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Bioactivity in an aggrecan 32mer fragment

Bioactivity in an aggrecan 32mer fragment is mediated via Toll-like receptor 2

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Bioactivity in an aggrecan 32mer fragment

Objectives

The aim of this study was to determine whether an aggrecan 32mer fragment derived from dual ADAMTS and MMP cleavage in the aggrecan interglobular domain was bioactive, and if so, to elucidate its mechanism of action.

Methods

Mouse primary chondrocytes, synovial fibroblasts or peritoneal macrophages, human primary chondrocytes, and cells or cell lines from MyD88-deficient and TLR-2-deficient mice were stimulated with synthetic mouse or human 32mer peptide or scrambled peptide or native, glycosylated 32mer peptide. Cells stimulated with 32mer were analysed for changes in mRNA expression by qPCR. Conditioned medium was analysed for levels of IL-6 protein by AlphaLISA or MMP-3 and MMP-13 protein by Western blot. NF κ B activation was measured in a luciferase reporter assay.

Results

Treatment of mouse cells or cartilage explants with 32mer peptide or scrambled peptide revealed that the 32mer, but not the scrambled peptide, had anti-anabolic, pro-catabolic and pro-inflammatory bioactivity *in vitro*. Chondrocytes, synovial fibroblasts and macrophages from *Myd88*-deficient mice failed to respond to 32mer stimulation. A macrophage cell line derived from *Tlr2*-deficient mice also failed to respond to 32mer stimulation. Stimulation of human chondrocytes with human 32mer increased the expression of catabolic markers at the mRNA and protein level. Mouse and human chondrocytes stimulated NF- κ B activation in a TLR2-dependent reporter assay, and the response of chondrocytes from both species to native, glycosylated 32mer was similar to the response to synthetic peptides.

Conclusions

The aggrecan 32mer is a novel, endogenous ligand of TLR2 with the potential to accelerate cartilage destruction *in vivo*.

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Cartilage is a unique, avascular tissue comprised mostly of a collagen II-rich and aggrecan-rich extracellular matrix, with only a sparse population of resident chondrocytes. Aggrecan degradation by metalloproteinases is a property of all arthritides, but is a particular hallmark of osteoarthritis (OA). Similarly, collagen degradation by collagenases, primarily MMP-13 (1), is a critical feature of late-stage OA and is widely considered to mark an irreparable phase of cartilage damage. The aggrecanases and collagenases that degrade cartilage matrix are produced by chondrocytes, synovial fibroblasts (SF) and also infiltrating macrophages, the latter representing as much as 60% of the inflammatory infiltrate in OA (2).

Toll-like receptors (TLR) have central roles in the innate immune response to pathogens. Pathogen-derived ligand binding to TLRs activates the NF- κ B and MAP kinase pathways which drive production of inflammatory mediators such as IL-1 α , IL-6, and TNF α (3, 4). In addition, endogenous proteins, nucleic acids and carbohydrates released from injured cells or tissues are also TLR ligands, known as damage-associated molecular patterns (DAMPs) (5). In injured tissues, the DAMP-induced inflammatory response can promote tissue repair, however in disease, self-perpetuating cycles of tissue destruction can lead to episodes of severe, chronic inflammation.

The results from *in vivo* studies on TLRs and their ligands in the synovium of mice with inflammatory arthritis suggest a critical role for TLRs in the pathogenesis of rheumatoid arthritis (RA)-like disease (6-12). Fewer studies have examined TLRs and their ligands in cartilage and OA, however it is clear that human chondrocytes express TLRs 1-5 (13, 14), that there is differential regulation of TLR2 and TLR3 in normal and OA human cartilage (13) and that pro-arthritic agents can regulate TLR2 activation *in vitro* (14). The down-regulation of TLR2 expression in human OA is thought to confer protection against an inflammatory insult

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(13). Several fragmented components of the cartilage matrix, released into the synovial space, are recognised TLR ligands in cartilage (7, 15), including low molecular weight HA (16, 17), tenascin C (18, 19) and fibronectin (14, 20). Other matrix molecules such as the small leucine-rich proteoglycans decorin and biglycan (21-23) and the aggrecan-related proteoglycan versican (24) have been identified as TLR ligands in other cell and tissue types, and may also be active in joint tissues (reviewed in (25)). The calcium-binding proteins S100A8 and S100A9 were recently identified as DAMPs present at high concentration in OA joints (26). To date, there are no reports of TLR ligands derived from the major cartilage components, aggrecan or type II collagen.

Aggrecan is a large proteoglycan that, together with type II collagen, confers the weight-bearing properties of cartilage, where it is almost exclusively expressed. Several aggrecan fragments comprising the N-terminal globular domains accumulate in cartilage with age (27, 28). Following aggrecanolysis, large glycosaminoglycan-bearing fragments from the aggrecan C-terminus are separated from their anchor to HA, and lost from the cartilage matrix, whereas a range of fragments from the aggrecan N-terminus (29) are retained in the tissue by binding to HA via the first globular (G1) domain. G1-containing fragments produced by proteinase cleavage in the interglobular domain (IGD) include G1-EGE³⁷³ produced by ADAMTS cleavage at E³⁷³↓³⁷⁴A (30, 31) and G1-PEN³⁴¹ produced by MMP cleavage at N³⁴¹↓³⁴²F (32-34). The G1-EGE³⁷³ fragment is the substrate for 32mer production and the 32mer is the product of MMP activity.

Primary bovine chondrocytes cultured in the presence of exogenous G1-EGE³⁷³ internalise this G1 fragment via HA-mediated binding to CD44 (35). Thereafter, epitopes associated with the G1 domain localise in intracellular vesicles, whereas EGE³⁷³ neoepitopes are detected in the

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nucleus (35), independent of the G1 domain. This intriguing finding led us to hypothesise that a G1-deficient, EGE³⁷³-containing peptide(s), most likely the aggrecan 32mer, has a biological function *in vivo*. In the present study, we have identified the aggrecan 32mer fragment as a naturally-occurring TLR2 ligand in chondrocytes, synovial fibroblasts and macrophages.

