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C-type natriuretic peptide signalling drives homeostatic effects in human chondrocytes.

Peake N¹, Bader DL¹, Vessillier S², Ramachandran M³, Salter DM⁴, Hobbs AJ⁵ and Chowdhury TT^{1*}

¹Institute of Bioengineering, School of Engineering and Materials Science, Queen Mary University of London, Mile End Road, London E1 4NS, UK.

²National Institute for Biological Standards and Control, Biotherapeutics Group, South Mimms, Potters Bar, Hertfordshire EN6 3QG, UK.

³Department of Orthopaedics and Trauma, The Royal London Hospital and Barts & The London School of Medicine & Dentistry, Queen Mary University of London, Whitechapel Road, London E1 1BB, UK.

⁴Centre for Genomics and Experimental Medicine, MRC Institute of Genetics and Molecular Medicine, University of Edinburgh, Crew Road, Edinburgh EH4 2XU, UK.

⁵William Harvey Research Institute, Barts and The London School of Medicine and Dentistry, QMUL, Charterhouse Square, London EC1M 6BQ, UK.

Address for correspondence: Dr Tina Chowdhury, SEMS, QMUL, Mile End Road, London E1. 4NS, UK. Tel: +44(0)207 8827560. Email: t.t.chowdhury@qmul.ac.uk

Abstract

Signals induced by mechanical loading and C-type natriuretic peptide (CNP) represent chondroprotective routes that may potentially prevent osteoarthritis (OA). We examined whether CNP will reduce hyaluronan production and export via members of the multidrug resistance protein (MRP) and diminish pro-inflammatory effects in human chondrocytes. The presence of interleukin-1 β (IL-1 β) increased HA production and export via MRP5 that was reduced with CNP and/or loading. Treatment with IL-1 β conditioned medium increased production of catabolic mediators and the response was reduced with the hyaluronan inhibitor, Pep-1. The induction of pro-inflammatory cytokines by the conditioned medium was reduced by CNP and/or Pep-1, α CD44 or α TLR4 in a cytokine-dependent manner, suggesting that the CNP pathway is protective and should be exploited further.

Keywords: C-type natriuretic peptide, interleukin-1 β , chondrocyte, mechanical loading, osteoarthritis.

Introduction

The importance of the C-type natriuretic peptide (CNP) pathway is evident in regulating joint homeostasis [1-3]. In chondrocytes, CNP binds to natriuretic peptide receptor (Npr) 2 leading to synthesis of cyclic guanosine-3',5'-monophosphate (cGMP) and homeostasis. CNP also binds to Npr3 that degrades the peptide and reduces beneficial signalling via Npr2 [4]. CNP treatment of cell lines, monolayers or pellet culture increased chondrocyte differentiation, proliferation and matrix synthesis, mediated by Npr2/cGMP [5-9]. In the chondrocyte/agarose model, the protective effects of CNP were enhanced by mechanical loading [10-11]. Taken

together, the evidence indicates that the effects of CNP are mediated via Npr2 and its antagonism inhibits the protective actions of CNP in cartilage.

We previously reported that CNP or mechanical loading upregulates intracellular and extracellular levels of cGMP [11], but the way in which it is exported in chondrocytes is unclear. In fibroblasts, the multidrug resistance protein 5 (MRP5) was reported to export both HA and cGMP [12-13]. However, agents designed to inhibit HA export by increasing cGMP levels prevent proteoglycan and collagen loss in OA affected cartilage explants [14-15]. Since we reported that cGMP levels were also influenced by CNP, IL-1 β and mechanical loading [11], the action of cGMP on preventing HA export could potentially be chondroprotective and might reduce the downstream actions of HA signalling and its breakdown. However, HA fragmentation is influenced by mechanical loading or the presence of hyaluronidases or free radicals typically found in the OA joint [16-18]. It was reported that the HA binding receptors, CD44 or Toll-Like Receptor 4 (TLR4) promotes inflammatory effects induced by hyaluronidase treatment [19-24]. We hypothesised that induction of cGMP by CNP and mechanical loading will reduce HA production and export that prevents the catabolic effects induced by HA fragmentation. These impacts may alter both quantity and size distribution of HA, and play an important role in balancing the protective and inflammatory effects mediated by CNP and HA fragmentation.

