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## Long non-coding RNAs in the regulation of inflammatory pathways in rheumatoid arthritis and osteoarthritis

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Long non-coding RNAs in the regulation of inflammatory pathways in rheumatoid arthritis and osteoarthritis

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Abstract
Long non coding RNAs (lncRNAs) are a functionally diverse family of non-coding RNAs, which have been implicated in modulating gene expression through transcription, translation and DNA epigenetic modifications. As a relatively nascent field, the number of known functional lncRNAs is currently small. However, increasing numbers of lncRNAs are now being identified as candidate regulators of inflammatory pathways that are relevant to osteoarthritis and rheumatoid arthritis joint pathology. Furthermore, several lncRNAs have been identified which are differentially expressed in osteoarthritis or rheumatoid arthritis joint tissue.

In this review, we highlight those lncRNAs for which functional effects have been demonstrated within the major pathways associated with inflammation, including NFkB signalling, p38 MAPK pathway, arachidonic acid pathway and TLR signalling. We also highlight those lncRNAs which have been shown to be differentially expressed in inflammatory joint tissue, and are therefore strong candidates for future investigative work.

Determining the expression and functional role of lncRNAs in mediating inflammation within the joint will not only advance our understanding of the epigenetics which underlie the pathology of inflammatory joint disease, but may also ultimately aid in the identification of novel targets for therapeutic intervention.
1. Introduction

It was initially envisaged that upon the completion of the human genome project our understanding of human disease would lead to an abundant development of new targeted therapies by which to modify disease progression or prevent disease onset. Furthermore, advances in Next Generation Sequencing (NGS) have allowed for large-scale DNA and RNA transcriptome analysis of multiple tissue and cell types encompassing a myriad of disease states, leading to an increased understanding of the composition and regulation of the human genome. However, as a result of such studies it has become increasingly apparent that many chronic diseases are likely to be the result of the interaction between intrinsic genes and the external environment, which can result in modification to the DNA sequence and impact on gene expression.

This understanding has led to the emergence of the research field known as epigenetics, which includes the analysis of DNA modifications (including methylation and histone acetylation), as well as the transcription of non-coding RNAs. Indeed, the protein coding elements of the human genome are now known to be restricted to just 2% of the total genetic material present. Initially the remaining DNA was thought to be mostly “junk”, but much of this so called “genomic dark matter” is now known to transcribe multiple families of non-coding RNAs(1), many of which have been shown to modulate gene expression (Figure 1).

One such family of non-coding RNAs is the microRNA family(2), which bind to and prevent the translation of target mRNAs (Figure 2A). Importantly, there is now overwhelming evidence that microRNAs are regulators of the inflammatory
response(3), and several have been associated with multiple inflammatory diseases including osteoarthritis (OA)(3), rheumatoid arthritis (RA)(4).

However, recent NGS studies have now identified a new family of non-coding RNA, known as long non-coding RNAs (IncRNAs)(5), which are defined as transcripts >200 nt in length (Figure 1A). Over 14,000 have thus far been identified in humans(6), and they are currently sub-classified, based on their position relative to protein coding genes (Figure 1B), into antisense IncRNAs, pseudogene IncRNAs, enhancer RNAs, intronic IncRNAs and long intergenic ncRNA (lincRNA). These multiple variants may in part explain the functional diversity that has thus far been assigned to IncRNAs(7), including inhibition of transcriptional machinery, functioning as miRNA sponges, affecting mRNA stability and epigenetic DNA modifications (Figure 2B, C, D). Many of these functions have been attributed to the theory that IncRNAs form flexible scaffolds, which can bind proteins and RNAs.

Importantly, increasing evidence suggests that IncRNAs are important regulators of pathological and physiological processes, and evidence is now emerging to suggest that IncRNAs are central regulators of the inflammatory response. Therefore, in this review, we examine the evidence for the role of IncRNAs as regulators of inflammatory joint disease by highlighting studies which have reported differential expression of IncRNAs in diseased inflammatory joint tissue of patients with RA and OA, as well as studies which have implicated IncRNAs as regulators of known inflammatory pathways relevant to joint pathology. LncRNAs are poorly conserved across species, which has limited studies to determine their function. This poor conservation places great importance on the requirement to validate the function of
IncRNAs in disease-relevant human cells and tissue(8). For this reason, this review will focus on IncRNA data generated from human tissue/cells.

2. LncRNAs regulate inflammatory pathways

Inflammation is a central hallmark of RA joint disease, and evidence for inflammatory pathway dysregulation and cell dysfunction has been found in almost all innate and adaptive immune cells including. In addition, despite historically being seen as a “wear and tear” disease of the cartilage, there is now substantial evidence for inflammation also being a key contributor to OA joint pathology(9). OA synovial inflammation (synovitis) has been shown histologically, as well as by ultrasound and MRI imaging(10, 11), and there is evidence for increased cellular infiltration of activated B cells and T lymphocytes, and elevated levels of pro-inflammatory cytokines in the synovial fluid(12). Furthermore, ex-vivo stimulation of cartilage tissue with pro-inflammatory cytokines mimics several pathological features of the OA joint(13), and inhibitors of inflammatory pathways have modified OA disease in preclinical models(14).

Below we examine the current evidence for the role of IncRNAs in regulating canonical inflammatory pathways that are relevant to inflammatory joint pathology. Of the major signaling pathways that are known to govern joint inflammation four pathways were currently highlighted as having IncRNAs associated with their regulation, namely NFκB signalling, p38 MAPK pathway, the arachidonic acid pathway and TLR signalling.

2.1 NFκB Pathway
NFκB is considered a master regulator of inflammation, and its activity has been shown to regulate the production of several pro-inflammatory cytokines implicated in OA and RA joint pathology, including IL-1β, IL-6, IL-17 and TNF-α(12, 15). Therefore, inhibition of NFκB signalling has long been considered an attractive pathway to develop a therapeutic for chronic inflammatory disorders(15). However, therapeutic targeting of NFκB presents a challenge since NFκB may also have an anti-inflammatory role and be important for the resolution of inflammation(16). Therefore, identifying new approaches to appropriately target the pro-inflammatory activity of NFκB within the joint(17), so that systemic effects are avoided and patients are not immunocompromised, is important.

