FN-F a 10-fold increase in catabolic cytokine release, including TNF-α, IL1α, IL-1β was observed.[56] This implies that the destruction of cartilage is dependent on high concentrations of 29-kDa FN-Fs. At a concentration of 1 uM, it was found the 29- and 50-kDa FN-Fs cause over a 50-fold increase in the release of gelatinolytic and collagenolytic proteinase release, and over 23-fold increase in the release of proteoglycans.[57] This suggests that upon cleave, 29-kDa FN-Fs stimulate further cleavage of

fragments by producing MMPs, and maintains inflammation in the synovium to mediate cartilage degradation. Additionally, it appears that FN-Fs mediated proteoglycan release and cartilage degradation is dose-dependent. However, evidence does not yet indicate a role for 29-kDa FN-Fs in activating the synovial macrophages or fibroblasts.

In vivo, it has been found that FN levels in the SF of OA patients are greatly increased compared to healthy patients, and fragments of 100- and 200-kDa at concentrations of 1uM have been observed.[58] An increased concentration of FN-Fs likely implies greater production of proteases and thereby increased cartilage degradation. In the synovium, 30-kDa FN-Fs were found to inhibit monocyte attachment to fibrin, inhibiting macrophage clearance of fibrin in the inflamed joints, implying a role for FN-Fs in the initiation of synovial inflammation.[38] Furthermore, removal of FN-Fs from synovial fluids in OA samples reduced cartilage damage, thereby implying a need for FN-Fs to be produced consistently to mediate inflammation.[59]

Therefore, various conclusions can be drawn regarding the contribution of FN-Fs to the inflammatory response. Through a positive feedback loop, FN-Fs essentially mediate their own fragmentation by binding to various receptors, inducing production of pro-inflammatory cytokines and protease. Eventually, this results in cartilage degradation.[34] Each FN-F size has different biological effects, which eventually results in either i) MMP production; ii) an increase in proteoglycan release and/or iii) mediation of pro-inflammatory cytokine release to maintain inflammation in the synovium. This implicates FN-Fs in the maintenance of low-grade synovial inflammation observed in OA.

3.2. Tenascin-C

Tenascin-C (TN-C) is a glycoprotein found in the ECM with high-molecular weight subunits ranging from 200-400 kDa.[60] TN-C is notably absent in adult organism tissues, but is released upon injury, and serves to regulate cell migration, proliferation and signalling by inducing the production of pro-inflammatory cytokines.[61–63] This is further confirmed by increased TN-C in the early stages of synovial inflammation.[64,65] TN-C contains four domains: the tenascin assembly domain, epidermal growth factor-like (EGF-L) repeats, FN type III-like repeats and the fibrinogen-like globe (FBG) domain.[60] TN-C has been confirmed to exist in various isoforms that are due to alternative splicing in the FNIII repeats. While FNIII 1-8 are always present, the nine FNIII repeats found between FNIII 5 and FNIII 6 may be alternatively spliced, which could theoretically produce 511 different TN-C isoforms.[63] Full-length TN-C has been found to be involved in cartilage repair, whereas TN-C with FBG, FNIII3, and FNIII1-5 domains has been implicated in synovitis via TLR4 binding and upregulation of pro-inflammatory cytokines.[66] The first study to characterize the role of TN-C fragments in articular cartilage identified two sizes of fragmented TN-C: 100-kDa and 150-kDa. These fragments were mapped to display cleavage in the EGF-L and FNIII domains 3-8.[67]

The isoform significantly impacts TN-C functionality, as the different FNIII repeats have their own binding partners, proteolytic cleavage sites and post-transcriptional modification sites. TN-C has been reported to undergo cleavage by MMPs, and *in vitro* cleavage of purified TN-C via MMPs-1, -2, -3, and -7, showed that cleavage is mediated primarily through sites located in the alternatively spliced domains. Additionally, MMP-7 and ADAMTS5 can also cleave the tenascin assembly domain.[68] While there is also evidence pointing to the accumulation of TN-C levels in the synovium, many studies do not consider the different TN-C fragments. This is likely due to the high variability in TN-C fragments that can exist due to alternative splicing.