Materials and Methods

Cell isolation and 3D agarose culture: Human cartilage was obtained from sixteen patients with ethical approval (East London and The City Research Ethics Committee) and informed patient consent, undergoing total knee arthroplasty at the Royal London Hospital, Barts Health NHS Trust, London, UK. Cartilage was

removed from the femoral condyles and tibial plateaux with experiments repeated with cells from 3-4 donors. Chondrocytes were isolated from tissue explants and resuspended in DMEM + 20% FCS at a cell concentration of 8×10^6 cells/ml using well-established protocols [10-11]. The cell suspension was added to an equal volume of molten 6% (wt/vol) agarose type VII in Earle Balanced Salt Solutions (EBSS) to yield a final cell concentration of 4×10^6 cells/ml in 3% (wt/vol) agarose (Sigma-Aldrich, Poole, UK). The chondrocyte/agarose suspension was allowed to gel at 4°C for 20 min in a sterile stainless steel mould, containing holes 5 mm in diameter and 5 mm in height. Constructs were equilibrated in culture in DMEM + 10% FCS at 37°C in 5% CO₂ for 24 hours (Sigma-Aldrich, Poole, UK).

Effect of agents that interfere with the MRP/Npr pathway: The effect of IL-1 β and CNP on HA production was investigated in free-swelling culture. Equilibrated constructs were cultured with 0 or 10ng/mL IL-1 β and/or 100nM CNP or 1 μ M MK571 (inhibits MRP5), 0.5 μ M cyclic gly-24-ser (P19, selective Npr2 antagonist, Gentaur Molecular Products, Whetsone, UK) or 1 μ M c-Atrial Natriuretic Factor (cANF⁴⁻²³, selective Npr3 agonist, Bachem AG, Bubendorf, Switzerland) [11]. In separate experiments, constructs were subjected to 15% dynamic compression at 1Hz frequency (10min every 6 hours) using well-established protocols [10-11] for 48 hours. Media was supplemented with 0 or 10ng/ml IL-1 β and/or 100 nM CNP and/or 0.5 μ M P19 and/or 1 μ M cANF⁴⁻²³. Controls were unstrained in the bioreactor and cultured for the same time period.

The effect of conditioned media and agents that influence the HA pathway: The role of HA and its fragments on promoting inflammatory effects was investigated with

conditioned medium, and/or specific pharmacological agents that inhibit downstream effects on two HA receptors, CD44 and TLR4. Constructs were cultured with 0 or 10 ng/ml IL-1 β and/or 100nM CNP for 48 hours. 300 μ L of the supernatant was used as conditioned medium to treat patient-matched constructs for the following conditions:

- Untreated conditioned media (UT^{CM})
- CNP conditioned media (CNP^{CM})
- IL-1 β conditioned media (IL-1 β ^{CM})
- IL-1 β + CNP conditioned media (IL-1 β + CNP^{CM})

Conditioned medium was supplemented with HA binding peptide Pep-1 (30 μ M, AnaSpec), which blocks binding of small HA fragments and prevents downstream signalling [25-26]. For analysis of the HA binding mechanism, conditioned media was treated with specific antibodies that blocked HA binding to CD44 (clone Bu52, AbDSerotec, Kidlington, UK) or TLR4 (anti-CD284 clone HTA125, AbDSerotec, Kidlington, UK). Both antibodies were used at 2 μ g/mL and were shown to block activity [26-27]. Constructs were cultured with conditioned media with P19 and cANF for a further 48 hours.

Gene expression analysis: Total RNA was isolated with the QIAquick Spin gel extraction and RNeasy kits (Qiagen, West Sussex, UK), reverse transcribed (200 ng) with Enhanced Avian RT First Strand cDNA synthesis kit (Sigma Genosys, Cambridge, UK) and real-time PCR reactions performed on the Mx3000P quantitative PCR instrument (Stratagene, Amsterdam, The Netherlands) using well established protocols [28]. The following specific primer sequences were used:

MRP2 sense: 5'CGTTGTTGCCATCTTAGG-3', antisense: 5'-CAAACATCATTGCTGGGTAA-3'; MRP4 sense: 5'-GCAGTTCTAATCATTCTC-3',

antisense: 5'-AAATCTCCTTCTTTCTCA-3'; MRP5 sense: 5'-CTTGTCTCCTGGAAGATGTT-3', antisense: 5'-GAAGATGTCATTCACTAGC-3' (Sigma Genosys, Cambridge, UK). The real-time PCR efficiencies (E) of amplification for each target were defined according to the relation, $E = 10^{[-1/\text{slope}]}$, and revealed efficiency values ranging from 1.94 to 2.03 for optimal primer pairs concentrations (0.3 μM) derived from standard curves ($n = 3$). The C_t values for GAPDH remained stable, with no changes detected under all culture conditions, suggesting its suitability as a reference gene. Relative quantification of MRPs were normalized to the target ΔC_t and reference GAPDH ΔC_t , and to the calibrator sample by a comparative C_t approach, as described [28].