MATERIALS AND METHODS**Reagents**

Synthetic mouse 32mer peptide (FFGVGGEDDITIQTVTWPDLELPLPRNVTEGE) and a scrambled peptide (LPTFGEVEVWLLGEDQDFDIPTTVGPRTGEIN) with the same amino acids but with no homology to any known protein were from Auspep, Parkville, Australia. The human 32mer peptide (FFGVGGEEEDITVQTVTWPDMELELPLPRNITEGE) was prepared with free N- and C-termini using Fmoc chemistry on a microwave-assisted Liberty automatic peptide synthesizer. The crude peptide was purified on a C8 reverse-phase column and the structure was confirmed by electrospray mass spectrometry and from amino acid analysis. Peptides in PBS were used at the concentrations shown. Native, glycosylated 32mer was isolated from pig articular cartilage as described previously (36). IL-1 α was from Peprotech, (NJ, USA). TK-Renilla-luciferase expressing plasmid, Reporter lysis buffer and reagent kit were from Promega corporation, Madison, USA. Eugene 6 was from Roche Diagnostic. Lipopolysaccharide (LPS) was from Invitrogen (San Diego, USA).

Primary cells, cell lines & human tissue

Procedures involving mice were approved by the Murdoch Childrens Research Institute Animal Ethics Committee. Mouse epiphyseal chondrocytes were prepared as described previously (37). Mouse synovial fibroblasts were prepared as outgrowth cultures from

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explanted mouse patellae harvested from knee joints at 11-22wks of age. The patellae were cultured in DMEM with 10% FCS and antibiotics for 3-4 weeks to allow the synovial fibroblasts to migrate away from the explants and form monolayers. Non-migratory chondrocytes were not detected in the cell monolayers. The synovial fibroblasts were expanded in culture for ~3 weeks, then 0.5×10^6 cells were seeded into 48 well plates prior to treatment.

Peritoneal macrophages were harvested at 11-22wk of age by flushing the peritoneum of culled mice. After gently agitating the abdomen, 8-10mls of ice cold PBS with 2% FBS was injected and peritoneal cells were collected and centrifuged at 1500rpm. Cells (10^6 cells/500 μ L) in RPMI with 10%FBS were incubated for 2 hours at 37°C in 48 well plates. Non-adherent contaminating cells were discarded and adherent macrophages cultured overnight at 37°C in fresh medium, prior to treatment. Immortalised macrophage cell lines derived from the bone marrow of wildtype or *Tlr2*-deficient mice were those described previously (38) and made available by Dr Eicke Latz, University of Bonn, Germany, via Dr Ashley Mansell, Monash Institute for Medical Research, Australia. The cells were cultured in DMEM with 10% FBS for 4-5 days before treating for 24 hours with peptides, IL-1 α or LPS.

Human femoral head cartilage with normal, healthy appearance was obtained from a 13 year old male patient undergoing joint replacement surgery for spondyloepiphyseal dysplasia at the Royal Children's Hospital, Melbourne, Australia. Chondrocytes prepared from fresh cartilage slices were cultured for 3 days prior to treatment for 24 hours.

Quantitative reverse transcription (RT-PCR) analysis of mRNA expression

mRNA expression was analysed by real-time quantitative PCR (qPCR) with probes and primers from the Universal Probes Library (UPL) (Roche, Basel, Switzerland). The $\Delta\Delta C_p$

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values, derived from the C_p values calculated by the Roche Light Cycler 480, were calculated for each gene and normalized against the geometric mean of two cell type-specific reference genes (39). The relative $\Delta\Delta C_p$ values for mouse chondrocytes were normalized against the geometric mean of the genes *Rpl10* and *Rpl26*, for synovial fibroblasts, against the geometric mean of *Mrps16* and *Atp5b* and in peritoneal macrophages against *Hprt1* and *Ppia*. The relative $\Delta\Delta C_p$ values for human chondrocytes were normalized against the geometric mean of the genes *RPL10* and *ATP5B*. The primer sequences used for the genes specified are shown in Table 1.

IL-6 ELISA, Western blotting and luciferase reporter assay

The concentration of mouse IL-6 in conditioned media was assayed by the mouse IL-6 AlphaLISA (Perkin Elmer, MA, USA). The range of the assay was 1.6-300,000 pg/mL. A luciferase reporter assay was as described previously (40). Sheep polyclonal antibodies against human MMP-3 and human MMP-13 were generous gifts from Prof Gillian Murphy and Dr Vera Knäuper as described previously (41).

RESULTS

To test our hypothesis that the 32mer was a bioactive aggrecan fragment, we treated primary mouse chondrocytes with mouse 32mer peptide for 24 hours in a dose-response experiment (1-300 μ M) and measured changes in mRNA expression of matrix proteins, metalloproteinases, signalling molecules, cytokines and chemokines by qPCR. We predicted that the 32mer would stimulate an anabolic response in chondrocytes, to redress the catabolic event(s) that had caused its release. Surprisingly, the results revealed a dose-dependent increase in the expression of pro-catabolic and pro-inflammatory genes including *Mmp13*, *Mmp12*, *Il6* and *Adamts5* in the presence of 1-30 μ M 32mer (**Figure 1A**). Other up-regulated genes detected by

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qPCR included *Inos*, *Mmp14*, *Mmp8* and *Ccl2* (data not shown). To confirm that the effects of the 32mer on mRNA expression were specific, we compared expression levels relative to treatment with a 32mer scrambled peptide. **Figure 1B** shows that the scrambled peptide had a negligible effect, or no effect, on mRNA expression, confirming that the response of mouse chondrocytes to mouse 32mer peptide was specific. Importantly, the level of up-regulated gene expression in IL-1 α -treated samples was similar to the level induced by the 32mer peptide (**Figure 1B**). Since native 32mer detected in human synovial fluid (42) or purified from pig articular cartilage(43) contains a substantial amount of keratan sulphate (KS) we also analysed mouse chondrocytes for their response to native, glycosylated pig 32mer and found that it also increased the expression of pro-catabolic and pro-inflammatory genes, including *Mmp13* and *Ccl2* (**Figure 1C**).