It has been hypothesized that TN-C fragments with the FBG domains serve as endogenous inducers of synovitis via TLR4 binding.[66] This was confirmed by a study that observed TN-C as an endogenous activator of TLR4 *in vivo* via intra-articular injection of the FBG domain in murine models.[64] Furthermore, it was also found that via signalling of TN-C via the FBG domain increased synthesis of TNF-α, IL-6, and IL-8 in primary human macrophages and IL-6 in synovial fibroblasts via the TLR4-MyD88-dependent signalling pathways.[11,61]

Synovial fibroblasts also express the integrin α_9 receptor, which plays an important role in recruiting inflammatory cells. It has been shown that there is an autocrine and paracrine interaction of TN-C with the α_9 receptor on synovial cells. This results in the production of pro-inflammatory mediators. IL-6, IL-1α, CCL2, and CXCL5 are thought to be produced by synovial fibroblasts and macrophages, whereas IL-1β, TNF-α, CCL3, CCL4, and CXCL2 production is thought to only be produced by synovial macrophages. In turn, these activate osteoclasts and lead to further cartilage degradation and synovial hyperplasia.[69] Further research regarding the role of the integrin α_9 receptor found that rheumatoid arthritis fibroblast-like synoviocytes autonomously produced pro-inflammatory mediators, and disruption of the integrin α_9 receptor inhibited TN-C expression, implying a positive feedback loop where TN-C self-amplifies its own signalling to progress synovial hyperplasia.[70]

Furthermore, human TN-C has recently been identified as an activator of latent TGF-β *in vitro* through its FBG-like domain.[71] Furthermore, the FBG-like domain has been shown to associate with the TGF-β1 small latent complex (SLC) *in vitro*, which promotes the presentation of the mature TGF-β to cells and is a known stimulator of chondrogenesis.[71] Findings regarding the interaction of the FBG-domain and TGF-β1 have not been studied explicitly in the context of synovial inflammation. However, TGF-β1 is known to stimulate fibrotic changes via cell proliferation and accumulation of type I collagen, while also promoting differentiation of OA synoviocytes into myofibroblast-like phenotypes.[72] Studies using rheumatoid arthritis (RA) models of synovitis demonstrated that monoclonal antibodies for the FBG-domain of TN-C inhibited cytokine release by the synovial cells and prevented RA progression.[65] This therefore implies a role of the FBG domain of TN-C in maintaining the low-grade inflammatory response observed in OA SF via stimulation of pro-inflammatory cytokines.

An *in vivo* study performed by Midwood et al., demonstrated that *Tnc-/-* mice with acute synovitis induced by zymosan demonstrate a quick resolution of joint inflammation, whereas mice which were administered an intra-articular injection of TN-C demonstrated joint inflammation.[61] This implies a role for TN-C with regards to maintaining joint inflammation *in vivo*, but does not directly imply a role directly in the initiation of inflammation.[61] In both human knee injury and a comparable canine joint injury model, elevated levels of TN-C were observed in the SFs.[73] This was increased 7-fold in the acute injury phase and a more than 3-fold increase persisted even 1 year post-injury in the human model.[73] In the canine model, the same trend was observed, where elevated TN-C levels were observed for at least 6 months after the initial injury.[73]

Therefore, it can be concluded that TN-C plays a role in the inflammatory response in OA. It appears that biomechanical injury to the joint may be what initiates inflammatory processes by TN-C, which implying TN-C as an ECM-DAMP involved early in OA pathophysiology. Additionally, it appears that the inflammatory actions by TN-C are mediated by its FBG-domain, making this an especially interesting therapeutic target.

3.3. Low-Molecular Weight Hyaluronic Acid

Hyaluronic acid (HA) is a natural, linear disaccharide polymer and member of the non-sulfated GAG family, and identified as a critical component of the ECM.[25,74] In the SF, HA contributes to the viscoelastic properties observed and allows for lubrication of the joints.[75,76] Physiologically healthy systems contain native HA, which is synthesized by the synovial fibroblasts and chondrocytes via the HA synthase (HAS) 1-3 enzymes located on the plasma membrane.[17,74,77] There is usually a high turn-over rate of HA, where degradation of HA is mediated by hyaluronidase (HYAL) enzymes.[77] The degradation of HA into smaller fragments is mediated specifically by HYAL1 and HYAL2, which act in an acidic microenvironment.[77] When the rates of synthesis and degradation are in equilibrium, homeostasis is maintained, since this results in the HA being replenished and the fragments degraded completely. However, when ECM homeostasis is disturbed in pathological conditions, the native, high-molecular weight (HMW)-HA can be degraded by HYALs and ROS into low molecular weight LMW-HA fragments, which can then be further depolymerized to oligosaccharide HA (oHA).[77,78] As a

consequence of the HA degradation, it loses its lubricating properties, enabling it to contribute to the painful symptoms associated with synovitis.[79]