Biochemical analysis: NO, PGE₂ and HA production were determined in supernatant by Griess, EIA (GE Healthcare, Buckinghamshire, UK) or ELISA (R&D Systems, UK), using well established methods [10-11]. Total MMP activity was measured with fluorogenic MMP substrate at excitation and emission values of 340 and 440nm, respectively (Enzo Life Sciences, Exeter, UK). GAG synthesis was measured by DMMB assay and normalised to DNA values using Hoescht 33258. Cytokines were measured with Human Th1/Th2 10-plex tissue culture MSD plates according to manufacturer's instructions (Meso Scale Discovery, Rockville, USA).

Statistics: Statistical analysis was performed by a two-way analysis of variance (ANOVA) and the multiple post hoc Bonferroni-corrected t-tests to compare differences between the various treatment groups as indicated in the figure legend. For gene-expression data, ratio values were log transformed before analysis by a

two-way ANOVA and a *post hoc* Bonferroni-corrected *t* test. In all cases, a level of 5% was considered statistically significant ($P < 0.05$).

Results

MRP transporters are regulated by IL-1 β and CNP: MRP5 gene expression was reduced with CNP when compared to untreated controls ($p < 0.05$, Fig. 1A). IL-1 β enhanced MRP5 ($p < 0.001$) but was reduced with CNP ($p < 0.01$). In the absence of IL-1 β , the pattern of MRPs2 and MRP4 gene expression was similar to MRP5, with a significant downregulation with CNP when compared to untreated controls (both $p < 0.001$). In the presence of IL-1 β , MRP2 and MRP4 expression was increased (both $p < 0.05$) but reduced with CNP (Fig. 1B and C).

IL-1 β increased HA and the response was inhibited by CNP and/or dynamic compression: MRP5 mediates export of cGMP, but is also a HA exporter, an activity inhibited by cGMP. We therefore considered whether the production of cGMP in response to CNP could inhibit the export of HA (Fig. 2). In the presence of IL-1 β , HA production was significantly increased when compared to untreated controls and the response was reduced with CNP or the MRP inhibitor MK571 (all $p < 0.001$; Fig. 2A). The ability of CNP to block the cytokine-induced production of HA was diminished in constructs cultured with the Npr2 antagonist P19, resulting in a two-fold increase in HA when compared to constructs treated with IL-1 β alone ($p < 0.001$). In the absence of CNP, the Npr3 agonist cANF⁴⁻²³ abolished HA production in cytokine treated constructs, ($p < 0.001$).

We examined whether dynamic compression could influence HA production in constructs treated with IL-1 β . In the absence of the cytokine, dynamic compression did not influence HA production. In unstrained constructs, the cytokine enhanced HA production and the response was reduced with CNP or dynamic compression (both $p < 0.001$; Fig. 2B). HA production was reduced further with CNP and/or compression ($p < 0.05$), with the magnitude of inhibition similar to constructs treated with the Npr3 agonist cANF⁴⁻²³. In contrast, the Npr2 antagonist P19 blocked CNP-induced inhibition of HA production resulting in high levels of HA in constructs subjected to dynamic compression ($p < 0.001$).

Treatment of constructs with IL-1 β ^{CM} produced catabolic factors mediated by

HA: In order to assess whether HA production and its fragments in response to IL-1 β are catabolic, we generated conditioned medium from constructs treated in the presence or absence of IL-1 β and/or CNP, and used the conditioned medium to stimulate patient matched control constructs. By using this approach, we observed upregulation of NO and PGE₂ release and MMP activity with constructs cultured with IL-1 β ^{CM} when compared to UT^{CM} (all $p < 0.001$, Fig. 3A, B and C, respectively). The presence of CNP^{CM} marginally downregulated IL-1 β -stimulated NO generation ($p < 0.01$; Fig. 3A) and MMP activity ($p < 0.05$; Fig. 3C) but did not significantly influence PGE₂ production.