Thus far, several lncRNAs have been identified and implicated in targeting different components of the NFκB signalling pathway (Figure 3A). Of most immediate interest for inflammatory joint disease is lincRNA-p21, whose ability to inhibit NFκB signalling by sequestering RelA (the p65 subunit of NFκB) in T cells has been demonstrated following methotrexate treatment in RA patients. LincRNA-p21 expression was shown to be upregulated by methotrexate via a DNA-dependent protein kinase catalytic subunit (DNA PKcs)-dependent mechanism (18).

Another lncRNA which has been implicated as regulating NFκB activity is the pseudogene Lethe, which has been shown to be expressed in fibroblasts. Functional studies have demonstrated that Lethe interacts with and blocks the DNA binding of RelA (p65). It is believed that Lethe acts as part of a negative feedback loop which acts to regulate the inflammatory response, whereby following NFκB activation by pro-inflammatory cytokines, upregulation of Lethe inhibits NFκB
signalling. Of interest, the tissue expression of Lethe has been shown to be downregulated in aged mice (19). If the same is true in human ageing, the reduction in the expression of this potential brake on NFκB activity could be a contributing factor to age-associated inflammatory joint disorders such as RA and OA. Finally, studies conducted in human inflammatory breast epithelial cells have shown that the IncRNA NKILA (NFκB-interacting lncRNA) binds to and blocks phosphorylation sites on IκB, thereby inhibiting IKK-induced IκB phosphorylation and NFκB activation (20). Thus far, there are no studies which have reported the functional role of lincRNA-p21, Lethe or NKILA in RA or OA joint tissues or cells.

2.2 Arachidonic Acid Pathway

Arachidonic acid metabolites (prostaglandins and leukotrienes), are known mediators of the inflammatory response (21), and several have been found to be differentially expressed in the joints of patients with either OA or RA (22). Therefore, targeted inhibition of the arachidonic acid pathway has long been considered an important area of research for developing a therapeutic that combats joint inflammation and pain. Indeed, inhibition of the prostaglandin synthase enzymes (COX enzymes) is the basis for the mode of action of non-steroidal anti-inflammatory drugs (NSAIDs) such as Ibuprofen (23) and selective PTGS2 (COX2) inhibitors such as Valdecoxib (24), which have shown efficacy in reducing joint inflammation in several clinical trials.

Of importance therefore was the recent finding that the expression of PTGS2 (COX2) was positively regulated by a p50-associated COX-2 extragenic IncRNA (Figure 3B), referred to as PACER (25). PACER was shown to bind to and remove
the repressive action of the p50 homodimer (of NFκB), at the COX2 promoter, leading to activation of COX-2 gene expression. Furthermore, we recently found that the expression of PACER was associated with the IL-1β-mediated inflammatory response in primary human OA chondrocytes, and differentially expressed in human OA cartilage compared to non-OA cartilage (26).

Unfortunately, long-term administration of non-selective NSAIDs are associated with gastrointestinal toxicity(27), whilst selective COX2 inhibitors increase the risk of cardiovascular adverse events(28), attributed to a resulting imbalance between Thromboxane and prostacyclin levels. Therefore, identifying new regulators of prostaglandin production, such as PACER, is important, since it may lead to new targeted approaches that are capable of selectively inhibiting the production of inflammation-associated arachidonic acid metabolites, without any resulting toxicity.

2.3 p38 MAPK Signalling

Several studies have provided evidence that the p38 MAPK pathway is central to mediating both the production of pro-inflammatory cytokines and their signal transduction in the joint(13). Furthermore p38 small molecule inhibitors have shown efficacy both in reducing cartilage degeneration in OA preclinical models(29, 30), and reducing inflammatory pain in RA models(14, 31), albeit clinical trials of p38 inhibitors in patients with RA have thus far failed in the clinic.

With regards to IncRNA regulation of the p38 pathway, recent studies suggest the involvement of the metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) (32) (Figure 3C). MALAT1 is one of the most abundantly expressed
lncRNAs, with high levels of conservation across multiple tissue types. It has previously been well characterised in cancer where it is a transcription regulator implicated in cell cycle control and metastasis. However, Liu and colleagues (2014) have now highlighted MALAT1 cross-talk with p38 MAPK as being pathogenic in diabetes mellitus. MALAT1 is believed to activate p38 MAPK, since siRNA mediated knockdown of MALAT1 reduced p38 phosphorylation (32). Furthermore, MALAT1 expression has been found to be implicated in inflammation, again in diabetes mellitus under hyperglycaemic conditions, where it was shown to contribute to increased IL-6 expression and reactive oxygen species (ROS) generation(33).

2.4 TLR Signalling

Innate immune cells such as macrophages and neutrophils are present in the inflamed joints of RA and OA patients, and it is known that Toll-like receptor (TLR) signalling plays a key role in the recruitment and activation of these cells. Therefore, understanding the regulation of cytokine and chemokine signalling by these cells will provide greater clues as to the onset and maintenance of chronic inflammation within the joint.

TNFα and hnRNPL related immunoregulatory LincRNA (THRIL) was recently identified in macrophages as a lncRNA which could form a complex with heterogeneous ribonucleoprotein L (hnRNPL) within the nucleoplasm (Figure 3D). hnRNPs are known to be important for the processing, function and stabilisation of mRNAs. Critically, it was demonstrated that the THRIL/hnRNPL complex binds to the promotor of TNFα and induces its expression following TLR2 activation(34).
Carpenter and colleagues (35) identified a lncRNA (lincRNA-COX2) that sits in close proximity to the PTGS2 gene but, unlike the aforementioned PACER, this lncRNA had no functional interaction with PTGS2. Instead, lncRNA-COX2 was shown to be upregulated following activation of the TLR2 pathway (Figure 3D). Furthermore, following knockdown of lincRNA-COX2 and under the challenge of a synthetic TLR2 antagonist, expression of IL-6 became undetectable, while CCL5 expression increased. Similarly to THRIL, lincRNA-COX2 forms complexes with hnRNPs, specifically hnRNP-A/B and hnRNP-A2/B1, and the formation of such complexes has been shown to modulate target gene expression.