At higher concentrations of 32mer (100-300 μ M) we detected a dose-dependent down-regulation of genes encoding matrix-building proteins, including *col2a1*, *Matl1*, *Col11a1* and *Fmod* (**Figure 2A**). Other genes down-regulated by high concentrations of 32mer included *Agcn*, *Col9a1*, *Col10a1*, *Pcolce2*, *Dlk1* (data not shown). Again, to confirm that the 32mer effects on mRNA expression were specific, we compared expression levels relative to treatment with a 32mer scrambled peptide and found that there was little or no effect of the scrambled peptide on the expression of matrix building genes including *Col2a1*, *Matl1*, *Agcn* and *Fmod* (**Figure 2B**), and also that the level of down-regulated mRNA expression induced by the 32mer, was similar to that induced by IL-1 α (**Figure 2B**).

The similarity in the mRNA expression profiles and the extent to which individual genes were up- or down-regulated by 32mer treatment compared with IL-1 α , suggested that 32mer bioactivity might be mediated by a member of the IL-1 receptor family, all of which are

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dependent on the adaptor protein MyD88. We therefore treated chondrocytes, synovial fibroblasts and peritoneal macrophages harvested from wildtype or *Myd88*-deficient mice with mouse 32mer and scrambled peptide for 24 hours and measured the mRNA expression of *Mmp12*, *Mmp13* and *Adamts* in mouse chondrocytes (**Figure 3A**), *Mmp12*, *Mmp13* and *Il6* in mouse synovial fibroblasts (**Figure 3B**), and *Il α* , *Il10* and *Ccl2* (**Figure 4I**) by qPCR. IL-1 α or LPS stimulations were included as positive controls.

The results of these experiments confirmed that for wildtype chondrocytes and synovial fibroblasts mRNA expression of *Mmp12*, *Mmp13*, *Adamts5* and *Il6* was increased in response to 32mer and IL-1 α treatment, however the response of these cell types to the scrambled peptide was absent or blunted, as expected. Macrophages from wildtype mice also increased their mRNA expression of selected genes, including *Il1a*, *Il10* and *Ccl2* (**Figure 3C**) in response to treatment with the 32mer or LPS, but they had little or no response to the scrambled peptide (**Figure 3C**). In contrast to the wildtype cells, chondrocytes, synovial fibroblasts and macrophages from the *Myd88*-deficient mice failed to respond to the mouse 32mer peptide (**Figure 3**). The *Myd88*-deficient macrophages also failed to respond to LPS as expected (**Figure 3**). These results raised the possibility that the 32mer is a hitherto unrecognised, cartilage-specific DAMP derived from proteolysis of aggrecan.

Since DAMPs mediate their actions via Toll-like receptors, and since TLR2 activation has previously been linked with arthritis pathology, we compared macrophage cell lines derived from wildtype or *Tlr2*-deficient mice for their response to the 32mer peptide by measuring changes in mRNA expression of *Il6* and *Inos* by qPCR (**Figure 4A,C**) and levels of IL-6 protein by ELISA (**Figure 4B**). The results showed clearly that the *Tlr2*-deficient cell line failed to respond to the 32mer peptide at both the mRNA and the protein level, and suggesting therefore

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that the aggrecan 32mer is a ligand for TLR2. Failure of the *Tlr2*-deficient macrophage cell line to respond to the 32mer also suggests that the 32mer is unlikely to be a TLR4 ligand, since TLR4 is not disrupted in the TLR2-deficient cells.

We next tested whether the human 32mer peptide is active in human cartilage. The results of a dose-response experiment showed that human chondrocytes increased their expression of mRNA encoding *IL6* and *MMP13* (**Figure 5A**) in response to human 32mer. Furthermore the native, glycosylated 32mer fragment also stimulated the increased expression of *CCL2*, *IL6* (**Figure 6D**), *MMP3* and *MMP13* (**Figure 5B**) in human chondrocytes. Western blot analysis of conditioned medium harvested from the human chondrocyte cultures after stimulation with human 32mer revealed increased levels of active MMP3 and MMP13(**Figure 5C**) protein to levels that were greater than the untreated explants, but not as high as samples treated with IL-1 α .

Finally, we measured 32mer-induced NF- κ B activation in a TLR2-dependent NF- κ B luciferase reporter assay, using both mouse and human 32mer peptides to stimulate HEK293 cells transfected with a TLR2-expressing plasmid. As predicted, cells in the reporter assay failed to activate NF- κ B in response to the scrambled peptide, however both mouse and human 32mer, at 12.5 μ M, stimulated NF- κ B reporter activity (**Figure 5D**). This experiment showed that the human peptide was more active than the mouse peptide, however the hierarchy of stimulation between mouse and human peptides varied slightly between different experiments (*data not shown*).