In contrast to HMW-HA which serves to scavenge reactive oxidative species (ROS) and 'protect' the cells, LMW-HA is characterized by its pro-inflammatory and immunostimulatory properties, which results in the elevation of cytokine and inflammatory mediator levels.[74] When the ECM homeostasis is disturbed, this results in an accumulation of HA fragments (HA-Fs) with a lower molecular weight, thereby leaving the cells without a first defense supported by HMW-HA.[78] It should be noted that there are also studies which found that HA-Fs do not appear to contain any DAMP properties in synovial fibroblast and chondrocyte cultures.[80] However, in this review studies that do report the DAMP properties of HA-Fs will be discussed.

HA-Fs and oHA in the synovium binds to several cell surface receptors, including CD44, RHAMM, and TLR2/4.[77] It was found LMW-HA serves as an activator of the innate immune response in murine alveolar macrophages by binding to TLR2. This activation occurs in an MyD88-IL-1R associated kinase-, TNFR-associated factor 6-, PKCζ-, and NF-κB-dependent pathway.[81] While this model was presented in alveolar macrophages, synovial fibroblasts and M2 macrophages also demonstrate increased expression of TLR2, thus implying DAMP recognition of HA-Fs may be mediated by TLR2 in the synovium.[82,83]

It appears that TLR signalling is dependent on the number of disaccharides in the HA-Fs. Stimulation of mouse chondrocytes with 4-mer HA-Fs demonstrated that the treated cells increased mRNA levels for TLR4, TNF-α, IL-1β, IL-6 and IL-18, as well as NF-κB activation.[84] 4-mer oHA stimulation on RA synovial fibroblasts (RASFs) demonstrated that the pro-inflammatory effects are mediated through interactions with TLR2/4. Upon binding to the TLRs, the transforming-growth factor activated kinase-1 (TAK-1), is activated, which subsequently activates p38-MAPK, resulting in translocation of NF-κB.[85] This resulted in the transcription of TNF-α, IL-1β, MMP-13, and induced nitric oxide synthase (iNOS).[85] The interaction of oHA with CD44 also enhanced MMP13 expression in chondrocytes via activation of p38-MAPK and subsequently NF-κB, implying a role of oHA directly in matrix degradation.[86]

Further studies also used a 6-mer oHA to determine its inflammatory consequences. It was found that upon addition of a 6-mer HA-F to chondrocyte cultures, the following effects were observed: i) increased expression of CD44 and TLR4; ii) activation of NF-κB; and iii) increased production of pro-inflammatory mediators, including TNF-α, IL-6, and IL-1β. This process was found to be CD44-mediated.[87] In a collagen induced arthritis (CIA) model using murine RASFs, administration of 6-mer oHA observed an interaction with both TLR4 and CD44 individually.[88] Consequently, this allowed for nuclear translocation of NF-κB and led to upregulation of IL-18 and IL-33 production.[88]

From literature, it appears that the ability for HA-Fs to enact their biological effects is influenced by their size. Different sizes of HA-Fs have different capacities to binding to their receptors, where the size of the HA-Fs appears capable of altering the biological effects of the HA-Fs. For instance, HMW-HA appears to bind to CD44 and increase receptor clustering, which is enabled by its long chains which contain multivalent binding sites. However, upon a subsequent addition of oHA, which has one or two binding sites, the clustering strength of HMW-HA is reduced, thereby implying that oHA acts as a HMW-HA antagonist and inhibits its positive biological effects *in vitro*.[77,89]

Thus, oHA can activate the TLR4 and CD44 receptors in chondrocytes, whereas TLR2/4 signalling is only implicated in the synovial fibroblasts. Furthermore, it also appears that 4-mer oHA interacts with TLR2/4, but not CD44, whereas 6-mer oHA binds to all three receptors. This increase in binding capacity is likely due to 6-mer oHA containing more binding sites than 4-mer oHA. This hypothesis is further supported by Yamasaki et al., who demonstrated involvement of NLRP3 receptor, whereby administration with 10-18-mer HA-Fs resulted in an increased in IL-1β.[90] This also implies the significance of receptor clustering by HMW-HA, which may be what allows it to enact its positive effects *in vivo*. In conclusion, it appears that HA-Fs play a direct role in the activation of synovial

inflammation via TLR and NLRP3 signalling, which is turn allows it to contribute to matrix degradation.