Activation of TLR3 has been shown to induce a lncRNA referred to as NEAT1 (nuclear enriched abundant transcript 1). NEAT1 was found to bind to a repressor of IL-8 transcription known as SFPQ (splicing factor proline/glutamine-rich) and relocate it to paraspeckle bodies (Figure 3D), resulting in IL-8 transcriptional activation (36). Ilott and colleagues (37) demonstrated that TLR4 activation in monocytes up-regulated expression of a lncRNA known as IL1β-RBT46. IL1β-RBT46 shares a promotor with the IL-1β coding gene and following pathway activation, both expression of the lincRNA and the IL-1β gene are induced (Figure 3D). LNA-mediated knockdown of IL1β-RBT46 led to a decrease in IL-1β mRNA expression, suggesting that the lincRNA promotes expression of the gene. A further consequence of IL1β-RBT46 knockdown was a reduction in expression of IL-8, a well-defined neutrophil chemoattractant.

Taken together, the lncRNAs described above represent a pool of lncRNAs, which have not as yet been associated with inflammatory joint disease. However, given
their prevalence in the regulation of central pathways of inflammation, determining their expression in joint tissues, and the relationship to OA and/or RA joint pathology would appear to be important studies.

The significance of genetic variations in the sequences of lncRNAs is currently not well understood and has been little studied to date. There is evidence that large alterations such as chromosomal rearrangements can affect lncRNA expression(38). However, as illustrated by the functional studies highlighted in this review, lncRNA function may largely be determined by its secondary structure, through binding to proteins and RNA. Therefore, smaller mutations and genetic variations such as SNPs may have little effect on lncRNA function. It is conceivable that SNPs could alter lncRNA function by altering lncRNA secondary structure, or causing alternative splicing of the lncRNA. Indeed, there is evidence of SNPs in the chromosomal region that harbour the lncRNA ANRIL being associated with a susceptibility to cardiovascular disorders(39). However, it is not currently known whether these SNPs impact on the functional role of ANRIL lncRNA, or simply affect its expression. Predominantly therefore, studies have thus far linked lncRNA to disease by expression analysis.

3. LncRNA expression in inflammatory OA and RA joint tissues

Transcriptional analyses by both NGS and microarray have identified several lncRNAs that are differentially expressed in inflammatory diseased joint tissue. Thus far, studies on OA patient samples have focussed on the cartilage tissue, whilst in RA disease other peri-joint tissues such as synovium and sub-chondral bone have been interrogated.
One lncRNA that has been found to be differentially expressed in both OA and RA joint tissue is HOTAIR. Located on chromosome 12, HOTAIR was the first lncRNA to be characterised, and has been shown to interact with polycomb repressive complex 2 (PRC2) which regulates the chromatin state, and the histone demethylase, LSD1. As such, it regulates epigenetic changes within its target transcripts(40).

In OA cartilage, HOTAIR was found to be upregulated compared to non-OA cartilage (41), although as yet its function in cartilage tissue or in isolated chondrocyte cells has yet to be reported. Song et al (42) have also identified differential expression of HOTAIR in RA peri-joint tissues, namely synovial fibroblasts, osteoclasts and bone mononuclear cells. Of note, Song et al (42) found that the function of HOTAIR differed depending on the RA cell studied. For example, in RA synovial fibroblasts and osteoclasts, HOTAIR expression was lower than in controls and its over-expression decreased the expression of MMP2 and MMP13. In contrast, HOTAIR was upregulated in RA bone mononuclear cells where it functioned to facilitate macrophage activation and migration towards the joint.

Another lncRNA for which differential expression has been reported in inflamed joint tissue is H19. H19 IncRNA has been found to be highly expressed in RA synovial tissues RA(43) and in OA cartilage(44). Previous studies have demonstrated that H19 is a developmental reservoir of the two miR-675 family members – miR-675-p3 and miR-675-p5, which have been shown to regulate Type II Collagen expression and mediate cellular development in multiple tissues (45-47). Of interest, numerous
studies have demonstrated that H19 controls the expression of several genes which are part of the Imprinted Gene Network (IGN) – a series of parentally inherited genes whose expression is epigenetically determined within the sperm or oocyte. Such “imprinting disorders” have been linked to a number of human pathologies. Currently, inflammatory disease isn't known to be affected by imprinting disorders, but given the inheritability of RA and other inflammatory diseases, studies which investigate the role of H19 in determining epigenetic susceptibility to disease could be of interest.

As previously mentioned, lncRNA analysis in OA has primarily focused on the cartilage tissue, and the lncRNAs GAS5(48) and MEG3(49) have been reported to be downregulated in OA diseased cartilage. Fu and colleagues(50) performed microarray analysis and identified 4714 lncRNAs which were differentially expressed in OA cartilage compared to non-OA cartilage. Although this study did not perform any modulation of lncRNA expression to determine lncRNA function, expression correlation patterns were utilised to predict candidate lncRNA regulated genes. This analyses highlighted lncRNAuc.343, which was upregulated in OA cartilage, as regulating in cis the expression of HOXC8(50), and that many of the differentially expressed lncRNAs worked in concert with the transcription factor, SP1, to modulate expression of trans target genes.

In a separate study, microarray analysis was used to identify a lncRNA with a specific role in the degradation of the cartilage matrix, named LncRNA-CIR. LncRNA-CIR was found to be upregulated in OA cartilage, compared to non-OA cartilage, and siRNA-mediated knockdown of its expression resulted in the increased
expression of collagen and aggrecan accompanied by reduced expression of MMP13 and ADAMTS5 (51).

Furthermore, we recently identified IncRNAs associated with the IL-1β-mediated inflammatory response in primary human OA chondrocytes, which were also differentially expressed in OA diseased cartilage. Of particularly note were HIVEP2AS and IL-7AS, two intergenic IncRNAs proximal to the Hivep2 and IL-7 protein coding genes respectively(26). Both HIVEP2AS and IL-7AS were found to be upregulated in both Knee OA and hip OA cartilage compared to non-OA control cartilage(26). Furthermore, LNA-mediated knockdown of HIVEP2AS and IL-7AS expression in human chondrocytes resulted in an increase in the production of pro-inflammatory cytokines, including IL-6(26), suggesting that these IncRNAs may function to control aberrant joint inflammation.

