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TNF-α and IL-1β Dependent Induction of CCL3 Expression by Nucleus Pulposus Cells Promotes Macrophage Migration through CCR1

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Abstract

Objective—To investigate TNF-α and IL-1β regulation of CCL3 expression in nucleus pulposus (NP) cells and in macrophage migration.

Methods—qRT-PCR and immunohistochemistry were used to measure CCL3 expression in NP cells. Transfections were used to determine the role of NF-κB, C/EBP-β and MAPK on cytokine mediated CCL3 promoter activity. Effect of NP-conditioned medium on macrophage migration was measured using a transwell system.

Results—*An increase* in CCL3 expression and promoter activity was observed in NP cells after TNF-α or IL-1β treatment. Treatment of cells with NF-κB and MAPK inhibitors abolished the effect of the cytokines on CCL3 expression. The inductive effect of p65 and C/EBP-β on CCL3 promoter was confirmed through gain- and loss-of-function studies. Noteworthy, co-transfection of p50 completely blocked cytokine and p65 dependent induction. In contrast, c-Rel and RelB had little effect on promoter activity. Lentiviral transduction with Sh-p65 and Sh-Ikkβ significantly decreased TNF-α dependent increase in CCL3 expression. Analysis of degenerate human NP tissues showed that CCL3, but not CCL4 expression correlated positively with the grade of tissue degeneration. Importantly, treatment of macrophages with conditioned medium of NP cells treated with TNF-α or IL-1β promoted their migration; pretreatment of macrophages with antagonist to CCR1, primary receptor for CCL3 and CCL4, blocked cytokine mediated migration.

Conclusions—By controlling the activation of MAPK, NF-κB and C/EBPβ signaling, TNF-α and IL-1β modulate the expression of CCL3 in NP cells. The CCL3-CCR1 axis may play an important role in promoting macrophage infiltration in degenerate, herniated discs.

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INTRODUCTION

The intervertebral disc (IVD) is a unique tissue that that permits rotation, as well as flexion and extension of the spine. It consists of a gel-like nucleus pulposus (NP) surrounded circumferentially by a fibrocartilagenous annulus fibrosus (AF). NP cells secrete a complex extracellular matrix that contains fibrillar collagens and the proteoglycan aggrecan. The initial phase of disc degeneration is characterized by increased expression of catabolic enzymes, decreased proteoglycan synthesis, and an overall shift towards synthesis of a fibrotic matrix and events that compromise the structural integrity of the tissue (1–4). Structural failure of the NP and AF lead to herniation of NP tissue that is often followed by an inflammatory phase, characterized by invasion of immune cells in the tissues (2, 5, 6).

It has been reported that during degeneration, resident NP and AF cells produce high levels of the cytokines TNF-α and IL-1β (7, 8). These cytokines stimulate production of NGF, BDNF and VEGF, molecules associated with nerve ingrowth into the NP and angiogenesis (9). Moreover, in response to high cytokine levels, disc cells also produce chemoattractive proteins such as MCP-1 and IL-8 (10). However, mechanisms that control expression of these chemokines during disc degeneration have received little attention.

Chemokines and their receptors have been shown to be involved in many inflammatory diseases including rheumatoid arthritis (RA) and osteoarthritis (11, 12). Of chemokine receptors, C-C chemokine receptor 1 (CCR1) is directly linked to the pathogenesis of RA. Moreover, a recent study showed inflammatory cytokine IL-1β induced the expression of CCL3 and CCL4 in human chondrocytes (13). High levels of CCR1-expressing macrophages and chemokines CCL3 and CCL4 have been identified in RA synovial fluid and tissues (14–17). *In-vitro* migration studies have shown that CCR1-mediated monocyte migration induced by RA synovial fluid can be blocked with either a CCR1 blocking antibody or a small molecule CCR1 antagonist (18). A clinical study using a specific CCR1 antagonist in patients with RA has confirmed the potential of this approach (15).

While CCL3 has been reported to be expressed in herniated intervertebral discs (10), it was noted that reactivity was associated with fibroblasts, endothelial cells and infiltrating macrophages in the granulation tissues. Aside from this study, little is known about the expression and regulation of CCL3 in NP cells during disc degeneration. Since disc cells are known to mount a robust inflammatory response, we advance the notion that secretion of chemokines such as CCL3 by NP cells in response to inflammatory cytokines promotes tissue infiltration of macrophages and T cells. Herein, we show for the first time that in NP cells TNF- α as well as IL-1β control CCL3 transcription in MAPK, NF-κB and CEBP/β dependent fashion. Importantly, our results show that CCL3, through its receptor CCR1, may play an important role in promoting the cytokine dependent migration of macrophages into the disc and exacerbation of the inflammatory state.

EXPERIMENTAL PROCEDURES

Reagents and Plasmids

Human CCL3 promoter constructs were a kind gift from Dr. Linda Sandell, Washington University, St. Louis. pCMX-IκBM (catalog #12330), RelA/p65-cFLAG-pcDNA3 (#20012), p50-cFLAG-pcDNA3 (#20018) from Dr. Inder Verma, pCMV-FLAG-LAP2 (15738), pCMV-FLAG-LIP (15737) from Dr. Joan Massague, psPAX2 (# 12260) and pMD2G (#12259) from Dr. Didier Trono, RelB-cFlag-pcDNA3 (#20017) and c-Rel-cFlag-pcDNA3 (#20013) were obtained from Addgene repository. Plasmids Sh-p65 and Sh-Ikkβ in lentiviral FSVsi vector that co-expresses YFP were kindly provided by Dr. Andree Yeremian, Univeristy of Lleida, Spain. As an internal transfection control, vector pRL-TK

(Promega) containing *Renilla reniformis* luciferase gene was used. Transfection methodology has been optimized for rat NP cells (19). Wild type and p65 null cells were a kind gift from Dr. Denis Guttridge, University of Ohio, Columbus. Antibodies against p-p38, p38, ERK1/2 and pERK1/2 were from Cell Signaling. Antibodies against CCL3, CCL4 and CD11b were from abcam, Cambridge, UK. β-tubulin was from DSHB, University of Iowa and GAPDH was from Novus Biologicals. TNF-α and IL-1β were purchased from Peprotech, NJ.

Harvesting rat disc tissues, isolation of NP cells and treatments

NP and AF tissues and NP cells were isolated from adult Wistar rats (350 g) using a method reported earlier by Risbud *et al.* (19). NP cells were maintained in Dulbecco's Modified Eagles Medium (DMEM) and 10% FBS supplemented with antibiotics. To investigate effect of cytokines, cells were treated with IL-1β (5–20 ng/ml) and TNF-α (25–100 ng/ml) for 24 h.

Human tissue collection and grading

Human lumbar IVD tissue was obtained either at surgery or post-mortem (PM) examination with informed consent of the patient or relatives (Sheffield Research Ethics Committee # 09/H1308/70). Eight PM IVDs were recovered from 2 donors. They consisted of intact IVDs within the complete motion segment from which the IVDs were removed. Thrity one surgical IVD tissue samples were obtained from patients undergoing micro-discectomy procedures for the treatment of low back pain and root pain as caused by prolapse of the IVD (see Suppl. Table 1 for sample details). NP tissue was divided into two, one half was fixed in 10% neutral buffered formalin and processed for histological and immunohistochemical examination. The remaining tissue was used for RNA isolation. H&E stained sections were used to score the degree of morphological degeneration according to previously published criteria (7). Briefly, tissue sections were scored for the presence of cell clusters, the presence of fissures, loss of demarcation and loss of haematoxophilia. A score of 0 – 3 indicates a histologically normal (non-degenerate) IVD and a grade of 4 – 12 indicates evidence of degeneration. Tissue samples were also examined for the presence of infiltrating cells based on both cell morphology and CD11b staining, multiple sections throughout each tissue block were examined for infiltrating cells, and classed as infiltrating if these were observed on any section. Gene expression study samples were classified as non-degenerate (0–3), degenerate but free of infiltrating cells (4+) or 'Infiltrated' based on histological examination. Grading was performed independently by two researchers and grades averaged.