3.4. Small Leucine-Rich Proteoglycan Family

Proteoglycans have been classified into five families, dependent on the structural properties of the core protein.[91] One of these families is the small leucine-rich proteoglycan (SLRP) family, which is composed of a small core protein of 36- to 42-kDa, and has a unique structural organization of tandem leucine-rich repeats.[91] This unique structure allows SLRPs to interact with other proteins, which can regulate fibril formation, as supported by *SLRP-/-* mice, which show disorganized collagen fibrils in their ECM networks, implying less capacity to withstand mechanical loading.[92] More generally, SLRPs have the capacity to bind to ECM components, cytokines, and growth factors, allowing them to regulate cellular functions.[91]

There are 18 members of the SLRP family which are grouped into five classes. Of these 18 members, several have been observed to have increased fragmentation in OA articular cartilage compared to healthy controls by collagenases, including biglycan (BGN), decorin (DCN), fibromodulin (Fmod), and lumican (LUM).[91,93] Each of these will be discussed in detail individually. It is proposed that SLRPs interact with the ECM collagen fibrils to form a 'coating', thereby limiting MMP access to cleavage sites and protecting against fragmentation. However, SLRP cleavage may precede destruction of the collagen network and thereby interfere with any compensatory repair mechanisms.[91] Each of the SLRPs enacts different biological functions, which may in part due to their varying levels of fragmentation, as observed by Melrose et al..[93]

3.4.1. Biglycan

BGN is a 42-kDa protein-core SLRP which exists as either ECM-bound of ECM-released soluble BGN (sBGN).[94] Under normal physiological conditions, BGN is sequestered to the ECM where it serves to interact with collagens or elastins. However, upon injury or in inflammatory conditions, BGN is by ADAMTS-4 and ADAMTS-5 and released into the SF, resulting in sBGN. Additionally, upon stimulation of human primary chondrocytes with sBGN, an increased mRNA and protein expression or MMP-1,-9, -13 was observed.[20,95] This further implies MMPs in the cleavage of BGN as well. Lastly, sBGN can also be produced *de novo* by activated macrophages.[94,96]

In the SF, sBGN is a high-affinity ligand for TLR2/4 and acts as a DAMP in macrophages.[94] More specifically, TLR4-mediated signalling triggers NF-κB activation, as confirmed by a TLR4 specific siRNA knockdown.[95] Recent research has also identified sBGN as a novel high-affinity ligand for CD14, a known co-receptor for TLR-mediated signalling, and that its presence is required for inducing TLR2/4 dependent inflammatory signalling pathways in macrophages.[97] The CD14 receptor is also present on chondrocytes, but specific studies on the necessity of the receptor to induce signalling have not been elucidated.[98] In TLR2/TLR4/CD14 signalling, sBGN can induce TNF-α and CCL2 production, heat shock protein (HSP) 70 via TLR2/CD14 signalling and CCL5 via TLR4/CD14 signalling.[97] This was mediated by sBGN-induced phosphorylation and nuclear translocation of p38, p44/42, and NF-κB.[97]

A murine model of renal ischemia/reperfusion (I/R) injury *CD14-/-* mice also blocked BGN-mediated cytokine expression macrophage recruitment and M1 macrophage polarization.[97] A similar murine renal ischemia/reperfusion injury model demonstrated that sBGN can signal through TLR2/CD14, TLR4/CD14 or TLR4/CD44, the hyaluronan receptor, and this may result in macrophage polarization.[98] Macrophage polarization is thought to be involved in OA and has been proposed as a therapeutic target to mend the imbalance in the synovial microenvironment, and specifically reprogramming macrophages to the M2 type may modulate inflammation-related OA symptoms.[99] Furthermore, sBGN was also

found at increased levels in the SF of OA patients compared to healthy controls.[20] Altogether, current research implicates sBGN as a critical ECM-derived DAMP in mediating and directly activating synovial inflammation, but more relevant physiological methods are required to further elucidate the mechanisms underlying this.

