Inhibition of the Extracellular Signal-regulated Kinase Pathway Restores the Discogenic Phenotype of Inflammatory Intervertebral Disc Cells

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Abstract

Background: Intervertebral disc (IVD) degeneration is a spinal disease caused by trauma and/or repetitive mechanical overloading of the spine which triggers inflammatory response pathways. Long-term disc inflammation may lead to development of spinal pseudoarthrosis. The aim of the present study was to elucidate the role of the extracellular signal-regulated kinase (ERK) pathway in inflammation-induced IVD cells.

Methods: Inflammatory human nucleus pulposus cells (NPC) were stimulated using tumor necrosis factor alpha (TNFα) and the ERK pathway was blocked using a selective molecule-based inhibitor U0126. Gene expression of catabolic and anabolic events, pro-inflammatory, and NPC markers were investigated. The enzymatic activity of matrix metalloproteinases (MMP)2/9 were determined by gelatin zymography. The cytoxicity of U0126 concentrations on NPC was quantified using resazurin assay, and the specificity of U0126 on ERK1/2 signaling was determined.

Results: The pro-inflammatory cytokines like MMP3/13 and interleukin 6 in nucleus pulposus (NP) inflammatory conditions were down-regulated by U0126 and a trend towards an increase of the NP-specific collagen type 2, aggrecan and keratin 19 was observed suggesting a recovery of the NP phenotype. U0126 does not seem to have effect on prostaglandin production, aggrecanases and some anabolic genes. We confirmed that U0126 selectively blocks the ERK phosphorylation and U0126 affects the cells metabolic activity only for high concentrations.

Conclusions: Inhibition of ERK signaling down-regulates important metalloproteinase, pro-inflammatory cytokines, and up-regulates NP markers in order to restore the discogenic phenotype of inflammatory NPC.

Background

Low back pain affects millions of people in industrialized societies every year and represents, therefore, a leading cause of disability with significant economic and social burdens [1–3]. Chronic low back pain is strongly associated with intervertebral disc (IVD) degeneration and consists mainly in increasing pain during the execution of daily spinal movements. The IVD consists of an inner nucleus pulposus (NP) surrounded by the annulus fibrosus (AF) tissue, and a hyaline articular cartilage is located at the endplates between the IVD and two adjacent vertebral bodies. The NP is a gelatinous-like and avascular connective tissue containing a highly organized extracellular matrix rich in proteoglycans and collagens with few numbers of cells. The NP cells (NPC) actively regulate the homeostasis in the extracellular matrix by several growth factors and cytokines acting in an autocrine and paracrine fashion. The early stage of disc degeneration is often asymptomatic [4] and tends to develop a chronic inflammation within the spine. This produces high stress in the disc tissue, which begins with synthesis of catabolic enzymes [5], resulting in degradation and loss of the extracellular matrix (ECM) structures present in the IVD. The

loss of IVD structures leads to leakage of the connective tissue from the NP through the AF, which causes compression of peripheral nerves, resulting in pain and inability of spinal movements.

Non invasive interventions including medications, steroid injection and physical therapy are most commonly recommended, and often of limited efficacy [6–7] since they do not maintain or restore the native tissue structure in degenerative discs. Currents surgical treatments are aimed to remove discogenic pain by replacement of the injured tissue with a functional biological substitute or prosthesis, however, surgery for disc degeneration also yielded to mixed clinical outcomes and often results in incomplete interbody fusion [8].

Interleukin 1 beta (IL1β) and tumor necrosis factor alpha (TNFα) are pro-inflammatory cytokines know as key mediators of the development/progression of disc degeneration and low back pain [5]. It has been shown, for instance, in many studies that TNFα is highly expressed in degenerative IVD tissues [9–10]. Surgical samples obtained from patients with history of low back pain revealed higher levels of TNFα-positive cells than autopsy from healthy controls [11]. It causes an up-regulation of ECM-degrading enzymes and decreases the expression of matrix-specific matrix proteins [12]. Thus identifying and targeting signaling pathways responsible for the TNFα-mediated inflammation could be considered as a promising therapeutic option to regenerate the IVD tissues.

Extracellular signal-regulated kinase (ERK), a downstream of the mitogen-activated protein kinase (MAPK) signaling cascade, is an important inflammatory pathway that plays a critical role in the production of inflammatory cytokines and the activation of procatabolic responses induced by TNFα in chondrocytes-lineages cells [13]. Inhibition of MAPK signaling attenuates the decrease of collagen type II and aggrecan without inducing apoptosis in primary rat and immortalized chondrocytes [14]. In agreement with this, inhibition of MAPK/ERK activity enhances chondrogenesis of mesenchymes [15], and TNFα-induced NF-κB DNA binding in chondrocytes is reduced by inhibition of MAPK signaling [14]. Recent studies suggested a catabolic pathway of ERK in the degeneration of intervertebral disc. In AF cells, ERK was shown to mediate IL1-induced upregulation of cyclooxygenase [16], important aggrecanses and metalloproteinases [17], while ERK inhibition decreased the IL1β-induced apoptosis [18].

The role of the ERK pathway in effecting the action of TNFα in NPC, however, has not been fully elucidated. The aim of the present study is to clarify the role of ERK in inflammation-induced human NP cell culture model.

Methods

Human NP isolation and culture

Human IVD were collected from trauma patients undergoing spinal fusion surgery. The procedure was performed with patients' written consent and was approved by the local ethical committee of the canton of Bern, Switzeland. The disc materials were collected from patients aged between 17 and 51 years old (34 ± 16.9 [mean ± SD]). The NP tissue was separated from the outer AF of the disc by an experienced

surgeon and subsequently processed within 24 h after the surgery. Data were anonymized and solely the sex and age were recorded from each donor. The NPC were isolated by sequential digestion of the NP tissue fragments with 1.9 mg/mL pronase (Roche Basel, Switzerland) for 1 h followed by collagenase II (Worthington, London, UK) at 37°C overnight on a plate shaker. The remaining undigested NP tissue debris was removed by filtration through a 100 µm cell strainer (Falcon, Becton Dickinson, Allschwil, Switzerland) and cell viability was determined by Trypan blue exclusion.

The NPC were expanded in proliferation medium (Dulbecco's Modified Eagle Medium [DMEM, Sigma-Aldrich, Basel, Switzerland] containing 10% Fetal Bovine Serum [FBS, Sigma-Aldrich] and penicillin/streptomycin [P/S, 100 U/mL and 100 µg/mL, respectively, Merck, Darmstadt, Germany]) until confluency. In order to amplify the low number of NPC obtained after digestion of the biopsy, the NPC were expanded in the proliferation medium until reaching several passages and a cell stock was cryopreserved for further analysis. Low-passage (less than 3 passages) NPC were used in this study.

Induction of pro-inflammatory environment and ERK inhibition

NPC were seeded at a density of 5×10^4 cells/well in 24-well plates in the proliferation medium and left overnight for cell adherence. Induction of a pro-inflammatory environment in NPC was performed by addition of 10 ng/mL of human recombinant TNFα (Peprotech, London, UK) to the wells. Control cultures represented NPC with (positive control) or without (negative control) TNFα.

