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Cytokine-mediated Differential Regulation of Cyclooxygenase-2, High Mobility Group Box 1 Protein and Matrix Metalloproteinase-9 Expression in Fibroblast-like Synovial Cells

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Abstract

Persistent joint inflammation and pain with concomitant joint erosion, characterize Rheumatoid Arthritis (RA). We used a Fibroblast-like Synovial (FLS) cell line derived from a female rabbit, as a model system for studying the initiation and attenuation of conditions of RA *in vitro*. We used two pro-inflammatory cytokines, TNFα and IL-1β to examine potential inflammatory responses and cartilage erosion exerted by each cytokine alone and/or in combination. We determined the expression levels of cytokine-induced expression of Cyclooxygenase-2 (COX-2), production of Prostaglandin E2 (PGE2), release of high mobility group box-1 (HMGB1) protein and the activity of Metalloproteinase-9 (MMP-9) in FLS cells. Treatment with TNFα alone increased HMGB1 release levels, MMP-9 activity, COX-2 expression and PGE2 production in both concentration- and exposure time-dependent manner. But treatment with a low concentration of TNF-α in combination with an equivalent concentration of IL-1β produced similar levels of COX-2 expression and PGE2 production compared with the same concentration of TNF-α alone. This suggests that the effects observed could only be due to the TNFα. IL-1β did not affect COX-2 expression in a concentration-dependent manner compared to media control. Treatment with indomethacin or NS392 significantly decreased TNFα-induced COX-2 expression coupled with decreased PGE2 production and MMP-9 expression. In addition, anti-TNFα decreased HMGB1 release level, PGE2 production and MMP-9 expression, which support a critical role for TNFα-induced TNF Receptor (TNFR) activation for these effects. Overall, our results support treatment approaches in RA that attenuate the effects of TNFα-induced TNFR stimulation on MMP-9 and PGE2 production with HMGB1 release for a more efficacious therapy.

Keywords: Fibroblast-like Synovial (FLS); TNF-α; IL-1β; Cyclooxygenase-2; Matrix metalloproteinase 9

Abbreviations:

RA: Rheumatoid Arthritis; FLS: Fibroblast-like synoviocytes; TNF-α: Tumor Necrosis Factor-α; IL-1β: Interleukin-1β; COX-2: Cyclooxygenase-2; PGE2: Prostaglandin E2; MMP-9: Membrane Metalloproteinase-9; HMGB1: High Mobility Group Box 1; NSAIDS: Non-Steroidal Anti-Inflammatory Drugs; TIMPs: Tissue Inhibitors of Matrix Metalloproteinases; SDS: Sodium Dodecyl Sulfate; OD: Optical Density; ELISA: Enzyme-Linked Immunosorbent Assay

Introduction

Rheumatoid Arthritis (RA) is a systemic chronic autoimmune inflammatory disorder [1] that causes more disability than any other disease [2]. While the exact cause of RA still remains elusive, genetic and environmental factors are associated with the tendency to develop the disease [3]. RA is characterized by synovial hyperplasia, pathophysiological immune responses and progressive erosion of joint tissue. Different types of cells are involved in the pathogenesis of RA, which include T helper (Th) cells, antigen presenting cells, endothelial cells and resident fibroblasts of the synovial membrane [4]. However, our experimental focus here is on the role of Fibroblast-like Synovial (FLS) cells that may be engaged in initiation and maintenance of RA and that can destroy articular cartilage independent of ongoing inflammation [5-7].

The cytokines are important mediators involved in the immune reaction and maintenance of homeostasis. An imbalance in the cytokine network may lead to inflammation and autoimmune diseases such as RA [8,9]. The excessive production of proinflammatory cytokines such as tumor necrosis factor (TNFα), interleukin-1 (IL-1β) and IL-6 by intra-articular macrophages appears to occupy a critical pathogenic role in the development, progression and maintenance of the disease. These cytokines, which also include the high mobility group box 1 (HMGB1) protein [10,11] and IL-17 [12], induce inflammation of the joints and destruction of bone and cartilage via activation of macrophages, FLS, Th cells and osteoclasts.

FLS cells play a crucial role in joint damage as well as in propagation of inflammation [13]. It appears that in response to potent pro-inflammatory cytokines such as TNFα, FLS cells can produce large amounts of Matrix Metalloproteinases (MMP), which are key drivers of Extracellular Matrix (ECM) destruction [14]. There is also growing evidence that activation of FLS (e.g., by responses of the innate immune system), is an early step in the development of RA [15,16]. Once activated, FLS can attach to cartilage and bone to cause progressive erosion of articular structures by producing a variety of cytokines, chemokines, and extracellular matrix-degrading enzymes that mediate interactions with the microenvironment of neighboring cells. It is established that endogenous factors in RA can transform FLS to a tumor-like phenotype, which is directly or indirectly linked to RA

development and progression that lead eventually to bone erosion [17]. While the pathogenesis of RA is only partially understood, the involvement of immune cells and their respective proinflammatory mediators remains a common hallmark of the disorder [18,19].

Two main proinflammatory cytokines have definitively been shown to contribute to RA, TNF-α and IL-1β [20,21]. Both cytokines are pleiotropic with multiple biological effects on different cell types, many of which are not yet fully understood. The master cytokine that triggers inflammation and joint destruction is TNF-α as systemic overexpression of TNF-α gene in transgenic mouse model hTNFtg [22] is sufficient to initiate chronic synovitis, cartilage destruction and bone erosion [23].

