

Inhibiting IL-1 signaling pathways to inhibit catabolic processes in disc degeneration

DANIELS, Jodie, BINCH, Abbie A. L. and LE MAITRE, Christine L.
<<http://orcid.org/0000-0003-4489-7107>>

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1 **Title: Inhibiting IL-1 signalling pathways to inhibit catabolic processes in disc**
2 **degeneration.**

3 **Jodie Daniels¹, Abbie LA Binch¹, Christine L Le Maitre¹**

4 1: Biomolecular Sciences Research Centre, Sheffield Hallam University, Sheffield, UK, S1
5 1WB.

6 **Corresponding Author:** Dr Christine Le Maitre, Biomolecular Sciences Research Centre,
7 Sheffield Hallam University, Howard Street, Sheffield, South Yorkshire, S1 1WB, UK.

8 E-mail: C.LeMaitre@shu.ac.uk

9 Telephone: 0114 225 6163

10 Fax: 0114 225 3064

11 **Running title:** Intracellular signalling in IVD degeneration.

12 **Author contributions:** JD & AB performed all laboratory work, and initial data analysis,
13 contributed to study design and helped draft the manuscript. CLM conceived the study,
14 participated in its design and coordination, secured funding and assisted in data and
15 statistical analysis, and co-wrote the manuscript. All authors read and approved the final
16 manuscript.

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18 The funding source had no involvement in study design, collection, analysis or interpretation
19 of data, writing of the manuscript or in the decision to submit the manuscript for publication.

20 **CONFLICT OF INTERESTS**

21 The author(s) declare that they have no conflicts of interests.

22

23 **KEY WORDS:**

24 Intervertebral disc, Interleukin 1, intracellular signalling, GDF-5, NFκB.

25

1 **Abstract:**

2 Intervertebral disc degeneration is characterised by an imbalance between catabolic and
3 anabolic signalling, with an increase in catabolic cytokines particularly IL-1 β , a key regulator
4 of IVD degeneration. This study aimed to investigate intracellular signalling pathways
5 activated by IL-1 β , and GDF-5 in the degenerate IVD to identify potential new therapeutic
6 targets.

7 Human NP cells were cultured in alginate beads to regain in vivo phenotype prior to
8 stimulation with IL-1 β or GDF-5 for 30 minutes, a proteasome profiler array was initially
9 utilised to screen activation status of 46 signalling proteins. Immunofluorescence was used
10 to investigate activation of the NF κ B pathway. Cell based ELISAs were then deployed to
11 confirm results for ERK1/2, p38 MAPK, c-jun and I κ B signalling. IHC was utilised to
12 investigate native activation status within human IVD tissue between grades of
13 degeneration. Finally cells were stimulated with IL-1 β in the absence or presence of p38
14 MAPK, c-jun, JNK and NF κ B inhibitors to investigate effects on MMP3, MMP13, IL-1 β , IL-6
15 and IL-8 mRNA expression.

16 This study demonstrated three key signalling pathways which were differentially activated by
17 IL-1 β but not GDF-5; namely p38 MAPK, c-jun and NF κ B. Whilst ERK 1/2 was activated by
18 both GDF-5 and IL-1. Immunohistochemistry demonstrated p38 MAPK, c-jun and NF κ B
19 were activated during human IVD degeneration and inhibition of these pathways reduced or
20 abrogated the catabolic effects of IL-1 β , with inhibition of NF κ B signalling demonstrating
21 most widespread inhibition of IL-1 β catabolic effects.

22

1 **Introduction:**

2 The intervertebral disc (IVD) is an important component of the spinal column enabling
3 bending, flexion and torsion of the spine, composed of the cartilaginous end plates (CEP),
4 the annulus fibrosus (AF) and the central gelatinous nucleus pulposus (NP). The major
5 components are water, collagens, proteoglycans, non-collagenous proteins and elastins.
6 The collagen fibres of the IVD provide a strong durable framework supporting the cells and
7 confine the highly hydrated proteoglycan aggregates¹. The normal human IVD contains
8 chondrocyte-like cells within the NP and inner AF termed NP and AF cells, and fibroblast-like
9 cells in the outer AF. These cells are responsible for the synthesis and regulation of the
10 matrix in the disc and control the homeostasis between extracellular matrix (ECM) synthesis
11 and ECM degradation². Within the normal adult IVD the anabolic and catabolic processes
12 which regulate the ECM are thought to be kept in balance by a number of growth factors
13 (e.g. TGF, FGF, IGF, GDF) and cytokines (e.g. IL-1, IL-6, IL-8, TNF α)³⁻⁵. If this balance is
14 disrupted in favour of catabolic processes, the IVD begins to degrade, resulting in disc
15 degeneration which can lead to low back pain (LBP)³. An improved understanding of the
16 homeostatic mechanisms and those seen during degeneration of the disc are crucial to
17 understanding the biology of this important tissue.

18 Increasing data suggests that inflammatory cytokines produced by NP cells within the IVD
19 during degeneration are responsible for matrix degradation and induction of painful stimuli⁶⁻
20 ¹², with a particular role for IL-1 β hypothesized^{6, 7, 9-11}. IL-1 β has been shown to be increased
21 in degenerate IVDs without a concordant increase in its antagonist (IL-1Ra)⁶. IL-1 β has also
22 been shown to regulate a pleathora of events linked to IVD degeneration such as increased
23 matrix degradation, decreased matrix synthesis, increased pro inflammatory cytokine
24 production and increased production of neurotrophic and angiogenic factors⁶⁻¹⁰.
25 Spontaneous degeneration was seen in a mouse model where the natural inhibitor of IL-1 β
26 (IL-1Ra) was knocked out¹¹, which together with increased risks of LBP in patients with IL-1 β
27 polymorphisms¹³⁻¹⁶, suggests a major role for IL-1 β in the pathogenesis of disc degeneration.

1 A number of methods to inhibit the catabolic processes (e.g. matrix degradation via TIMPs)
2 have been proposed, however whilst these have shown some promise, many target
3 individual catabolic cytokines (e.g. IL-1, TNF), which carries a major shortcoming as many
4 cytokines and other catabolic factors (e.g. NO, PGEs) are produced within a degenerate disc
5 and other cytokines may replace the degenerate effects if one such as IL-1 β is removed^{3, 5}.

6 One alternative would be to inhibit the intracellular signalling mechanisms common to
7 catabolic processes, however many of these mechanisms are also deployed by the anabolic
8 factors including growth factors within the disc. Anabolic factors have been proposed as
9 alternative therapeutic strategies to adjust the balance towards matrix synthesis and of these
10 GDF-5/6 (CDMP-1/2) appear to be an attractive growth factor¹⁷⁻²⁰ as its receptors are
11 expressed by NP cells even in degenerate discs however unlike the majority of the growth
12 factors which have been investigated are not expressed by the blood vessels which are
13 found in degenerate discs²¹. It is important to target anabolic factors which do not have off
14 target effects, such as potential detrimental angiogenesis and accompanied nerve ingrowth
15 which could result from the use of growth factors whose receptors are also expressed by the
16 blood vessels in the IVD.

17 Thus identification of intracellular signalling pathways activated by catabolic factors but not
18 anabolic factors is essential to identify potential targets to inhibit degeneration without
19 inhibiting anabolic processes. Recently p38 MAPK (Mitogen activated protein kinase)^{22, 23}
20 and NF κ B (Nuclear factor kappa beta)²⁴⁻²⁷ have been identified within IVD cells, as signal
21 transduction pathways following IL-1 β stimulation and inhibition of these pathways have
22 shown partial inhibition of the catabolic mediators induced in response to IL-1 β ^{22, 23}. However
23 importantly these studies were investigated using IVD cells cultured in monolayer culture
24 which do not represent the phenotype of NP cells in vivo, indeed very limited studies have
25 investigated signalling molecule activation in chondrocyte like cells in a 3D culture system
26 which more closely mimics the in vivo phenotype.