Alternatively, the cells were pretreated with a selective molecule-based inhibitor of ERK, U0126 (Selleck Chemicals, Houston, USA) at 0.5 µM and 5 µM for 1 hour to inhibit ERK pathway activation. The cultures were maintained for 3 days and collected thereafter for downstream applications.

NP cells mitochondrial activity

The NPC were seeded at a density of 2×10^3 cells/well in 96-well plates in the proliferation medium and left overnight for cell adherence. To address whether blocking the ERK pathway and a combination of TNFα and ERK inhibition may induce a cytotoxic effect on IVD cells, NPC were stimulated with 10 ng/mL TNFα and increasing concentration of a U0126 ranging from 0.1 µM to 10 µM. The cultures were collected after 3 days and the cells viability was determined with a resazurin red solution (Sigma-Aldrich) as previously described [19]. Briefly, the cultures were incubated with 50µM resazurin red solution in a humidified atmosphere (5% CO_2, 37°C) for 2 hours. The absorbance was measured at 580 nm on a microplate reader (SpectraMax M5, Bucher Biotec, Basel, Switzerland).

Analysis of gene expression

Total RNA was extracted as previously described [20] from the NPC after stimulation with TNFα and inhibition of the ERK signaling. NP hall markers, including aggrecan (ACAN), collagen type II (COL2A1) and cytokeratin 19 (KRT19), anabolic markers including insulin-like growth factor 1 (IGF1) and transforming growth factor beta 1 (TGFβ1), catabolic markers including matrix metalloproteinase 3

(MMP3), MMP13 and a disintegrin and metalloproteinase with thrombospondin motifs 5 (ADAMTS5), pro-inflammatory markers including IL6 and cyclooxygenase-2 (COX2), and the ribosomal 18S RNA as reference gene, were determined. Human-specific oligonucleotide primers (Table 1) (Microsynth, Balgach, Switzerland) were designed with Beacon DesignerTM (Premier Biosoft, California, Palo Alto, USA) based on the sequences of the nucleotides from GenBank. The efficiency and melting curves of the amplicons were tested to determine specific amplification.

Table 1

Custom-designed DNA primers used in real-time qPCR study. Amplicons were generated using a 2-step amplification cycling (95° for 15s and 57°C for 30s for 45 cycles) and SYBR-green mastermix.

Gene	Forward sequence	Reverse sequence
18S	CGATGCGGCGGCGTTATTC	TCTGTCAATCCTGTCCGTGTC C
ACAN	TCTGGAGTAGAGGACATC	AGGAAGTTCACTGACATC
COL2	AGCAGCAAGAGCAAGGAGAA	GTAGGAAGGTCATCTGGA
KRT19	TCTTGCTGCTGATGACTT	CCTCTTCGTGGTTCTTCT
IGF1	CAGACAGGCATCGTGGAT	TGACTTGGCAGGCTTGAG
TIMP1	TCAACCAGACCACCTTATACCA	ATCCGCAGACACTCTCCAT
TGFβ1	CGTGCTAATGGTGGAAAC	GCTCTGATGTGTTGAAGAAC
MMP3	CAAGGCATAGAGACAACATAGA	GCACAGCAACAGTAGGAT
MMP13	AGTGGTGGTGATGAAGAT	CTA AGG TGT TAT CGT CAA GTT
ADAMTS5	GCTGTGCTGTGATTGAAGA	TGCTGGTAAGGATGGAAGA
IL6	GCCACTCACCTCTTCAGAAC	GCAAGTCTCCTCATTGAATCCA
COX2	GTCTGGTGCCTGGTCTGA	GTCTGGAACAACTGCTCATCAC

Gelatin zymography

A gel zymography was performed in cell lysates after treatment of NPC with TNFα and/or inhibition of the ERK signaling pathway to analyze the pattern of MMP2/9 in different treatment groups. Briefly, the cell layers were washed with PBS and homogenized with 0.5% Triton-X (Sigma-Aldrich) containing a protease inhibitor (Sigma-Aldrich). The extracts were applied to a gelatin-containing 10% SDS-PAGE and the gel was incubated in a developing buffer composed of 50mM Trizma Base and 5mM $CaCl_2$ (pH 8.0) overnight at 37°C. Subsequently, the polyacrilamide gel was stained with 0.1% Coomassie blue R250 solution (Sigma-Aldrich) for 2 hours.

Western Blot analysis

Primary human NP cells (250’000 cells/well) were seeded into 6-well culture plates and grown for one week in the proliferation medium without additional factors to allow for cell adherence. The medium was changed and treatment of the cells with TNFα, TNFα together with U0126 (0.1 µM, 0.5 µM, 1 µM and 5 µM) and DMSO (control) was initiated. The cells were pre-treated with various concentrations of U0126 for 1 hour and subsequently TNFα (10 ng/mL) was applied to the cultures in order to simulate an inflammatory environment. After 30 min of treatment, adherent cells were rinsed with PBS and immediately lysed in the cell lysis buffer (CelLytic, Sigma-Aldrich) containing a protease inhibitor cocktail (25µg/ml, Sigma-Aldrich) and phosphatase inhibitor (1 mM NaF, 1 mM sodium orthovanadate, 4 mM sodium tartrate, 1.15 mM sodium molybdate and 2 mM imidazole, Biotool, USA). The total protein content in the cell layers was determined by Bradford protein assay. The samples (10 µg) were subjected to 12% SDS-PAGE and transferred to PVDF membrane. Non-specific binding sites were blocked using 5% skim milk in Tris-buffered saline with 0.05% Tween (TBST) for one hour at room temperature. The membrane was washed several times with TBST followed by incubation with a rabbit anti-phospho-ERK1/2 and/or ERK1/2 primary antibodies (Cell Signaling Technology, USA) at 4°C overnight. Detection was achieved using a goat anti-rabbit antibody (LI-COR Biosciences GmbH, Germany), and the band intensity was quantified with Odyssey Software (LI-COR).

Statistical analysis

Differences in real time PCR and resazurin sodium salt assay were evaluated by a one-way ANOVA with Bonferroni’s post-hoc test using GraphPad Prism (version 6.0 f for Mac OS; GraphPad Software Inc., La Jolla, CA USA). $p < 0.05$ was considered significant.

Results

U0126 effect on the NP metabolic activity

The cellular metabolic activity was analyzed using the rezasurin assay to verify whether U0126 induces any cytotoxic effect on the cells. There was a dose-dependent decrease in the NPC viability with increasing concentrations of U0126 as compared to the control group (TNFα). The NPC metabolic activity was significantly reduced ($p = 0.0016$, Fig. 1) by U0126 at 0.5µM and beyond 1µM ($p < 0.0001$). These data suggested that U0126 is a toxic component at high concentrations (micro-molar range). In addition, TNFα (10ng/ml) alone significantly reduced ($p = 0.001$) the NPC metabolic activity as compared to untreated cells. The TNFα-stimulated NPC were morphologically different, spindle shaped with fibroblast-like appearance and less dense compared to untreated cells as observed microscopically.

Gene expression

The effect of TNFα stimulation followed by ERK inhibition was investigated on the transcript levels of NPC. The gene expression of catabolic, anabolic genes and NPC markers including matrix components and anticatabolic, and inflammatory markers were therefore considered. The addition of TNFα (10ng/ml) to the NPC cultures resulted in increased expression with many significant differences of important

catabolic and pro-inflammatory pathway markers including MMP3 ($p < 0.0001$, 317-fold, Fig. 2A), COX2 (5-fold, Fig. 2B), and IL6 ($p = 0.002$, 580-fold). While a decrease in the anabolic and NPC markers including ACAN (2-fold, Fig. 2C), COL2 (7-fold), IGF1 (4-fold), TGFβ1 (2-fold), and KRT19 (10-fold, Fig. 2D) was observed.