HMGB1 chromosomal protein, a nuclear DNA-binding protein and a potent dual action cytokine [24,25] is implicated as an important mediator of RA [10]. HMGB1 is passively released from necrotic or stressed cells. But, inflammatory cells can actively secrete HMGB1 to function as an extracellular signaling molecule for cell migration and tissue regeneration. Thus, HMGB1 is secreted by cells destined to die or by activated cells of the innate immunity. Once released, HMGB1 can function as Damage Associated Molecular Pattern (DAMP) to activate pattern recognition receptors including Toll like receptors 2, 4, and the receptor for advanced glycation end products (RAGE) [26,27]. Increased levels of HMGB1 are found in the joints of RA patients [28], and its injection into the joints of naïve mice induced RA-like conditions [29]. Previously, it has been shown that Interferon-γ (IFN-γ) plays a role in the regulation of HMGB1 release partially through a TNFα-dependent mechanism [30].

The biological effects of TNF-α are mediated through two structurally distinct high affinity membrane receptors expressed on target cells-TNFR1 (also known as p55) with a molecular weight of 55 kDa, and TNFR2 (or p75) with a molecular size of 75 kDa. These receptors activate two separate intracellular signaling pathways to gene transcription [31,32]. TNFR1 is expressed on nearly all cells in the body, including the entire lymphoid system, whereas TNFR2 exhibits more restricted expression, being found on certain subpopulations of immune cells and a few other cell types. The majority of the biological actions of TNF-α are mediated through TNFR1 [33] since it is widely expressed. The biological activities of TNF-α account for the pathological processes that contribute to RA, including recruitment of inflammatory T cells, B cells, macrophages, synovial cell proliferation, augmentation of matrix degrading metalloproteinase activity leading to bone and cartilage destruction [34].

IL-1β, a 17 kDa peptide, is the predominant form of IL-1 and shares approximately 26% amino acid sequence homology with IL-1α. IL-1β is produced predominantly by macrophages and monocytes. Other cells, including endothelial cells, keratinocytes, astrocytes, B lymphocytes and activated T lymphocytes may also produce IL-1β [35,36]. There are two types of IL-1 receptors, type I IL-1R and type II IL-1R. The type I receptor is the functional receptor that exerts the biological effects of IL-1β [37]. The type II IL-1R acts as a decoy receptor. The systemic effects of IL-1β are exerted in many physiological processes in the CNS, bone marrow, blood vessels etc., but its local effects on immune system are important in RA. Thus, IL-1β augments production of T and B-lymphocytes, production of prostaglandin E2 (PGE2) and proliferation of fibroblasts [38].

Prostaglandin E2 is synthesized from arachidonic acid (AA) through the Cyclooxygenase (COX) pathway. As the rate-limiting enzyme that catalyzes the conversion of AA to prostaglandins, there are two COX isoforms, COX-1 and COX-2 [39]. Both enzymes share at least 60% homology in their amino acid sequence but differ in their regulation and expression. Primarily, COX-1 (or prostaglandin synthase H1) is referred to as a housekeeping enzyme, which is constitutively expressed in almost all tissues and regulates normal homeostatic functions. COX-2 (or prostaglandin synthase H2) is the inducible form of COX and is usually undetectable in most normal (unstimulated) tissues. However, it is constitutively expressed in certain areas like the cortex and hippocampus of the brain. The role of COX-2 expression in exacerbating inflammation and pain has been established as a key perpetrator in RA [40]. Animal models of RA suggest that increased COX-2 expression is responsible for increased PGE2 production in the inflamed synovial tissue [41]. This is the rationale for the advantageous use of Non-steroidal Anti-inflammatory Drugs (NSAIDS) to target COX enzymes, which relieves the symptoms of inflammation and pain in RA [42].

The mechanisms by which bone erosion occurs in RA are not clear. However, Th17 immune cells play an important role in RA pathogenesis through several mechanisms. Th17 cells can activate osteoclasts through IL-17 production. Th17 cells can also activate the pro-osteoclastogenic cytokines such as IL-1 and TNF-α. Thus, IL-1 and TNFα can increase receptor activator of nuclear factor kappa-B ligand (RANKL) [also known as tumor necrosis factor ligand superfamily member 11 (TNFSF11)], RA expression and promote osteoclastogenesis [43]. Furthermore, Th17 polarization is activated with increased TNFα [44].

MMP-9 belongs to a family of zinc-containing endopeptidases that are involved most prominently in tissue remodeling, but its expression is not constitutive. It is expressed in macrophages, neutrophils, chondrocytes and a variety of transformed cell lines [45]. MMP-9 catalytic activity is finely counter-regulated by the activity of endogenous inhibitors, the Tissue Inhibitors of Matrix Metalloproteinases (TIMPs), of which four are identified to date. TIMP-1 can bind specifically to MMP-9 to inhibit its activity. MMP-9 also acts as an important regulatory molecule on the expression of cytokines and adhesion molecules. Several studies have shown that there is up regulation of MMPs enzymes especially MMP-9 [46,47] in the serum and synovial fluid of RA patients. Many extracellular stimuli such as TNF-α [43,44] and IL-1β [43] regulate MMP-9 expression in various cell types. Thus, the expression of TNF-α-induced MMP-9 [44] can be integrated into the signaling networks that augment FLS activation by degradation of the ECM.

Recently, much attention is placed on the endogenous factors within FLS, which are directly or indirectly responsible for FLS activation. Amongst these are inflammatory cytokines, MMP-9, HMGB1 release and COX-2/PGE2 production as representative endogenous factors. Based upon the role of FLS, we propose a triad of signaling cross talk function between cytokines, HMGB1, COX-2/PGE2 and MMP-9 to maintain and propagate inflammation and pain phenotype in RA [48]. We have designed elegantly simple experiments to test the hypothesis that proinflammatory cytokines such as TNF-α/IL-1β will enhance the expression of COX-2 that leads to increased PGE2 production, HMGB1 release and MMP-9 expression in FLS cells.

