Medical Research Archives

ESMED
European Society of Medicine

OPEN ACCESS

Published: September, 30, 2022

Citation: Alsousi AA, and Igwe OJ, 2022. Mechanistic role of redox-active trace metals/Toll like receptor 4-coupled activation in AP-1 signalling pathway in fibroblast-like synovial cells, Medical Research Archives, [online] 10(9). https://doi.org/10.18103/mra.v10i9.3040

DOI
https://doi.org/10.18103/mra.v10i9.3040

ISSN: 2375-1924

RESEARCH ARTICLE

Mechanistic role of redox-active trace metals/Toll like receptor 4-coupled activation in AP-1 signalling pathway in fibroblast-like synovial cells

Asmaa A. Alsousi[1] and Orisa J. Igwe*[1]

[1]Division of Pharmacology and Pharmaceutical Sciences, 2464 Charlotte Street, HSB # 2247, School of Pharmacy, University of Missouri-Kansas City (UMKC), Kansas City, MO 64108-2718, USA.

* igweo@umkc.edu

ABSTRACT

Reactive oxygen species (ROS) are implicated in playing a role in initiating and in propagating the pathogenesis of rheumatoid arthritis (RA). We investigated the mechanism(s) by which essential redox-active trace metals (RATM) may activate gene transcription in synovial fibroblasts. The rabbit fibroblast-like synovial cells which express Toll-like receptor 4 (TLR4), were used as a model system for potentially initiating RA through oxidative stress. Potassium peroxychromate (PPC, Cr^{5+}), ferrous chloride ($FeCl_2$, Fe^{2+}), and cuprous chloride (CuCl, Cu^{+}) at the indicated valency states were used as exogenous pro-oxidants. These trace metals can induce oxidative stress through TLR4 activation to release inflammatory cytokines and high mobility group box 1 protein. We measured the total expression levels of mitogen-activated protein kinase (MAPK) in the synovial cells and examined the effect of the redox-active trace metals on the time-course production of phosphorylated moieties of MAPK by fluorescence cell-sorting flow cytometry. TLR4 siRNA was used to examine the role of TLR4 in the activator protein -1 (AP-1) signalling activity, and western blots were used to measure the time-course phosphorylation levels of AP-1-activation-related proteins. While the redox-active trace metals increased intracellular ROS that can induce oxidative stress, they also induced MAPK kinases to upregulate the expression of AP-1 proteins in synovial cells. Our results show that redox-active trace metal/TLR4-coupled activation may contribute to the pathogenesis of RA. The signaling pathway by which inflammation and its destructive sequel may occur in RA through synovial cells underlies the need for developing therapeutic agents to serve in individualized RA therapy with a consideration for the underlying mechanism(s) of its pathogenesis.

Keywords: Redox-active trace metals; Oxidative stress; Rabbit synovial fibroblasts; Activator protein -1; Mitogen-activated protein kinases

Abbreviations: AP-1: Activator protein 1; DAMP: Damage-associated molecular pattern; ERK: Extracellular signal regulated kinase; FC: Flow cytometry; FLS: Fibroblast-like synovial cells; HMGB1: High mobility group box 1; JNK: c-Jun N-terminal kinases; LBP LPS binding protein; LDH: Lactate dehydrogenase; RATM: Redox-active trace metals; LPS: Lipopolysaccharide; MAPKs: Mitogen-activated protein kinases; MTT: 3- (4,5- di methylthiazol -2-yl)-2,5-di phenyl tetrazolium bromide; PPC: Potassium peroxychromate; RASF: Rheumatoid arthritis synovial fibroblasts; ROS: Reactive oxygen species; RS: Reactive species; WB: Western Blot

1. Introduction

Rheumatoid arthritis (RA), as a chronic systemic inflammatory disease of the joints, is a leading cause of disability that results in significant health care costs[1]. As the most common type of arthritis in the USA, affecting 1.3 million adults [2], RA frequently occurs during the fourth and fifth decades of life, although it can occur at any age [3]. It is a disease of synovial joints which predominantly affects women [4], and is characterized by synovial inflammation, hyperplasia and invasive growth of the synovial lining, leading to tissue, cartilage, and bone destruction[5]. Studies have established a strong correlation between increased oxidative damage and causative agents such as ROS and OH radicals, and that measurement of oxidative stress (peripheral blood and/or SF) is an effective biomarker for monitoring RA activity [6,7].