There was a 1.5-fold and 1.8-fold increase of COL2, 1.8-fold and 1.2-fold increase of ACAN transcript levels following ERK inhibition by U0126 at 0.5μM and 5μM, respectively, in TNFα-treated NPC cells as compared to TNFα alone group. The gene expression of IGF1 was increased by 4-fold and 6-fold by addition of U0126 to the cultures at 0.5μM and 5μM in TNFα-treated cells. Similarly, the cytokeratin 19 (KRT19) was up-regulated by 17-fold and 170-fold while treating the TNFα NPC cultures by U0126 at 0.5μM and 5μM.

The catabolic marker MMP3 transcript levels was significantly reduced following treatment of the TNFα-stimulated NPC cultures by U0126 at 0.5μM ($p = 0.02$) and 5μM ($p = 0.0008$). The MMP13 transcript levels was decreased by 2-fold and 4-fold ($p = 0.048$) following addition of U0126 at 0.5μM and 5μM, respectively. Similarly, the pro-inflammatory cytokine IL6 was significantly reduced by addition of U0126 at 0.5μM and 5μM ($p = 0.03$) to the inflamed NPC cultures. The COX2 mRNA levels remained almost the same in the U0126 treated cultures as compared to the TNFα alone group.

Gelatinases expression

Gel zymoraphy was performed in cell layer lysates for the detection and assessment of the gelatinases (MMP2/9) expressed in the different treatment groups. It was able to detect both the MMP2 and MMP9 and their respective pro-MMP2/9 in the lysates (Fig. 3). However, the band intensities were almost the same in the lysates with insignificant variations upon the treatment groups.

Activation of ERK signalling

Western blots were carried out to assess the effects of TNFα and U0126 on the ERK mitogenic dependent intracellular signaling. TNFα induced the ERK signaling pathway in human NPC as demonstrated by the phosphorylation of ERK1/2 (Fig. 4A). We found that the peak ERK phosphorylation was achieved after 30 minutes of TNFα treatment in NPC. The cytokine-mediated activation of the ERK pathway was typically transient with a maximal TNFα stimulation obtained followed by decline of p-ERK dynamics after the first 30min of TNFα treatment (Fig. 4B). Further western blots investigating the effect of U0126 on ERK pathway in TNFα-stimulated NPC were carried out at 30min of cytokine exposure in order to benefit from the maximal TNFα stimulation effect. U0126 abolished the the TNFα-mediated ERK phosphorylation in a dose-dependet manner (Fig. 5A). U0126 treated cells at 1μM along with TNFα resulted in phosphorylated ERK results comparable to the untreated control group (Fig. 5B).

Discussion

Current treatments to alleviate chronic low back pain are principally surgical with very variable outcomes. For example, a 10–40% failure rate was observed in lumbar surgeries with or without spinal fusion, and

similar results were obtained in patients whom underwent a discectomy of herniated disc with recurrence after two years of surgery [21–24]. Innovative strategies for IVD with regenerative medicine and/or pharmacological inhibitors of disc degeneration are urgently wanted.

The present study investigated the contribution of ERK signaling in *in vitro* inflammation-induced human NPC by TNFα and whether ERK inhibition using selective mitogen-activated protein kinase kinase enzyme (MEK) inhibitor can reverse the inflammatory phenotype of these cells. We found that TNFα stimulation in NPC induced a pro-inflammatory microenvironment of human IVD cells, in particular NPC, characterized by increased expression of important catabolic events and pro-inflammatory mediators. Herein, the expression of matrixproteinases including MMP3 and MMP13 was upregulated in TNFα-stimulated NPC. MMP are believed to be the major proteolytic enzymes responsible for ECM degradation in the IVD leading to disc degeneration [25]. The expression of numerous metalloproteinases at the transcript and protein levels has been studied in several human IVD as well in experimental animal models revealing the catabolic changes and their mediation in the progression of IVD degeneration [26–28].

This study further supports that the TNFα cytokine is a potent mediator of inflammatory response in IVD and in particular in NPC leading to ECM degradation through increased catabolism and therefore to disc degeneration. Inflammatory processes exacerbated by TNFα and/or IL1β are believed to trigger disc degeneration and in later stages low back pain. For instance, surgical samples obtained from patients with history of low back pain revealed higher levels of TNFα-positive cells than autopsy from healthy controls [11]. In addition, these intradiscal pro-inflammatory cytokines are implicated in the onset and progression of IVD degeneration and discogenic pain, and are produced by native IVD cells including NPC and AFC as well to infiltrating macrophages [5, 9–10]. Taken together, TNFα is an essential initiator of a pro-inflammatory environment in IVD tissue and cells which leads to the tissue ECM degradation and disorganization, and therefore to disc degeneration and painful spine. The importance of TNFα cytokine in discogenic pain led to multiple clinical trials using TNFα inhibitors, which resulted in mixed results [29–31] and highlighting, therefore, for further research studies [12]. In particular, monoclonal antibodies against TNFα have shown promise for mitigating disc degeneration and relieving low back pain. Anti-TNFα treatment significantly decreases the concentration and activity of MMP1 and MMP3 in *ex vivo* IVD tissues isolated from patients with herniated discs [32]. Despite obvious benefit of TNFα mAbs, some patients do not respond to them and/or many will develop recurrent disease despite continuing dosing which hampers the clinical use of these antibodies. [33]. In addition, Infliximab which is a TNFα blocker and a chimeric IgG1 antibody did not appear to interfere with spontaneous resorption of disc herniation over a prolonged period based on MRI diagnosis in a randomized controlled study [34]. Therefore, further research to elucidate the mechanism by which inflammatory cascade is initiated through TNFα is required for targeted pharmacological treatment of IVD degeneration.

Within this study, we aimed to block the TNFα downstream signaling pathway by targeting the ERK from the MAPK family. First, we reproduced an *in vitro* inflammatory environment in NPC and subsequently the ERK pathway was blocked through U0126. The current study sought to elucidate the role of ERK1/2 signaling pathway in TNFα-mediated catabolic effect in NPC.

We used the resazurin red assay to examine the possibility of any cytotoxic effect of the ERK inhibitor on NPC. It was found that U0126 was cytotoxic in NPC at large concentrations translated by a dose-dependent reduction of cells' viability with increasing U0126 molarity. This finding further supports the critical role of MAPK, in particular ERK1/2 pathway, in the regulation of mammalian cell proliferation as previously documented [35].