*Bioactivity in an aggrecan 32mer fragment***DISCUSSION**

DAMPs and Toll-like receptors are considered important modulators of the disease process in inflammatory arthritis (reviewed in (6, 8)). Several models of inflammatory arthritis in mice involve TLR-mediated synovial activation (9, 10, 44) that is thought to drive the disease process, and based on the results of these studies, TLR antagonists are in development for the management of RA (6). However, interest in TLR activation by endogenous ligands in OA is relatively recent, and emerges from the new paradigm that OA is associated with chronic, low-levels of inflammation and synovitis (45-47). Our finding that the aggrecan 32mer is a TLR2 ligand that induces MMP and cytokine expression is consistent with other studies that have identified TLR2, 3 and 4-dependent increases in collagenase activity and cartilage resorption, as well as TLR4-dependent down regulation of aggrecan and collagen expression using traditional bacterial ligands such as LPS (13, 48). The most novel and important aspect of the present study is that the activating ligand that stimulates these same catabolic and inflammatory pathways (albeit to lower levels than for LPS) is an endogenous peptide fragment, derived directly, and possibly exclusively, from cartilage. Further work is required to determine whether the aggrecan 32mer might also signal through other TLRs, interact with other accessory proteins, for example soluble CD14 (49), and synergise with other catabolic agents, such as oncostatin M (13), to induce even greater levels of cartilage resorption.

A recent mouse study comparing the role of TLR2 in contrasting models of experimental OA showed that whereas *Tlr2*-deficiency exacerbated disease severity in the highly inflammatory collagenase-induced model of OA, the non-inflammatory, destabilisation of the medial meniscus (DMM model) showed no difference in disease severity between wildtype and *Tlr2*-deficient mice (50). These results, which suggest a protective role for TLR2 in collagenase-induced OA, seemed counter-intuitive at the time, however it now seems likely that the

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different outcomes reflect the different inflammatory load each model delivers to the affected joint. In the future it will be important to investigate activation of TLR2, and indeed other Toll-like receptors, in longer-term models of non-inflammatory and trauma-induced OA that more closely resemble the human situation.

In contrast to the aggressive inflammation that typifies RA, the hallmark of OA is radiographic joint space narrowing caused by proteinase-driven cartilage erosion in a relatively mild inflammatory environment. Cartilage erosion in OA can progress for many years as subclinical disease. Since type II collagen in mature human cartilage has a half-life of ~117 years (51), any significant damage to the collagen network is widely considered to be irreparable (52). Data from mouse models of arthritis have confirmed that cartilage can withstand only a limited degree of type II collagen degradation before the onset of irreversible damage (53). In contrast, large glycosaminoglycan-bearing aggrecan fragments lost from cartilage following normal or stimulated aggrecanolysis *in vivo*, are readily replaced by newly synthesised aggrecan (54). Because aggrecanases cleave at G1-EGE^{373,374}ARG, and because G1-EGE³⁷³ (32mer substrate) is retained in the cartilage matrix, repeated cycles of glycosaminoglycan loss coupled with new aggrecan synthesis will lead to an accumulation of G1-EGE³⁷³, and the rate of accumulation will increase proportional with the rate of aggrecanolysis. Accordingly, since the concentration of aggrecan (glycosaminoglycan) in normal cartilage is approximately 25μM, the micromolar concentrations of 32mer used in this study might be physiological under certain conditions. The half-life of aggrecan G1-domains in adult cartilage is ~24 years (27), nearly 7 times longer than for intact aggrecan (28).

Native 32mer present in human synovial fluids (42, 55) ranges in size from Mr 35,000-70,000, depending on the extent of KS substitution present on amino acids T³⁵², T³⁵⁷, N³⁶⁸ and T³⁷⁰;

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these residues are strictly conserved in mammalian aggrecan (56). KS in the aggrecan IGD of young pigs is uniquely under-sulphated compared with that in the KS-rich region of aggrecan (36), however KS in the IGD of other species has not been examined. The significance of the sulphation patterns on native 32mer, and its influence on ligand binding to TLR2, and indeed other potential binding partners in the OA joint is presently unclear. Further studies to examine the level and pattern of KS sulphation in the human 32mer, whether it changes with age (as for the KS-rich region in humans) (57) or disease, and the impact of different glycosylation profiles on TLR activation is warranted.

In summary, we have discovered that a naturally-occurring aggrecan fragment is an NF- κ B-activating, TLR2 ligand that stimulates pro-inflammatory and pro-catabolic activities. The aggrecan 32mer is an important addition to the list of joint-relevant and OA-relevant DAMPs, especially because it is renewable within the cartilage matrix and is derived from a precursor with a long tissue half-life. Based on its signalling via TLR2, and activation of NF- κ B *in vitro*, the aggrecan 32mer has a putative role in exacerbating inflammation in joint disease and potentially, in other aggrecan-expressing tissues such as nerves and brain. We speculate that MMP-13 inhibitors in development as collagen II-protective agents in OA may find additional utility in reducing joint inflammation and synovitis by blocking MMP-dependent 32mer production.

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Table 1. qPCR primers

Species	Gene	Primer	Sequence
Mouse	<i>Adamts5</i>	F	5'-atgcagccatcctgtttcac-3'
		R	5'-cattcccagggtgtcacat-3'
	<i>Mmp12</i>	F	5'-ttgtggataaacactactggaggt-3'
		R	5'-aatcagctggggtaagca-3'
	<i>Mmp13</i>	F	5'-tcaaggctatgcacactgggt-3'
		R	5'-cactatggtctttcaatgcctaa-3'
	<i>Mmp3</i>	F	5'-ttgttcttgatgcagtcagc-3'
		R	5'-gatttgcgcaaaagtgc-3'
	<i>Il6</i>	F	5'-tgatggatgctacaaactgg-3'
		R	5'-ttcatgtactccaggtagctatgg-3'
	<i>Inos</i>	F	5'-tgaactgagcgaggagca-3'
		R	5'-ttcatgataacgtttctggctct-3'
	<i>Ccl2</i>	F	5'-catccacgtgttggtca-3'
		R	5'-gatcatcttgctggtgaatgagt-3'
	<i>Col2a1</i>	F	5'-ctgccagtggaaaattaggg-3'
		R	5'-ggaggtcctctgggtcctat-3'
	<i>Col11a1</i>	F	5'-ccagcagaaactgatttcacag-3'
		R	5'-ttcaaccacagccggttc-3'
	<i>Matl1</i>	F	5'-tccctgatagcctcagtcctg-3'
		R	5'-tcgacaacaaacaccaggtc-3'
	<i>Fmod</i>	F	5'-cagggcaacaggatcaatg-3'
		R	5'-ctgcagcttgagaagttcat-3'
	<i>Agn</i>	F	5'-agtgcactgagggctttgtc-3'
		R	5'-gtaggtgtggggtctgtgc-3'
Human	<i>IL6</i>	F	5'-ctgaccaaccacaaatgc-3'
		R	5'-ctgcagccactggttctgt-3'