Redox-active trace metal micronutrients such as Fe, Mn, Cr and Cu are essential for various biological functions including proper innate immune function. In inflammatory, immune cells of the innate and/or adaptive immune system are activated and recruited to the site of inflammation. Attraction and stimulation of immune cells is regulated by different cytokines and chemokines, which are predominantly regulated by transcription factors such as AP-1, NF-κB, NFATs and STATs [8]. Adapter molecules such as MyD88 and TRAF6 that eventually activate NF-κB and/or AP-1 integrate the signalling pathways of TLR4 activation that may lead to increased cytokine synthesis and release to maintain the inflammatory process. A major inflammatory component that affects the severity of RA is the activation of T cells. However, other cells such as synovial fibroblasts are the main culprits.

A key signalling molecule that was initially identified on activated T cells and as a regulator of T cell function is the receptor activator of NF-κB ligand (RANKL). The most important transcription factor complexes that are activated by RANKL/TRAF signals are NF-κB and Fos/AP-1. Oxidant-induced activation of TLR4 can initiate an MAPK signalling cascade through MyD88, resulting in the activation of AP-1 in macrophages[9] and human monocytes[10].

As a redox-regulated transcription factor, AP-1 is composed of homo and heterodimeric complexes of two oncogenic proteins, Fos and Jun activating transcription factor[11]. The AP-1 binding site is known as the tetra-deconyl phorbol acetate responsive element (TPARE). Fos proteins (Fos, FosB, Fra-1and Fra-2) can only heterodimerize with members of Jun family, while Jun family members (Jun, Jun B and Jun D) can homodimerize and heterodimerize with Fos members. Thus, AP-1 proteins consist of either Fos-Jun heterodimers or Jun-Jun homodimers[12]. Many important insights regarding specific functions of AP-1 proteins in development and disease have been obtained from genetically modified mice and cells derived from them. AP-1 activity affects the severity of RA at a level different from osteoclastogenesis. In addition to osteoclast-mediated bone erosion, synovial fibroblasts secrete many molecules that contribute to matrix degradation. Of particular importance are the matrix metalloproteinases (MMPs), which are regulated by AP-1. MMPs can degrade collagen, fibronectin or other components of the extracellular matrix. Signals that lead to activation of Jun have been implicated in RA. In particular, JNK is highly activated in synovial fibroblasts of RA patients.

Stress-activated protein kinase/mitogen-activated protein kinase (SAPK/MAPK) family proteins are enzymes of highly conserved serine/threonine kinases that are involved in signaling cascades in all eukaryotic cell regulation. Different MAPK pathways coordinate and integrate responses to various stimuli via a cascade of sequential kinase activations through simultaneous Tyr and Thr phosphorylation within conserved Thr-X-Tyr motifs in the kinase subdomain VIII activation loop. These stimuli include: hormones, growth factors, cytokines, transforming growth factor (TGF)-β-related agents, agents that act through G-protein–coupled receptors and through pathogen associated molecular patterns (PAMPs), damage associated molecular patterns (DAMPs), and environmental stresses. Once activated, MAPKs exert effects on cell physiology where they regulate cell proliferation, differentiation, survival and apoptosis, motility and senescence [13,14]. MAPKs are key regulators of proinflammatory cytokine production in the signaling cascades downstream of Toll-like receptors (TLRs), as well as B-cell antigen receptor (BCR) [15], T-cell antigen receptor (TCR)/CD28[16], IL-1, IL-17[17] and TNFα receptors.