We investigated whether the induction of catabolic mediators by IL-1 β ^{CM} could be due to HA and its fragments by using Pep-1, which binds to HA fragments or by targeting CD44 and TLR4 receptor activation with blocking antibodies. The inclusion of the HA binding peptide to constructs cultured with IL-1 β ^{CM} significantly inhibited NO and PGE₂ release when compared to constructs treated with IL-1 β ^{CM} alone (both

$p < 0.001$), implicating that Pep-1 prevents the catabolic action of HA (Fig. 3A and B). The presence of monoclonal antibodies that blocked activation of CD44 or TLR4 marginally reduced NO release (both $p < 0.05$) but not PGE_2 in $\text{IL-1}\beta^{\text{CM}}$ when compared to treatment with the $\text{IL-1}\beta^{\text{CM}}$ alone (Fig. 3A and B).

MMP activity was enhanced with $\text{IL-1}\beta^{\text{CM}}$ but reduced with CNP^{CM} ($p < 0.05$) or Pep-1 ($p < 0.01$; Fig. 3C). Anti-CD44 or anti-TLR4 inhibited MMP activity similar to controls (both $p < 0.001$; Fig. 3C). GAG synthesis was upregulated with CNP^{CM} when compared to UT^{CM} ($p < 0.001$; Fig. 3D). $\text{IL-1}\beta^{\text{CM}}$ reduced GAG synthesis ($p < 0.05$) and was marginally reversed with constructs treated with $\text{IL-1}\beta + \text{CNP}^{\text{CM}}$ ($p < 0.05$) or Pep-1 ($p < 0.01$). Anti-CD44 and anti-TLR4 marginally reversed GAG synthesis when compared to constructs treated with $\text{IL-1}\beta^{\text{CM}}$ (both $p < 0.05$; Fig. 3D).

Pro-inflammatory cytokine production was reduced by HA inhibitor: Analysis of cytokine production by multiplex assay demonstrated up-regulation of $\text{TNF}\alpha$, $\text{IL-1}\beta$, IL-8 , IL-10 , IL-13 and $\text{IFN}\lambda$ in $\text{IL-1}\beta$ -treated conditioned media when compared to untreated controls (Fig. 4 and Table 1). The presence of Pep-1 significantly reduced $\text{TNF}\alpha$ and $\text{IL-1}\beta$ production (both $p < 0.001$) and the response to the $\text{IL-1}\beta^{\text{CM}}$ was influenced with blocking antibodies that prevented receptor activation of CD44 and TLR4 (Fig. 4). We observed increases for IL-10 , IL-13 and $\text{IFN-}\gamma$ levels in constructs stimulated with $\text{IL-1}\beta^{\text{CM}}$ (all $p < 0.001$, Table 1) but not $\text{IL-1}\alpha$, IL-2 , IL-5 and IL-12 p70 (data not shown). In addition, the upregulation of IL-8 , IL-10 , and $\text{IFN-}\gamma$ by the presence of the $\text{IL-1}\beta^{\text{CM}}$ was marginally influenced by the interventions tested, suggesting that this upregulation may be due to soluble factors related to multiple pathways involving HA and other intracellular signalling mechanisms (Table 1).

Discussion

It is well established that HA fragments will contribute to the pathological process by activating catabolic events involving cytokines, MMPs, aggrecanases leading to chondrolysis, apoptosis and tissue breakdown [19-20, 29]. However, high molecular weight HA was reported to be exported through MRP5 and exerts a protective effect via CD44 activation, thereby offering protection in response to the inflammatory environment [22, 30-33]. In contrast, HA oligosaccharides found in diseased cartilage or synovial joint tissues will promote catabolic signalling via CD44 or TLR4 [22-24, 30, 32-35]. Since we reported homeostatic effects of the CNP/Npr2/cGMP pathway, we hypothesised that cGMP could inhibit HA export function of MRP, thereby preventing downstream inflammatory effects induced by HA fragmentation.