Materials and Methods

Cytokines, antibodies and reagents

Recombinant human tumor necrosis factor-α and anti-human TNF-α were purchased from Peprotech Inc. (Rocky Hill, NJ). COX-2 and β-actin affinity-purified goat polyclonal antibodies (C terminus) were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA), whereas recombinant human IL-1β was purchased from R&D Systems Inc. (Minneapolis, MN). Indomethacin (≥99% purity) and NS-398 (purity ≥ 98%) were purchased from Cayman Chemical (Ann Arbor, MI). Micro BCA™ Protein assay reagent kit was obtained from Pierce, Rockford, IL. Anti-HMGB1 was obtained from Proteintech (Rosemont, IL, USA).

Cell culture and treatments

The cell line used for these experiments was HIG-82 rabbit synovial fibroblast derived from the intra-articular soft tissue of the knee joint of a young female New Zealand rabbit. Cells were purchased from the American Tissue Culture (ATCC® CRL-1832™) (Manassas, VA, USA). Cells were expanded and maintained in HAM F-12 Kaighn's modification (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS), Penicillin (100 units/ml) and fungizone (2.5 μg/ml), at 37°C in an atmosphere of 5% CO_2/95% air/100% humidity. We initiated treatments after the plated cells reached ~75-80% confluency. Cells were incubated with OPTI-MEM® (Invitrogen Life Technologies, Carlsbad, CA), a reduced serum medium, for 48 h before treatment with cytokines to allow time for decay of preformed proteins, following initial growth in 10% FBS. In the cytokine stimulation studies, TNF-α and/or IL-1β were dissolved in the OPTI-MEM®. When using inhibitors and the neutralizing antibody, these were added at least 2 h before stimulation with TNF-α and/or IL-1β.

Extraction of whole cell protein

Approximately 10^6 cells were plated per dish 60 mm dish and at 75-80% confluent, media containing 10% FBS was removed and replaced with OPTI-MEM®, a reduced serum medium. After 48 h, cells were treated with TNF-α or IL-1β alone or in combination, following which total protein was extracted. Cells were first rinsed with 0.5 ml PBS followed by the addition of 300 ul of RIPA buffer [l × PBS, 1% (wt/vol) Igepal CA-630, 0.5% (wt/vol) sodium deoxycholate and 0.1% (wt/vol) Sodium Dodecyl Sulfate (SDS)] to each dish. RIPA buffer was supplemented just before use with l × protease inhibitor cocktail (AEBSF hydrochloride, aprotinin, protease inhibitor E-64, disodium EDTA and leupeptin hemisulfate) and sodium orthovanadate (10 μl/ml). Cells were scraped into the buffer with a sterile plastic cell scraper and transferred into microfuge tubes. Each dish was rinsed once with additional 200 ul RIPA buffer and combined with the original cell lysate. Combined lysates were incubated on ice for 45 min. The supernatant was collected and stored at -80°C as total protein extract. Total protein was quantitated spectrophotometrically using Micro BCA protein Assay reagent kit (Cat. #23235 Pierce; Rockford, Illinois) and absorbance was measured at 562 nm.

Western blot analysis of COX-2

Protein aliquots containing 10 μg total protein were denatured and fractionated on precast Tris-glycine gels (4-12%) (Life Technologies, Pittsburgh), using a Novex X-Cell II electrophoresis cell run at 125 volts for 90 min. Following fractionation, samples were transferred to PVDF membrane (Millipore) using an X-Cell II blot module electrophoretic transfer cell. Immunoblot was commenced by blocking non-specific binding sites on the membrane with blocking buffer [5% Carnation milk in PBS with Tween (0.05%) (PBS-T) for 90 min at room temperature followed by washing with (PBS-T) for 30 min. Membrane was incubated overnight at 4°C with affinity purified goat anti-COX-2 pAb (1:250-1:1000), and goat anti-β-actin pAb (1:5000) which was used for normalization. Blot was washed again in PBS-T for 30 min and incubated for 1 h at room temperature with HRP-coupled anti-goat IgG (1:5000) in 1% Carnation milk in PBS-T. After incubation, blot was washed and total immunoreactivity was detected using Supersignal West Pico Chemiluminescent substrate on CL-Xposure™ X-ray film. The Optical Density (OD) ratio of COX-2 protein to β-actin protein was obtained by densitometry analysis (Molecular Dynamics personal densitometer SI, model 375-A, Molecular Dynamics Inc., Sunnyvale, CA). β-actin signal was used to normalize for gel loading and PVDF membrane transfer errors.

Measurement of prostaglandin E2 (PGE2)

We used ELISA STAT PGE2 kit to quantify PGE2 secreted into the media (Cayman Chemical; Ann Arbor, MI). 24 h following different treatments, culture media was collected, treated with Indomethacin to inhibit oxidative production of PGE2 and stored at -20°C. This assay was carried out according to the manufacturer's instructions. The intensity of the yellow color, which is inversely proportional to the amount of PGE2 coated on the wells, was allowed to develop following the addition of para-nitrophenyl phosphate, and was determined spectrophotometrically at 405 nm using a microplate reader (Power Wave with KCA v3.0 software; Bio-Tek Instruments, Inc., Winnooski, VT).