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Figure Legends

Figure 1. Increased mRNA expression of pro-inflammatory and pro-catabolic genes induced by 32mer treatment of primary mouse chondrocytes. A) qPCR was used to

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measure mRNA expression of *Mmp13*, *Il6*, *Mmp12* and *Adamts5* following stimulation of primary mouse chondrocytes with 1-30 μ M mouse 32mer peptide ($n = 3$ independent pools of cells assayed in duplicate). **B)** qPCR was also used to measure chondrocyte mRNA expression of *Mmp13*, *Inos*, *Mmp12* and *Adamts5* following stimulation with mouse 32mer peptide, scrambled peptide or 10ng/mL IL-1 α . The concentration of 32mer and scrambled peptide was 30 μ M ($n = 4$ independent pools of cells assayed in duplicate). **C)** mRNA expression of *Mmp13* and *Ccl2* in mouse chondrocytes stimulated with native, glycosylated 32mer was measured by qPCR. The results for each gene are representative of two or more separate experiments.

Figure 2. Decreased mRNA expression of matrix-building genes induced by 32mer treatment of primary mouse chondrocytes. **A)** qPCR was used to measure mRNA expression of *Col2a1*, *Matl1*, *Col11a1* and *Fmod* following stimulation of primary mouse chondrocytes with 30-300 μ M mouse 32mer peptide ($n = 2$ independent pools of cells assayed in duplicate). **B)** qPCR was also used to measure chondrocyte mRNA expression of *Col2a*, *Matl1*, *Agcn* and *Fmod* following stimulation with mouse 32mer peptide, scrambled peptide or 10ng/mL IL-1 α . The concentration of 32mer and scrambled peptide was 300 μ M ($n = 2$ independent pools of cells assayed in duplicate). The results for each gene are representative of two or more separate experiments.

Figure 3. Mouse 32mer activity is *Myd88*-dependent. qPCR was used to measure mRNA expression of **A)** *Mmp12*, *Mmp13* and *Adamts5* in mouse chondrocytes, **B)** *Mmp12*, *Mmp13* and *Il6* in mouse synovial fibroblasts and **C)** *Il1 α* , *Il10* and *Ccl2* (I) in mouse peritoneal macrophages derived from wildtype ($n = 1$ or 2 independent pools of cells assayed in duplicate) or *Myd88*-deficient ($n = 1-3$ independent pools of cells assayed in duplicate) mice, and treated

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with mouse 32mer peptide, scrambled peptide, IL-1 α or LPS. The results are representative of 2 separate experiments.

Figure 4. Mouse 32mer activity is TLR2-dependent. The levels of **A)** *Il6* mRNA expression, **B)** IL6 protein expression and **C)** *Inos* mRNA expression were measured in 3 separate cultures, assayed in duplicate, of macrophage cell lines derived from wildtype or TLR2-deficient mice, treated with mouse 32mer peptide, scrambled peptide, IL-1 α or LPS. The results are representative of 2 separate experiments.

Figure 5. Human 32mer is pro-catabolic in human chondrocyte cultures and activates NF- κ B via TLR2. **A)** qPCR was used to assess the effect of human 32mer and scrambled peptide on the expression of mRNA for human IL6 and human MMP13. **B)** qPCR was also used to analyse the effect of native, glycosylated 32mer on the mRNA expression of human CCL2, IL6, MMP3 and MMP13. **C)** Western blotting was used to assess the effects of human 32mer and scrambled peptide on MMP3 and MMP13 protein expression. **D)** The stimulatory effects of scrambled, mouse and human 32mer peptides were compared in a TLR2-dependent NF- κ B luciferase reporter assay, using the TLR2 agonist, Pam2Cys, as a positive control.