Blocking the ERK pathway in inflammation-induced human NPC resulted in a down-regulation of MMP3 and MMP13 to similar levels observed in control NPC, at least for the mRNA levels. It was previously demonstrated that CCAAT/enhancer binding protein beta (C/EBPβ) in the TNFα promoter region was suppressed in the presence of an ERK inhibitor PD98059 and the p38-MAPK inhibitor SB202190, but not the JNK inhibitor SP600125 in rat NPC [36]. In addition, the C/EBPβ and MMP13 expression was co-localized in chondrocytes in inflammatory arthritic patients [37]and that pro-inflammatory cytokines such as IL1β and TNFα binds to MMP3 and MMP13 promoter regions and stimulates their expression [37–38]. Similarly, treatment of rat NPC with ERK1/2 inhibitors (PD98059 and U0126) abolished the antagonistic effect of TGF-β1 on TNFα mediated MMP3 catabolic response [39], which further supports our finding on the implication of ERK pathway in inflammatory human NPC. Taken together, TNFα induces an inflammatory cascade in mammalian cells, in particular NPC, by up-regulation and modulation of MMP family members such as MMP3/MMP13 through ERK1/2 pathway, and inhibition of ERK signaling can reverse this catabolic effect. ERK1/2 is, therefore, considered a downstream signaling pathway of TNFα and this MAPK might be a target for the increased MMP enzymatic activity. Although, the enzymatic activities mediated by the gelatinases MMP2/9 were visible on gel zymography in NPC cell layer lysates, we could not detect significant variations of MMP2/9 following treatment of NPC with TNFα. This could be explained by an inappropriate model for the detection of MMP2/9, the intracellular gelatinases MMPs are normally secreted in the extracellular compartment for a variety of cell lineages [40–42]. Therefore, it is more relevant to assess the incorporated gelatinases in culture supernatant rather than within the cells lysates [43] as also detected previously for IVD cells [44].

The activation of an inflammatory microenvironment through stimulation of NPC with TNFα resulted in an increased ADAMTS5 expression as compared to controls. Blocking the ERK1/2 pathway with U0126, however, did not alter the expression of ADAMTS5. This observation might be explained by a differentiational regulation of aggrecanase-mediated proteoglycan degradation including ADAMTS4-5 which is mediated through NF-kB activation and not ERK1/2 in bovine NPC [45]. Similarly, it was suggested that ADAMTS4 expression and promoter activity increased in NPC following TNFα and IL-1β treatments [46] and treatment of the cells with NF-kB inhibitor abolished this inductive effect of the cytokines on ADAMTS4 mRNA and protein expression. This further supports that modulation of ADAMTS5 in NPC is mediated through NF-kB which might explain our observation on the role of ERK1/2 in ADAMTS5 expression.

The results of this study revealed a trend towards an increase in transcripts levels of the NP-specific markers including COL2 and ACAN, but also a significant increase of KRT19 in inflammatory NPC that were treated with U0126. In addition, increased expression of anabolic genes like IGF1 was observed

suggesting a restoration of the NPC phenotype following the inhibition of the ERK pathway in inflammatory cells. Wei et al. [17] observed an imbalance between anabolic and catabolic events in rat AFC activated with IL1, and ERK inhibition significantly blocked the catabolic and inflammatory effects of IL1 in AFC.

Within this study, we provided evidence that TNFα-mediated inflammation in human NPC is triggered through the ERK1/2 pathway which in terms increased pro-inflammatory mediators like MMPs and decreased anabolic genes characteristic of a degenerated IVD cells. The ERK1/2 pathway was modulated through TNFα as observed by Western Blot assay and simultaneous treatment of NPC with TNFα and U0126 abolished this effect. We also showed that U0126 is a specific inhibitor of ERK1/2 pathway.

There are some limitations of the current study. First, due to the difficulty and availability in obtaining healthy and non-degenerated human disc tissues samples, the results were limited to gene expression analysis of some anabolic and catabolic genes, and we did not investigate their protein levels more in details. Second, we did not address the contribution of other MAPK like p38-MAPK and JNK in inflammation, although some pilot data were generated (data not shown). In addition, we used *in vitro* monolayer cultures of NPC, which might differ from 3D cultures like cells-seeded scaffolds or *ex vivo* IVD samples and the combined effects of inflammatory mediators with biomechanical stimuli was not addressed which is the aim for further investigations.

Conclusions

In conclusion, the current study provides evidence that ERK pathway is implicated in NPC inflammatory process and ERK1/2 inhibition could provide some protection against the adverse effect of TNFα.

List Of Abbreviations

18S 18S ribosomal RNA

ACAN Aggrecan

ADAMTS A disintegrin and metalloproteinase with thrombospondin motifs

AF Annulus fibrosus

C/EBPβ CCAAT enhancer binding protein beta

CaCl2 Calcium chloride

COL2A1 Collagen Type II Alpha 1 Chain

COX2 Cyclooxygenase-2

DMEM Dulbecco's Modified Eagle Medium

DNA Deoxyribonucleic acid

ECM Extracellular matrix

ERK Extracellular signal-regulated kinase

FBS Fetal Bovine Serum

IGF1 Insulin-like growth factor 1

IL1β Interleukin 1 beta

IVD Intervertebral disc

KRT19 Cytokeratin 19

mAbs Monoclonal antibodies

MAPK Mitogen-activated protein kinase

MEK Mitogen-activated protein kinase kinase enzyme

MMP3 Matrix metalloproteinase

MRI Magnetic resonance imaging

NaF Sodium fluoride

NF-κB Nuclear factor kappa-light-chain-enhancer of activated B cells

NP Nucleus pulposus

P/S Penicillin/streptomycin

PBS Phosphate-buffered saline

PCR Polymerase chain reaction

PVDF Polyvinylidene fluoride

RNA Ribonucleic acid

TBST Tris-buffered saline Tween

TGFβ1 Transforming growth factor beta 1

TNFα Tumor necrosis factor alpha

Declarations

Ethics approval and consent to participate

The procedure was approved by Ethics Office of the Canton of Bern, Switzerland.

Consent for publication

Written informed consent was obtained from the patients/participants for publication of the manuscript and figures.

Availability of data and materials

Data of this study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

AT designed and conducted the experiments, collected the data, and wrote the manuscript draft. AM performed additional experiments and assisted in statistical analysis. BG prepared the research plan, conducted additional experiments, provided the funding and reviewed the manuscript. All the authors read and approved the final manuscript.

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References

1. Balague F, Mannion AF, Pellise F, Cedraschi C. Non-specific low back pain. Lancet. 2012;379:482-91.
2. Hoy D, March L, Brooks P, Blyth F, Woolf A, Bain C, et al. The global burden of low back pain: estimates from the Global Burden of Disease 2010 study. Ann Rheum Dis. 2014;73:968-74.
3. Fourney DR, Andersson G, Arnold PM, Dettori J, Cahana A, Fehlings MG, et al. Chronic low back pain: a heterogeneous condition with challenges for an evidence-based approach. Spine (Phila Pa 1976). 2011;36:S1-9.