1 This study aimed to identify intracellular signalling pathways differentially expressed between
2 catabolic (IL-1 β) and anabolic (GDF-5) factors in human IVDs and determined the activation
3 status of these pathways in native IVD tissues highlighting potential pathways for new
4 therapies.

5 **Materials and Methods:**

6 **Human IVD samples:**

7 Human IVD tissue was obtained from surgery for micro discectomy or post mortem
8 examination (processed within 72 hours after death) with informed consent of the patient or
9 relatives. Ethical approval was obtained from Sheffield Research Ethics Committee
10 (09/H1308/70).

11 **Histological Grading:**

12 Representative tissue samples were fixed in 10% v/v neutral buffered formalin (Leica, Milton
13 Keynes, UK) and processed to paraffin wax. Haematoxylin and eosin stained sections were
14 evaluated independently by two researchers (AB & CLM) to determine the extent of
15 degenerative tissue changes. Sections were scored numerically between 0 and 12 based on
16 the presence of cell clusters, fissures, loss of demarcation and haematoxophilia; a score of 0
17 to 3 indicates histologically non-degenerate IVDs; ≥ 4 indicates evidence of degeneration, as
18 described previously⁶.

19 **NP cell isolation and culture:**

20 Surgical NP tissue samples were finely minced and digested with 2U/ml protease (Sigma,
21 Poole, UK) in DMEM (Gibco, Paisley, UK) for 30 minutes at 37°C, followed by 2mg/mL
22 collagenase type I (Sigma) in DMEM for 4 hours at 37°C. Cells were maintained in DMEM
23 supplemented with 10% heat inactivated foetal bovine serum (Gibco), 200U/mL
24 penicillin/200 μ g/mL streptomycin (Gibco), 500ng/mL amphotericin B (Sigma), 2mM L-
25 glutamine (Gibco) and 50 μ g/mL ascorbic acid (Sigma) (complete media). NP cultures were

1 used for experimental purposes upto and including passage 2, note no NP samples which
2 displayed histological evidence of infiltration were used for culture experiments, all cultures
3 were completed with NP cells from individual patients no pooling of patient samples was
4 performed. Cultures were maintained at all times at 37°C and 5% CO₂ in a humidified
5 environment.

6 **Alginate Culture and stimulation regimes**

7 Following expansion in monolayer, NP cells were re-suspended in 1.2%w/v medium
8 viscosity alginic acid (Sigma) in 0.15M sodium chloride (Fisher Scientific, Loughborough,
9 UK) at a density of 4.0 x 10⁶ cells/ml (which mimics native NP cell density). Alginate beads
10 were formed via extrusion through a 19 gauge needle into 200mM CaCl₂ (Fisher Scientific),
11 washed in 0.15M NaCl and media and maintained in complete media for 14 days prior to IL-
12 1β or GDF-5 stimulation with culture media changed twice weekly. To investigate signalling
13 molecule activation all treatments were performed with either 10ng/ml recombinant IL-1β
14 (Peprotech, London, UK), 10ng/ml recombinant GDF-5 (Peprotech) or left untreated as
15 controls. Stimulations were performed for 30 minutes prior to downstream analysis (30
16 minutes was selected from a preliminary time course experiment where 10,20,30,60 and 180
17 mins were investigated for NFκB activation (data not shown for time course)). Following
18 stimulation alginate beads were dissolved in alginate dissolving buffer (55mM C₆H₆Na₂O₇
19 (Sigma); 30mM EDTA (Fisher); 0.15M NaCl; pH 6.0) for 10 minutes at 37°C to release cells
20 for downstream analysis with arrays, immunofluorescence or in cell ELISAs.

21 **R&D Proteome Array for intracellular signalling molecules:**

22 NP samples derived from 2 patients (Grade 6, age 21 and 36) were used for this component
23 of the study (Table S-1). Forty alginate beads were used for each treatment in duplicate.
24 Following treatment and release from beads, cells were lysed with cell lysis solution
25 containing protease inhibitors, and protein quantified, activation of 46 signalling molecules

1 investigated using the Human Phospho-Kinase Proteome profiler array as per manufactures
2 instructions (Cat: ARY003; R&D systems, Abingdon, UK).

3 **NFκB Immunocytochemistry**

4 NP samples derived from 3 patients (Age 29,32,36; Grade 6,7,10) were used for this study
5 (Table S-1) were treated for 30 mins with 10ng/ml IL-1β, 10ng/ml GDF-5 or left untreated as
6 controls. Following treatment and release from beads cell pellets were fixed in 4% w/v
7 paraformaldehyde/PBS and cytopins formed spinning 300,000 cells per slide via 1500rpm
8 30 min cytopsin (Shandon cytopsin). Cells were washed in tris buffered saline (TBS) for 5
9 minutes. Non-specific binding sites blocked with 25% v/v goat serum (Abcam) in 1% w/v
10 BSA in TBS for 1 hour at room temperature. Cells were incubated with rabbit polyclonal
11 antibody against p65 NFκB (1/50) (Abcam) overnight at 4°C. Cells were washed 3 times for
12 5 minutes each in 0.1% v/v Tween 20/TBS for 5 minutes, cells were then incubated with
13 FITC conjugated goat anti-rabbit IgG (1/250) (Abcam) at room temperature for 30 minutes.
14 Cells were washed 3x5 minutes in 0.1% v/v Tween 20/TBS, mounted in 90% v/v
15 glycerol/TBS and coverslips sealed with nail varnish. Cells were visualised and images
16 captured on the Zeiss laser scanning confocal microscope at a magnification of 630X
17 magnification using the Zen 2009 operating system.

18 **Cell Based ELISAs: ERK1/2, p38 MAPK, IκB, c-Jun:**

19 To confirm results from R&D proteome array on additional patients on key signalling
20 molecules identified to be upregulated by IL-1β cell based ELISAs were utilised to
21 investigate quantitative expression of the signalling molecules identified from the array (ERK
22 1/2, p38 MAPK, c-jun) together with investigating activation of the NFκB signalling pathway
23 which is not included in the array, but demonstrated to be potentially differentially activated
24 via immunofluorescence. This was investigated by determining levels of phosphorylated IκB
25 which is the first stage in the activation of this signalling pathway. The cell based ELISAs
26 enable quantification of total and phosphorylated proteins in cells in situ without the need to

1 perform protein extraction. A further 3 patients were utilised for this component of the study
2 (Age 29,32,36; Grade 6,7,10) (Table S-1), Alginate beads were transferred 1 bead per well
3 of four black 96 well microplates (R&D Systems) in complete media (separate plates were
4 used for detection of each signalling molecule). Following stimulations beads were
5 solubilised in alginate dissolving buffer and plates centrifuged for 10 mins 400g to deposit
6 cells on the base of the culture wells, and fixed in 4% v/v formalin/PBS. The amount of
7 phosphorylated ERK1/2, p38 MAPK, c-Jun or IκB was measured using cell based ELISA as
8 per manufactures instructions (R&D systems, UK). The plate was read fluorometrically using
9 the Tecan Infinite 200 Pro with excitation at 540 nm and emission at 600 nm, then with
10 excitation at 360 nm and emission at 450 nm. Expression of phosphorylated protein was
11 then assessed between treatment groups.