The present study confirmed induction of MRP gene expression and HA production in response to IL-1 β that was diminished with CNP. Our data is consistent with previous studies in chondrocytes and synoviocytes cultured in alginate beads or monolayer, which also showed an upregulation of HA production and fragmentation in response to IL-1 α , IL-1 β or TNF α [36-38]. HA oligosaccharides stimulate HA synthase 2 (HAS-2), CD44 and aggrecan gene expression leading to proteoglycan loss [15, 22]. In the present study, blockade of HA export with the MRP5 inhibitor MK571 diminished HA production in response to IL-1 β and correlates with previous studies in human chondrocytes or bovine cartilage explants [14-15]. Interventions designed to mimic the actions of cGMP or interfere with MRP5 export or block HA breakdown with inhibitors that affect the action of hyaluronidases was shown to reduce proteoglycan and collagen degradation and MMPs in cartilage explants treated with IL-1 β [16, 17-18]. HA export was inhibited with ODQ which increased intracellular levels of cGMP implying that specific HA export inhibitors could be used

to attenuate matrix loss. Since a number of transport inhibitors are used clinically, the efficacy of the HA exporters should be explored to potentially treat matrix disorders. In addition, the production of HA in response to IL-1 β was reduced with CNP and/or mechanical loading or the presence of agents that induce CNP signalling (cANF⁴⁻²³). There is growing evidence that enhanced signalling via the CNP/Npr2/cGMP route will induce homeostatic effects in chondrocytes [5-11]. In contrast, the Npr2 antagonist P19 had the opposite effect and enhanced HA production, confirming that blockade of the CNP/Npr2 signalling system has an impact on HA export. The data also complements our previous observations demonstrating a protective role for physiological mechanical loading in maintaining cartilage homeostasis. In concert with the present findings, the application of cyclic tensile strain to synovial fibroblasts influenced the expression and activity of HA production and hyaluronidase enzymes [40], emphasising the critical role of mechanical loading in maintaining cartilage homeostasis.

To test whether chemical agents that interfered with HA binding (Pep-1) or inhibit HA receptor activation (α CD44 or α TLR4), we generated IL-1 β ^{CM} in anticipation the inflammatory environment promotes the generation of HA oligosaccharides that could be blocked by the interventions examined. Culturing constructs with IL-1 β ^{CM} increased production of NO, PGE₂ and MMPs that was associated with an inhibition of GAG synthesis. We also observed increased production of pro-inflammatory (TNF- α , IL-1 β , IL-8), anti-inflammatory (IL-10) and immunoregulatory cytokines (IFN- γ) that was reduced with CNP^{CM}, implying protective effects of CNP against cytokine induction. Few studies have examined the effects of CNP on cytokine and immunoregulatory function in chondrocytes.

The HA binding peptide, Pep-1 diminished cytokine production in response to constructs treated with IL-1 β ^{CM} and this reduction was greater in comparison to agents that prevent CD44 and TLR4 receptor activation. Since we did not examine the combined effect of the two blocking agents in our conditioned media model, it is possible that low molecular weight HA will induce signalling events through both receptors. For example, binding of small HA oligosaccharides to CD44 and TLR4 results in NF- κ B activation, and cytokine production involving TNF α , IL-1 β , IL-6 and IL-18 [22-23]. Further studies are needed to explore the interactions of the CNP/cGMP pathway on HA signalling in chondrocytes.

The cytokine-treated conditioned media model provides an inflammatory environment that could be influenced with interventions that blocked HA binding or activation of the HA receptors. The present study suggest that CNP will module the pro-inflammatory actions of cytokines and protective effects of the Npr2/cGMP signalling pathway should be exploited further.

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Figure Legends

Fig. 1. Effect of IL-1 β and/or CNP on MRP gene expression. Constructs were cultured with 0 or 10 ng/ml IL-1 β and/or 100 nM CNP for 6 hours. Error bars represent the mean and SEM values of 6 replicates from two separate experiments. (*, ** or ***) indicates significant comparisons for the multiple treatment conditions.

Fig. 2. Effect of free-swelling culture and/or dynamic compression on HA production. Constructs were cultured with 0 or 10 ng/ml IL-1 β and/or 100 nM CNP or 0.5 μ M MK571 or 0.5 μ M P19 or 1 μ M cANF⁴⁻²³ for 48 hours (A). In separate experiments, constructs were subjected to dynamic compression (15%, 1Hz) for 48 hours under similar treatments (B). Error bars represent the mean and SEM values of between 12 and 18 replicates from six separate experiments. (*, ** or ***) indicates significant comparisons for the multiple treatment conditions.