4. Perspectives

LncRNA research in inflammatory joint disease is very much a nascent field. However, several IncRNAs have now been identified as being either differentially expressed in diseased joint tissue, or as candidate central regulators of inflammatory pathways relevant to joint pathology. Therefore, future studies to determine the functional role and mode of action of these disease-associated IncRNAs will be insightful, as will joint tissue expression profiling of IncRNAs for which functional roles within key inflammatory pathways have been determined. To this end, it will be important to conduct further transcriptomic analysis of joint tissues (including subchondral bone, cartilage, and synovium), where diseased joint tissues are
compared with age-matched non-inflammatory control joint tissue, and to conduct functional mode-of-action studies in disease relevant cells.

It should be noted that currently the expression analysis of lncRNAs in the joint tissues from patients with other inflammatory joint diseases including gout, psoriatic arthritis and juvenile idiopathic arthritis has not yet been reported. Clearly, such studies could also prove to be informative in building a broader pool of inflammatory joint disease-associated lncRNAs. Indeed, since inflammatory joint diseases share several common pathways, such studies are likely to identify lncRNAs that are dysregulated across multiple inflammatory joint disorders. Such findings will add to the recent paradigm shift in our understanding of the dysregulation of chronic disease, whereby the disease is no longer classified solely by its gross pathology but by the involvement of a particular dysregulated pathway.

It is currently too early to determine whether disease modifying therapeutics could be developed that are designed to directly target and modulate lncRNA function. Recent progress has been achieved using siRNA RNAi-based therapeutics, whereby targeted knockdown of TNF-α (52) or NFκB (53) has modified disease progression in animal models of RA and OA. Therefore, it is conceivable that lncRNAs could provide a new class of targets for RNAi based therapeutics. Regardless, ultimately determining the mode of action of lncRNAs that regulate inflammatory pathways, and determining their relationship to joint pathology will provide a better understanding of how inflammation is epigenetically regulated within the joint. This improved understanding will likely be important in aiding the identification of new
targets for therapeutic intervention, as well as in identifying at risk patient populations.
COMPETING INTERESTS

The authors declare no competing interests

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References

### Table 1: LncRNA involvement in inflammatory pathways

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Figure Legends

Figure 1. Classification of non-coding RNAs and IncRNA sub-types

(A) LncRNAs are classified as being >200 nt in length, in contrast to miRNAs which are <200 nt long. (B) LncRNAs are further classified by their position relative to protein-coding genes. Intronic IncRNAs are transcribed from intronic transcription start sites (TSS) and can be in either sense of antisense orientation. They do not intersect any exons. In contrast, Antisense IncRNAs are transcribed across protein-coding exons on the opposite strand. Intergenic IncRNAs (lincRNAs) are transcribed from TSS between protein-coding genes. Pseudogenes can be classified as a type of lincRNA and originate when a gene loses its coding ability, for example through a mutation. Enhancer RNAs (eRNAs) can also be considered a type of lincRNA, but are transcribed from enhancer regions (identified by the presence of high H3K4me1 histone marks). In contrast, other IncRNAs are transcribed from regions associated with the presence of high H3K4me3 histone marks, similar to protein-coding genes.

Figure 2. Functional diversity attributed to IncRNAs.

(A) Post-transcriptional regulation of gene expression by non-coding miRNAs. miRNAs bind to the 3’UTR of target mRNAs in the cytoplasm. Assembly of a RNA induced silencing complex (RISC), composed of Ago2, Dicer and R2D2, leads to mRNA degradation. Conversely, IncRNAs can modulate gene expression through both pre- and post-transcriptional regulation mechanisms. (B) LncRNAs can act as scaffolds for the assembly of complexes that epigenetically modify the DNA (e.g histone acetylation and methylation), leading to modulation of transcription. (C) LncRNAs can bind to transcription factors and mediate assemble of transcription
initiation complexes, leading to modulation of transcription. (D) LncRNAs can bind to miRNAs, acting as either sponges to block their activity, or affect miRNA stability.

**Figure 3. Functional IncRNAs in inflammatory pathways.**

(A) **Canonical NFκB signalling.** The IncRNA *NKILA* blocks the IkBα phosphorylation site preventing its activation. The IncRNAs *Lethe* and *LincRNA-p21* both bind to RelA and prevent NFκB binding to NFκB response elements. (B) **Arachidonic acid pathway.** The IncRNA *PACER* binds to and removes the repressive action of the p50 homodimer at the COX2 promoter, leading to activation of COX-2 gene expression. (C) **p38 MAPK signalling.** The IncRNA *MALAT1* activates p38 phosphorylation. ARE represents Adenine Rich Elements in mRNA transcripts, which are stabilised via downstream MK2 activation. (D) **TLR signalling.** The IncRNAs *THRIL* and *LincRNA-COX2* form complexes with hnRNPLs, which then bind to and activate the promoters of pro-inflammatory cytokines TNF-α and CCL5 respectively. The IncRNA *NEAT1* binds to SFPQ and relocates it to paraspeckle bodies, relieving its repression of the IL-8 promoter. The *IL1β-RBT46* is induced by TLR4 signalling and promotes induction of IL-8 and IL-1β via an as yet unknown mechanism.
Long non-coding RNAs in the regulation of inflammatory pathways in rheumatoid arthritis and osteoarthritis

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Abstract

Long non coding RNAs (lncRNAs) are a functionally diverse family of non-coding RNAs, which have been implicated in modulating gene expression through transcription, translation and DNA epigenetic modifications. As a relatively nascent field, the number of known functional lncRNAs is currently small. However, increasing numbers of lncRNAs are now being identified as candidate regulators of inflammatory pathways that are relevant to osteoarthritis and rheumatoid arthritis joint pathology. Furthermore, several lncRNAs have been identified which are differentially expressed in osteoarthritis or rheumatoid arthritis joint tissue.

In this review, we highlight those lncRNAs for which functional effects have been demonstrated within the major pathways associated with inflammation, including NFkB signalling, p38 MAPK pathway, arachidonic acid pathway and TLR signalling. We also highlight those lncRNAs which have been shown to be differentially expressed in inflammatory joint tissue, and are therefore strong candidates for future investigative work.