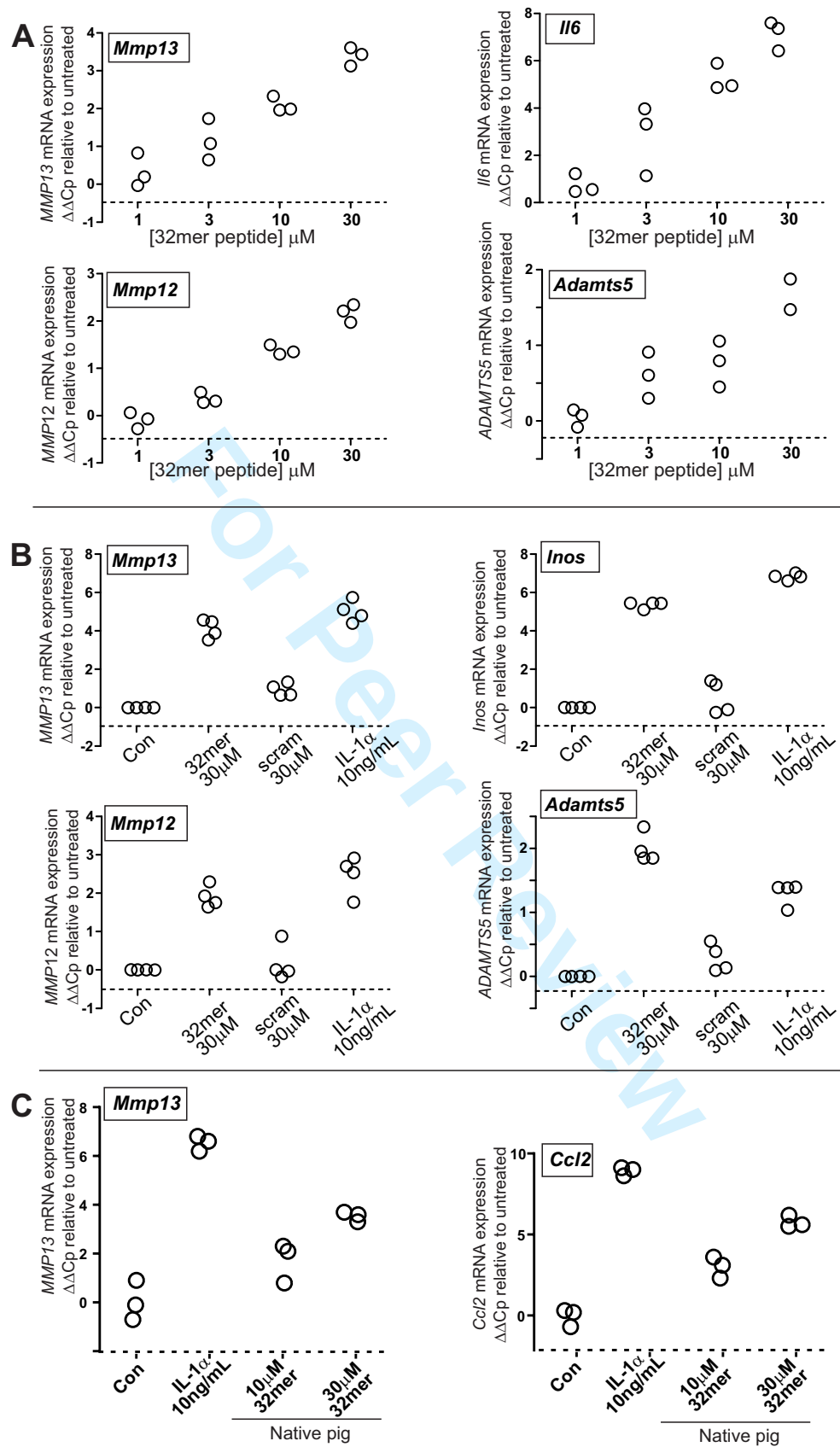


Figure 1

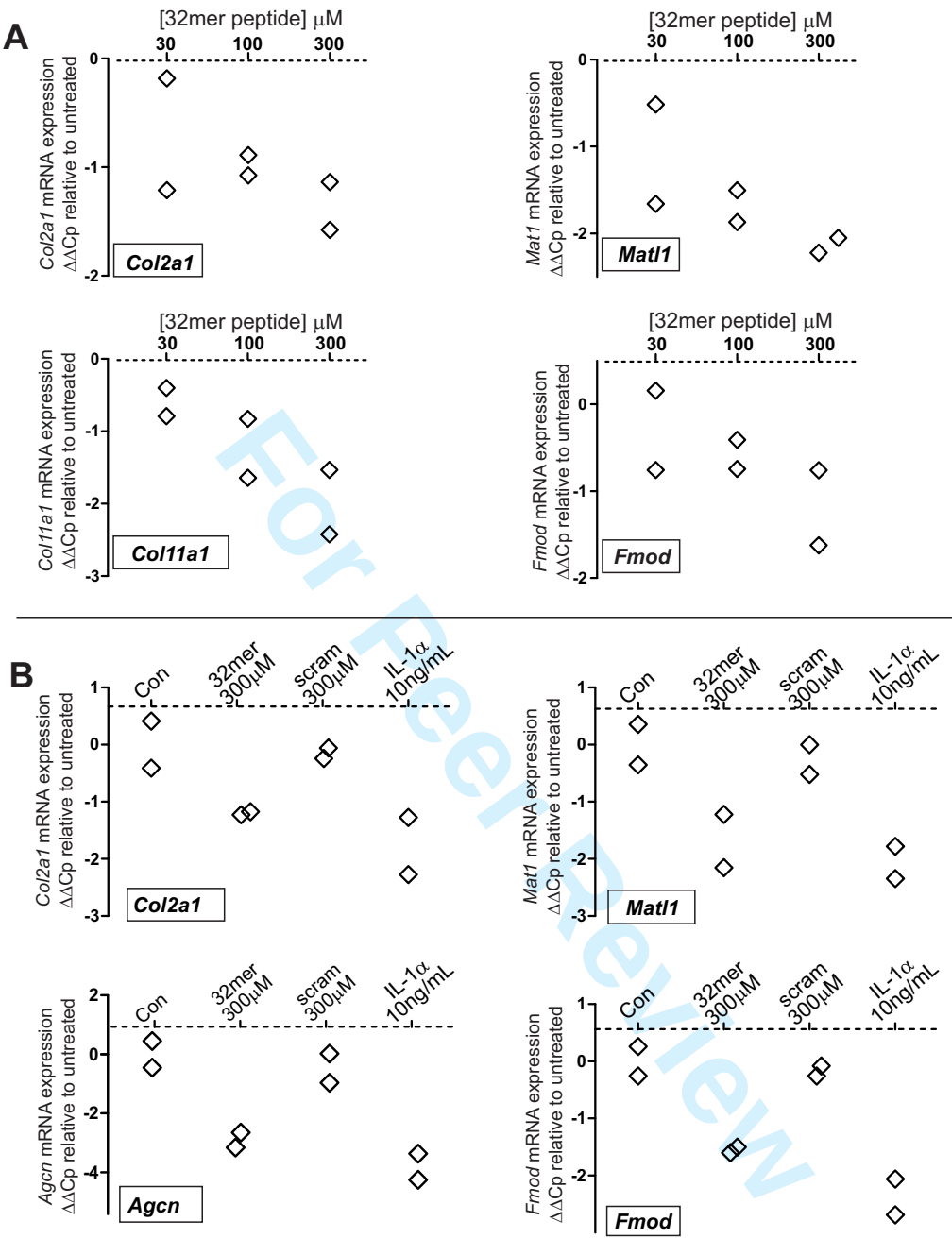