Fig. 3. Effect of conditioned media and HA pharmacological agents on catabolic activities. Constructs were cultured with conditioned media for IL-1 β (IL-1^{CM}), CNP (CNP^{CM}) or both (IL-1 β + CNP^{CM}) and treated with inhibitors for HA signalling (Pep-1, α CD44 or α TLR4 blocking antibodies) for a further 48 hours, on NO release (A), PGE2 release (B), Total MMP activity (C) and GAG synthesis (D). Error bars represent the mean and SEM values of between 8 and 10 replicates from 3-4 separate experiments. (*, ** or ***) indicates significant comparisons for the multiple treatment conditions.

Fig. 4. Effect of conditioned media and HA pharmacological agents on pro-inflammatory cytokine production. Constructs were cultured with conditioned media for IL-1 β (IL-1^{CM}), CNP (CNP^{CM}) or both (IL-1 β + CNP^{CM}) and treated with inhibitors for HA signalling (Pep-1, α CD44 or α TLR4 blocking antibodies) for a further 48 hours, on TNF α (A) and IL-1 β production (B) Error bars represent the mean and SEM values of 10 replicates from 4 separate experiments. (*, ** or ***) indicates significant comparisons for the multiple treatment conditions.

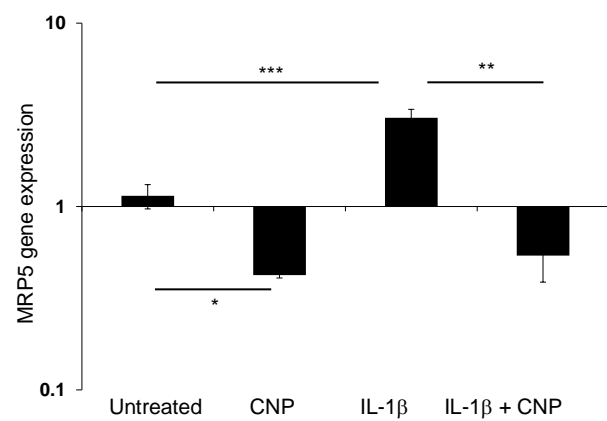
Table 1: The effect of HA inhibitors on cytokine production in constructs cultured with IL-1 β and/or CNP conditioned media

	UT vs CNP ^{CM}	UT vs IL- 1 β ^{CM}	IL-1 β ^{CM} vs IL-1 β + CNP ^{CM}	IL-1 β ^{CM} vs IL-1 β ^{CM} + Pep-1	IL-1 β ^{CM} vs IL-1 β ^{CM} + α CD44	IL-1 β ^{CM} vs IL-1 β ^{CM} + α TLR4
TNFα	5.9 (\pm 9.5)	187.1 (\pm 23.9)	-48.5 (\pm 6.3)	-63.8 (\pm 6.1)	-36.7 (\pm 7.4)	-35.4 (\pm 7.0)
IL-1β	8.3 (\pm 13.5)	5362.9 (\pm 508.2)	-16.6 (\pm 22.6)	-59.4 (\pm 28.5)	-10.2 (\pm 8.4)	-11.0 (\pm 8.2)
IL-8	12.4 (\pm 10.6)	95.9 (\pm 6.0)	-4.5 (\pm 4.5)	8.2 (\pm 4.2)	10.6 (\pm 5.4)	-10.8 (\pm 3.9)
IL-10	32.6 (\pm 16.0)	737.8 (\pm 79.0)	-53.7 (\pm 13.0)	-25.1 (\pm 23.6)	-7.9 (\pm 27.4)	-9.9 (\pm 25.4)
IL-13	-3.1 (\pm 1.4)	39.2 (\pm 2.8)	-14.5 (\pm 4.9)	-10.6 (\pm 6.1)	-17.2 (\pm 2.6)	3.7 (\pm 6.2)
IFN-γ	17.3 (\pm 2.6)	237.3 (\pm 24.3)	-2.1 (\pm 8.5)	-1.4 (\pm 5.6)	-4.3 (\pm 6.1)	7.5 (\pm 4.8)