Determining the expression and functional role of lncRNAs in mediating inflammation within the joint will not only advance our understanding of the epigenetics which underlie the pathology of inflammatory joint disease, but may also ultimately aid in the identification of novel targets for therapeutic intervention.
1. Introduction

It was initially envisaged that upon the completion of the human genome project our understanding of human disease would lead to an abundant development of new targeted therapies by which to modify disease progression or prevent disease onset. Furthermore, advances in Next Generation Sequencing (NGS) have allowed for large-scale DNA and RNA transcriptome analysis of multiple tissue and cell types encompassing a myriad of disease states, leading to an increased understanding of the composition and regulation of the human genome. However, as a result of such studies it has become increasingly apparent that many chronic diseases are likely to be the result of the interaction between intrinsic genes and the external environment, which can result in modification to the DNA sequence and impact on gene expression.

This understanding has led to the emergence of the research field known as epigenetics, which includes the analysis of DNA modifications (including methylation and histone acetylation), as well as the transcription of non-coding RNAs. Indeed, the protein coding elements of the human genome are now known to be restricted to just 2% of the total genetic material present. Initially the remaining DNA was thought to be mostly “junk”, but much of this so called “genomic dark matter” is now known to transcribe multiple families of non-coding RNAs(1), many of which have been shown to modulate gene expression (Figure 1).

One such family of non-coding RNAs is the microRNA family(2), which bind to and prevent the translation of target mRNAs (Figure 2A). Importantly, there is now overwhelming evidence that microRNAs are regulators of the inflammatory
response(3), and several have been associated with multiple inflammatory diseases including osteoarthritis (OA)(3), rheumatoid arthritis (RA)(4).

However, recent NGS studies have now identified a new family of non-coding RNA, known as long non-coding RNAs (lncRNAs)(5), which are defined as transcripts >200 nt in length (Figure 1A). Over 14,000 have thus far been identified in humans(6), and they are currently sub-classified, based on their position relative to protein coding genes (Figure 1B), into antisense lncRNAs, pseudogene lncRNAs, enhancer RNAs, intronic lncRNAs and long intergenic ncRNA (lincRNA). These multiple variants may in part explain the functional diversity that has thus far been assigned to lncRNAs(7), including inhibition of transcriptional machinery, functioning as miRNA sponges, affecting mRNA stability and epigenetic DNA modifications (Figure 2B, C, D). Many of these functions have been attributed to the theory that lncRNAs form flexible scaffolds, which can bind proteins and RNAs.

Importantly, increasing evidence suggests that lncRNAs are important regulators of pathological and physiological processes, and evidence is now emerging to suggest that lncRNAs are central regulators of the inflammatory response. Therefore, in this review, we examine the evidence for the role of lncRNAs as regulators of inflammatory joint disease by highlighting studies which have reported differential expression of lncRNAs in diseased inflammatory joint tissue of patients with RA and OA, as well as studies which have implicated lncRNAs as regulators of known inflammatory pathways relevant to joint pathology. LncRNAs are poorly conserved across species, which has limited studies to determine their function. This poor conservation places great importance on the requirement to validate the function of
In this review, we will focus on IncRNA data generated from human tissue/cells.

2. LncRNAs regulate inflammatory pathways

Inflammation is a central hallmark of RA joint disease, and evidence for inflammatory pathway dysregulation and cell dysfunction has been found in almost all innate and adaptive immune cells including. In addition, despite historically being seen as a “wear and tear” disease of the cartilage, there is now substantial evidence for inflammation also being a key contributor to OA joint pathology(9). OA synovial inflammation (synovitis) has been shown histologically, as well as by ultrasound and MRI imaging(10, 11), and there is evidence for increased cellular infiltration of activated B cells and T lymphocytes, and elevated levels of pro-inflammatory cytokines in the synovial fluid(12). Furthermore, *ex-vivo* stimulation of cartilage tissue with pro-inflammatory cytokines mimics several pathological features of the OA joint(13), and inhibitors of inflammatory pathways have modified OA disease in preclinical models(14).

Below we examine the current evidence for the role of IncRNAs in regulating canonical inflammatory pathways that are relevant to inflammatory joint pathology. Of the major signaling pathways that are known to govern joint inflammation four pathways were currently highlighted as having IncRNAs associated with their regulation, namely NFκB signalling, p38 MAPK pathway, the arachidonic acid pathway and TLR signalling.

2.1 NFκB Pathway
NFκB is considered a master regulator of inflammation, and its activity has been shown to regulate the production of several pro-inflammatory cytokines implicated in OA and RA joint pathology, including IL-1β, IL-6, IL-17 and TNF-α(12, 15). Therefore, inhibition of NFκB signalling has long been considered an attractive pathway to develop a therapeutic for chronic inflammatory disorders(15). However, therapeutic targeting of NFκB presents a challenge since NFκB may also have an anti-inflammatory role and be important for the resolution of inflammation(16). Therefore, identifying new approaches to appropriately target the pro-inflammatory activity of NFκB within the joint(17), so that systemic effects are avoided and patients are not immunocompromised, is important.

Thus far, several lncRNAs have been identified and implicated in targeting different components of the NFκB signalling pathway (Figure 3A). Of most immediate interest for inflammatory joint disease is lincRNA-p21, whose ability to inhibit NFκB signalling by sequestering RelA (the p65 subunit of NFκB) in T cells has been demonstrated following methotrexate treatment in RA patients. LincRNA-p21 expression was shown to be upregulated by methotrexate via a DNA-dependent protein kinase catalytic subunit (DNA PKcs)-dependent mechanism (18).

Another lncRNA which has been implicated as regulating NFκB activity is the pseudogene Lethe, which has been shown to be expressed in fibroblasts. Functional studies have demonstrated that Lethe interacts with and blocks the DNA binding of RelA (p65). It is believed that Lethe acts as part of a negative feedback loop which acts to regulate the inflammatory response, whereby following NFκB activation by pro-inflammatory cytokines, upregulation of Lethe inhibits NFκB
signalling. Of interest, the tissue expression of Lethe has been shown to be downregulated in aged mice (19). If the same is true in human ageing, the reduction in the expression of this potential brake on NFκB activity could be a contributing factor to age-associated inflammatory joint disorders such as RA and OA. Finally, studies conducted in human inflammatory breast epithelial cells have shown that the lncRNA NKILA (NFκB-interacting lncRNA) binds to and blocks phosphorylation sites on IκB, thereby inhibiting IKK-induced IκB phosphorylation and NFκB activation (20). Thus far, there are no studies which have reported the functional role of lincRNA-p21, Lethe or NKILA in RA or OA joint tissues or cells.