3.4.2. Decorin

DCN is 57% homologous in its amino acid sequence to BGN, and is typically secreted by the synovial fibroblasts and has various biological functions, such as participating in collagen formation, acting as a ligand to mediate signal transduction pathways and blocking various cytokines and growth factors[100] Despite its similarity to BGN, DCN is not a substrate for ADAMTS-4 and -5, and instead minor cleavage of DCN by MMP-13 has been observed in human articular cartilage, thereby releasing its soluble form.[96,101] Currently, research on DCN cleavage in the joint is lacking, and beyond this nothing is known about DCN cleavage

Soluble DCN (sDCN) has been found to have bind to both TLR2/4, as well as to the C1q component of the complement cascade.[100,102] The C1q component is the recognition protein for C1, the major activator for the classical pathway of the complement cascade.[103] It has been found that sDCN can activate TLR2/4 and activate p38, MAPK, and NF-κB to enhance the production of IL-12p70 and TNF-α.[100] When sDCN binds to the C1q component of the classical complement pathway, it thereby inhibits the cascade and has a pro-inflammatory effect.

The role of DCN on cartilage and chondrocytes has been better characterized compared to the effect on synovial cells. For instance, DCN has been found to activate the pro-inflammatory response in chondrocytes via TLR4 signalling, which results in the upregulation of OA inflammatory factors including IL-6, IL-8, MMPs and NO levels.[20] This would imply that DCN mediates cartilage destruction by facilitating further cleavage of ECM components and production of ROS. Subsequent research, using an inducible gene knockout model for DCN with a surgical model of post-traumatic OA (PTOA) found that the absence of DCN accelerated joint degeneration.[104] These results therefore directly contradict the previous results, and would imply a protective role for DCN. In a different forced-exercise OA model in *DCN-/-* and WT mice and the loss of DCN lead to interrupted TGF-β signalling, increased stiffness of the articular cartilage and made joints more resistant to OA.[105] These differences may be attributed to the type of knockout model used by Han et al., as the inducible knockout model may serve to be more physiologically similar to OA progression than the constitutive knockout model used by Gronau and colleagues.[104,105]

DCN levels in the SF did not differ significantly between OA patients and healthy controls.[106] However, in another study by Baretto et al. low levels of soluble DCN (sDCN) could be detected in OA SF. Furthermore, mRNA levels of DCN were found to be 1.2 higher in a canine model of induced OA.[107] Therefore, the effects both *in vitro* and *in vivo* appear to contradict one another. While more research is required regarding the role of sDCN in OA, it does appear that sDCN mediates inflammation via TLR4 in synovial macrophages and facilitates the release of various cytokines into the SF, further enabling SF inflammation.

3.4.3. Fibromodulin

Fmod is a 59-kDa protein which exists in the cartilage as bound to the surface of collagen I and II fibrils and has been studied primarily regarding its role in collagen fibrillogenesis.[108]Additionally, it exhibits significantly increased protein fragmentation in inflammatory conditions.[103,108] Studies have found that Fmod degradation occurs both when stimulated with IL-1 and with MMP-13.[109] Cleavage with IL-1 and MMP-13 yield similar Fmod fragments (Fmod-Fs), which are approximately 10-kDa.[40] Upon cleavage of Fmod, there may be an exposure of cleavage sites on the collagen fibrils, allowing

proteinases to further cleave the collagen network and thereby damage the ECM.[40] This also implies its role as a key early event in disruption of the collagen fibril network critical to the ECM.

While DCN also binds to C1q, only Fmod-Fs are capable of complement activation.[40] Fmod can also engage with Factor H, which inhibits complement activation. Fmod-Fs was found to have different binding sites for both C1q and Factor H, implying that depending on the site of proteolytic cleavage, this can influence either activation of inhibition of the complement system.[40] Fmod-Fs may activate the classical complement pathway by binding to factor H, but upon degradation, the N-terminal of the Fmod fragment binds to C1q and activates the complement system.[108] However, it is also worth noting that the complement system is activated in OA, and directly activated after knee injury and associated with inflammation, as shown by an immunoassay of the Cd3, C3bBbP and soluble terminal complement complex (sTCC) in the SF of OA patients.[110] However, this is an association and does not imply causality. Lastly, mRNA levels of Fmod are increased 2.4 times in canine cartilage in a model of experimental OA, providing evidence that the increased expression of Fmod may be to compensate for a deteriorating collagen network.[107] To conclude, it does not appear that Fmod is involved in instigating the inflammatory response in OA, but rather that its actions are a consequence of the inflammatory response, where it plays a role primarily in disrupting the collagen network and therefore ECM integrity.