Quantitative Real-Time RT-PCR

Human tissues were processed as described previously. Extracted RNA was subjected to treatment with DNase (Qiagen, Crawley, UK) and purified using Qiagen MinElute Cleanup kit prior to cDNA synthesis using MMLV Reverse Transcriptase (Bioline, London, UK) and random hexamers. Real-time PCR analysis was performed using pre-designed, FAM-labeled Taqman® Gene Expression Assays (Applied Biosystems). A total of 35 IVDs were used for this component of the study (Supplementary table 1); 6 non-degenerate (2 post-mortem and 4 surgical samples): 23–45 years, mean age 37 years, 16 degenerate (16 surgical samples): 25–52 years, mean age 39 years and 13 infiltrated (13 surgical samples): 20–63 years, mean age 38 years. For rat tissues or cultured NP cells, 1–2 μg of total DNA free-RNA was used to synthesize cDNA using SuperScript III cDNA synthesis kit (Invitrogen). Reactions were set up in triplicate in 96 well plate using cDNA and appropriate PCR Master Mix (Applied Biosystems). PCR reactions were performed in a StepOnePlus real-time PCR system (Applied Biosystems) according to the manufacturer's instructions. Human pre-designed primer probe mixes (GAPDH, 18s, CCL3 and CCL4) were purchased from Applied

Biosystems (Warrington, UK), rat gene primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA).

Transfections and dual luciferase assay

Rat NP cells were transferred to 48-well plates at a density of 2×10^4 cells/well one day before transfection. To investigate the effect of NF-κB and C/EBP-β/LAP2 on CCL3 promoter activity, cells were cotransfected with 50–150 ng of IκBM or p65 or p50 or p65 plus p50 or RelB or cRel or LAP2 or dominant-negative (DN) – C/EBP-β/LIP with or without appropriate backbone vector and 175 ng CCL3 reporter and 175 ng pRL-TK plasmid. In some experiments, cells were transfected with 250 ng of CCL3 reporter with 250 ng pRL-TK and treated with the inhibitors for NF-kB: SM7368 (10 μM), p38: SB203580 (10μM) or ERK: PD98059 (10 μM) or JNK: SP60025 (10 μM) (Calbiochem) in presence of absence of TNF-α or IL-1β. Lipofectamine 2000 (Invitrogen) was used as a transfection reagent. 48 h after trasfection, the cells were harvested and a Dual-LuciferaseTM reporter assay system (Promega) was used for sequential measurements of firefly and Renilla luciferase activities. At least three independent transfections were performed, and all analyses were carried out in triplicate.

Lentiviral particle production and viral transduction

HEK 293T cells were seeded in 10 cm plates (1.3×10^6 cells/plate) in DMEM with 10% heat-inactivated FBS two days before transfection. Cells were transfected with 2.5 μg of Sh-Ctr or Sh-p65 or Sh-IKKβ plasmids along with 1.875 μg psPAX2 and 0.625 μg pMD2.G. After 16 h, transfection media was removed and replaced with DMEM with 5% heat-inactivated FBS and penicillin-streptomycin. Lentiviral particles were harvested at 48 and 60 h post-transfection. NP cells were plated in DMEM with 5% heat-inactivated FBS one day before transduction. Cells in 10 cm^2 plates were transduced with 5 ml of conditioned media containing viral particles along with 6 μg/ml polybrene. After 24 h, conditioned media was removed and replaced with DMEM with 5% heat-inactivated FBS. Cells were harvested for protein extraction 5 days after viral transduction.

Protein extraction and Western blotting

Cells were placed on ice immediately following treatment and washed with ice-cold HBSS. All wash buffers and final re-suspension buffer included 1X protease inhibitor cocktail (Pierce, IL), NaF (5 mM) and Na_3VO_4 (200 μM). Total cell proteins were resolved on 8–12% SDS-polyacrylamide gels and transferred by electroblotting to PVDF membranes (Bio-Rad, CA). The membranes were blocked with 5% non-fat dry milk in TBST (50 mM Tris, pH 7.6, 150 mM NaCl, 0.1% tween 20) and incubated overnight at 4 °C in 3% non-fat dry milk in TBST with the p38, Pp38, ERK1/2 and pERK1/2 (1:1000, Cell Signaling), anti-β-tubulin (1:3000, Developmental Studies Hybridoma Bank). Immunolabeling was detected using the ECL reagent (Amersham Biosciences).

Immunohistochemical analysis

Immunohistochemistry was used to confirm and localize production of CCL3 and CCL4 in 30 IVDs; 8 PM and 22 surgical samples (Supplementary Table 1), 25 – 66 years, mean age 48 years. 4 μm tissue sections were dewaxed, rehydrated, endogenous peroxidases quenched and following heat antigen retrieval, blocked in goat serum. Sections were incubated overnight at 4°C with rabbit polyclonal antibodies against human CCL3 (1:4000), CCL4 (1:2000) (Abcam) and CD11b (1:50) (Abcam). Pre-immune rabbit IgG (Abcam) was used as a negative control. After washing, sections were incubated with biotinylated goat anti-rabbit antiserum (1:500 dilution; Abcam) and binding detected by the formation of streptavadin-biotin complex (Vector Laboratories, Peterborough, UK) with 3,3'-diaminobenzidine

tetrahydrochloride solution (Sigma-Aldrich). Sections were counterstained with Mayers Haematoxylin (Leica Microsystems, Milton Keynes, UK), dehydrated, cleared and mounted in Pertex (Leica Microsystems). Sections were visualized images captured using an Olympus BX60 microscope and QCapture Pro v8.0 software (MediaCybernetics, Marlow, UK). A total of 200 NP cells were counted in each section and the number of immuno-positive cells expressed as a percentage of total count. Linear regression analysis was performed to investigate correlation between measured percentage immuno-positivity and histological grade of degeneration.

Collection of conditioned medium and Cell migration assay

NP cells cultured in 6 cm plates (5×10^5/ plate) were treated with TNF-α (50 ng/ml) or IL-1β (10 ng/ml) for 24 h in Serum-free medium. Following the cytokine treatment, conditioned medium (CM) was collected, centrifuged at 2000 rpm for 10 min to remove any cells and debris and used immediately for cell migration assays. Macrophage migration was measured using 24-well cell culture insert system (VWR Scientific), in which the top and bottom compartments were separated by a filter with 8-μm pores. RAW264.7 cells were plated in the upper chamber of 24-well plates inserts at a density of 2×10^5 cells/well. The next day, RAW264.7 cells were exposed to control medium or CM from NP cells that were treated for 24 h with or without TNF-α or IL-1β in presence or absence of CCR1 antagonist J-113863, (Tocris Bioscience). After 24 h, RAW264.7 cells were removed from the upper side of membranes and cells were measured on the lower side of the membrane and in bottom chamber by MTT assay to determine the migration rate.