Figure 2

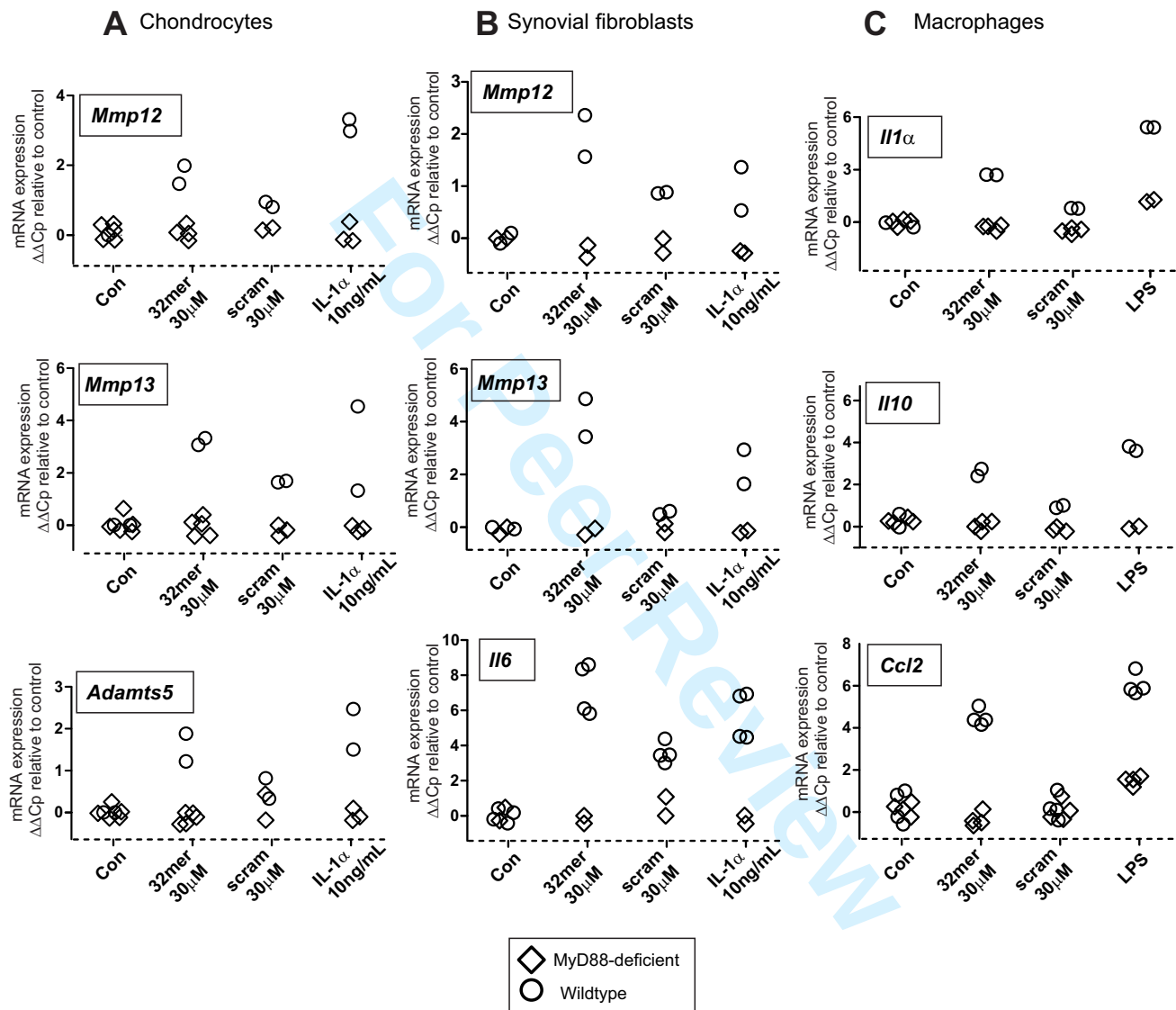


Figure 3