2.2 Arachidonic Acid Pathway

Arachidonic acid metabolites (prostaglandins and leukotrienes), are known mediators of the inflammatory response (21), and several have been found to be differentially expressed in the joints of patients with either OA or RA (22). Therefore, targeted inhibition of the arachidonic acid pathway has long been considered an important area of research for developing a therapeutic that combats joint inflammation and pain. Indeed, inhibition of the prostaglandin synthase enzymes (COX enzymes) is the basis for the mode of action of non-steroidal anti-inflammatory drugs (NSAIDs) such as Ibuprofen (23) and selective PTGS2 (COX2) inhibitors such as Valdecoxib (24), which have shown efficacy in reducing joint inflammation in several clinical trials.

Of importance therefore was the recent finding that the expression of PTGS2 (COX2) was positively regulated by a p50-associated COX-2 extragenic IncRNA (Figure 3B), referred to as PACER (25). PACER was shown to bind to and remove
the repressive action of the p50 homodimer (of NFκB), at the COX2 promoter, leading to activation of COX-2 gene expression. Furthermore, we recently found that the expression of PACER was associated with the IL-1β-mediated inflammatory response in primary human OA chondrocytes, and differentially expressed in human OA cartilage compared to non-OA cartilage (26).

Unfortunately, long-term administration of non-selective NSAIDs are associated with gastrointestinal toxicity(27), whilst selective COX2 inhibitors increase the risk of cardiovascular adverse events(28), attributed to a resulting imbalance between Thromboxane and prostacyclin levels. Therefore, identifying new regulators of prostaglandin production, such as PACER, is important, since it may lead to new targeted approaches that are capable of selectively inhibiting the production of inflammation-associated arachidonic acid metabolites, without any resulting toxicity.

2.3 p38 MAPK Signalling

Several studies have provided evidence that the p38 MAPK pathway is central to mediating both the production of pro-inflammatory cytokines and their signal transduction in the joint(13). Furthermore p38 small molecule inhibitors have shown efficacy both in reducing cartilage degeneration in OA preclinical models(29, 30), and reducing inflammatory pain in RA models(14, 31), albeit clinical trials of p38 inhibitors in patients with RA have thus far failed in the clinic.

With regards to IncRNA regulation of the p38 pathway, recent studies suggest the involvement of the metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) (32) (Figure 3C). MALAT1 is one of the most abundantly expressed
lncRNAs, with high levels of conservation across multiple tissue types. It has previously been well characterised in cancer where it is a transcription regulator implicated in cell cycle control and metastasis. However, Liu and colleagues (2014) have now highlighted MALAT1 cross-talk with p38 MAPK as being pathogenic in diabetes mellitus. MALAT1 is believed to activate p38 MAPK, since siRNA mediated knockdown of MALAT1 reduced p38 phosphorylation (32). Furthermore, MALAT1 expression has been found to be implicated in inflammation, again in diabetes mellitus under hyperglycaemic conditions, where it was shown to contribute to increased IL-6 expression and reactive oxygen species (ROS) generation(33).

2.4 TLR Signalling

Innate immune cells such as macrophages and neutrophils are present in the inflamed joints of RA and OA patients, and it is known that Toll-like receptor (TLR) signalling plays a key role in the recruitment and activation of these cells. Therefore, understanding the regulation of cytokine and chemokine signalling by these cells will provide greater clues as to the onset and maintenance of chronic inflammation within the joint.

TNFα and hnRNPL related immunoregulatory LincRNA (THRIL) was recently identified in macrophages as a lncRNA which could form a complex with heterogeneous ribonucleoprotein L (hnRNPL) within the nucleoplasm (Figure 3D). hnRNPs are known to be important for the processing, function and stabilisation of mRNAs. Critically, it was demonstrated that the THRIL/hnRNPL complex binds to the promotor of TNFα and induces its expression following TLR2 activation(34).
Carpenter and colleagues (35) identified a lncRNA (lincRNA-COX2) that sits in close proximity to the PTGS2 gene but, unlike the aforementioned PACER, this lncRNA had no functional interaction with PTGS2. Instead, lincRNA-COX2 was shown to be upregulated following activation of the TLR2 pathway (Figure 3D). Furthermore, following knockdown of lincRNA-COX2 and under the challenge of a synthetic TLR2 antagonist, expression of IL-6 became undetectable, while CCL5 expression increased. Similarly to THRIL, lincRNA-COX2 forms complexes with hnRNPs, specifically hnRNP-A/B and hnRNP-A2/B1, and the formation of such complexes has been shown to modulate target gene expression.

Activation of TLR3 has been shown to induce a lncRNA referred to as NEAT1 (nuclear enriched abundant transcript 1). NEAT1 was found to bind to a repressor of IL-8 transcription known as SFPQ (splicing factor proline/glutamine-rich) and relocate it to paraspeckle bodies (Figure 3D), resulting in IL-8 transcriptional activation (36). Ilott and colleagues (37) demonstrated that TLR4 activation in monocytes up-regulated expression of a lncRNA known as IL1β-RBT46. IL1β-RBT46 shares a promoter with the IL-1β coding gene and following pathway activation, both expression of the lncRNA and the IL-1β gene are induced (Figure 3D). LNA-mediated knockdown of IL1β-RBT46 led to a decrease in IL-1β mRNA expression, suggesting that the lncRNA promotes expression of the gene. A further consequence of IL1β-RBT46 knockdown was a reduction in expression of IL-8, a well-defined neutrophil chemoattractant.

Taken together, the lncRNAs described above represent a pool of lncRNAs, which have not as yet been associated with inflammatory joint disease. However, given
their prevalence in the regulation of central pathways of inflammation, determining their expression in joint tissues, and the relationship to OA and/or RA joint pathology would appear to be important studies.

The significance of genetic variations in the sequences of lncRNAs is currently not well understood and has been little studied to date. There is evidence that large alterations such as chromosomal rearrangements can affect lncRNA expression\(^{(38)}\). However, as illustrated by the functional studies highlighted in this review, lncRNA function may largely be determined by its secondary structure, through binding to proteins and RNA. Therefore, smaller mutations and genetic variations such as SNPs may have little effect on lncRNA function. It is conceivable that SNPs could alter lncRNA function by altering lncRNA secondary structure, or causing alternative splicing of the lncRNA. Indeed, there is evidence of SNPs in the chromosomal region that harbour the lncRNA ANRIL being associated with a susceptibility to cardiovascular disorders\(^{(39)}\). However, it is not currently known whether these SNPs impact on the functional role of ANRIL lncRNA, or simply affect its expression. Predominantly therefore, studies have thus far linked lncRNA to disease by expression analysis.