4. Boden SD, Davis DO, Dina TS, Patronas NJ, Wiesel SW. Abnormal magnetic-resonance scans of the lumbar spine in asymptomatic subjects. A prospective investigation. J Bone Joint Surg Am. 1990;72:403-8.
5. Le Maitre CL, Hoyland JA, Freemont AJ. Catabolic cytokine expression in degenerate and herniated human intervertebral discs: IL-1beta and TNFalpha expression profile. Arthritis Res Ther. 2007;9:R77.
6. Qaseem A, Wilt TJ, McLean RM, Forciea MA. Noninvasive Treatments for Acute, Subacute, and Chronic Low Back Pain: A Clinical Practice Guideline From the American College of Physicians. Ann Intern Med. 2017;166:514-30.
7. Savage RA, Whitehouse GH, Roberts N. The relationship between the magnetic resonance imaging appearance of the lumbar spine and low back pain, age and occupation in males. Eur Spine J. 1997;6:106-14.
8. Watkins Rt, Watkins R, 3rd, Hanna R. Non-union rate with stand-alone lateral lumbar interbody fusion. Medicine (Baltimore). 2014;93:e275.
9. Yoshida M, Nakamura T, Sei A, Kikuchi T, Takagi K, Matsukawa A. Intervertebral disc cells produce tumor necrosis factor alpha, interleukin-1beta, and monocyte chemoattractant protein-1 immediately after herniation: an experimental study using a new hernia model. Spine (Phila Pa 1976). 2005;30:55-61.
10. Weiler C, Nerlich AG, Bachmeier BE, Boos N. Expression and distribution of tumor necrosis factor alpha in human lumbar intervertebral discs: a study in surgical specimen and autopsy controls. Spine (Phila Pa 1976). 2005;30:44-53; discussion 4.
11. Bachmeier BE, Nerlich AG, Weiler C, Paesold G, Jochum M, Boos N. Analysis of tissue distribution of TNF-alpha, TNF-alpha-receptors, and the activating TNF-alpha-converting enzyme suggests activation of the TNF-alpha system in the aging intervertebral disc. Ann N Y Acad Sci. 2007;1096:44-54.
12. Risbud MV, Shapiro IM. Role of cytokines in intervertebral disc degeneration: pain and disc content. Nat Rev Rheumatol. 2014;10:44-56.
13. Klooster AR, Bernier SM. Tumor necrosis factor alpha and epidermal growth factor act additively to inhibit matrix gene expression by chondrocyte. Arthritis Res Ther. 2005;7:R127-38.
14. Seguin CA, Bernier SM. TNFalpha suppresses link protein and type II collagen expression in chondrocytes: Role of MEK1/2 and NF-kappaB signaling pathways. J Cell Physiol. 2003;197:356-69.
15. Oh CD, Chang SH, Yoon YM, Lee SJ, Lee YS, Kang SS, et al. Opposing role of mitogen-activated protein kinase subtypes, erk-1/2 and p38, in the regulation of chondrogenesis of mesenchymes. J Biol Chem. 2000;275:5613-9.
16. Pratsinis H, Papadopoulou A, Neidlinger-Wilke C, Brayda-Bruno M, Wilke HJ, Kletsas D. Cyclic tensile stress of human annulus fibrosus cells induces MAPK activation: involvement in proinflammatory gene expression. Osteoarthritis Cartilage. 2016;24:679-87.
17. Wei Y, Zhi-Hong W, Gui-Xing Q, Bin Y, Jun C, Yi-Peng W. Extracellular signal-regulated kinase inhibition modulates rat annulus fibrosus cell response to interleukin-1. Spine (Phila Pa 1976). 2013;38:E1075-

81.

18. Yang X, Wang L, Yuan ZQ, Zhou PH, Chu GL, Li B, et al. Interleukin-1beta induces apoptosis in annulus fibrosus cells through the extracellular signal-regulated kinase pathway. Connect Tissue Res. 2018;59:593-600.
19. Xiao J, Zhang Y, Wang J, Yu W, Wang W, Ma X. Monitoring of cell viability and proliferation in hydrogel-encapsulated system by resazurin assay. Appl Biochem Biotechnol. 2010;162:1996-2007.
20. May RD, Tekari A, Frauchiger DA, Krismer A, Benneker LM, Gantenbein B. Efficient Nonviral Transfection of Primary Intervertebral Disc Cells by Electroporation for Tissue Engineering Application. Tissue Eng Part C Methods. 2017;23:30-7.
21. Thomson S. Failed back surgery syndrome - definition, epidemiology and demographics. Br J Pain. 2013;7:56-9.
22. Skolasky RL, Wegener ST, Maggard AM, Riley LH, 3rd. The impact of reduction of pain after lumbar spine surgery: the relationship between changes in pain and physical function and disability. Spine (Phila Pa 1976). 2014;39:1426-32.
23. Baber Z, Erdek MA. Failed back surgery syndrome: current perspectives. J Pain Res. 2016;9:979-87.
24. Dvorak J, Valach L, Fuhrimann P, Heim E. The outcome of surgery for lumbar disc herniation. II. A 4-17 years' follow-up with emphasis on psychosocial aspects. Spine (Phila Pa 1976). 1988;13:1423-7.
25. Vo NV, Hartman RA, Yurube T, Jacobs LJ, Sowa GA, Kang JD. Expression and regulation of metalloproteinases and their inhibitors in intervertebral disc aging and degeneration. Spine J. 2013;13:331-41.
26. Roughley PJ. Biology of intervertebral disc aging and degeneration: involvement of the extracellular matrix. Spine (Phila Pa 1976). 2004;29:2691-9.
27. Blain EJ. Mechanical regulation of matrix metalloproteinases. Front Biosci. 2007;12:507-27.
28. Podichetty VK. The aging spine: the role of inflammatory mediators in intervertebral disc degeneration. Cell Mol Biol (Noisy-le-grand). 2007;53:4-18.
29. Sainoh T, Orita S, Miyagi M, Inoue G, Kamoda H, Ishikawa T, et al. Single Intradiscal Administration of the Tumor Necrosis Factor-Alpha Inhibitor, Etanercept, for Patients with Discogenic Low Back Pain. Pain Med. 2016;17:40-5.
30. Cohen SP, Wenzell D, Hurley RW, Kurihara C, Buckenmaier CC, 3rd, Griffith S, et al. A double-blind, placebo-controlled, dose-response pilot study evaluating intradiscal etanercept in patients with chronic discogenic low back pain or lumbosacral radiculopathy. Anesthesiology. 2007;107:99-105.
31. Tobinick E, Davoodifar S. Efficacy of etanercept delivered by perispinal administration for chronic back and/or neck disc-related pain: a study of clinical observations in 143 patients. Curr Med Res Opin. 2004;20:1075-85.
32. Genevay S, Finckh A, Mezin F, Tessitore E, Guerne PA. Influence of cytokine inhibitors on concentration and activity of MMP-1 and MMP-3 in disc herniation. Arthritis Res Ther. 2009;11:R169.