3.4.4. Lumican

LUM is a proteoglycan synthesized by chondrocytes that is well-known for interacting with fibrillar collagens, similarly to Fmod.[111] The primary sequences of Fmod and LUM are 47% homologous, and thought to compete for collagen binding.[112] Upon degeneration of the OA joints, LUM is released in its soluble form (sLUM) into the SF. LUM has been observed to be a ligand for TLR4, but only upon interaction with LPS, a well-known TLR4 ligand.[111,113] It is assumed that this is sLUM, but this distinction is not made in the literature. sLUM binds to LPS, where they bind to TLR4 and induces NF-κB translocation. Additionally, LPS and sLUM induced a shift in synovial macrophages towards the M1 phenotype as confirmed by a concurrent increase in the protein secretion levels of IL-10 and TNF-α.[114,115] Additionally, *LUM-/-* macrophages demonstrate impaired response to LPS, resulting in decreased production of IL-6 and TNF-α.[91]

In vivo, it has been sLUM has been observed at increased levels in the SF of OA patients compared to knee meniscectomy patients.[111] When cartilage explants are stimulated with LPS or IL-1β, sLUM is decreased in the articular cartilage, which is likely linked to the increased sLUM levels in the SF. Altogether, this demonstrates that sLUM independently does not appear to mediate the inflammatory response in OA, but is an immunologic adjuvant that enables secretion of pro-inflammatory cytokines via TLR4/NF-κB signalling pathway. However, LUM and sLUM remain understudied in OA, and therefore no robust conclusions can be drawn regarding the role for this SLRP in OA.

3.5. Collagen

The structural and functional integrity of articular cartilage is dependent on primarily the collagen type II (Col2) molecules in the ECM, which can then act with collagens type III, VI, IX, X, and XI, matrix FN, and members of the SLRP family.[116] Collagens are critical for providing tensile strength, regulating cell adhesion and supporting migration.[117] Col2 is produced by the articular chondrocytes and subsequently deposited in the cartilage matrix.[116] As previously described, members of the SLRP family can interact with Col2 fibrils to prevent their cleavage. However, if these protective measures are absent due to SLRP cleavage, cleavage of Col2 is possible.

Wu et al. demonstrated that cleavage of Col2 occurs in the pericellular and superficial sites of the articular cartilage, where they found MMP-1 and -13 localization, and found a close correlation between the cleavage of Col2 and the sites of localization.[118] It was found in a culture of primary human chondrocytes that upon incubation with Col2, Col2 induces fragments of pro-inflammatory cytokines, which results in Col2 fragmentation and accelerates matrix degradation.[119] This therefore implies MMP-1/-13 as primarily responsible for Col2 cleavage, and they are produced by Col2 via MAPK p38 and NF-κB signalling.[119] A 24-mer synthetic Col2 peptide (CB12-II) was also found to stimulate Col2 cleavage via induction of MMP-13 expression in healthy bovine articular cartilage explants.[122] Therefore, it appears that cleavage of Col2 is dependent on a feed-forward mechanism, and this cleavage and subsequent release into the SF is due to the exposure of cryptic epitopes allowing Col2 to act as DAMPs.[120,121] A 29-mer Col2 fragment with the N-terminal of Col2 (N-telo) was found to increase the mRNA and protein levels of MMP-2, -3, -9, and -13 in cultured bovine articular knee chondrocytes and their explants.[122] Various studies have been performed using varying lengths of Col2 peptides to determine the effect on the inflammatory response in OA, and each has distinct consequences for the articular cartilage.