Matrix metalloproteinase-9 (MMP-9) analysis

We assessed the expression of MMP-9 by ELISA with matrix metalloproteinase-9 (MMP-9) activity assay (Biotrak system (Cat. #RPN-2634, GE Healthcare Life Sciences, Pittsburgh, PA). The assay recognizes both pro and active forms of human MMP-9, but also cross-reacts with rabbit and mouse samples. However it does not cross react with other MMPs and Tissue Inhibitors of Matrix Metalloproteinases (TIMPs). The assay was performed using cell culture supernatant, collected 24 h after different treatments and stored at -20°C. It is a non-radioactive microtiter plate based assay that uses the pro-form of a detection enzyme that can be activated by captured active MMP-9. The natural activation sequence in the pro-detection enzyme has been replaced with an artificial sequence recognized by specific MMP-9. MMP-9 activated detection enzyme was then measured using a specific chromogenic peptide substrate. Standards and samples were incubated overnight in microtiter plate coated with anti-MMP-9 antibody.

This allows the MMP-9 present to bind to the wells; the rest of the sample is removed by washing following overnight incubation. Total levels of free MMP-9 are measured by activating the pro-MMP-9 standards and samples using p-amino-phenylmercuric acetate (APMA). After 90 min incubation with the detection reagent (as specified in the kit) absorbance was read at 405 nm (using a microplate reader power wave, with KC4 v3.0 software) and concentration of active MMP-9 was determined by extrapolation from the standard curve.

Determination of the effect of TNF-α neutralizing antibody on PGE2 production and MMP-9 activity

A polyclonal antibody, antihuman TNF-α was used in the study at 100 fold the concentration of TNF-α (the antigen) to determine its effect on TNF-α-induced PGE2 production and MMP-9 activity. Approximately 10^6 cells were plated per petri-dish (60 mm dish). At 75-80% confluency cells were treated with OPTM-MEM® for 48 h prior to the treatment with anti-TNF-α. Cells were treated with actinomycin (1 μg/ml); a transcription inhibitor for 1 h, following which the medium containing actinomycin was removed. Cells were rinsed with PBS and the cells were treated with anti TNF-α for 1 h following which TNF-α at a concentration of 1 ng/ml was added. Cells were incubated for a period of 24 h and cell media was collected. ELISA as described above was used to determine MMP-9 and PGE2 in media. The media containing PGE2 were analyzed promptly or treated with indomethacin (10 μg/ml) before storage to prevent oxidation of PGE2 contents.

Determination of the effect of TNF-α on HMGB1 level and release

Immunofluorescence of HMGB1: FLS cells were seeded in 8-well Lab-TekR II chamber slide (Nalge Nunc International, NY) and grown overnight, followed by incubation in Opti-MEM® I Reduced Serum Medium. Cells were then treated with TNF-α at 4 and 8 ng/ml or pretreated with anti-TNF-α antibody for 24 h. After fixing in 4% formaldehyde in PBS for 10 min at Room Temperature (RT), cells were permeabilized with 0.2% Triton X-100 in PBS for 1 h. Cells were then rinsed in PBS, blocked in 5% BSA at RT for 1 h followed by overnight incubation with gentle shaking at 4°C with primary antibody (1:100, rabbit polyclonal, anti-HMGB1). After rinsing in PBS, we incubated cells with FITC conjugated goat anti-rabbit IgG for 1 h and NucBlue R live cell stain for 15 min. After subsequent washes with PBS, images were acquired using fluorescence microscopy (Axiovert 200 M; Zeiss) at excitation and emission wavelengths: ~495/519 nm for FITC and 405/410-550 nm for NucBlue R.

Quantification of total HMGB1 levels by flow cytometry: FLS cells were grown overnight in 6-well plates at a density of 5 X 10^5 cells/well. Cells were treated in Opti-MEM® I Reduced Serum Medium (control), or TNF-α 4 or 8 ng/ml or pretreated with anti-TNF-α antibody for 24 h. Cells were washed and then incubated with PBS for 15 min at 37°C followed by transfer into 1.5 ml vials for flow cytometer. Cells were fixed in 4% formaldehyde in PBS for 10 min at RT, and permeabilized with 0.2 % Triton X-100 in PBS for 30 min on ice. After rinsing in PBS, cells were blocked in 5% BSA at room temperature (RT) for 30 min followed by incubation for 3 h at 4°C with primary antibody (1:100, rabbit polyclonal, anti-HMGB1) gentle rocking. After rinsing in PBS, cells were incubated with FITC conjugated goat anti-rabbit IgG for 1 h. Acquisition and analysis of flow cytometric data were conducted on FACSCanto™ II flow cytometer (BD Biosciences, San Jose, CA). The fluorescence intensity corresponding to HMGB1 antibody was determined using (FITC) filter at excitation/emission of 495/519 nm. We used the unstained cells as negative controls for HMGB1. For each parameter investigated, at least 10^4 events (cells) were analyzed per sample. The fluorescence intensities data were compared between different treatments.

Statistical analysis

Data was analyzed with GraphPad Prism 6. Differences between treatments were assessed by one-way ANOVA followed by Tukey' test of multiple comparisons. Results of statistical tests were considered significant if $p<0.05$. All the values are expressed as mean ± S.E.M.

Results and Discussion

TNFα, but not IL-1β, increased COX-2 expression in a concentration and time dependent manner in FLS

Cells were treated for 24 h. The resultant COX-2 expression was TNF-α concentration-dependent with maximum expression at 10 ng/ml TNF-α (Figure 1). The cytokine significantly increased the expression of COX-2 at all the five concentrations examined. Increase in COX-2 expression using 4 ng/ml was significantly higher than at 1 ng/ml, but not different from 8 and 10 ng/ml. Thus concentrations of 1.0 and 4 ng/ml were used in all subsequent experiments. Among the time points examined (Figure 2), we observed maximum expression of COX-2 at 24 h and 48 h after treatment on the basis of which we chose 24 h for the time point. Decreased COX-2 expression was observed after 72 h of treatment leading to the conclusion that TNF-α induction of COX-2 increased with time up to 48 h before it declined, which was consistent in all experiments. The Western blot analysis of COX-2/β-actin expression ratios (Figure 3) upon treatment with different concentrations of (IL-1β) 1-10 ng/ml showed that treatments were not significantly different from the control. We chose concentrations of 1 ng/ml and 4 ng/ml for subsequent experiments. Furthermore, IL-1β treatment at 1-10 ng/ml did not affect IL-1β-mediated COX-2 expression in a concentration-dependent manner.