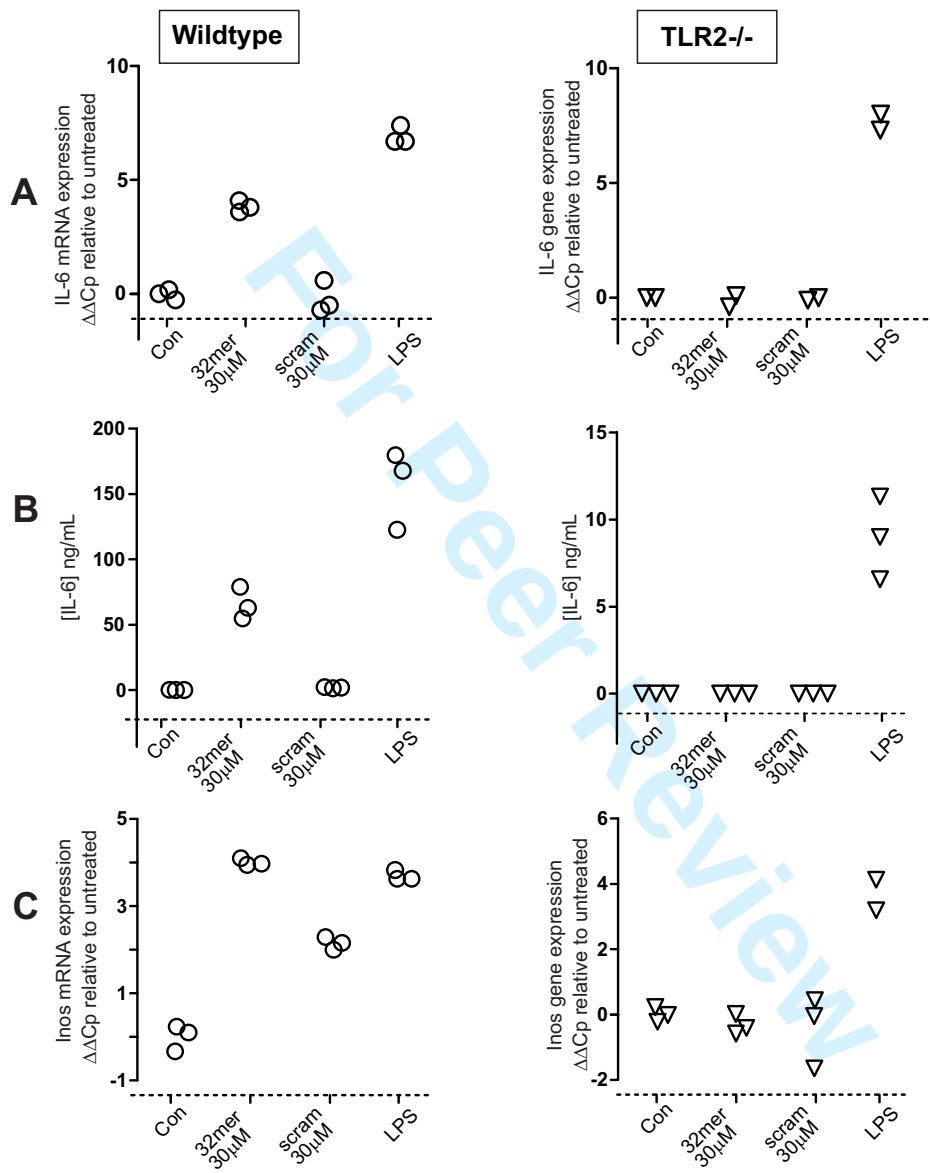


Figure 4

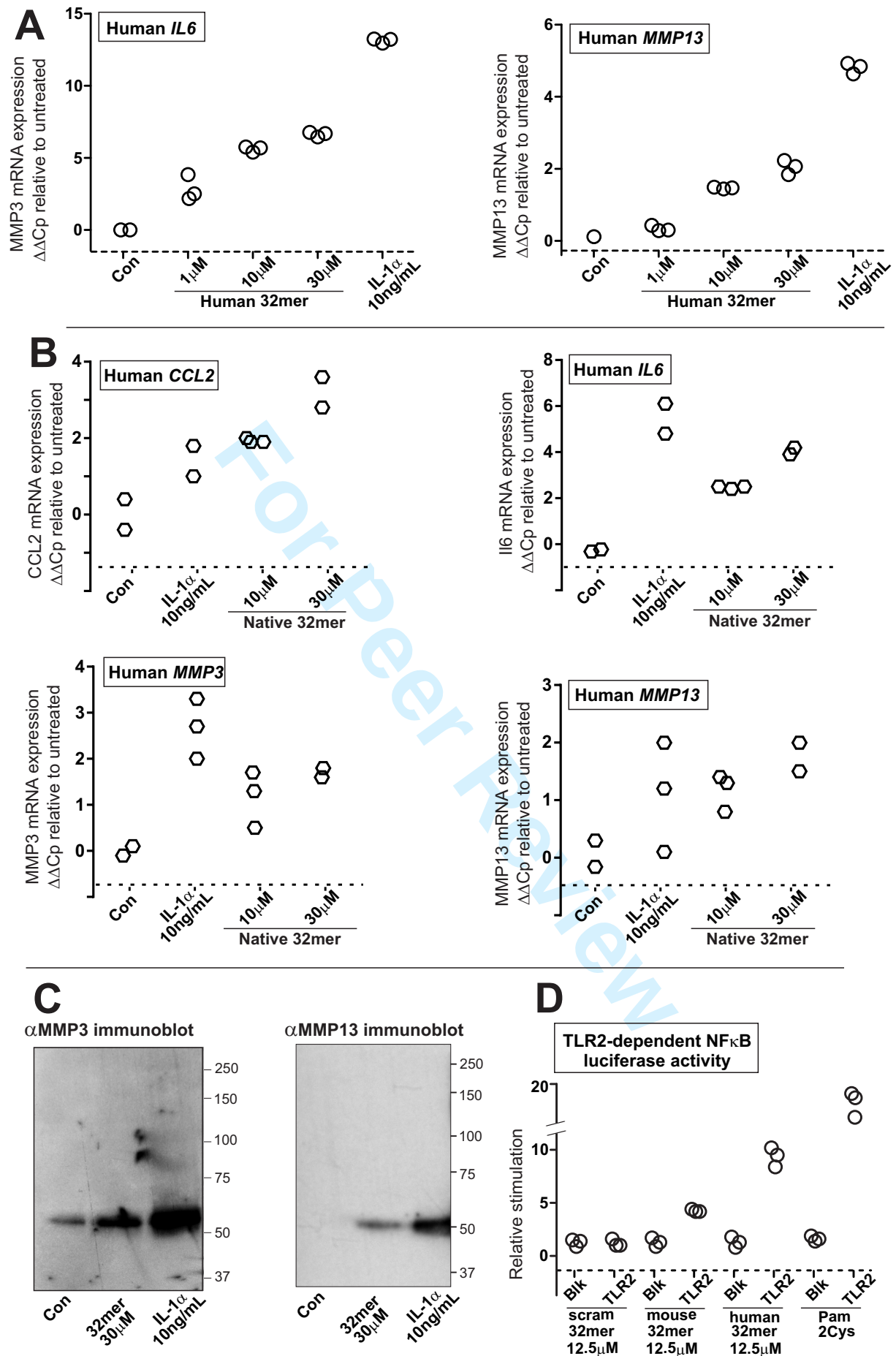


Figure 5