12 **Immunohistochemical identification of activation status in vivo**

13 Immunohistochemistry (IHC) was utilised to identify expression levels of phosphorylated-p38
14 MAPK, phosphorylated-c-jun and phosphorylated-NFκB in native NP cells from different
15 grades of degeneration to investigate activation status in vivo and whether this altered during
16 degeneration. IHC was utilised rather than western blotting to ensure expression was
17 quantified only within the native NP cells not any infiltrating cells, in addition IHC has the
18 advantage that multiple patient samples can be investigated. Expression of each
19 phosphorylated signalling molecule was investigated in 30 IVD tissue samples (Age:
20 45.5 ± 14.4) (Table S-1), divided between three study groups: non-degenerate; histologically
21 graded as intermediate degeneration and histologically graded as severe degeneration.
22 Briefly, 4µm paraffin sections were dewaxed, rehydrated and endogenous peroxidase
23 blocked using hydrogen peroxide. After washing in TBS, sections were subjected to heat
24 induced antigen retrieval (10 minute microwave irradiation in 0.05M tris buffer, pH 9.5).
25 Following TBS washing, non-specific binding sites were blocked at room temperature (RT)
26 for 2hrs with 10%v/v normal goat serum in 1%w/v BSA/TBS. Sections were incubated
27 overnight at 4°C with rabbit polyclonal primary antibodies against human phosphorylated -

1 p38 MAPK (1:800;Abcam:ab4822), phosphorylated-c-jun (1:400;Abcam:ab32385) and
2 phosphorylated-NFκB (1:100;Abcam:ab31481). Negative controls in which rabbit IgGs
3 (Abcam) replaced the primary antibody at an equal protein concentration were used.
4 Following TBS washes, sections were incubated in biotinylated goat anti-rabbit antiserum
5 (1:300;Abcam) for 30 minutes at RT. Disclosure of secondary antibody binding was by HRP-
6 streptavidin-biotin complex (Vector Laboratories, Peterborough, UK) with 0.08% v/v
7 hydrogen peroxide in 0.65 mg/mL 3,3'-diaminobenzidine tetrahydrochloride (Sigma) in TBS.
8 Sections were counterstained with Mayer's Haematoxylin (Leica), dehydrated, cleared and
9 mounted in Pertex (Leica).

10 All slides were visualised using an Olympus BX60 microscope and images captured using a
11 digital camera and software program QCapture Pro v8.0 (MediaCybernetics, Marlow, UK).
12 Evaluation of IHC was performed by counting 200 NP cells, with immunopositive cells
13 expressed as a percentage of total count.

14 **Stimulation of NP cells in alginate with IL-1β in presence of signalling inhibitors:**

15 NP samples derived from 3 patients (Age 29,32,36; Grade 6,7,10) (Table S-1), 2 alginate
16 beads per well were treated with DMSO at equivalent concentrations as those used for
17 signalling inhibitors or with signalling inhibitors (p38MAPK inhibitor: 40μM SB203580²⁸
18 (Sigma, Poole, UK); c-jun inhibitor: 800μM c-jun peptide²⁹ (Tocris cat: 1989); JNK inhibitor:
19 20μm SP600125³⁰ (Sigma); or NFκB inhibitor: 10μM Helenalin³¹ (ENZO, Exeter, UK)) for 1
20 hr prior to stimulation with 10ng/ml IL-1β for 48hrs or left unstimulated to act as signalling
21 inhibitor only controls. All treatments were performed in triplicate on each patient
22 independently.

23 **RNA extraction, cDNA synthesis and Real time PCR:**

24 Following stimulation NP cells were recovered from alginate culture and re-suspended in
25 0.06%w/v type I collagenase and incubated for 10 minutes at 37°C and cells recovered by

1 centrifugation. RNA was extracted using Qiagen RNeasy Mini kit (Qiagen, Crawley, UK) as
2 per manufacturers' protocol. cDNA was reverse transcribed using Moloney Murine
3 Leukaemia Virus reverse transcriptase (Bioline) and random hexamers (Applied Biosystems,
4 Warrington, UK). cDNA samples were interrogated by qRT-PCR for the expression of
5 MMP3, MMP13, IL-1 β , IL-6, IL-8 and aggrecan (Pre-designed primer/probe mixes Applied
6 Biosystems). Plates were ran on a StepOnePlus real-time PCR machine for 50 cycles
7 (Applied Biosystems). Relative gene expression levels were calculated using the $2^{-\Delta\Delta CT}$
8 method and normalised against two internal reference genes (GAPDH and 18S) and un-
9 stimulated control samples. Stable expression of internal reference genes was confirmed by
10 geNorm algorithm.

11 **Statistical Analysis**

12 All data from this study was shown to be non-parametric and as such statistical analysis for
13 assays was performed using the Kruskal-Wallis test with Conover-Ingman post hoc tests
14 used to investigate significant differences between treatment groups or grades of
15 degeneration. All Statistical analysis was performed using statistical software: Stats-Direct.

16 **Results:**

17 **Identifying differential signalling pathways between anabolic and catabolic factors:**

18 Proteome profiler arrays were deployed to enable screening of the phosphorylation status of
19 46 intracellular signalling proteins following stimulation of human NP cells with either IL-1 β or
20 GDF-5 for 30 minutes which was shown from preliminary experiments to be the optimum
21 time frame. Significant increases in phosphorylated forms of 3 signalling proteins were seen
22 in cells following stimulation with IL-1 β (c-jun, ERK1/2 and p38)(Figure 1, Table 1) and 2
23 following stimulation with GDF-5 (STAT 1 and 4)($P < 0.05$)(Table 1). Interestingly IL-1 β
24 stimulation resulted in the significant down regulation of 12 signalling proteins: including
25 STATs (STAT 3, 5A/B and 6); src family members (src, YES, Fyn, Hck, Lck); and other
26 signalling proteins involved in focal adhesions (FAK, Paxillin)(Table 1). In addition decreased

1 activation of MEK1/2 and mTOR was observed ($P < 0.05$)(Table 1). Whilst GDF-5 only
2 significantly inhibited 1 signalling protein (Lck) ($P < 0.05$)(Table 1). Of particular interest
3 pERK1/2 was increased following GDF-5 (6 fold) and IL-1 β (30 fold) stimulation although
4 only significantly so following IL-1 β ($P < 0.05$) (Table 1, Figure 1), whilst FAK was down
5 regulated by both GDF-5 and IL-1 β although again only significantly so following IL-1 β
6 stimulation ($P < 0.05$) (Table 1, Figure 1). Surprisingly from the 46 signalling proteins
7 investigated only three were shown to be differentially activated by IL-1 β but not GDF-5,
8 these were p38 MAPK, JNK and its downstream target c-jun ($P < 0.05$) (Table 1, Figure 1).
9 Caution should be taken when utilising these results as they were completed on 2 patients
10 each in quadruplet, thus the biological significance cannot be concluded from this
11 component of the study. Hence this component of the study was utilised to identify potential
12 signalling pathways for further investigation in additional patient samples.

13 As the proteome profiler array did not include NF κ B signalling this was investigated initially
14 using immunofluorescence using a phosphorylated antibody which would only detect
15 activated NF κ B. NP cells cultured in alginate to maintain NP cell phenotype were stimulated
16 with IL-1 β or GDF-5. Cells which were unstimulated or stimulated with GDF-5 showed
17 punctate staining only, whilst cells stimulated with IL-1 β showed increased staining across
18 the cell, including nuclear staining, suggesting activation of the NF κ B pathway was more
19 pronounced with IL-1 β stimulation (Figure 2).

20 Cell based ELISAs were then utilised to confirm the results from profiler and
21 immunofluorescence on further patient samples. Phosphorylated ERK 1/2 was significantly
22 increased following GDF-5 and IL-1 β stimulation although induction was significantly more
23 following IL-1 β stimulation ($P < 0.05$)(Figure 3A). GDF-5 stimulation had no stimulatory effect
24 on the phosphorylation status of p38 MAPK, c-jun or I κ B, although a significant decrease
25 was seen in phosphorylated c-jun ($P < 0.05$)(Figure 3), however IL-1 β stimulation induced

1 phosphorylation of p38 MAPK, c-jun and I κ B, which was significant for p38 MAPK and c-jun
2 (P<0.05)(Figure 3B,C,D).