3. LncRNA expression in inflammatory OA and RA joint tissues

Transcriptional analyses by both NGS and microarray have identified several lncRNAs that are differentially expressed in inflammatory diseased joint tissue. Thus far, studies on OA patient samples have focussed on the cartilage tissue, whilst in RA disease other peri-joint tissues such as synovium and sub-chondral bone have been interrogated.
One lncRNA that has been found to be differentially expressed in both OA and RA joint tissue is HOTAIR. Located on chromosome 12, HOTAIR was the first lncRNA to be characterised, and has been shown to interact with polycomb repressive complex 2 (PRC2) which regulates the chromatin state, and the histone demethylase, LSD1. As such, it regulates epigenetic changes within its target transcripts(40).

In OA cartilage, HOTAIR was found to be upregulated compared to non-OA cartilage (41), although as yet its function in cartilage tissue or in isolated chondrocyte cells has yet to be reported. Song et al (42) have also identified differential expression of HOTAIR in RA peri-joint tissues, namely synovial fibroblasts, osteoclasts and bone mononuclear cells. Of note, Song et al (42) found that the function of HOTAIR differed depending on the RA cell studied. For example, in RA synovial fibroblasts and osteoclasts, HOTAIR expression was lower than in controls and its over-expression decreased the expression of MMP2 and MMP13. In contrast, HOTAIR was upregulated in RA bone mononuclear cells where it functioned to facilitate macrophage activation and migration towards the joint.

Another lncRNA for which differential expression has been reported in inflamed joint tissue is H19. H19 lncRNA has been found to be highly expressed in RA synovial tissues RA(43) and in OA cartilage(44). Previous studies have demonstrated that H19 is a developmental reservoir of the two miR-675 family members – miR-675-p3 and miR-675-p5, which have been shown to regulate Type II Collagen expression and mediate cellular development in multiple tissues (45-47). Of interest, numerous
studies have demonstrated that H19 controls the expression of several genes which are part of the Imprinted Gene Network (IGN) – a series of parentally inherited genes whose expression is epigenetically determined within the sperm or oocyte. Such “imprinting disorders” have been linked to a number of human pathologies. Currently, inflammatory disease isn't known to be affected by imprinting disorders, but given the inheritability of RA and other inflammatory diseases, studies which investigate the role of H19 in determining epigenetic susceptibility to disease could be of interest.

As previously mentioned, lncRNA analysis in OA has primarily focused on the cartilage tissue, and the lncRNAs GAS5(48) and MEG3(49) have been reported to be downregulated in OA diseased cartilage. Fu and colleagues(50) performed microarray analysis and identified 4714 lncRNAs which were differentially expressed in OA cartilage compared to non-OA cartilage. Although this study did not perform any modulation of lncRNA expression to determine lncRNA function, expression correlation patterns were utilised to predict candidate lncRNA regulated genes. This analyses highlighted lncRNAuc.343, which was upregulated in OA cartilage, as regulating in cis the expression of HOXC8(50), and that many of the differentially expressed lncRNAs worked in concert with the transcription factor, SP1, to modulate expression of trans target genes.

In a separate study, microarray analysis was used to identify a lncRNA with a specific role in the degradation of the cartilage matrix, named lncRNA-CIR. LncRNA-CIR was found to be upregulated in OA cartilage, compared to non-OA cartilage, and siRNA-mediated knockdown of its expression resulted in the increased
expression of collagen and aggrecan accompanied by reduced expression of MMP13 and ADAMTS5 (51).

Furthermore, we recently identified IncRNAs associated with the IL-1β-mediated inflammatory response in primary human OA chondrocytes, which were also differentially expressed in OA diseased cartilage. Of particularly note were HIVEP2AS and IL-7AS, two intergenic IncRNAs proximal to the Hivep2 and IL-7 protein coding genes respectively(26). Both HIVEP2AS and IL-7AS were found to be upregulated in both Knee OA and hip OA cartilage compared to non-OA control cartilage(26). Furthermore, LNA-mediated knockdown of HIVEP2AS and IL-7AS expression in human chondrocytes resulted in an increase in the production of pro-inflammatory cytokines, including IL-6(26), suggesting that these IncRNAs may function to control aberrant joint inflammation.

4. Perspectives
LncRNA research in inflammatory joint disease is very much a nascent field. However, several IncRNAs have now been identified as being either differentially expressed in diseased joint tissue, or as candidate central regulators of inflammatory pathways relevant to joint pathology. Therefore, future studies to determine the functional role and mode of action of these disease-associated IncRNAs will be insightful, as will joint tissue expression profiling of IncRNAs for which functional roles within key inflammatory pathways have been determined. To this end, it will be important to conduct further transcriptomic analysis of joint tissues (including subchondral bone, cartilage, and synovium), where diseased joint tissues are
compared with age-matched non-inflammatory control joint tissue, and to conduct functional mode-of-action studies in disease relevant cells.

It should be noted that currently the expression analysis of IncRNAs in the joint tissues from patients with other inflammatory joint diseases including gout, psoriatic arthritis and juvenile idiopathic arthritis has not yet been reported. Clearly, such studies could also prove to be informative in building a broader pool of inflammatory joint disease-associated IncRNAs. Indeed, since inflammatory joint diseases share several common pathways, such studies are likely to identify IncRNAs that are dysregulated across multiple inflammatory joint disorders. Such findings will add to the recent paradigm shift in our understanding of the dysregulation of chronic disease, whereby the disease is no longer classified solely by its gross pathology but by the involvement of a particular dysregulated pathway.