33. Slevin SM, Egan LJ. New Insights into the Mechanisms of Action of Anti-Tumor Necrosis Factor-alpha Monoclonal Antibodies in Inflammatory Bowel Disease. Inflamm Bowel Dis. 2015;21:2909-20.
34. Autio RA, Karppinen J, Niinimaki J, Ojala R, Veeger N, Korhonen T, et al. The effect of infliximab, a monoclonal antibody against TNF-alpha, on disc herniation resorption: a randomized controlled study. Spine (Phila Pa 1976). 2006;31:2641-5.
35. Zhang W, Liu HT. MAPK signal pathways in the regulation of cell proliferation in mammalian cells. Cell Res. 2002;12:9-18.
36. Hiyama A, Hiraishi S, Sakai D, Mochida J. CCAAT/enhancer binding protein beta regulates the expression of tumor necrosis factor-alpha in the nucleus pulposus cells. J Orthop Res. 2016;34:865-75.
37. Hayashida M, Okazaki K, Fukushi J, Sakamoto A, Iwamoto Y. CCAAT/enhancer binding protein beta mediates expression of matrix metalloproteinase 13 in human articular chondrocytes in inflammatory arthritis. Arthritis Rheum. 2009;60:708-16.
38. Tsushima H, Okazaki K, Hayashida M, Ushijima T, Iwamoto Y. CCAAT/enhancer binding protein beta regulates expression of matrix metalloproteinase-3 in arthritis. Ann Rheum Dis. 2012;71:99-107.
39. Yang H, Gao F, Li X, Wang J, Liu H, Zheng Z. TGF-beta1 antagonizes TNF-alpha induced up-regulation of matrix metalloproteinase 3 in nucleus pulposus cells: role of the ERK1/2 pathway. Connect Tissue Res. 2015;56:461-8.
40. Jobin PG, Butler GS, Overall CM. New intracellular activities of matrix metalloproteinases shine in the moonlight. Biochim Biophys Acta Mol Cell Res. 2017;1864:2043-55.
41. Solli AI, Fadnes B, Winberg JO, Uhlin-Hansen L, Hadler-Olsen E. Tissue- and cell-specific co-localization of intracellular gelatinolytic activity and matrix metalloproteinase 2. J Histochem Cytochem. 2013;61:444-61.
42. Sariahmetoglu M, Crawford BD, Leon H, Sawicka J, Li L, Ballermann BJ, et al. Regulation of matrix metalloproteinase-2 (MMP-2) activity by phosphorylation. FASEB J. 2007;21:2486-95.
43. Toth M, Fridman R. Assessment of Gelatinases (MMP-2 and MMP-9 by Gelatin Zymography. Methods Mol Med. 2001;57:163-74.
44. Kao TH, Peng YJ, Salter DM, Lee HS. Nerve growth factor increases MMP9 activity in annulus fibrosus cells by upregulating lipocalin 2 expression. Eur Spine J. 2015;24:1959-68.
45. Seguin CA, Bojarski M, Pilliar RM, Roughley PJ, Kandel RA. Differential regulation of matrix degrading enzymes in a TNFalpha-induced model of nucleus pulposus tissue degeneration. Matrix Biol. 2006;25:409-18.
46. Tian Y, Yuan W, Fujita N, Wang J, Wang H, Shapiro IM, et al. Inflammatory cytokines associated with degenerative disc disease control aggrecanase-1 (ADAMTS-4) expression in nucleus pulposus cells through MAPK and NF-kappaB. Am J Pathol. 2013;182:2310-21.

Figures

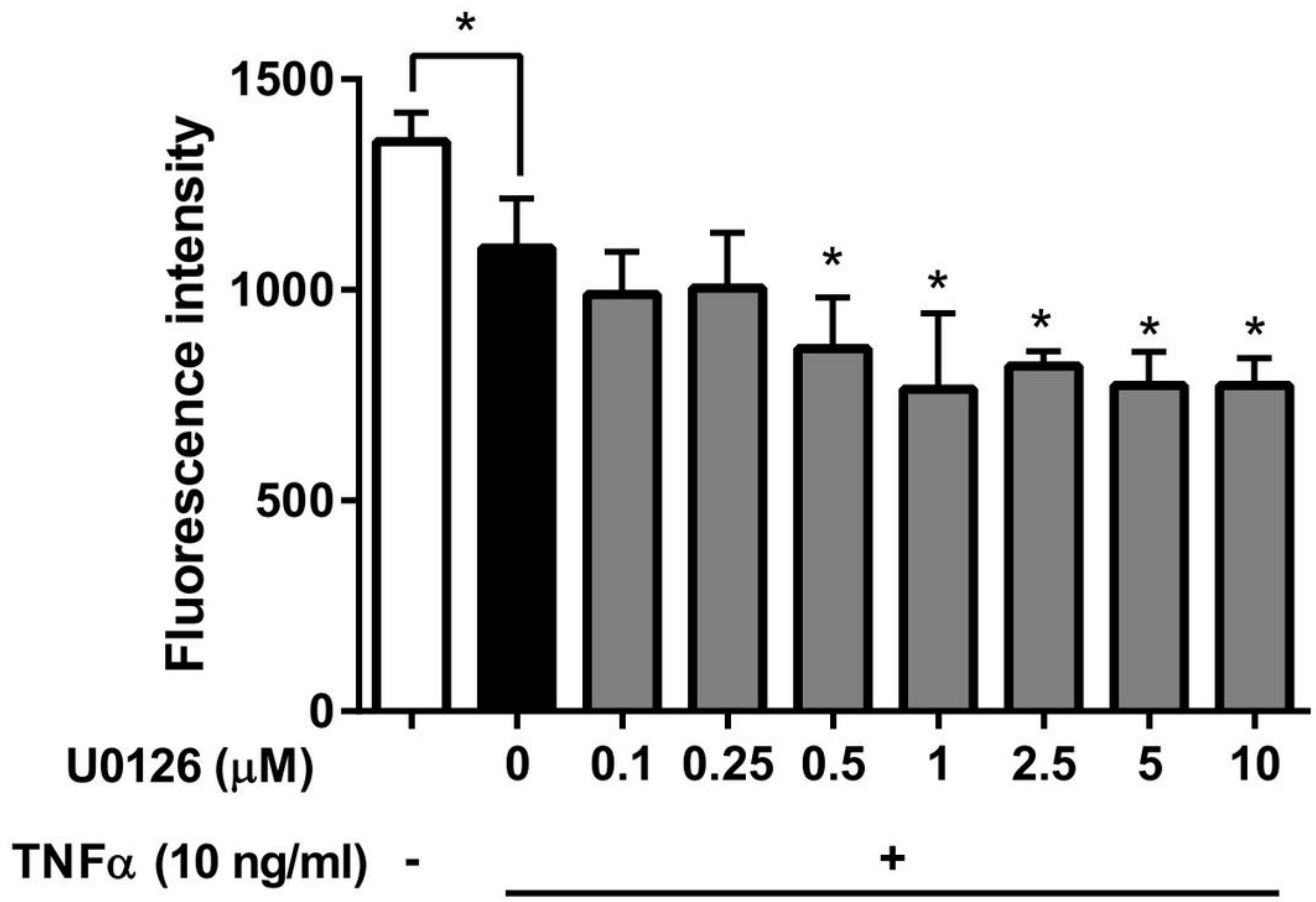

Figure 1

Cell activity following treatment of NPC with TNFα and different concentrations of U0126 after 2 h of incubation with resazurin sodium salt assay. A dose-dependent reduction in NPC cell activity with increasing U0126 concentrations was observed. *$p < 0.05$ as compared to positive control (TNFα treatment) and bars represent average values of N = 5, biological triplicate with standard deviations.