3 **Activation status of IL-1 β induced intracellular signalling pathways in vivo.**

4 To determine whether IL-1 β induced signalling pathways: p38 MAPK, c-jun and NF κ B are
5 active within human IVDs and whether these are differentially activated within degenerate
6 discs, human IVDs from differing grades of histological degeneration were investigated for
7 IHC staining with phosphor specific antibodies. Cytoplasmic staining for phospho-p38
8 MAPK, c-jun and NF κ B were identified in NP cells, particularly those cells in clusters (Figure
9 4). Percentage immunopositive cells for phosphorylated p38 MAPK and phosphorylated
10 NF κ B were significantly increased in discs with intermediate histological degeneration
11 compared to histologically non-degenerate discs (P<0.05)(Figure 4). Whilst percentage
12 immunopositive cells for phosphorylated c-jun was significantly higher in both high grade and
13 intermediate grades of histological degeneration compared to histologically non degenerate
14 discs (P<0.05)(Figure 4).

15 **Effects of inhibitors of intracellular signalling on native mRNA expression in NP cells**

16 Inhibition of p38 MAPK signalling decreased mRNA expression of MMP3, MMP13, IL-1 β and
17 IL-8 but not IL-6 in cells not stimulated with IL-1 β although this only reached significance for
18 MMP3 and IL-1 β (P<0.05)(Figure 5). Inhibition of JNK or its downstream target c-jun in the
19 absence of IL-1 β resulted in significant decrease in native mRNA expression of IL-1 β
20 (P<0.05)(Figure 5), whilst only c-jun inhibition decreased native expression of MMP3, and
21 JNK inhibition decreased native expression of IL-8 (P<0.05)(Figure 5), conversely native IL-6
22 mRNA expression was increased by inhibition of JNK or c-jun (P<0.05)(Figure 5). Inhibition
23 of NF κ B signalling significantly decreased mRNA expression of MMP13 (P<0.05)(Figure 5)
24 in the absence of IL-1.

25 **Inhibition of IL-1 β actions via inhibition of intracellular signalling.**

1 Real time PCR was utilised to investigate effects of signalling inhibitors on IL-1 β actions. IL-
2 1 β stimulation of NP cells significantly increased mRNA expression of MMP3, MMP13, IL-
3 1 β , IL-6 and IL-8 ($P < 0.05$)(Figure 5).

4 IL-1 β stimulation of NP cells in the presence of a p38 MAPK inhibitor induced MMP3, IL-6
5 and IL-8 mRNA although to a lesser extent than IL-1 β alone ($P < 0.05$)(Figure 5), whilst p38
6 MAPK inhibition prevented stimulation of MMP13 mRNA by IL-1 β but had no effect on the
7 induction of IL-1 β mRNA by IL-1 β (Figure 5).

8 IL-1 β stimulation of NP cells in the presence of a c-jun inhibitor induced MMP3, MMP13, and
9 IL-1 β mRNA although to a lesser extent than IL-1 β alone ($P < 0.05$)(Figure 5), whilst c-jun
10 inhibition had no effect on IL-6 or IL-8 mRNA induction by IL-1 β (Figure 5). IL-1 β stimulation
11 of NP cells in the presence of a JNK inhibitor induced MMP3, IL-1 β and IL-8 mRNA although
12 to a lesser extent than IL-1 β alone ($P < 0.05$)(Figure 5), whilst JNK inhibition had no effect on
13 MMP13 or IL-6 mRNA induction by IL-1 β (Figure 5).

14 IL-1 β stimulation of NP cells in the presence of an inhibitor of NF κ B signalling inhibited the
15 IL-1 β induced mRNA expression of MMP3, MMP13, IL-1 β , IL-6 and IL-8 mRNA expression
16 ($P < 0.05$)(Figure 5).

17 **Effect of signalling inhibitors on aggrecan mRNA expression.**

18 In order to determine the effect of signalling inhibitors on normal anabolic mRNA expression
19 the expression of aggrecan was investigated following inhibition of signalling factors.
20 Inhibition of JNK or c-jun had no effect on mRNA expression of aggrecan, however inhibition
21 of p38 MAPK or NF κ B resulted in down regulation of mRNA for aggrecan although this failed
22 to reach significance ($P > 0.05$)(Figure S-1).

23 **Discussion**

24 This study aimed to investigate the intracellular signalling pathways activated by IL-1 β in the
25 degenerate IVD which were not activated by the anabolic factor GDF-5 to identify potential

1 targets for new therapeutic strategies. It is important to identify signalling pathways which
2 are not activated by anabolic factors such as GDF-5 to ensure native growth factors are not
3 inhibited, whilst inhibiting catabolic processes.

4 Initial studies utilised the proteasome arrays to screen 46 signalling molecules to investigate
5 activation of a wide range of signalling molecules and determine if these signalling
6 molecules were differentially induced by IL-1 β and not by the anabolic factor GDF-5.
7 Interestingly, only a limited number of signalling molecules were activated by IL-1 β in these
8 cells and of these ERK1/2 was also induced by GDF-5, whilst p38, JNK and c-jun were
9 induced significantly more by IL-1 β than GDF-5, whilst this component of the study was
10 completed on two patients. These results were further confirmed in additional patients using
11 cell based ELISAs. IL-1 β has been previously shown to induce expression of
12 phosphorylated-ERK, p38 and JNK in human IVD cells in monolayer culture³², and together
13 with NF κ B signalling in rat NP cells³³. Furthermore as NF κ B signalling was not included
14 within the array, but previous studies have demonstrated this is a key signalling pathway
15 activated by IL-1 β in IVD cells²⁴⁻²⁷, this was investigated within human NP cells cultured in
16 alginate to maintain phenotype which demonstrated activation by IL-1 β but not GDF-5, which
17 was demonstrated using immunofluorescence and confirmed by cell based ELISAs.
18 Unfortunately performing these experimental procedures in alginate beads to maintain the
19 phenotype of the NP cells, alginate was shown to have high auto-fluorescence within
20 immunofluorescence experiments which prevented quantification of this data, however the
21 use of cell based ELISAs confirmed activation of the NF κ B signalling pathway.

22 GDF-5 stimulation resulted in activation of STAT 1 and 4 which has not been previously
23 demonstrated, however STAT 1 has been shown to be involved in the signalling of other
24 growth factors in chondrocytes, and has been shown to be a key signalling molecule in FGF
25 signalling³⁴, and IGFBP-3 signalling³⁵.

1 Interestingly IL-1 β stimulation resulted in the down regulation of 12 signalling proteins:
2 including STATs (STAT 3, 5A/B and 6), STAT3 has been shown to be expressed by human
3 NP cells previously³⁶, STAT3 is associated with IL-6 signalling in chondrocytes where it has
4 been shown to induce expression of cartilage matrix genes³⁷. These anabolic effects of IL-6
5 were inhibited by STAT3 knockdown³⁷. Thus the down regulation of STAT3 by IL-1 β seen in
6 the current study could be linked to the effects of IL-1 on matrix gene expression, however
7 its role in the IVD is poorly understood and requires further investigation.

8 The combined down regulation of the src family proteins, FAK and Paxillin demonstrate a
9 potential role for IL-1 β in the disruption of focal adhesion signalling, src family protein
10 kinases phosphorylate FAK and if these are inhibited integrin regulated adhesion to the
11 extracellular matrix is disrupted³⁸. Thus the inhibition of src family, FAK and paxillin signalling
12 could explain the altered integrin signalling seen during disc degeneration³⁹.

13 mTOR is a key signalling pathway involved in autophagy, inhibition of the mTOR complex
14 has been demonstrated to induce autophagy⁴⁰, thus the inhibition of mTOR shown here by
15 IL-1 β could provide a role for IL-1 β induced autophagy in the IVD⁴⁰. Additionally mTOR is
16 involved in regulation of cellular senescence and inhibitors of mTOR have been shown to
17 induce cellular senescence⁴⁰, which is also a key feature of disc degeneration⁴¹. As such the
18 modulation of these signalling pathways by IL-1 β requires further investigation particularly
19 whether abrogating the inhibition can be targeted by potential therapies.

20 It should be noted that activation of these signalling molecules reported within this study
21 were completed following 30 minutes, which was shown to be optimal from initial preliminary
22 studies, it was not possible to complete all studies at multiple time points but it should be
23 considered that as arrays investigated a wide range of signalling molecules further factors
24 may be activated by these factors at alternative times, however the majority of studies
25 investigating signalling factors do so over short time periods such as 30 mins.