Statistical analysis

All measurements were performed in triplicate, data is presented as mean + S.E. Differences between groups were analyzed by Student's t-test and ANOVA; *p< 0.05. RT- PCR expression data of human tissues was found to be non-parametric in distribution and so the Kruskal-Wallis test combined with post-hoc analysis by Conover-Inman test was used to determine significance between $2^{-\Delta CT}$ values from non-degenerate, degenerate and infiltrated samples. Two-sample proportion test was used to determine significance between the proportions of samples in each study group exhibiting expression of each target gene based only on whether or not expression was observed.

RESULTS

Expression of CCL3 is regulated by TNF-α and IL-1β in NP cells

Expression of CCL3 in mature rat tissues was studied using real-time PCR analysis. The basal expression of CCL3 mRNA in healthy mature NP as well as AF tissue is very low (Fig. 1A). To investigate if cytokines regulate CCL3 expression, NP cells were treated with TNF-α or IL-1β. Figure 1B and C indicates that treatment with TNF-α or IL-1β results in a peak increase in CCL3 mRNA expression at 4 h although expression stays significantly elevated compared to untreated control at 24 h. A similar, but a smaller increase in CCL4 expression was also seen when cells were treated by both of the cytokines (S. Fig. 1). To investigate if the CCL3 regulation is at the transcriptional level, we measured the cytokine dependent activity of 1.4 kb CCL3 promoter (Fig. 1D). CCL3 promoter contains three putative NF-κB binding motifs (20); the sequence, their location in the promoter relative to transcription start site, and relative JASPAR database score is as follows: AAAATTTCCC; −80/−71 bp; 78, GGGACTGACT;−289/−280 bp; 76.4, GGGAAATCAA; −1300/1291 bp; 76.1. In addition, several C/EBP-β binding sites are present in the promoter. Figure 1E and F shows that cytokines significantly increase promoter activity. Moreover, IL-1β dependent expression is dose dependent (Fig. 1F). The shorter 300 bp and 140 bp promoter fragments also evidenced induction in activity following cytokine treatment (Fig. 1G).

TNF-α and IL-1β promote CCL3 expression by activating NF-κB signaling

To ascertain if cytokine-induced CCL3 expression requires NF-κB signaling, NP cells were pretreated with the inhibitor SM7368. Pretreatment causes a significant suppression in TNF-α or IL-1β induction of CCL3 mRNA levels (Fig. 2A, B). When cells are treated with the NF-κB inhibitor SM7368 (Fig. 2C, D) or co-transfected with DN-NF-κB/IκBα (Fig. 2E, F), cytokine mediated induction in CCL3 promoter activity is completely abolished. In contrast, co-transfection with p65 results in a dose-dependent increase in CCL3 promoter activity (Fig. 3A). Surprisingly, co-transfection with p50 blocks the inductive effect of p65 on CCL3 promoter activity (Fig. 3B), addition of p50 alone has a small suppressive effect on promoter activity (Fig. 3B). Noteworthy, Fig. 3C and D show that p50 completely suppresses the inductive effect of cytokines on CCL3 promoter. We also determined if NF-κB subunits, RelB and cRel control CCL3 expression. Neither RelB (Fig. 3E) nor c-Rel (Fig. 3F) had a significant effect on CCL3 promoter activity. To determine if RelA controlled CCL3 promoter activity in a cell type specific manner, we measured CCL3 promoter activity in p65 wild type and null fibroblasts. Only in the wild type cells is the promoter activity cytokine inducible (Fig. 3G, H).

To further validate the role of p65/RelA in controlling CCL3 expression, we silenced the expression of p65 and its upstream controller Ikkβ and measured CCL3 expression in human NP cells. Silencing was achieved using lentivirus co-expressing YFP and p65-ShRNA or Ikkβ-ShRNA. Figure 4A shows that there is a robust YFP expression by the virally transduced cells, indicating a high level of transduction efficiency and transgene expression. As expected, in cells transduced with Sh-p65 and Sh-Ikkβ there is a significant decrease in the expression of p65 and Ikkβ respectively compared to cells transduced with control ShRNA (Fig. 4B) Suppression of individual NF-κB signaling components blocks the inductive effect of TNF-α on CCL3 mRNA expression (Fig. 4C).

C/EBP-β and MAPK signaling control CCL3 promoter activity in NP cells

We then examined if CCAAT enhancer-binding protein beta (C/EBP-β)/LAP2 controls cytokine dependent CCL3 expression in NP cells. We transfected NP cells with liver-enriched inhibitory protein (LIP), a functional LAP antagonist and measured cytokine dependent CCL3 promoter activity. Suppression of C/EBP-β function prevents TNF-α (Fig. 5A) or IL-1β (Fig. 5B) induction in promoter activity. Similarly, LIP causes a robust decrease in basal CCL3 promoter activity (Fig. 5C). On the other hand, co-transfection with LAP2 results in induction of promoter activity in the absence of exogenously added cytokines (Fig. 5D).

Since MAPK signaling controls the activity of both NF-κB and C/EBP-β, we determined if it is required for the cytokine dependent induction of CCL3 in NP cells. We first evaluated activation of p38 and ERK signaling pathway by the cytokines. TNF-α (Fig. 5E) or IL-1β (Fig. 5F) treatment results in a rapid increase in p-p38 and pERK1/2 (not shown). Pretreatment of NP cells with p38 and ERK inhibitors causes a significant suppression in TNF-α or IL-1β induction of CCL3 mRNA (Fig. 5G, H). In contrast, JNK inhibition selectively suppresses IL-1β response (Fig. 5H) but has a minimal effect on TNF-α (Fig. 5H). We then measured the effect of the p38 inhibitor on cytokine dependent CCL3 promoter activity. Again inhibitor treatment results in significant suppression in the IL-1β mediated induction of CCL3 promoter activity (Fig. 5I)

CCL3 and CCL4 expression is elevated in degenerate human intervertebral discs

Figure 6A shows that the CCL3 gene is expressed in 2 of 6 non-degenerate (0–3) samples, 8 of 16 degenerate (4+) samples and 10 of 13 infiltrated/herniated samples (Fig. 6A). The proportion of infiltrated samples expressing CCL3 is increased when compared to the

proportion of non-degenerate samples ($p<0.05$), but not significantly altered when compared to the degenerate (4+) group ($p>0.05$). Infiltrated samples are observed to express greater levels of CCL3 when compared to degenerate samples ($p=0.0383$), but are not significantly altered when compared to the non-degenerate (0–3) group ($P>0.05$). In the same study we also examined the expression of CCL4. Like CCL3, we found that CCL4 is present in 2 of 6 non-degenerate (0–3) samples, 5 of 16 degenerate (4+) samples and 9 of 13 infiltrated samples. Thus there was a significant increase in the proportion of disc samples containing infiltrated cells expressing CCL4 when compared to the degenerate (4+) group ($p<0.05$), but not when compared to the non-degenerate group (0–3) ($p>0.05$). No significant difference in the level of CCL4 gene expression is observed between study groups. Overall, CCL4 was expressed in 16 IVD samples in concert with CCL3. Only in 4 IVD samples was the CCL3 gene expressed independently of CCL4.