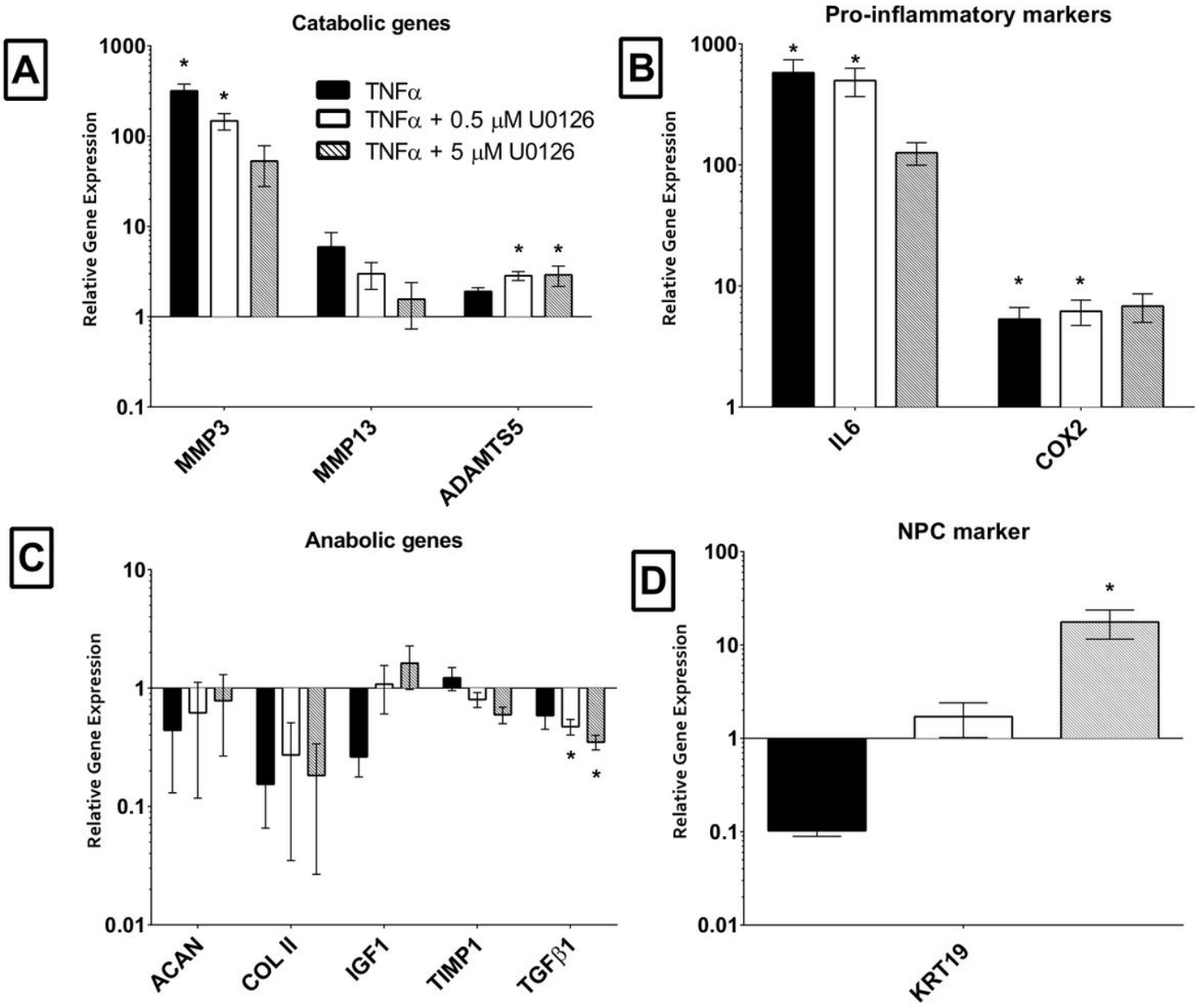

Figure 2

Modulation of human NPC transcript levels induced by TNFα (10 ng/ml) with or without ERK inhibition (0.5 μM or 5 μM of U0126). The change of catabolic genes (A), pro-inflammatory markers (B), anabolic genes (C), and NPC marker (D). Control values (without treatment) were set as 1 and different levels were normalized to their respective controls. Bars are average values with standard deviations (N = 6, duplicate). *p < 0.05 as compared to untreated controls.

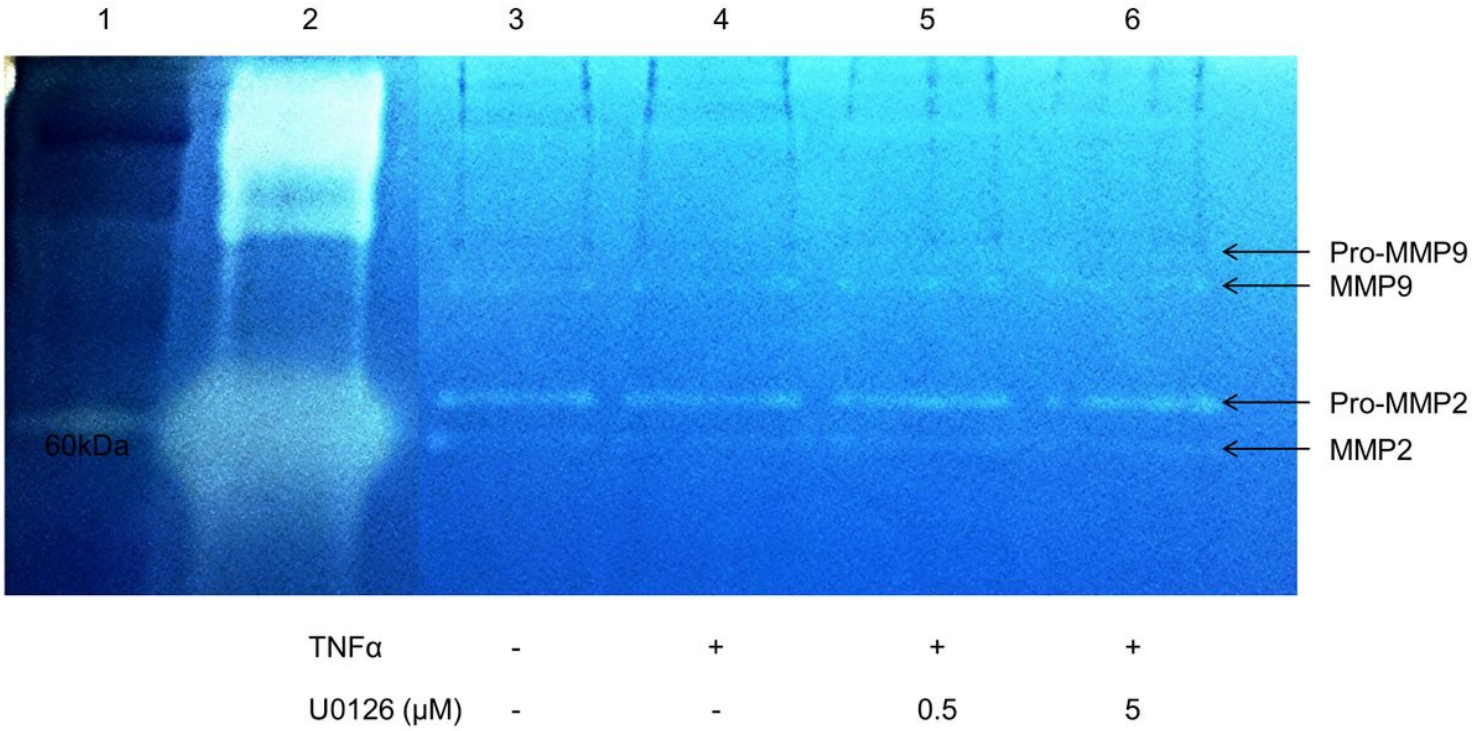

Figure 3

Gel zymography for the detection of the enzymatic activity of MMP2/MMP9 in NPC. Lane 1: ladder with a visible band being 60 kDa, lane 2: MMP2 standard at 100 ng/ml, lane 3-6: treatment groups of NPC with or without TNFα (10 ng/ml) and U0126. Pro- and active MMP2/9 are indicated by arrows on the gels.