Coll2-1, a Col2 peptide, was also used to stimulate human synoviocytes and chondrocytes from knee OA patients. Coll2-1 bound to TLR4 in both the synoviocytes and chondrocytes which led to subsequent NF-κB activation. This peptide has been found at increased concentrations in OA patients, increasing the physiological relevance of this model.[120] *In vitro* it was determined that in the synoviocytes, Coll2-1 induced production of IL-8 and H_2O_2. The IL-8 levels were elevated both in the SF and serum of OA patients. In human chondrocytes, Coll2-1 was determined to increase both MMP-3 and VEGF expression.[120] This therefore also implies a role for the Col2 peptide in inducing catabolic cytokines in both synoviocytes and chondrocytes.[17]

The 29-kDa fragment, N-telo, was also found to demonstrate enhanced mRNA, protein and activity levels of cathepsin B, L, and K production via PKC and p38 MAPK activation in cultured articular chondrocytes.[121] Cathepsins have been implicated in OA matrix degradation and synovial inflammation in RA.[123] Additionally, the CB12-II fragment also induced NF-κB activation via PI3K/Akt signalling.[124] However, this study did not further elaborate on the pro-inflammatory mediators that may be released into the SF as a consequence of this activation. However, both 24-mer and 29-mer Col2 peptides appear to be primarily involved in upregulating expression of pro-catabolic factors in articular chondrocytes, which could enable cleavage of other ECM-derived DAMPs and therefore contribute to the maintenance of inflammation.

Therefore, it appears that Col2 peptides of varying sizes are responsible for primarily inducing production of catabolic cytokines and facilitating degradation of the cartilage via inflammatory mediators. This implies a role for Col2 in progressing synovial inflammation and through this cartilage degradation, but this likely occurs later in the inflammatory response, as it appears that first SLRPs must be cleaved prior to Col2 cleavage.

4. Strategies Targeting ECM-Derived DAMP-Mediated Inflammation in Osteoarthritis Progression

Recently, the inflammatory component of OA has gained increasing attention due to its proposed role in early OA and initiation of synovitis. This review has characterized the role of different ECM-derived DAMP fragments in the context of OA-mediated inflammation. Based on current literature, it appears that DCN, Fmod, LUM, and Col2 serve to maintain and/or progress synovial inflammation. and therefore, cartilage degradation. On the other hand, FN-Fs, HA-Fs, TN-C, and sBGN appear to each play a role in the initiation of synovial inflammation in OA.

ECM-derived DAMPs enact inflammation in OA through PRRs. At first glance, it may therefore appear an attractive biological target to block the PRRs that ECM-DAMPs use, especially signalling occurs via the same receptors. However, the caveat in this is that PRRs are critical in host immune-response, and while this may prevent ECM-derived DAMP-mediated inflammation, it would also leave the patient susceptible to other infections. Therefore, this makes therapies involving TLR- or NOD-knockouts infeasible in practice. Additionally, anti-cytokine based treatments have been unsuccessful, and targeting other immune targets has also not shown significant clinical results.[125] Therefore, therapies that instead target the inflammation mediated by the ECM-derived DAMPs may be attractive therapeutic targets. By specifically targeting these fragments in novel therapies, this may serve to reduce the overall inflammatory response and provide a more specific manner in doing so compared to broader anti-inflammatory treatments. There are not yet any therapies focusing on inhibiting ECM-derived DAMP fragment production specifically. However, several other ways of targeting the products of ECM-DAMP induced inflammation are outlined below.

As HA-Fs were identified in this review as contributing to synovial inflammation in OA progression. Therefore, the supplementation of HMW-HA directly to the joint may serve to improve joint functionality, reduce pain, and hopefully decrease inflammation. However, differences between different molecular weights of HA have been reported.[126,127] A recent meta-analysis revealed that HMW-HA has the most promising effect. They found that the different molecular weights have different half-lives, with HMW-HA having a half-life of approximately 8.8 days. This means that at longer follow up, the beneficial effects are likely due to the effects of reactions that the injected HA causes, although evidence for this is limited.[128] For instance, this may promote endogenous HA synthesis by synovial fibroblasts, thereby increasing the ratio of HMW-HA:LMW-HA, therefore restoring the disrupted equilibrium that results in accumulation of excess LMW-HA. No studies appear to focus on the molecular consequences of HA injection though, likely since these studies are clinical in nature and focused on pain management. However, it would be relevant to measure protease and cytokine levels a prior to and after injection, as this would determine whether HMW-HA supplementation can shift this ratio.