We performed immunohistochemical staining to further analyze chemokine expression and localization in disc tissues (Fig. 6B, C). CCL3 and CCL4 staining is localized to NP cells within the IVD (Fig. 6C). Percentage immunopositivity for CCL3 was seen to correlate positively with the grade of tissue degeneration ($P=0.0171$). No correlation between CCL4 immunopositivity and grade of tissue degeneration was observed ($P=0.4175$) (Fig. 6B). Identity of infiltrating cells was also determined by immunostaining for CD11b. A large proportion of infiltrating cells were smaller in size (10–20 μm) and were localized as single cells at higher cell density (Fig. 6D) compared to the larger resident disc cells (20–60 μm) which were observed within lacuna either singly or in clusters, these smaller infiltrating cells together with some larger macrophage like cells stained positive for CD11b (Fig. 6E), whereas resident disc cells within lacuna were CD11b negative (Fig. 6E).

TNF-α and IL-1β promote NP-mediated migration of macrophages in CCR1 dependent fashion

We examined the effect of conditioned medium (CM) on NP cells treated with cytokines on macrophage migration. Figure 6E shows that CM of NP cells treated with TNF-α or IL-1β promotes chemotactic migration of macrophages compared to CM of untreated NP cells. Moreover, cytokine dependent macrophage migration is completely blocked when pretreated with J113863, a well-characterized antagonist of CCR1.

DISCUSSION

This investigation demonstrated for the first time that NP cells expressed both CCL3 and CCL4, and that expression of CCL3 was regulated by the inflammatory cytokines TNF-α and IL-1β through MAPK, NF-κB/p65 and C/EBP-β signaling pathways. We showed that in contrast to p65, NF-κB1/p50 was inhibitory to CCL3 expression. A second major observation was that by regulating CCL3 expression by NP cells, inflammatory cytokines promoted CCR1 dependent macrophage migration. Importantly, in terms of clinical relevance, analysis of human tissues indicated that the extent of CCL3 expression levels correlated positively with the grade of degeneration and that expression levels were higher in herniated tissues than degenerate but contained samples. Based on these observations we predict that if CCR1-CCL3 activity is blocked using therapeutic agents, then macrophage infiltration in NP and the associated inflammatory response into the herniated disc would be limited.

Expression studies showed that CCL3 was expressed in both human and rat NP tissues. Given the ability of normal NP cells to produce several cytokines and chemokines, it was not surprising to find that NP cells produced baseline levels of CCL3. The low level of expression in the rat tissues probably reflects the healthy state of the NP and suggests that CCL3 may have physiological functions other than chemotaxis. However, our investigation

clearly indicated that expression was induced by TNF-α as well as IL-1β, cytokines closely linked to degenerative disc disease. These results are in line with previous reports that showed that CCL3 expression is sensitive to cytokines including TNF-α and IL-1β (21–23). Moreover, promoter studies showed that regulation was at the transcript level and that the first 140 bases are sufficient to drive cytokine dependent transcription.

Although the mechanism of regulation is likely to be cell type and context specific, there is some evidence to indicate that CCL3 transcription may be controlled by NF-κB, C/EBP-β and MAPK signaling (13, 24). The presence of three putative κB motifs in the CCL3 promoter indicated that it was functionally involved in controlling transcription: Zhang *et al.* suggested that the first 300 bases of the promoter may control its activity (13). Noteworthy, the two κB motifs with the highest relative scores obtained using JASPAR database are contained within this region. Related to this finding, a recent study has also shown the presence of c-Rel binding sites in the CCL3 promoter and that IL-1β caused a higher induction in c-Rel mRNA than p65 in chondrocytes (13). Thus, our observation that the inductive effect is restricted to p65 and not RelB and c-Rel, further highlight the unique response of NP cells to environmental stimuli.

Another unique observation concerned the role of p50/RelA heterodimers. Lim and colleagues have shown that LPS causes a rapid recruitment of p50/RelA heterodimer along with E2F1 to the CCL3 promoter thereby enhancing gene transcription (24). In contrast to those studies, we found that in NP cells p50 not only suppressed the inductive effect of cytokines, but inhibited the activation of CCL3 promoter by p65. This result was unexpected, as our own recent studies have shown that p65 and p50 act synergistically to induce the expression of syndecan-4, one of the target genes of TNF-α and IL-1β in the NP cells (25). However, our findings are in line with previous studies that demonstrate repressive function of p50 homodimers in controlling expression of a number of chemokines and catabolic genes including CCL2, CxCL10, GM-CSF, and MMP-13 (26–28). Thus, formation of p50 homodimers and their binding to the κB motifs could promote recruitment of a transcriptional repressor, such as HDAC1, to the CCL3 promoter thereby suppressing RelA response (26). That RelA/p65 signaling modulated promoter function was supported by the observation that RelA null cells failed to induce CCL3 promoter activity, even when treated with cytokines. Moreover, the silencing studies that showed that there was inhibition of TNF-α dependent CCL3 expression following suppression of p65 and IKKβ signaling highlighted the importance of this pathway in controlling CCL3 gene expression.

Aside from RelA/p65, the current study indicated that C/EBP-β regulated CCL3 transcription. This finding was not unexpected as Zhang and colleagues showed that when chondrocytes were treated with IL-1β there was a cooperative regulation of CCL3 expression by both C/EBP-β and NF-κB (13). In a separate study, Choi *et al.* showed that AML-1A (acute myeloid leukemia 1A) and AML-1B/Runx1 controlled CCL3 expression in multiple myeloma (29); while a later study demonstrated importance of C/EBPβ in this regulation (30). Similarly, Grove and Plumb have shown that C/EBP, NF-κB, and cEts family members were important in LPS-induced expression of CCL3 and that the promoter sequence between −200/+36 bp was critical in conferring cell specific responses (31). With respect to cytokines, our data clearly shows that in NP cells C/EBP-β positively controlled TNF-α dependent CCL3 expression. Related to this observation, our recent investigation has clearly identified C/EBP-β as a negative regulator of cytokine dependent ADAMTS-4 transcription, again highlighting the importance of both context and target gene specificity in the regulatory machinery of NP cells (unpublished).

Turning to MAPK signaling, since the activity of this pathway is responsive to both NF-κB and C/EBP-β we investigated if it controlled CCL3 expression. We confirmed that both

TNF-α and IL-1β promoted MAPK activation and that the signaling pathways controlled CCL3 expression. We noted that there was differential sensitivity of CCL3 expression: thus, while p38, ERK and JNK positively controlled IL-1β dependent induction in CCL3, the JNK pathway was not involved in the TNF-α mediated response. Based on these findings, it is not unreasonable to assume that by controlling the activity of MAPK signaling, cytokines regulate the expression of CCL3 through NF-κB and C/EBP-β in NP cells.