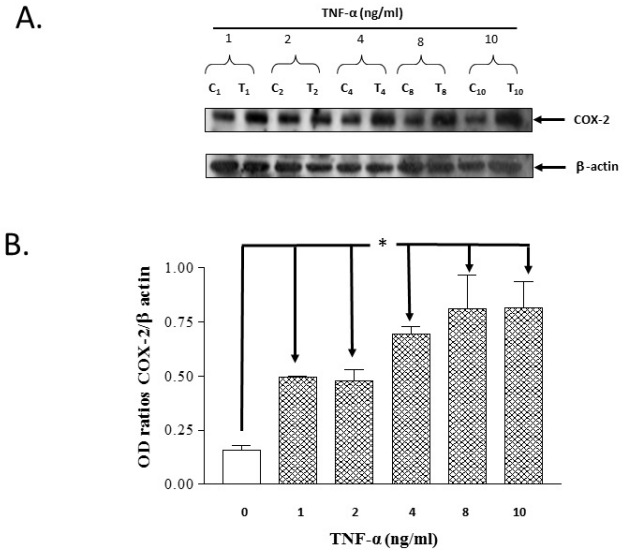

Figure 1: Western blot analysis showing TNF-α-concentration dependent induction of COX-2 expression (A), and semi-quantitative histograms of the Western blot analysis of the OD ratios of signals of COX-2 to β-actin (B). $^*p \leq 0.05$; n=5 independent experiments.

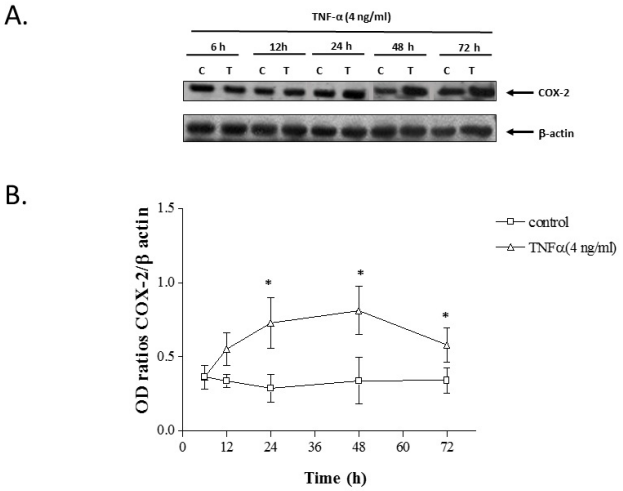

Figure 2: Western blot analysis of COX-2 (A) and time-course COX-2 expression (B) following treatment with TNF-α (4 ng/ml). $^{*}p \leq 0.05$ compared to their respective controls; n=5 independent experiments.

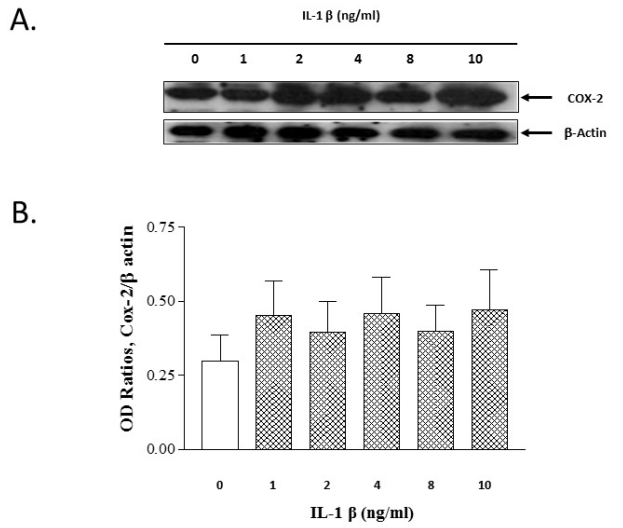

Figure 3: Western blot analysis (A), and semi-quantitative histograms of the Western blot analyses of the OD ratios of signals of COX-2 to β-actin (B) following treatment with different concentrations of IL-1β. n=4 independent experiments.

Combined treatment of TNFα and IL-1β increases COX-2 expression and PGE2 production

Western blot analysis for COX-2 expression (Figures 4A and 4B) following combined treatments with TNF-α and IL-1β showed that TNF-α alone at 1 ng/ml and 4 ng/ml significantly increased expression of COX-2 compared to control (P<0.05 and P<0.01) respectively. However, treatment with IL-1β alone at 1 ng/ml or 4 ng/ml did not significantly affect COX-2 expression compared to the control. Following combined treatment with TNF-α and IL-1β at equal concentrations (1 ng/ml each), there was a significant increase in in COX-2 expression (P<0.05), suggesting a dominant effect of TNF-α. The highest expression of COX-2 in the treatments was observed with combination of TNF-α (4 ng/ml)+IL-1β (1 ng/ml) (P<0.01). Treatment with TNF-α at 1 ng/ml and 4 ng/ml also resulted in a significantly higher production of PGE2 compared to control ($P \leq 0.05$ and $P \leq 0.01$) respectively (Figure 4C), whereas treatment with IL-1β did not. Consistent with increased COX-2 expression, combination of TNF-α and IL-1β at equal concentrations (1 ng/ml each), showed a higher production of PGE2 compared to control (P<0.05), suggesting again a dominant effect of TNF-α. Though, other investigators have shown synergism between IL-1β and TNF-α in primary cultures of articular chondrocytes and airway smooth muscle [49]. This synergistic effect is of particular relevance, as increased levels of both of these cytokines have been reported at the site of inflammation in RA [50]. Our data, while not in complete agreement with previous studies, may reflect a decreased expression of IL-1 R1 on HIG-82 cells. Nonetheless, our data appear to support a relationship between some malignancies and arthritis [51,52].