1 Following the identification of p38, c-jun and NF κ B signalling as potentially differentially
2 induced by IL-1 β , expression of phosphorylated forms of these proteins was determined in
3 native human IVDs, which demonstrated all three signalling proteins were active within
4 native NP cells with increased expression of phosphorylated-p38, c-jun and NF κ B in discs
5 with intermediate grades of histological degeneration compared to those discs graded as
6 non-degenerate, whilst c-jun was also increased in discs with severe histological
7 degeneration. Interestingly the discs with highest levels of expression are also those which
8 have previously been shown to express highest levels of IL-1 β i.e. discs with intermediate
9 grades of degeneration⁶. Tolonen et al., previously investigated protein expression of c-jun in
10 human IVD samples and demonstrated expression in 74% of patient samples investigated⁴²,
11 unfortunately it is not clear whether they utilised a phospho-specific antibody and they did
12 not investigate whether expression altered during degeneration, however in agreement with
13 the current study expression was seen within NP cell clusters⁴². Interestingly in stab induced
14 degeneration in rat IVDs an induction of p-ERK and p-p38 was observed associated with
15 increased immunopositivity for IL-1 β and IL-6, in contrast no increase in p-JNK was
16 observed⁴³. Nerlich et al., also demonstrated increased activation of NF κ B signalling in
17 human IVDs which increased with age and grade of degeneration⁴⁴. Increased NF κ B
18 signalling with degeneration has been further confirmed recently⁴⁵. Together this data
19 supports the potential role of inhibitors of these signalling pathways in IVD degeneration.

20 Thus, this study went on to investigate the potential use of inhibitors of these signalling
21 pathways within human NP cells derived from degenerate discs on a number of key targets
22 which have been shown previously to be regulated by IL-1 β . Interestingly inhibitors of p38
23 signalling and the JNK/c-jun pathway only partially abrogated effects of IL-1 β suggesting
24 these factors alone may not be sufficient to inhibit the plethora of effects induced by IL-1 β
25 (Figure S-2). Klawitter et al., demonstrated partial inhibition of IL-1 β effects via curcuma and
26 curcumin with inhibition of IL-1 β induced expression of MMPs and IL-6 but failed to inhibit
27 effects on IL-1 β , IL-8 and TNF expression, these effects were shown to be a result of JNK

1 inhibition³², whilst other studies have suggested curcumins actions are via inhibition of NFκB
2 signalling^{25, 46, 47}. Yu et al., demonstrated abrogation of IL-1 induced inhibition of Sox9 and
3 collagen type II expression by curcumin⁴⁶. Further to this, Ma et al., recently demonstrated
4 curcumin could reduce degenerative changes in a rat degeneration model²⁵, both studies
5 associating actions to inhibition of the NFκB pathway. Inhibition of p38 signalling in rabbit NP
6 cells abrogated the effects of IL-1 on inhibition of matrix synthesis and partly reduced the
7 effects on expression of Cox-2, MMP3, IL-1 and IL-6²³ and PGE₂²² which agrees with the
8 current study.

9 However inhibition of the NFκB signalling pathway demonstrated the most potential in the
10 current study with abrogation of all the effects of IL-1β investigated. Wang et al., also found
11 that NFκB inhibition could completely abrogate IL-1β induced MMP3, whilst inhibition of
12 p38/ERK/JNK only partially abrogated the effects of IL-1β²⁸. In contrast IL-1β induced
13 expression of ADAMTS4 could be blocked by inhibitors of NFκB, p38, JNK and ERK1
14 signalling³³, recently Sun et al., further demonstrated the important role for NFκB signalling
15 in the IL-1β induced expression of ADAMTS^{24, 45}. Inhibition of NFκB signalling has been
16 shown to abrogate a wide range of IL-1 induced effects, demonstrating the potential of this
17 signalling target for inhibiting the plethora of IL-1 effects in the IVD²⁶. The potential role of
18 NFκB as a target for treating disc degeneration is further supported by the finding that NFκB
19 signalling was increased in an accelerated aging mouse model, and catabolic effects could
20 be blocked by inhibiting NFκB⁴⁸. However, the current study demonstrated inhibition of the
21 NFκB signalling did induce a baseline decrease in the expression of aggrecan mRNA
22 expression, which although failed to reach significance should be treated with caution.

23 **Conclusion**

24 This study has highlighted three key pathways were identified which were differentially
25 activated by IL-1β but not GDF-5; namely p38, c-jun and NFκB. All pathways were shown to
26 be activated during human IVD degeneration and inhibition of these pathways reduced or

1 abrogated the catabolic effects of IL-1 β , with inhibition of NF κ B signalling demonstrating
2 more widespread inhibition of IL-1 effects and thus potentially holds most promise for future
3 investigations (Figure S-2).

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23

1 **Figure legends:**

2 **Figure 1:** Proteasome kinase array for 46 intracellular signalling molecules. Representative
3 arrays shown for control (A), IL-1 β (B) and GDF-5 (C) stimulated human NP cells. D: Box
4 and whisker plots shown for key kinases/transcription factor (ERK1/2 (Red); FAK (Blue); p38
5 MAPK (Green); JNK (Purple) and c-jun (Orange)) indicated on the representative images
6 with matching colour boxes, colours indicated on legend for graph. Box and whisker plots
7 show relative protein expression normalised to untreated controls in 2 patients each
8 stimulated in duplicate and tested on arrays in duplicate. Significant differences between
9 treatment groups shown with line above box and whiskers ($P < 0.05$).

10 **Figure 2:** Representative images for p65-NF κ B immunofluorescence from human NP cells
11 cultured in alginate and stimulated with IL-1 β (B) and GDF-5 (C) or unstimulated controls
12 (A). Human NP cells from 3 patients cultured in alginate and treated in triplicate with IL-1 β
13 and GDF-5 or unstimulated controls.

14 **Figure 3:** Cell based ELISAs for demonstrating relative phosphorylated ERK1/2 (A), p38
15 MAPK (B), c-jun (C) and I κ B (D) protein expression normalised to untreated controls. Human
16 NP cells from 3 patients cultured in alginate and treated in triplicate with IL-1 β and GDF-5 or
17 unstimulated controls. Statistical differences between groups demonstrated by bars and
18 *= $P < 0.05$.