As mentioned previously, TN-C is likely one of the ECM-derived DAMPs involved in early OA pathogenesis. As previously mentioned, the FBG domain is important for TN-C mediated activity, therefore making it an attractive target for therapeutics. Recently, the development of monoclonal antibodies that prevent binding of TN-C to TLR4 has been reported. In doing so, this also inhibits TN-C mediated inflammatory activations.[65] However, prophylactic administration of the anti-FBG antibodies to rats with collagen-induced arthritis did not lead to a reduction in joint inflammation. However, this did inhibit progression of the disease and prevented damage to the joint. This approach, while requiring further testing, might therefore be able to block other pro-inflammatory signals from ECM proteins/fragments and prevent progression of OA. To further identify sites on the TN-C molecule, research should also focus on elucidating the role of the different TN-C fragment sizes to determine additional sites significant for the inflammatory actions of TN-C. However, this may prove difficult due to the large number of TN-C fragments that can be generated due to alternative splicing. It is therefore critical that research focuses, for instance, on narrowing down which sites are especially sensitive to protease activity, for instance.

MMPs in the context of OA are responsible not only for the degradation of cartilage, but also for further generation almost all ECM-fragments mentioned in this review. This makes them an especially attractive target for inhibiting further fragmentation and therefore increasing the inflammatory response. Specifically, MMP-13 has been found to play a significant role in early OA and is a key mediator in OA-mediated inflammation, as shown in the previous sections. It was demonstrated that *MMP13-/-* mice demonstrate a decreased rate of progression in OA, therefore indicating their potential.[129] Baragi et al. demonstrated that the synthesis of non-hydroxamic acid-containing compounds demonstrated a high potency for MMP-13. This resulted in histological evidence of chondroprotection and reduced damage to the cartilage, all without demonstrating musculoskeletal toxicity.[130] However, it should also be noted that while they state these MMP-13 inhibitors as uniquely specific to MMP-13 and therefore distinct from previously developed broad-class MMP inhibitors, it does not appear that these inhibitors were ever used in clinical or pre-clinical models.

Further research by found another compound known to possess anti-inflammatory characteristics, paeoniflorin, inhibits IL-1β induced MMP secretion via the NF-κB pathway in rat chondrocytes.[131] Later work found that Maresin-1, a metabolite of docohexaenoid acid (DHA), was found to decrease MMP13 in a murine model of treadmill-induced OA.[132] It did so through activation of the PI3k/Akt pathway, which in turn inhibits NF-κB activation in fibroblast-like synovial cells stimulated with IL-1β. Currently, it appears there are not many, if any clinical trials using MMP inhibitors, and early clinical trials using broad-spectrum inhibitors generated unwanted side-effects for patients. Therefore, it is necessary to keep unravelling the mechanisms underlying MMP-13 production before it can be used as a therapeutic target, despite its high potential.[133] Additionally, other proteases such as ADAMTSs, which play a significant role in cleaving FN, TN-C, BGN, DCN, and collagen into fragments may also prove to be an attractive therapy for modulating inflammation.

This review illustrated that the complex initiation of inflammation in OA also means that existing studies have focused on the involvement of one ECM-derived DAMP at a time, but no studies appear to elucidate the crosstalk between fragments. This should be considered a critical aspect for improvement in future studies to not only understand the pathophysiology, but to understand the interactions between ECM-derived fragments better *in vivo.* To do so, novel methods such as a joint-on-a-chip or a microfluidic cartilage-injury-microtissue array could be used to study the progression of OA from an early stage to determine the contribution of the ECM-derived fragments. Of course, these methods are associated with their own limitations and due to their novelty may require much optimization but could be considered as a next step in modelling OA.

While there are therapies being developed, no definitive DMOADs are in the clinic yet for OA in any context, likely due to its complex pathophysiology. This makes understanding its pathophysiology even more critical. As illustrated in this review, there are still significant gaps of knowledge to be filled with regards to the role of inflammation in OA, but progress is being made. This highlighted a role for ECM-derived fragments in OA progression and their contribution to synovial inflammation. This could therefore be considered a next step in understanding the processes underlying OA initiation and to what extent this contributes to disease progression. This would, in turn, provide new therapeutic targets that may be capable of clinical translation that could significantly benefit millions of individuals suffering from OA worldwide.

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