Combined treatment with TNFα and IL-1β enhances MPP-9 activity

After treatment with varying concentrations of TNF-α and IL-1β at 1 ng/ml and 4 ng/ml and following combination treatment, we observed a significantly increased expression of MPP-9 compared to control (P<0.05) after treatment with TNF-α alone and following combination. However, MMP-9 expression after treatment with IL-1β was not significantly different from control (Figure 4D).

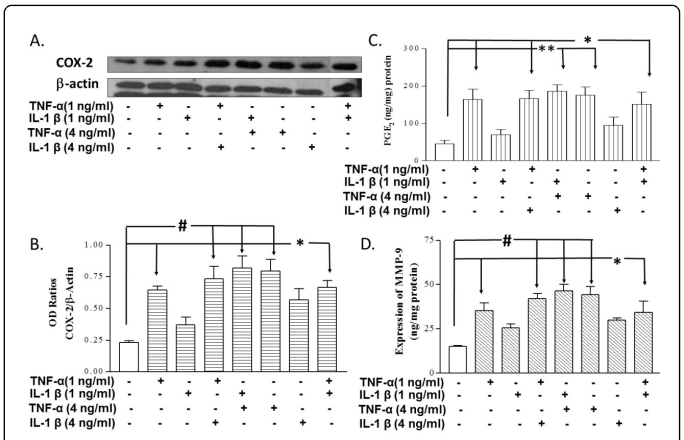

Figure 4: Combined effects of TNF-α and IL-1β on (A and B) COX-2 expression, (C) PGE2 production and (D) MMP-9 activity. $^{*}p<0.05$, $^{**}p \leq 01$, $^{\#}p \leq 0.001$; n=4 independent experiments.

Specific and non-specific blockage of COX-2 activity inhibits TNFα- and IL-1β-induced PEG2 and MMP-9 synthesis

Following treatment with either specific COX-2 inhibitor (NS-398), or nonspecific COX-1/COX-2 inhibitor (Indomethacin), PGE2 production was significantly decreased ($P \leq 0.01$) to the control level. The inhibitors also reduced the MMP-9 activity significantly (P<0.05)

compared to TNF-α treatment alone in the absence of the inhibitor. Currently Celebrex® (Celecoxib; Pharmacia) and Vioxx® (Rofecoxib; Merck) as relatively specific COX-2 are in the market. With this in mind, we designed our study with non-selective COX-1/COX-2 (Indomethacin) as well as a selective inhibitor of COX-2 (NS-398) [38] to delineate the contributions of COX-1 and COX-2 in TNF-α-mediated induction of COX-2, MMP-9 and PGE2. We assessed the effects of these inhibitors on PGE2 production and MMP-9 activity.

We incorporated in the experimental design a positive control by treating cells with TNF-α alone at a concentration of 1 ng/ml, thus enabling comparison of the inhibitor data to two controls, vehicle control (no treatment) and the treatment control (TNF-α 1 ng/ml). With the working Indomethacin concentration, we assumed that all of COX-1 activity would be inhibited and the PGE2 produced would be solely due to COX-2, whereas at 5 μM concentration of NS-398 [IC50 values for human recombinant COX-1 and -2 are 75 and 1.77 μM, respectively] the result would be solely due to COX-1 activity, as COX-2 would be completely inhibited. The data obtained (Figures 5A and 5B) indicated that there was a marked decrease in the production of PGE2 as well as in the expression of MMP-9. Compared to TNF-α (1 ng/ml), PGE2 levels were significantly reduced on treatment with the inhibitors supporting and confirming their use in anti-rheumatic therapy. Reduction in the MMP-9 levels upon treatment with the inhibitors broadened their therapeutic usefulness in RA. This study suggests that Indomethacin and NS-398 not only reduce inflammation and pain by decreasing PGE2 levels but also play a role in reducing matrix degradation and subsequent joint destruction by inhibiting MMP-9 activity.

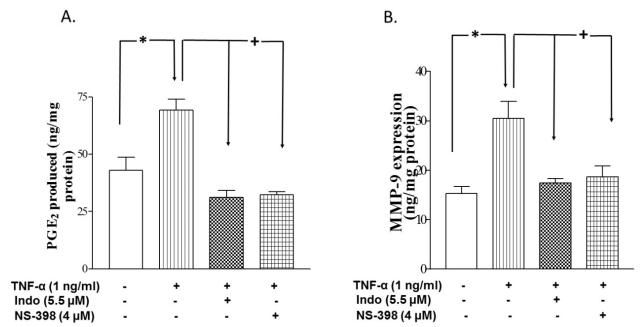

Figure 5: Histograms showing the effects of non-specific COX-1/COX-2 (Indomethacin) and relatively specific COX-2 (NS-398) inhibitors on PGE2 (A) and MMP-9 (B) production following treatment with TNF-α (1 ng/ml). *p<0.01 compared to the control, +p<0.05 compared to TNF-α (1 ng/ml); n=3 independent experiments.