In concert with CCL3 and CCL4 expression in RA synovial fluid and tissue (12–15), our studies confirmed that CCL3 is elevated in the degenerate human disc tissue and that staining is localized to NP as well as to the infiltrating cells. Importantly, CCL3 expression is positively correlated with the grade of tissue degeneration and was highest in tissues with infiltrating cells. It must be acknowledged that *in vitro* NP cells exhibit efficient phagocytosis and are capable of removing apoptotic cells, a classical behavior of macrophage like cells (32) and express CD68, a classic marker of a monocyte/macrophage (33). Thus, despite positive CD11b staining exact origin and identity of all of the infiltrating cells cannot be determined with certainty. However, results of our *in vitro* cell migration studies confirmed that chemotactic factors secreted by NP cells in response to TNF-α or IL-1β treatment promote macrophage migration. Noteworthy, pretreatment of macrophages with a selective CCR1 antagonist, J113863, completely inhibited NP induced macrophage migration, suggesting that CCR1 is critical for their migratory response. Since CCR1 is a receptor for CCL3, CCL4 as well as CCL5, further investigations will be necessary to elucidate the relative importance of the individual chemokines in this process. Based on these findings, it is plausible to consider that therapeutic blocking of CCR1-CCL3 activity would inhibit macrophage infiltration into the disc tissue and prevent the inflammatory response associated with degenerative disc disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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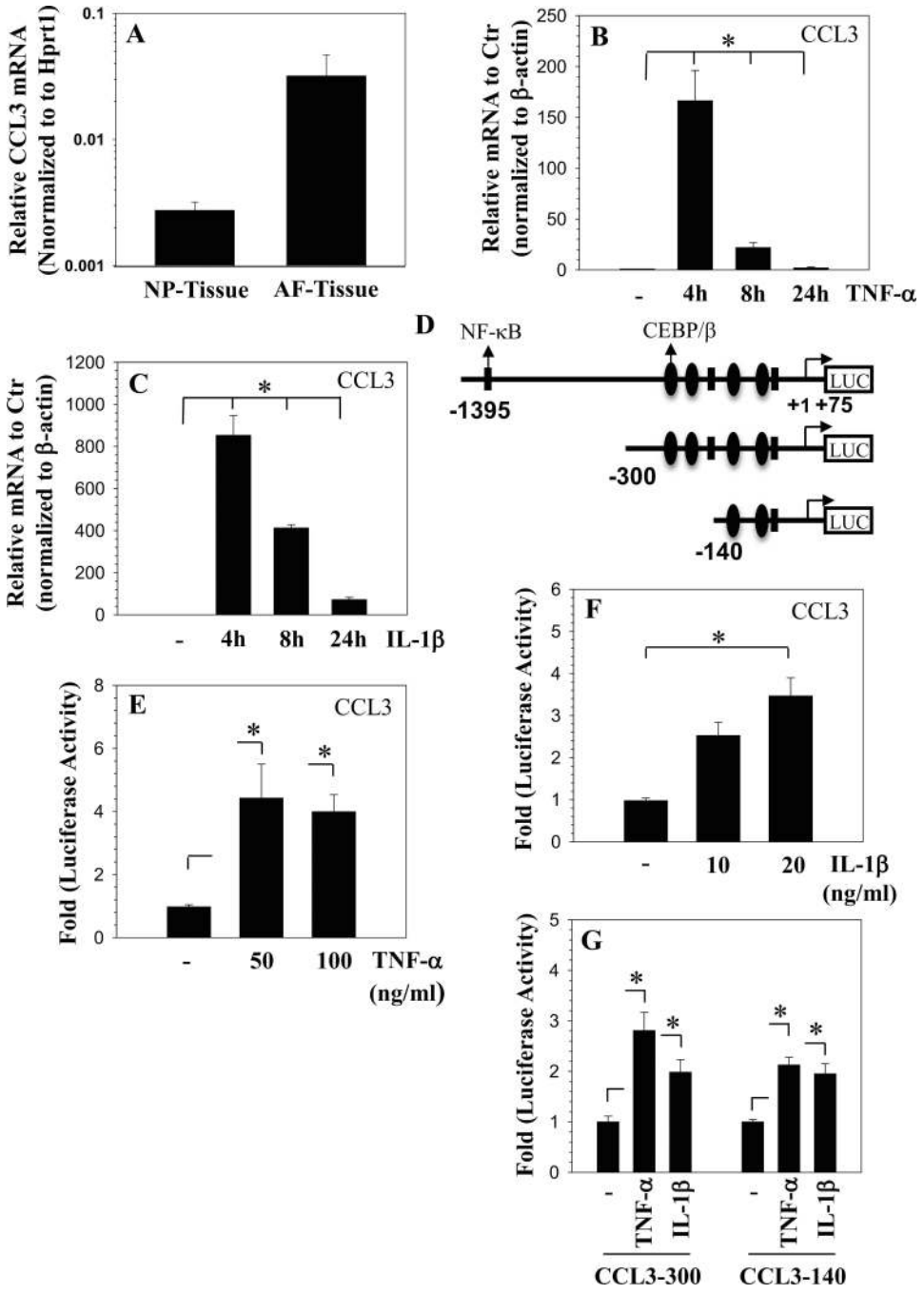

Fig. 1.

Expression and cytokine dependency of CCL3 in nucleus pulposus (NP) cells. A) Real-time RT-PCR analysis shows CCL3 was expressed at a very low level in adult rat NP and annulus fibrosus (AF) tissues. B, C) Real-time RT-PCR analysis of CCL3 expression by NP cells treated with TNF-α; 50 –100 ng/ml (B) or IL-1β; 10–20 ng/ml (C) for different time periods. Induction is maximal at 4 h and declines thereafter, although remaining elevated until 24 h. D) Schematic of different length CCL3 promoter constructs used in the study, putative NF-κB and CEBP/β elements are shown. E, F) NP cells transfected with a 1.4 kb CCL3 reporter construct were treated with increasing doses of TNF-α (E) or IL-1β (F). There was a dose-dependent increase in CCL3 promoter activity by cytokine treatment. G)

NP cells transfected with a 300 bp and a 140 bp reporter construct were treated with TNF-α or IL-1β. A significant induction in activity of both the CCL3 promoter fragments was seen when treated with the cytokines. Values shown are mean + SE, of 3 independent experiments; *p<0.05.