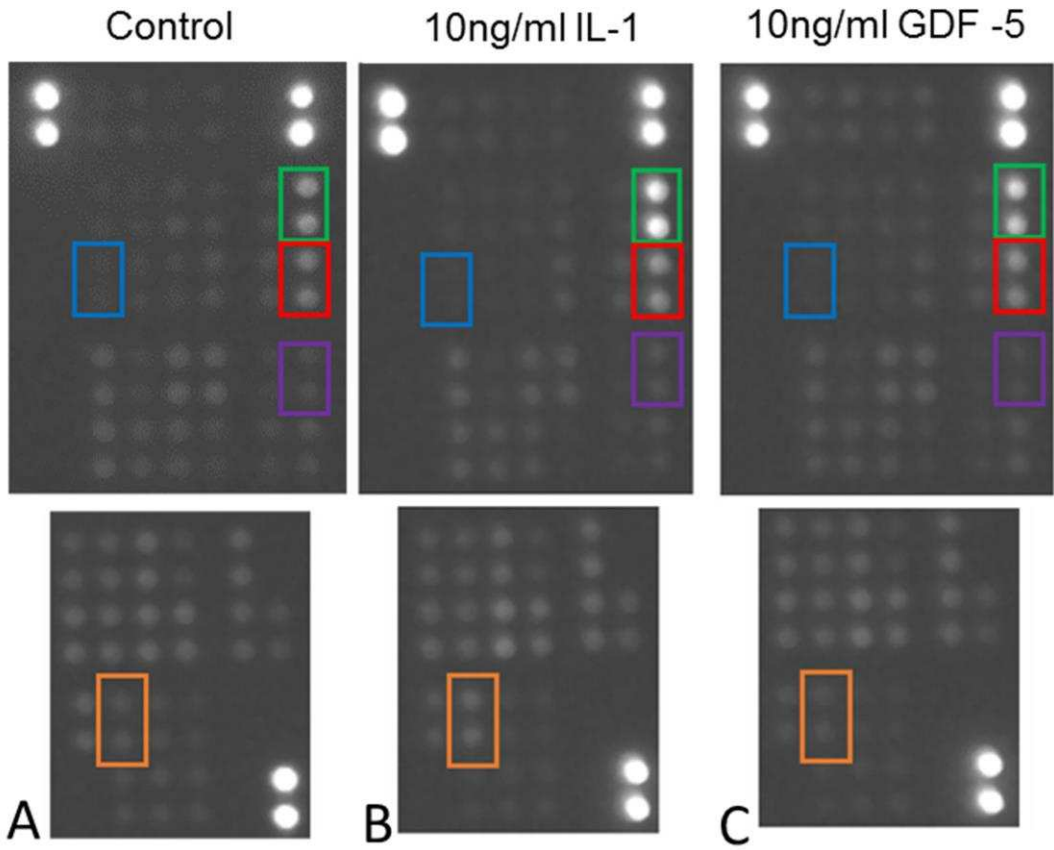
19 **Figure 4:** Immunohistochemical analysis for phosphorylated p38 MAPK (A-D); c-jun (E-H)
20 and NF κ B (I-L) in human IVD discs. Representative images of immunohistochemical staining
21 demonstrated for low grade degenerate discs (A,E,I); Intermediate grade discs (B,F,J); high
22 grade discs (C,G,K). Percentage immunopositivity within NP cells with differing grades of
23 degeneration (D,H,L). Statistical differences between immunopositivity for phosphorylated
24 signalling molecules in intermediate grades of degeneration (4-6) or severe degeneration
25 (≥ 7), compared to non degenerate discs (≤ 3) shown with *= $P < 0.05$. (n=30).

1 **Figure 5:** Relative mRNA expression for MMP3 (A), MMP13 (B), IL-1 β (C), IL-6 (D) and IL-8
2 (E) normalised to housekeeping genes (GAPDH and 18s) and untreated control cells. Data
3 shown generated from NP cells sourced from three independent patients cultured in alginate
4 and treated with or without IL-1 β , with or without prior treatment with inhibitors of signalling
5 molecules (p38 MAPK; c-jun, JNK and NF κ B), all treatments performed in triplicate within
6 each patient sample. * = significant difference between treated cells compared to untreated
7 controls (P<0.05); ^ = significant difference between IL-1 β stimulated cells following prior
8 treatment with signalling inhibitors compared to stimulation with IL-1 β alone P<0.05.

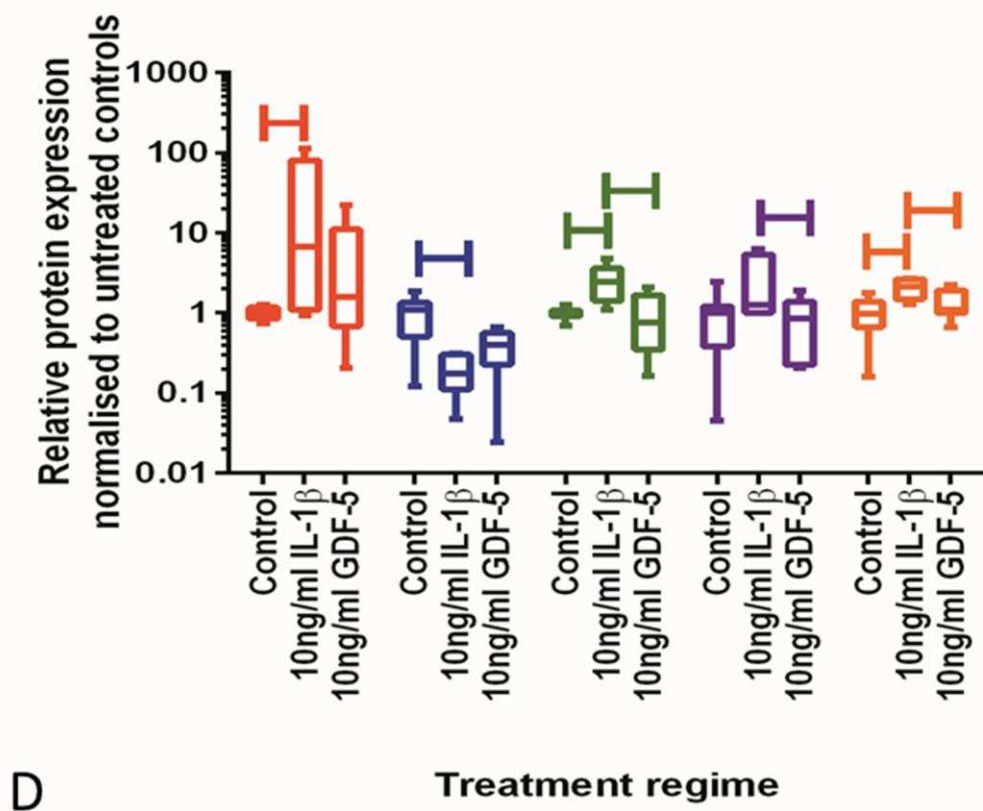
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10 **Figure S - 1:** Relative mRNA expression for aggrecan normalised to housekeeping genes
11 (GAPDH and 18s) and untreated control cells. Data shown generated from NP cells sourced
12 from three independent patients cultured in alginate and treated with or without signalling
13 inhibitors. All treatments performed in triplicate within each patient sample.

1 **Figure S - 2:** Schematic representing key findings.