It is currently too early to determine whether disease modifying therapeutics could be developed that are designed to directly target and modulate IncRNA function. Recent progress has been achieved using siRNA RNAi-based therapeutics, whereby targeted knockdown of TNF-α (52) or NFκB (53) has modified disease progression in animal models of RA and OA. Therefore, it is conceivable that IncRNAs could provide a new class of targets for RNAi based therapeutics. Regardless, ultimately determining the mode of action of IncRNAs that regulate inflammatory pathways, and determining their relationship to joint pathology will provide a better understanding of how inflammation is epigenetically regulated within the joint. This improved understanding will likely be important in aiding the identification of new
targets for therapeutic intervention, as well as in identifying at risk patient populations.
COMPETING INTERESTS

The authors declare no competing interests

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References


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Figure Legends

**Figure 1. Classification of non-coding RNAs and IncRNA sub-types**

(A) LncRNAs are classified as being >200 nt in length, in contrast to miRNAs which are <200 nt long. (B) LncRNAs are further classified by their position relative to protein-coding genes. Intronic IncRNAs are transcribed from intronic transcription start sites (TSS) and can be in either sense of antisense orientation. They do not intersect any exons. In contrast, Antisense IncRNAs are transcribed across protein-coding exons on the opposite strand. Intergenic IncRNAs (lincRNAs) are transcribed from TSS between protein-coding genes. Pseudogenes can be classified as a type of lincRNA and originate when a gene loses its coding ability, for example through a mutation. Enhancer RNAs (eRNAs) can also be considered a type of lincRNA, but are transcribed from enhancer regions (identified by the presence of high H3K4me1 histone marks). In contrast, other IncRNAs are transcribed from regions associated with the presence of high H3K4me3 histone marks, similar to protein-coding genes.

**Figure 2. Functional diversity attributed to IncRNAs.**

(A) Post-transcriptional regulation of gene expression by non-coding miRNAs. miRNAs bind to the 3'UTR of target mRNAs in the cytoplasm. Assembly of a RNA induced silencing complex (RISC), composed of Ago2, Dicer and R2D2, leads to mRNA degradation. Conversely, IncRNAs can modulate gene expression through both pre- and post-transcriptional regulation mechanisms. (B) LncRNAs can act as scaffolds for the assembly of complexes that epigenetically modify the DNA (e.g histone acetylation and methylation), leading to modulation of transcription. (C) LncRNAs can bind to transcription factors and mediate assembly of transcription
initiation complexes, leading to modulation of transcription. **(D)** LncRNAs can bind to miRNAs, acting as either sponges to block their activity, or affect miRNA stability.

**Figure 3. Functional lncRNAs in inflammatory pathways.** **(A)** Canonical NFκB signalling. The lncRNA *NKILA* blocks the IκBα phosphorylation site preventing its activation. The lncRNAs *Lethe* and *LincRNA-p21* both bind to RelA and prevent NFκB binding to NFκB response elements. **(B)** Arachidonic acid pathway. The lncRNA *PACER* binds to and removes the repressive action of the p50 homodimer at the COX2 promoter, leading to activation of COX-2 gene expression. **(C)** p38 MAPK signalling. The lncRNA *MALAT1* activates p38 phosphorylation. ARE represents Adenine Rich Elements in mRNA transcripts, which are stabilised via downstream MK2 activation. **(D)** TLR signalling. The lncRNAs *THRIL* and *LincRNA-COX2* form complexes with hnRNPLs, which then bind to and activate the promoters of pro-inflammatory cytokines TNF-α and CCL5 respectively. The lncRNA *NEAT1* binds to SFPQ and relocates it to paraspeckle bodies, relieving its repression of the IL-8 promoter. The *IL1β-RBT46* is induced by TLR4 signalling and promotes induction of IL-8 and IL-1β via an as yet unknown mechanism.
Figure 1. Classification of non-coding RNAs and lncRNA sub-types
(A) LncRNAs are classified as being >200 nt in length, in contrast to miRNAs which are <200 nt long. (B) LncRNAs are further classified by their position relative to protein-coding genes. Intronic lncRNAs are transcribed from intronic transcription start sites (TSS) and can be in either sense of antisense orientation. They do not intersect any exons. In contrast, Antisense lncRNAs are transcribed across protein-coding exons on the opposite strand. Intergenic lncRNAs (lincRNAs) are transcribed from TSS between protein-coding genes. Pseudogenes can be classified as a type of lincRNA and originate when a gene loses its coding ability, for example through a mutation. Enhancer RNAs (eRNAs) can also be considered a type of lincRNA, but are transcribed from enhancer regions (identified by the presence of high H3K4me1 histone marks). In contrast, other lncRNAs are transcribed from regions associated with the presence of high H3K4me3 histone marks, similar to protein-coding genes.
Figure 2. Functional diversity attributed to lncRNAs.

(A) Post-transcriptional regulation of gene expression by non-coding miRNAs. MiRNAs bind to the 3'UTR of target mRNAs in the cytoplasm. Assembly of a RNA induced silencing complex (RISC), composed of Ago2, Dicer and R2D2, leads to mRNA degradation. Conversely, lncRNAs can modulate gene expression through both pre- and post-transcriptional regulation mechanisms. (B) LncRNAs can act as scaffolds for the assembly of complexes that epigenetically modify the DNA (e.g., histone acetylation and methylation), leading to modulation of transcription. (C) LncRNAs can bind to transcription factors and mediate assembly of transcription initiation complexes, leading to modulation of transcription. (D) LncRNAs can bind to miRNAs, acting as either sponges to block their activity, or affect miRNA stability.
Figure 3. Functional lncRNAs in inflammatory pathways. (A) Canonical NFκB signalling. The lncRNA NKILA blocks the IκBα phosphorylation site preventing its activation. The lncRNAs Lethe and LincRNA-p21 both bind to RelA and prevent NFκB binding to NFκB response elements. (B) Arachidonic acid pathway. The lncRNA PACER binds to and removes the repressive action of the p50 homodimer at the COX2 promoter, leading to activation of COX2 gene expression. (C) p38 MAPK signalling. The lncRNA MALAT1 activates p38 phosphorylation. ARE represents Adenine Rich Elements in mRNA transcripts, which are stabilised via downstream MK2 activation. (D) TLR signalling. The lncRNAs THRIL and LincRNA-COX2 form complexes with hnRNPLs, which then bind to and activate the promoters of pro-inflammatory cytokines TNF-α and CCL5 respectively. The lncRNA NEAT1 binds to SFPQ and relocates it to paraspeckle bodies, relieving its repression of the IL-8 promoter. The IL1β-RBT46 is induced by TLR4 signalling and promotes induction of IL-8 and IL-1β via an as yet unknown mechanism.