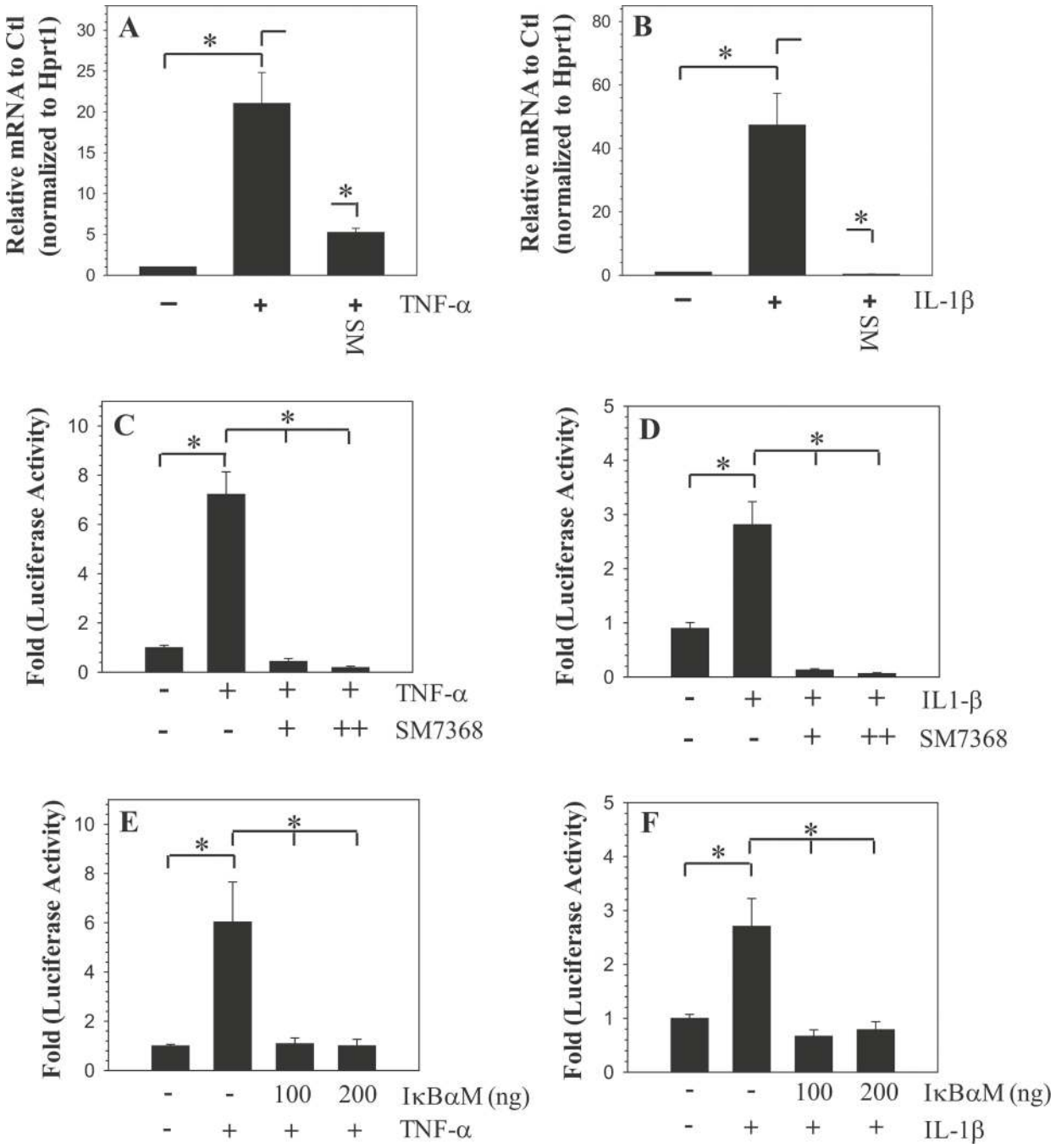

Fig. 2.

Modulation of cytokine dependent CCL3 expression by NF-κB signaling in NP cells. A, B) Real-time RT-PCR analysis of CCL3 expression by NP cells following TNF- α (A) or IL-1β (B) treatment for 24 h with or without NF-κB inhibitor (SM7368, 10 μM). Inhibition of NF-κB signaling resulted in a significant blocking of cytokine dependent induction in CCL3 mRNA expression. C, D) CCL3 promoter activity was measured following TNF-α (C, E) and IL-1β (D, F) treatment with or without NF-κB inhibitor SM7368 or co-transfection with dominant negative NF-κB/IκBαM. Cytokine mediated induction in promoter activity was completely blocked by inhibition of NF-κB signaling. Values shown are mean + SE, of 3 independent experiments; *p<0.05.

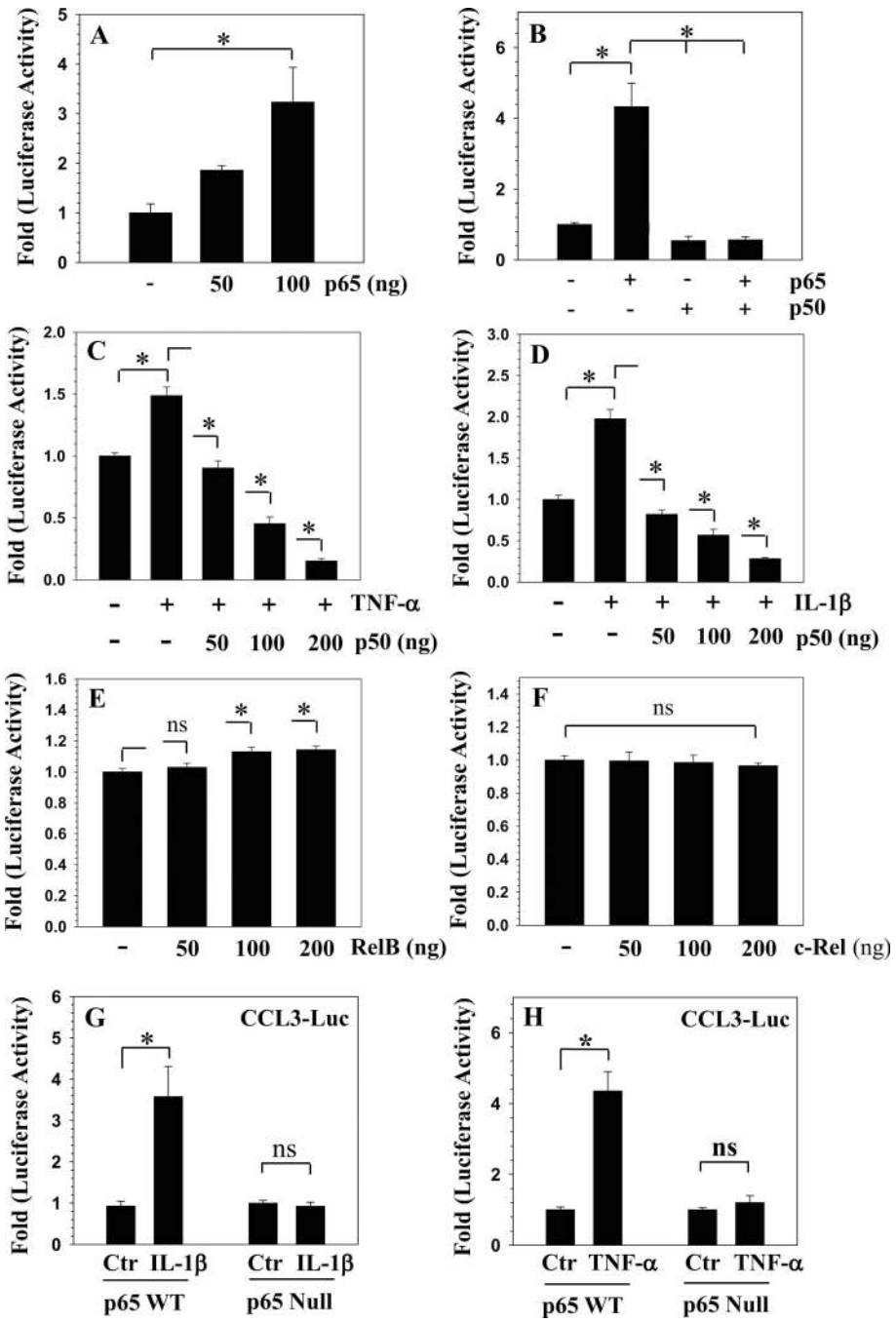

Fig. 3.

NF-κB regulation of CCL3 expression. A) NP cells were transfected with p65 and CCL3 promoter activity measured. There was a dose-dependent increase in CCL3 promoter activity. B) NP cells were co-transfected with p65 and/or p50 and promoter activity was measured. Transfection with p65, but not p50, resulted in increased promoter activity. Together, p50 significantly blocked p65-mediated induction in CCL3 promoter activity. C, D) Co-transfection with p50 completely abolished both C) TNF-α and D) IL-1β mediated induction in CCL3 promoter activity. E, F) In contrast to p65, RelB (E) and cRel (F) had little or no effect on CCL3 promoter activity. G, H) p65 WT and null cells were transfected with CCL3 reporter and treated with TNF-α (G) or IL-1β (H). Only wild type cells

evidenced an increase in CCL3 reporter activity. Values shown are mean + SE, of 3 independent experiments; *p<0.05.