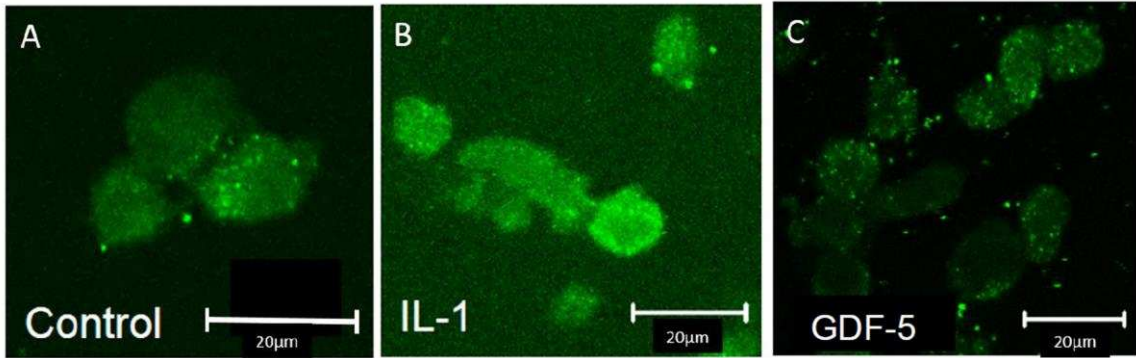


ERK1/2 FAK p38 MAPK JNK c-jun

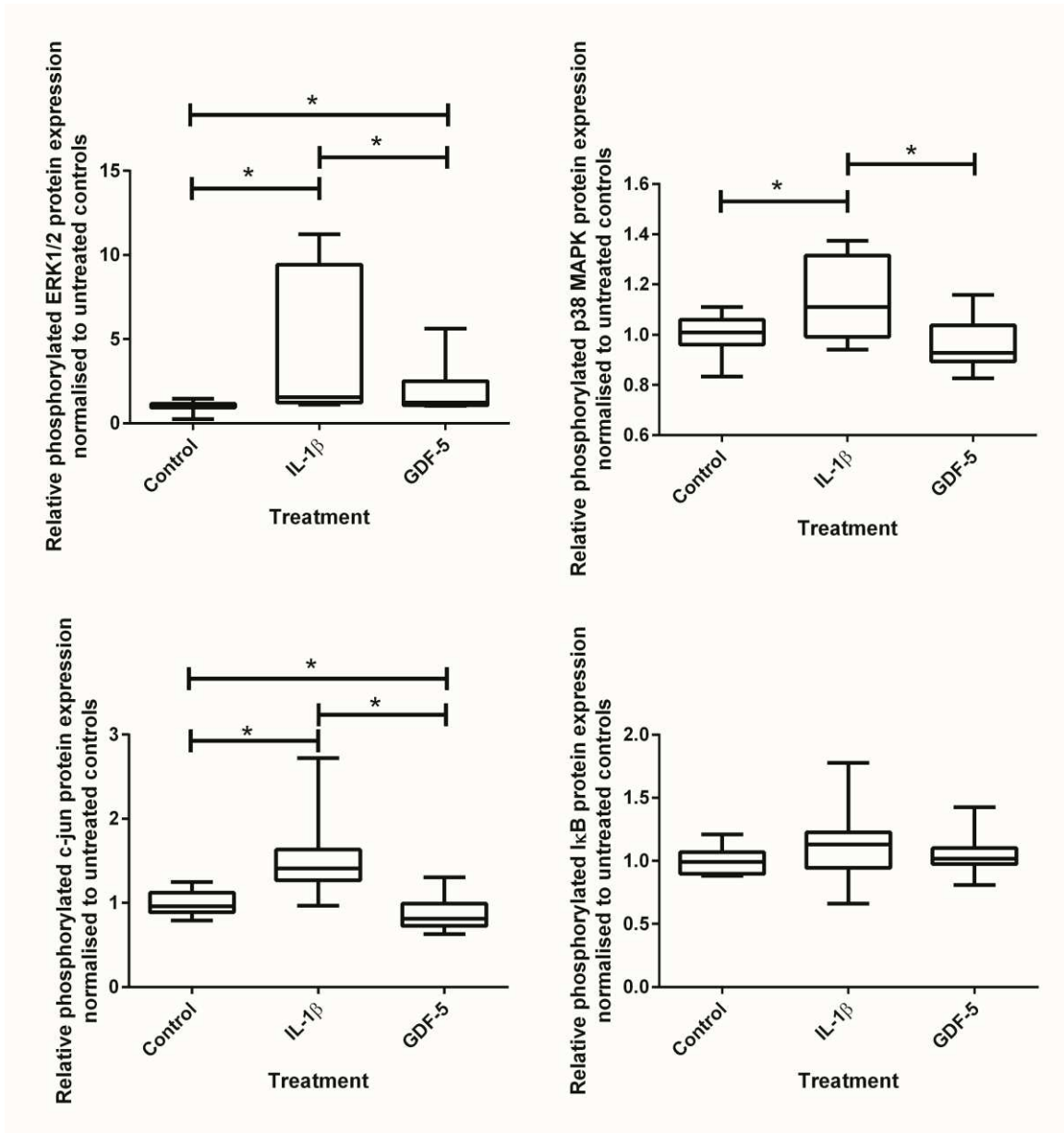


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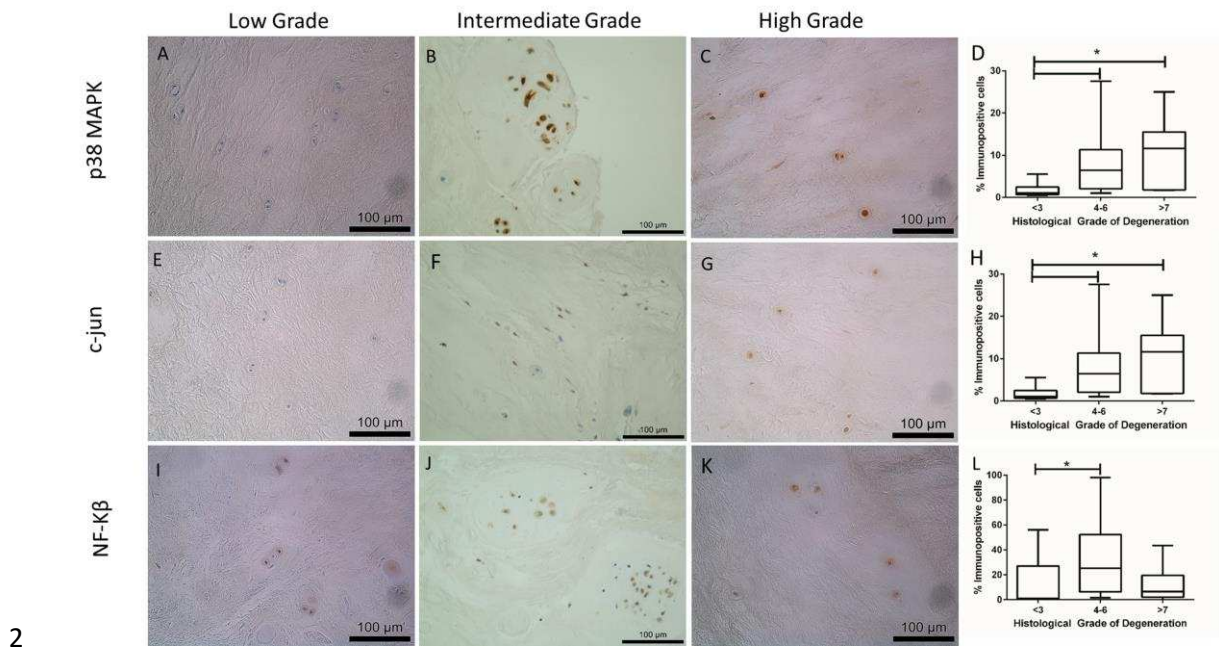