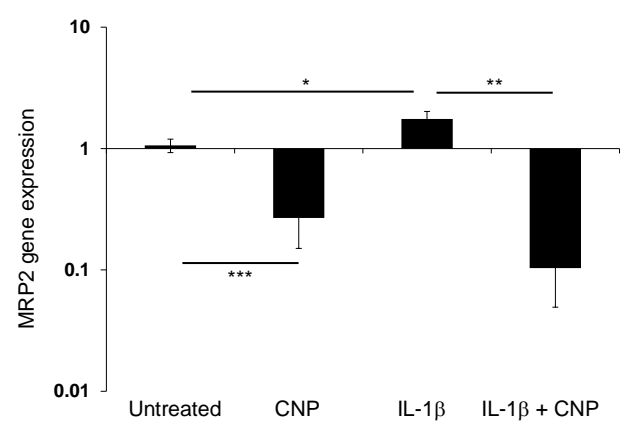
Note: Constructs were cultured with conditioned media for IL-1 β (IL-1^{CM}), CNP (CNP^{CM}) or both (IL-1 β + CNP^{CM}) and treated with agents that blocked HA binding (Pep-1) or HA receptor activation (α CD44 or α TLR4) for a further 48 hours. The production of pro-inflammatory (TNF α , IL-1 β and IL-8), anti-inflammatory (IL-10, IL-13) and immunoregulatory (IFN- λ) cytokines were examined. All values are expressed as a percentage difference (%) for the comparisons stated, where \pm SEM was calculated for n=8-10 constructs from 3-4 separate experiments.

Figure 1

A



B



C

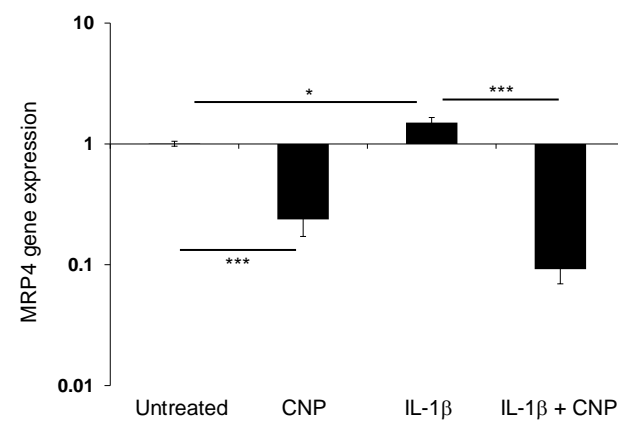
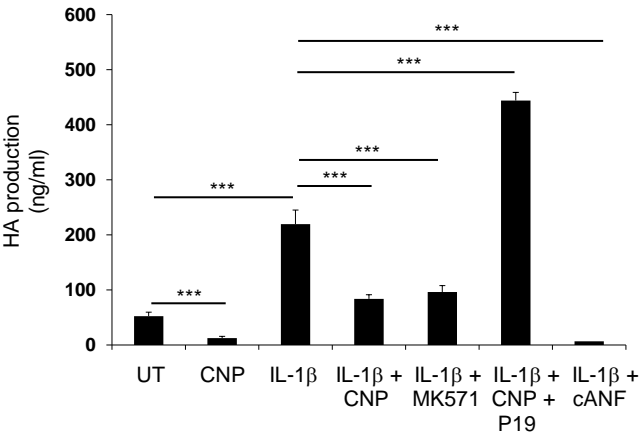