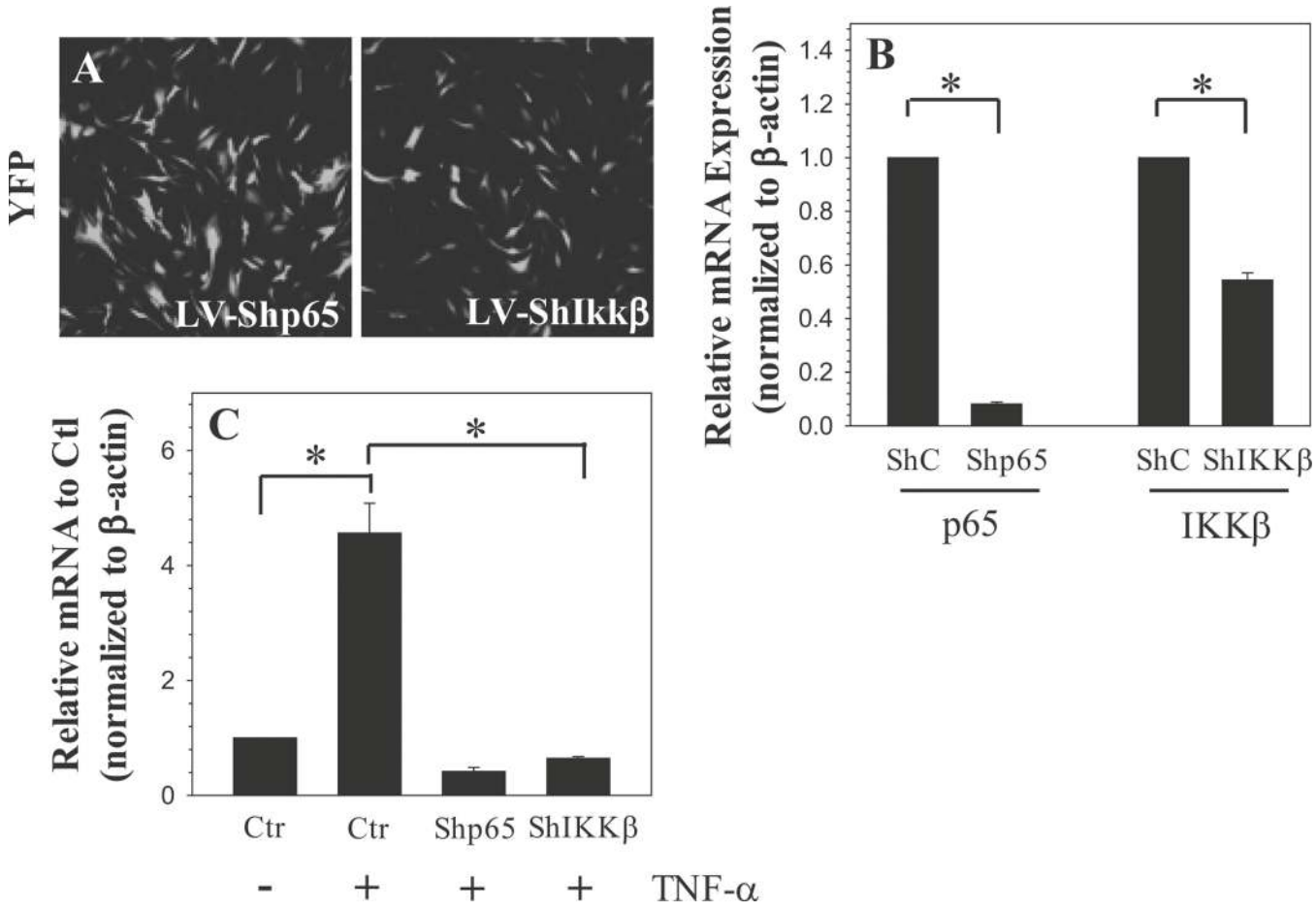

Fig. 4.

A) Immunofluorescence detection of YFP in human NP cells transduced with lentivirus co-expressing YFP and NF-κB pathway specific shRNAs (LV-shp65, LV-shIkkβ) show high transduction efficiency. Mag. X20. B) Real-time RT-PCR analysis of cells transduced with LV-shC, LV-shp65 and LV-shIkkβ. Expression of p65 and Ikkβ was significantly suppressed by the corresponding shRNAs when compared to cells transduced with a lentivirus expressing control-shRNA. C) Real-time RT-PCR analysis of CCL3 expression in cells infected with LV-shC and LV-shp65 and LV-shIkkβ following TNF-α treatment. Note that the TNF-α dependent induction in CCL3 mRNA levels is significantly blocked by suppression of components of the NF-κB signaling pathway. Values shown are mean + SE, of 3 independent experiments; *$p<0.05$.

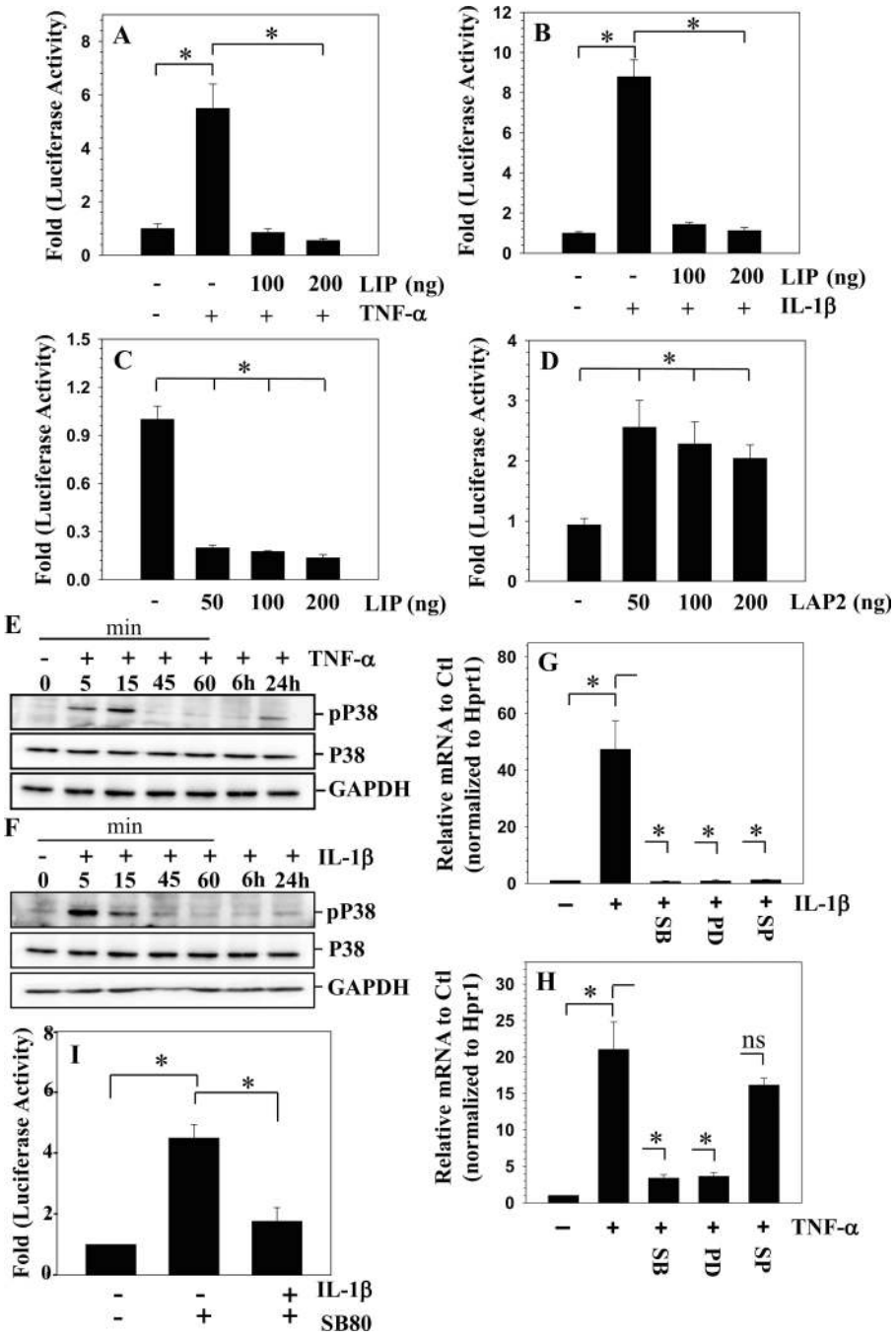

Fig. 5.