TNF-α neutralizing antibody inhibits PGE2 production and MMP-9 activity

We then evaluated the effect of neutralizing TNF-α with anti-TNF-α pAb on the PGE2 production and MMP-9 activity. A significant reduction in the production of PGE2 ($P \leq 0.05$) and in the MMP-9 levels was observed to control levels (Figures 6A and 6B). The data suggests that utilizing this technique may be more advantageous in reducing pain and joint destruction. The accompanying decrease in MMP-9 levels suggests that anti-TNFα can significantly alleviate matrix degradation and subsequent joint destruction.

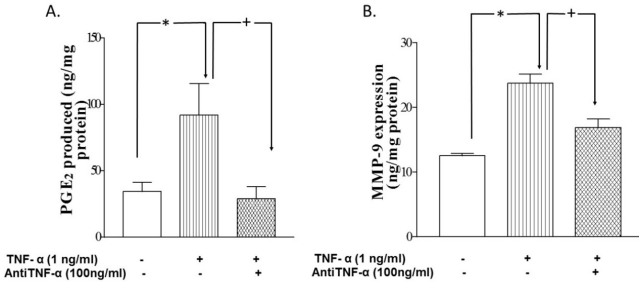

Figure 6: Histograms showing the effects of TNF-α neutralizing antibody on TNF-α-induced (A) PGE2 production and (B) MMP-9 activity. *p<0.001 compared to the control, +p<0.05 compared to TNF-α (1 ng/ml); n=4 independent experiments.

Treatment with TNF-α increased HMGB1 release, which is inhibited by TNF-α neutralizing antibody

Treatment of FLS with TNF-α increased the levels of extranuclear/cytoplasmic HMGB1 released in a TNF-α concentration-dependent manner (Figures 7A-7C). The TNFα-induced release is inhibited by prior or co-incubation with TNF-α neutralizing antibody suggesting that the release must be due to TNF-R activation/stimulation. Presumably, the released HMGB1 can also stimulate FLS cells directly by binding to toll-like receptor 4 (TLR4) [53].

In addition to potentially stimulating cells directly, HMGB1 can form immunostimulatory complexes with IL-1β, the TLR4 LPS and other endogenous and exogenous factors to promote inflammatory activity [54-56]. Furthermore, once released, HMGB1 might generate a positive feedback loop and induce production of several proinflammatory cytokines such as TNF-α, IL-1β and IL-6 by macrophages and dendritic cells, thereby sustaining prolonged inflammatory phenotype [57]. Curiously, HMGB1 released in rheumatoid synovitis was not consistently inhibited in vivo by TNFα receptor fusion proteins as well as novel TNFα mAb (Infliximab) blocking therapy [58]. Our present data leave doubt as for whether extra nuclear HMGB1 in synovitis represents TNFα-independent molecule that may be considered a separate pool of HMGB1. Therefore, the question regarding a functional relationship between TNF receptor stimulation and HMGB1 release while therapeutically pertinent in RA is not clarified in this model. Moreover, the complexity of FLS cell activation [6] raises the question as to what extent the inhibition of TNFα alone can influence the long-term outcome of RA.