3 Figure 2



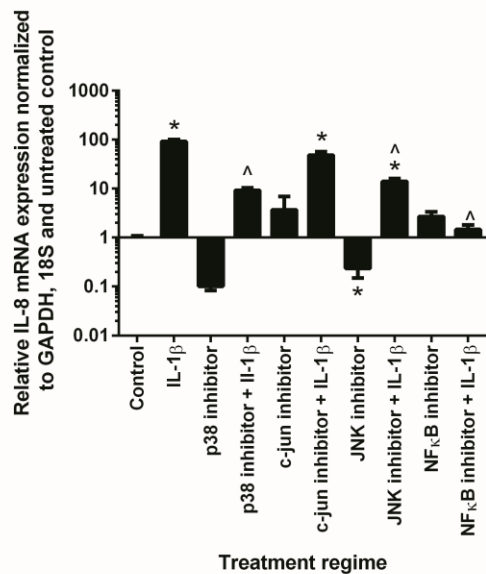
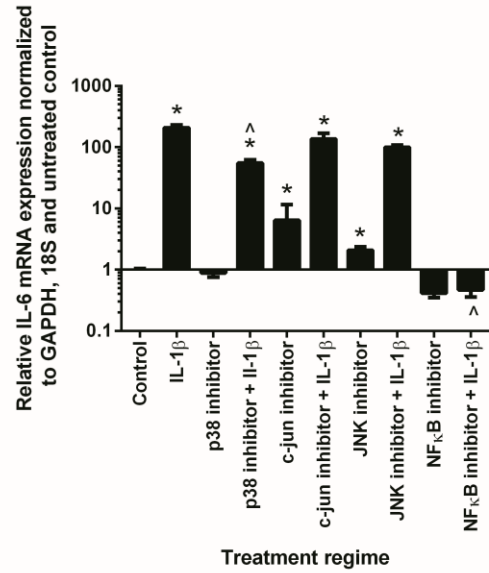
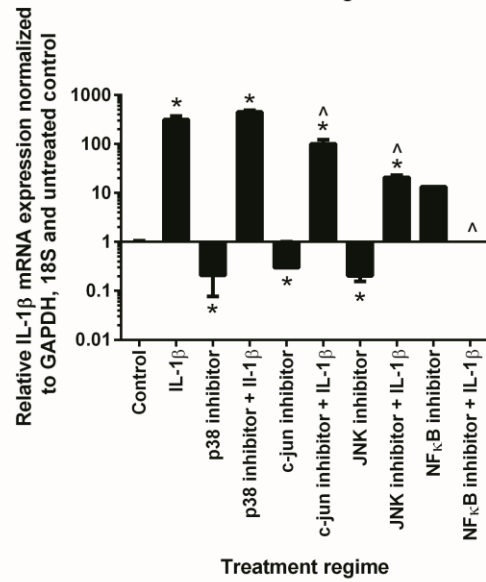
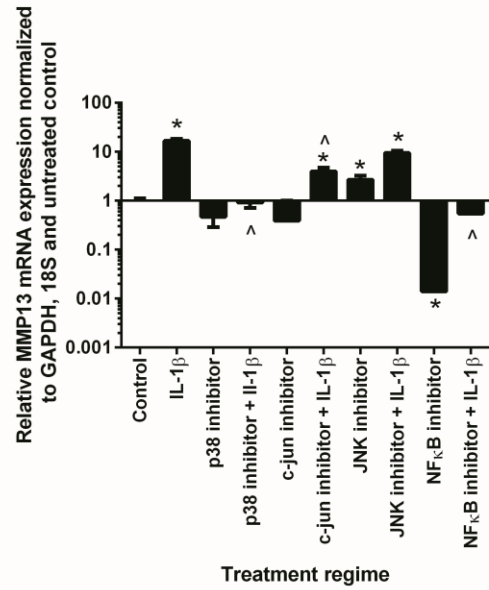
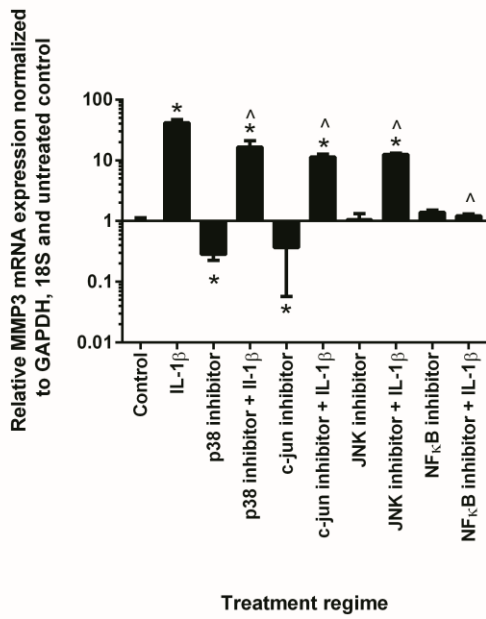
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1 **Figure 3**

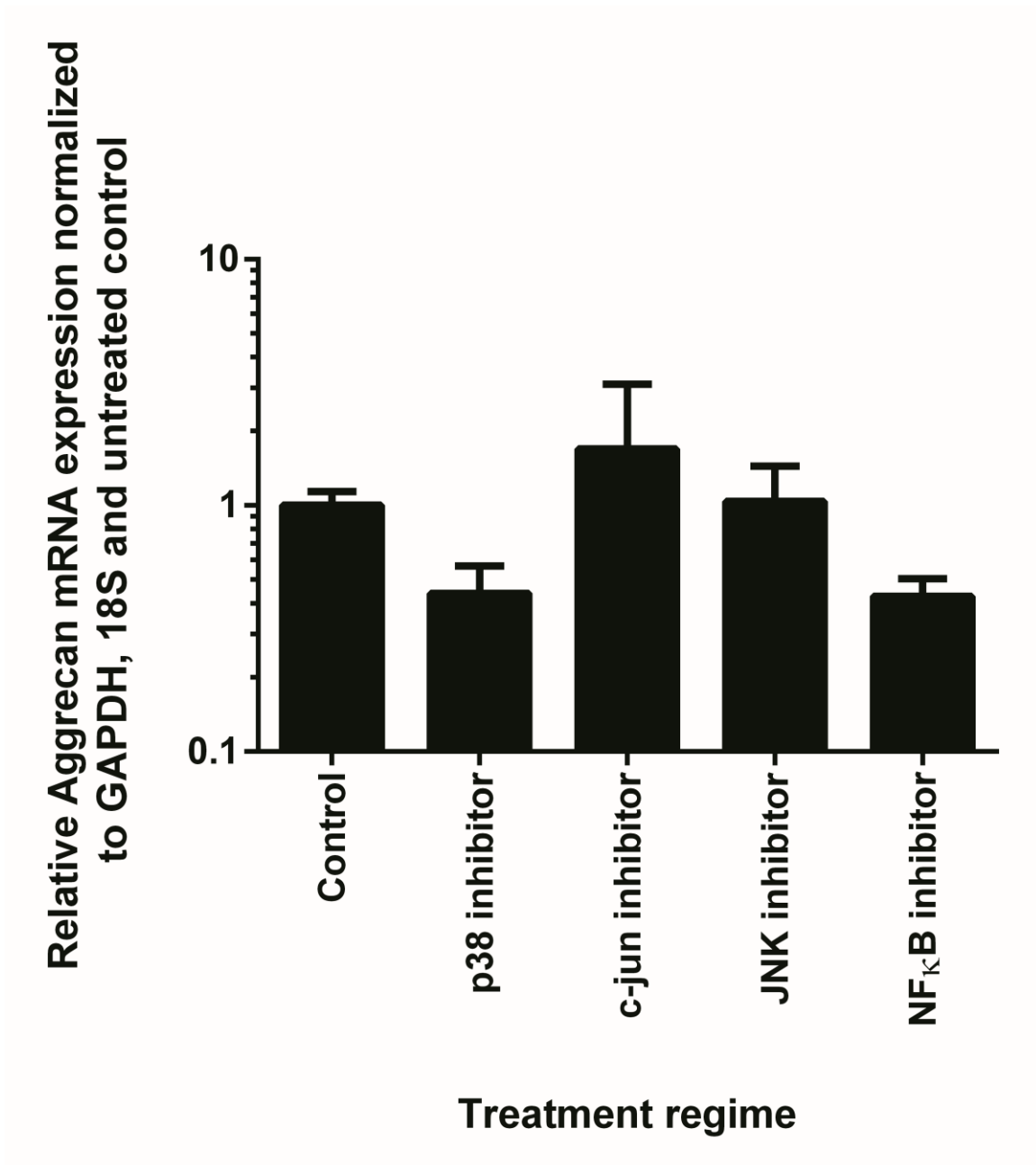


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3 **Figure 4**

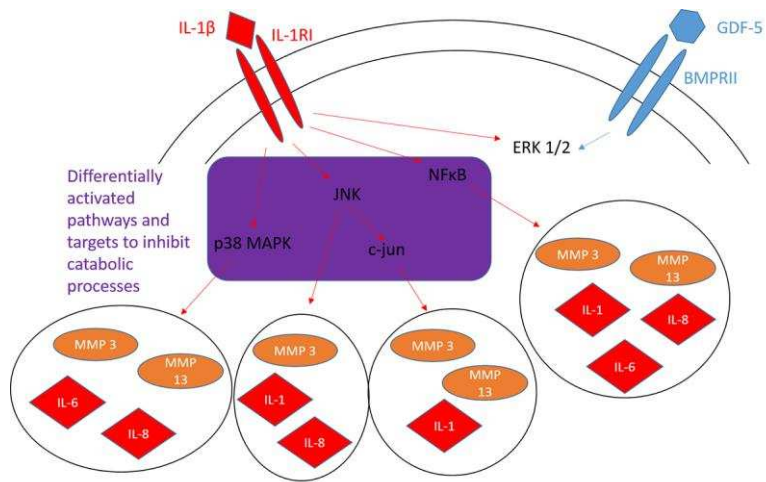


1 Figure 5



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3 Sup Fig 1



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2 **Sup fig 2**