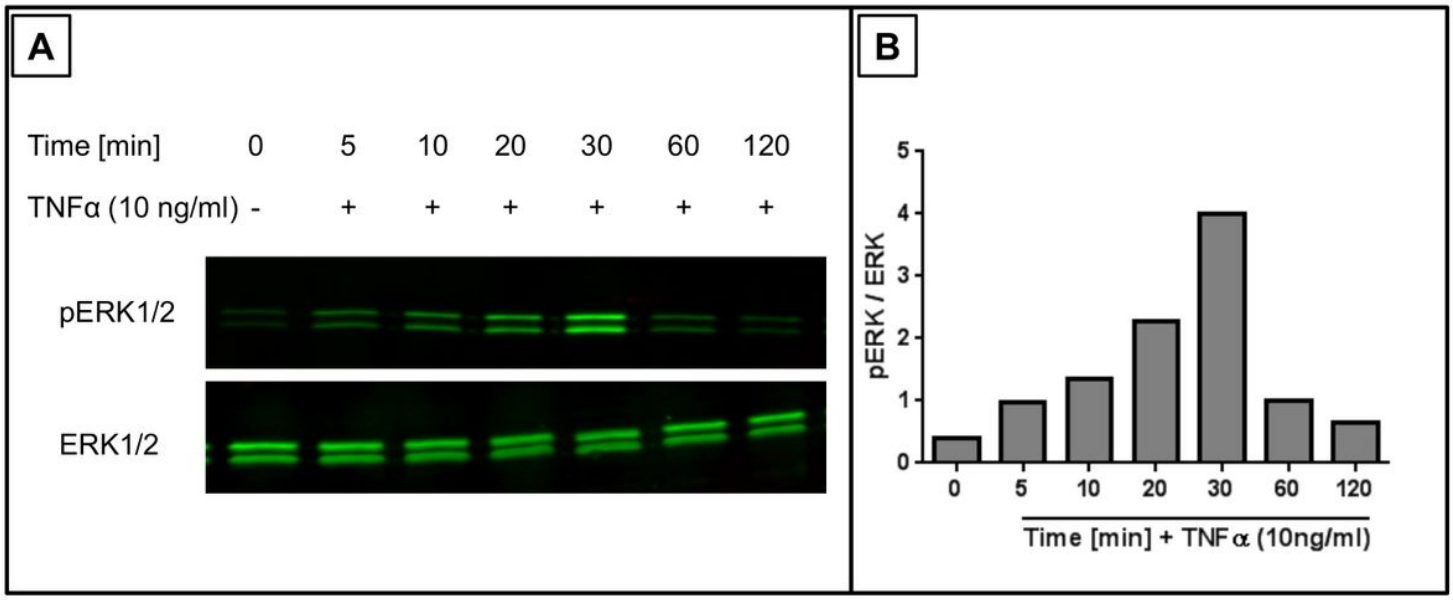

Figure 4

Western blot of phosphorylated ERK1/2 (p-ERK) and their respective total ERK1/2 in NPC after TNFα stimulation. The NPC were stimulated with TNFα (10 ng/ml) and the cells lysates were collected at

different time points (minutes until 2h) with control being time 0 min before stimulation (A). p-ERK and total ERK were determined using western blot, the signal intensities were quantified, and the p-ERK were normalized to total ERK (B).

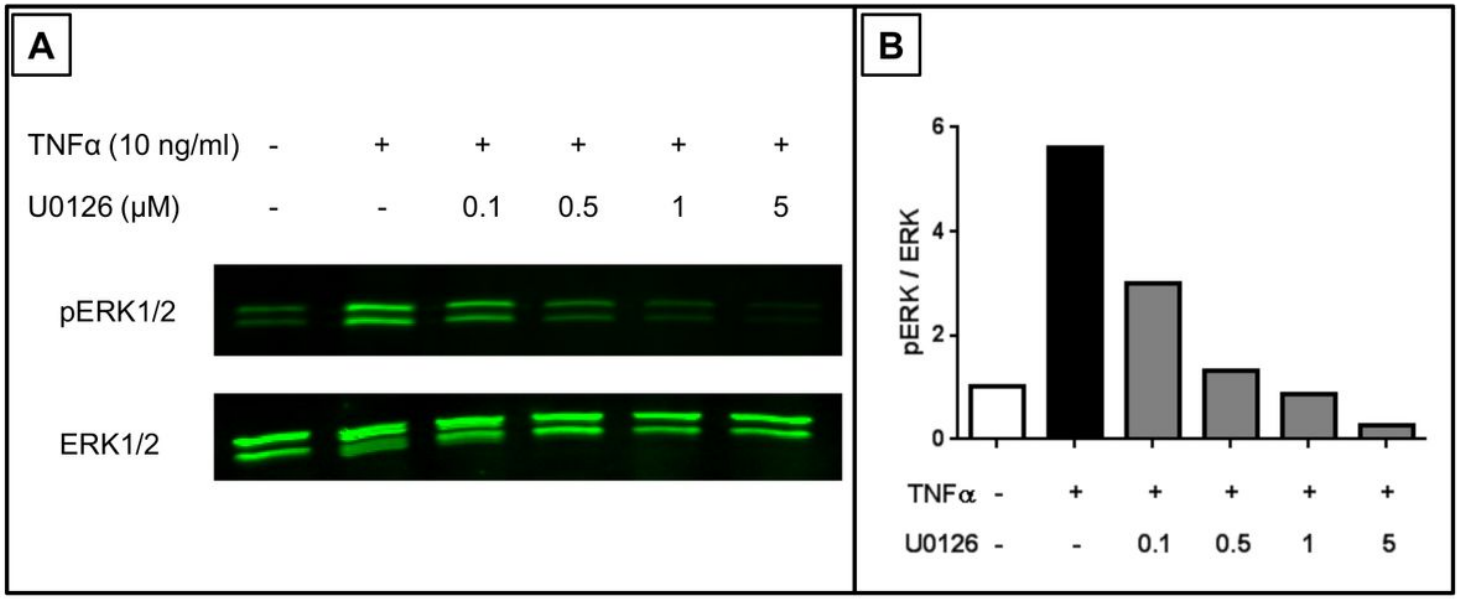

Figure 5

Western blot of phosphorylated ERK1/2 (p-ERK) and their respective total ERK1/2 in TNFα-stimulated NPC with or without ERK inhibition. The ERK pathway in NPC was blocked with U0126 (0.1, 0.5, 1 and 5μM) for 1h prior TNFα (10 ng/ml) treatment. (A) The cells were stimulated with TNFα and the cells lysates were collected 30 min of treatments. The negative control being the unstimulated cells (lane 1), the positive control being the TNFα-alone treated NPC (lane 2), and the treatments being simultaneous TNFα and U0126-treated samples (lane 3-6). (B) The p-ERK/ERK ratios were quantified in the different NPC samples.