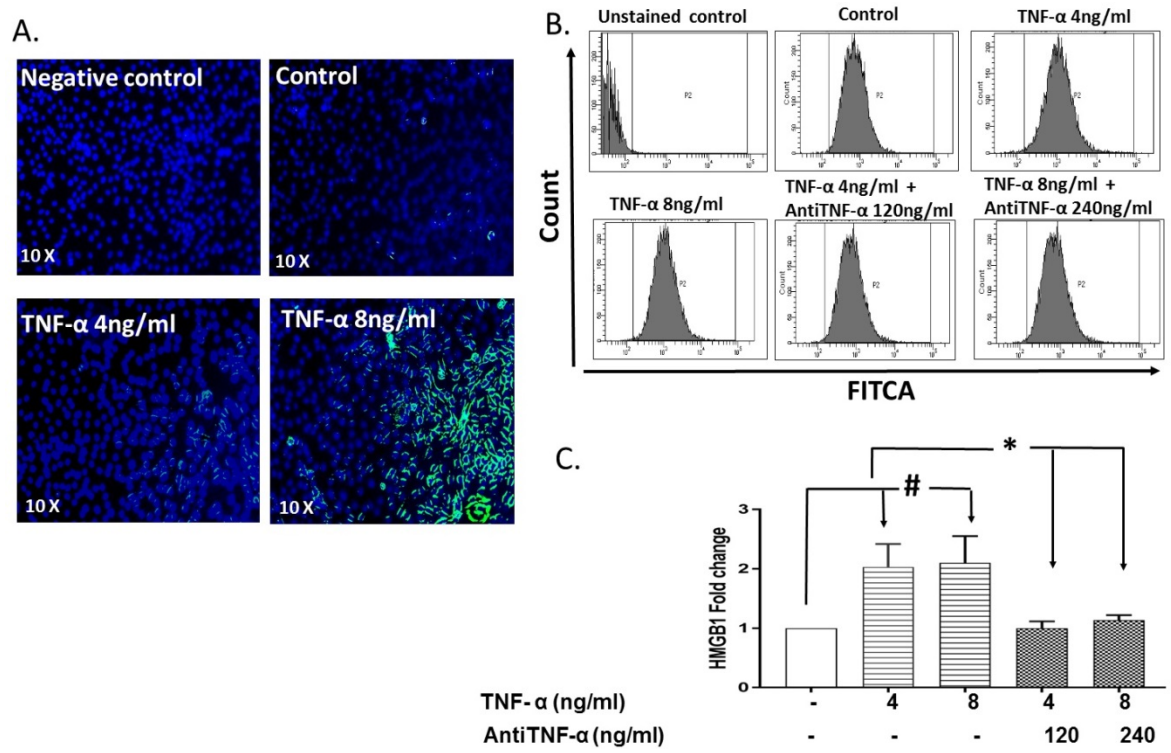

Figure 7: Effect of differing TNFα concentrations on HMGB1 release following incubations of FLS. (A) Immunofluorescence representation of the levels of HMGB1 released following treatments with 4 ng/ml and 8 ng/ml TNFα for 24 h, (B) Representative tracings of flow cytometric analyses of HMGB1 accumulation following preincubation with anti-TNFα followed by stimulation with different TNFα concentrations and (C) Quantitative analyses of tracings from (B) show changes in fluorescence intensity expressed as fold changes from control treatment. The data in (C) represent multiple independent experiments conducted in duplicates (n=4; $^{\#}p \leq 0.05$; $^{*}p \leq 0.1$).

Conclusion

We have shown a triad of signaling pathway (Figure 8) in which TNF-α up-regulates COX-2 expression with increased PGE2 production in the FLS cells leading to increased activation of MMP-9 expression and enhanced HMGB1 release to potentially propagate and maintain RA pathogenesis. Therefore, we have integrated TNFα-induced MMP-9 activity with cytokine/HMGB1 signaling networks that may augment joint inflammation by degradation of ECM that potentially maintain inflammation and pain in RA. We can thus conclude that TNF-α plays a dominant role in enhancing COX-2 expression, PGE2 production, MMP-9 activity and increased HMGB1 release in this fibroblast cell line. Our study supports a combination therapy of robust MMP-9 and COX-2 inhibitors as a viable therapeutic approach for RA, which remains a formidable clinical problem despite the remarkable advances of recent years. Whereas inflammatory cytokines are now firmly established as therapeutic targets, with blockade of TNF-α providing a benchmark for the development of new therapies, it is harder to define what constitutes a "good" clinical target from a preclinical modeling/perspective. TNF-α and IL-1β play a prominent role in the exacerbation of inflammation in RA, as evidenced by increased expression of COX-2 following direct treatment with either TNFα and/or IL-1β. TNF-α-induced COX-2 expression is time and concentration dependent in this rabbit synovial fibroblast model. This is in contrast to IL-1β-mediated COX-2, which was not, at least within the concentration range we studied, which may be attributed to low expression of IL-1 type 1 receptor density in this cell line. Combined treatment with TNF-α and IL-1β at various low concentrations displayed a TNFα-dependent dominant effect in inducing COX-2 expression, PGE2 production and enhancing the MMP-9 activity. Overall, in comparison to IL-1β, TNF-α exerted a more robust expression of COX-2/PGE2 and MMP-9, and may play a dominant role in initiating and maintaining the inflammatory cascade *in vivo* in the synovium. Non-specific COX-1/COX-2 inhibitor (Indomethacin) and specific COX-2 inhibitor (NS-398) significantly reduced TNF-α induced PGE2 production and MMP-9 activity, thereby supporting their role in pharmacotherapy to reduce joint inflammation, pain and subsequent joint destruction. Neutralization of TNF-α with anti-TNF-α yielded a marked decrease in the production of PGE2 and expression of MMP-9. This, once again, supports the potential role of TNF-α in inflammation and in inducing tissue damage, thus supporting the necessity for reducing TNF-α level in RA. Our overall data indicate that neutralizing TNF-α may be advantageous not only in alleviating pain and inflammation, but also in preventing the matrix degradation and joint destruction caused by MMP-9.

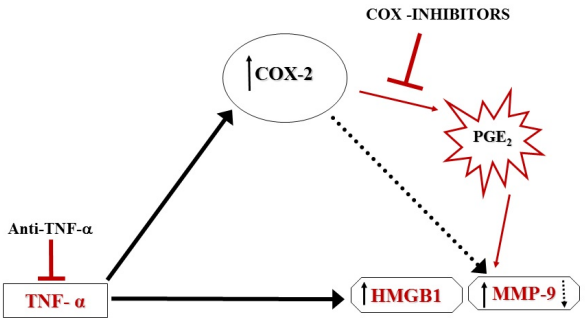

Figure 8: Schematic representation of a hypothetical triad of interaction between TNF-α, COX-2/PGE2, MMP-9 expression and HMGB1 release. The diagram shows that TNF-α treatment increases COX-2 expression, MMP-9 activity and HMGB1release, whereas inhibition of COX-2 activity reduces PGE2 production and MMP-9 activity. Furthermore, neutralizing TNFα with anti-TNFα antibody reduces PGE2 production, MMP-9 and HMGB1 levels. Inhibition of COX-2 expression and neutralization of TNF-α would reduce PGE2 production, and MMP-9 activity and HMGB1 release might be reduced directly by neutralizing TNF-α and via indirect action through COX-2 inhibition.

Finally, and crucially, the increasing number of cytokine inhibitors now available provides an invaluable tool to more carefully define RA pathogenesis, which in turn may lead to a more rational design of novel therapeutics in the future. The current RA therapy still fails to fully understand the interactions that subserve the cytokine networks *in vivo*. However, our results support therapeutic approaches in RA that will block the effects of TNFα/IL-1β on MMP-9 and PGE2 production with decreased extranuclear HMGB1 release for a more efficacious therapy.

Competing Interests

The authors declare that they have no competing interests.

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Authors' Contribution

AA-A and SS performed the experiments and contributed equally on the manuscript: OJI conceived and designed the study, and both AA-A and OJI wrote the paper.

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