Modulation of cytokine dependent expression of CCL3 expression by CEBP/β and MAPK in NP cells. A, B) NP cells were co-transfected with LIP (DN-C/EBPβ) and CCL3 reporter activity measured following TNF-α (A) and IL-1β (B) treatment. LIP inhibits cytokine dependent induction in CCL3 promoter activity. C) LIP significant inhibits basal CCL3 promoter activity. D) In contrast to LIP, significant induction in CCL3 promoter activity was seen with LAP2. E, F) Western blot analysis of p38 following treatment of NP cells with TNF-α (E) and IL-1β (F). Treatment induced phosphorylation of p38 within first 5 min and levels remained elevated for 24 h. No change in expression of p38 was seen. G, H) Real-time RT-PCR of CCL3 expression by NP cells following IL-1β (G) or TNF- α (H) treatment

for 24 h with or without inhibitors for p38 (SB203580, 10 μM), ERK (PD98059, 10 μM), JNK (SP60025, 10 μM). MAPK inhibition resulted in a blocking of cytokine-dependent induction in CCL3 mRNA with exception of JNK inhibition which had no effect on TNF-α dependent increase in CCL3 expression. I) Increase in CCL3 promoter activity by IL-1β was blocked by p38 inhibition. Values shown are mean + SE, of 3 independent experiments; *$p<0.05$.

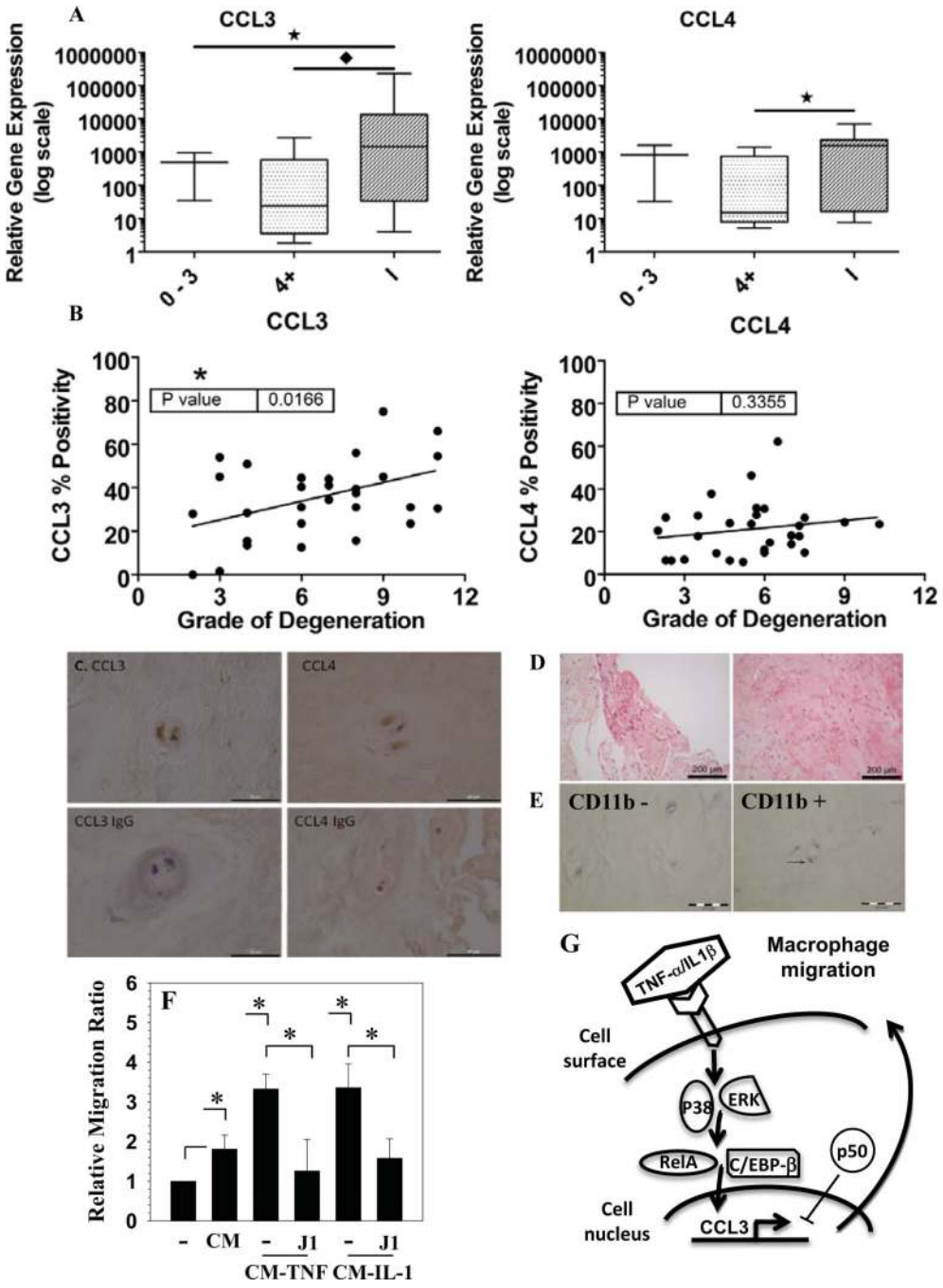

Fig. 6.

A–C: CCL3, CCL4 expression in human NP cells. A: Relative gene expression for CCL3 and CCL4, within non-degenerate discs (Graded 1–3), degenerate discs (4+), and in discs containing infiltrating cells. CCL3 was seen in a greater proportion of infiltrated discs compared to non-degenerate discs and at higher level to that seen in degenerate discs. CCL4 was seen in a greater proportion of infiltrated discs than degenerate discs. *P<0.05 proportionality test, ♦P<0.05 expression levels. B: Expression of CCL3 and not CCL4 by NP cells correlated with grade of degeneration, *P<0.05. C: CCL3 and CCL4 staining was localized to the NP cells within the disc. D) H&E staining showing morphology of infiltrating cells from two disc samples E) CD11b reactivity was absent from disc cells

found in single lacuna or within clusters, infiltrating cells displayed CD11b immunopositivity. Role of CCL3 on RAW264.7 macrophage migration. F) NP-condition medium (CM) enhanced macrophage migration rate, CM of TNF-α or IL-1β treated NP cells further increased migration rate and this increased migration was blocked by CCR1 antagonist J 113863 (J1). Mean + SE, n = 3; *p<0.05. G) A proposed model of relationship between inflammatory cytokines and CCL3 in NP cells.