Figure 2

A



B

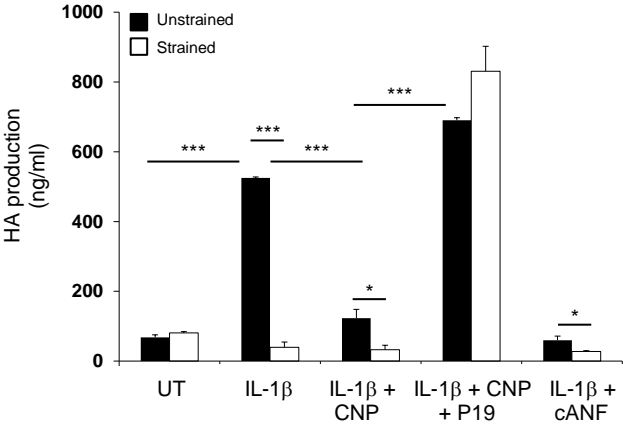


Figure 3

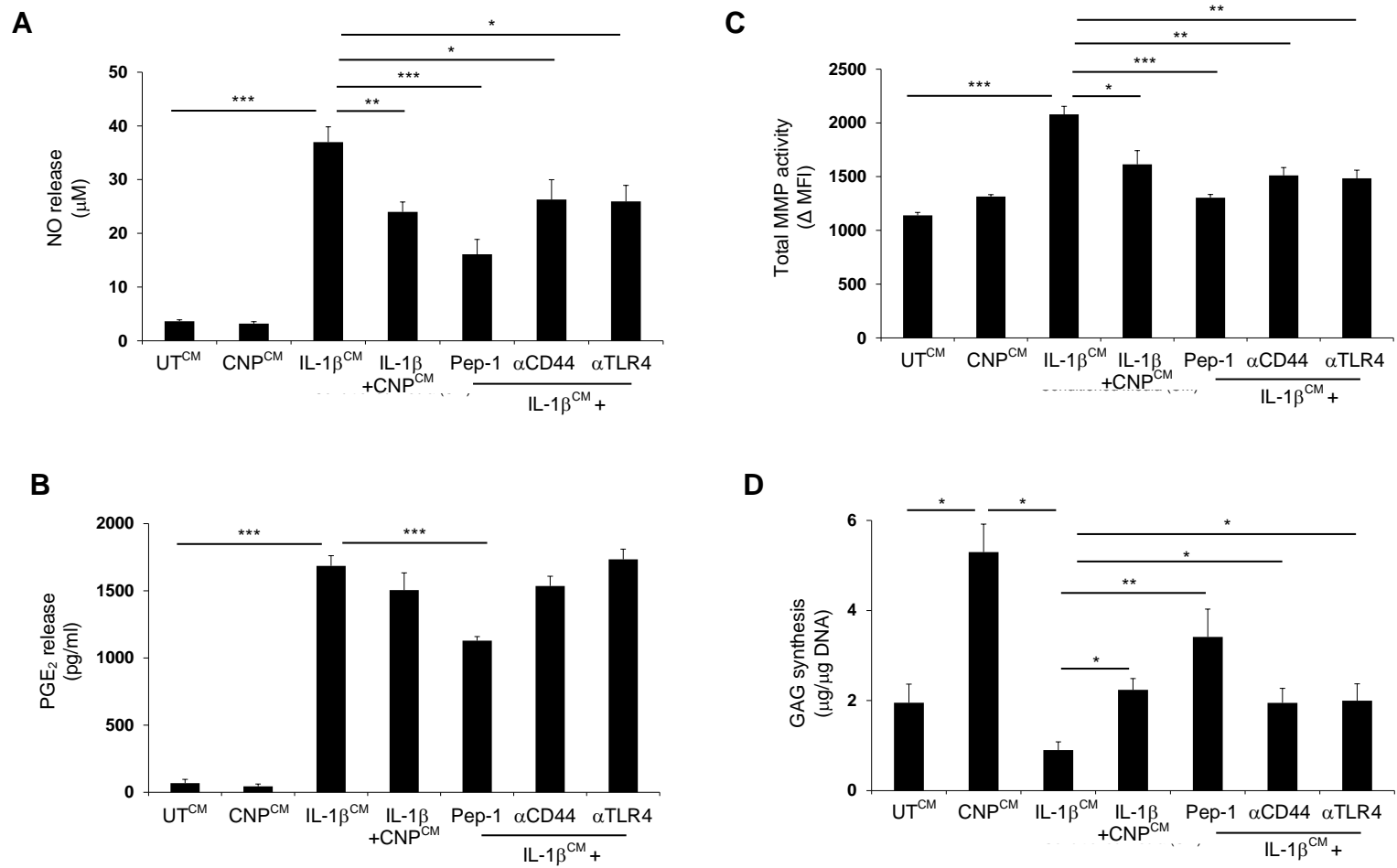
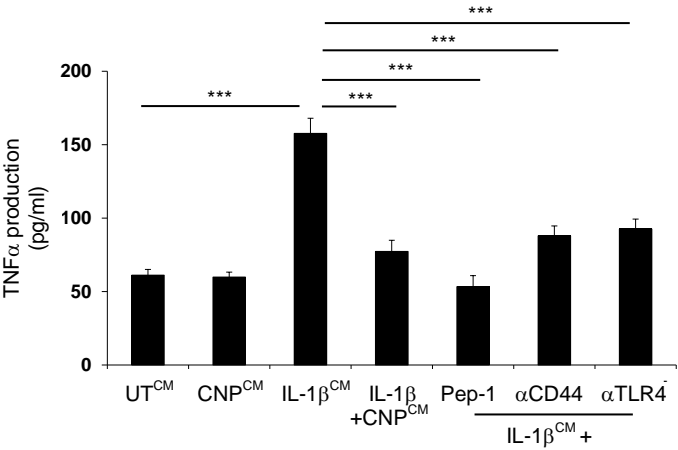


Figure 4

A



B