MAPKs are classified into distinctive groups. In mammalian cells, seven different MAPK pathways have been described: extracellular signal- regulated kinases 1 and 2 (ERK1/2) (P42/44MAPK), the c-Jun N-terminal Kinase (JNK) (also known as stress-activated kinase SAPK) pathway, P38 MAPK, ERK5, ERK3/4, ERK7/8, and the Nemo-like kinase(NLK)[18]. There are at least three main classes of MAPKs, namely (i) extracellular signal-regulated protein kinase (ERK) (P42/44MAPK) cascades, (ii) the c-Jun N-terminal

kinase (JNK) pathway, (iii) P38 MAPK[19] that are known by their (core signalling modules), and that play an important role in RA pathogenesis. They appear to control the response of cells to inflammation and stress. ERK1/2 has been shown to play an important role in regulating IL-6, IL-23 and TNF-α production in LPS- stimulated macrophages[20]. Furthermore, stimulation of FLS by epidermal growth factor (EGF) in RA patients resulted in regulation of the cyclooxygenase (COX)-2-dependent prostaglandin E2 (PGE2)[21]. ERK1/2 signaling has been associated with maintenance of RA. Of interest is the examination of ERK signaling cascade since it is implicated as a key mediator in arthritic diseases[22,23].

JNK MAPKs in RA appear to regulate MMP-mediated cartilage destruction downstream from IL-1 and TNFα. They promote extracellular matrix degradation by stimulating MMP expression in synovial fibroblasts and chondrocytes[24,25,26]. ROS has been implicated in JNK upregulation, which in turn activates Jun family proteins[27]. P38 MAPK is known as the mammalian homologue of the yeast high osmolarity glycerol (HOG) response kinase and is expressed throughout the body. There are four known P38 MAPK genes that encode four different proteins: P38α (MAPK14), β (MAPK11), γ (MAPK12), and δ (MAPK13). Each homologue has its distinct function and each can be activated in situ by environmental stresses, inflammatory stresses, cytokines, PAMPs, and DAMPs. P38 α (MAPK14) was the first isoform to be identified as a phosphorylated protein in response to LPS. cDNA cloning has revealed that it is related to *Streptomyces cerevesiae* MAPK HOG1[28, 29, 30]. The P38 MAPK pathway is known to contribute to RA pathogenesis through regulation of chemotaxis and angiogenesis [31]. Inhibition of P38 MAPK α using the orally-administered molecule P38 inhibitor, SCIO-469, to patients with active RA failed to show efficacy in adult RA clinical trials[32]. In view of the disappointing results of this clinical trial, further attention should be focused on ERK1/2 and JNK signaling in synoviocytes.

Overall, AP-1 induced activity in RA by inflammatory cytokines has a complex impact on osteoclast differentiation and production of soluble mediators of bone erosion. It is anticipated that several AP-1 components or signaling pathways leading to AP-1 activation may provide valuable drug targets for therapy of RA in the future.

Recently, we have shown that oxidants induce NF-kB activation through TLR4 signaling pathway and demonstrated that pro-oxidant activation of TLR4 is more robust in TLR4 than that of TLR2 [33]. We have also shown that RATM as oxidants in the form of (PPC, Cr^{+5}), ($FeCl_2$, Fe^{+2}), (CuCl, Cu^{+}), and [CuCl + Ascorbic acid combination treatment] (CuAA) generate ROS in FLS cells through TLR4 signaling pathway, and promote release of extracellular HMGB1 and inflammatory cytokines with a potential to initiate "sterile" inflammatory responses[34]. In our FLS model, we have shown that RATM upregulated HMGB1 expression whereby the released HMGB1 was primarily confined to the cytoplasm of FLS and to the extracellular milieu. HMGB1 had been implicated in the pathogenesis of RA by amplifying response to cytokines[35-37].

A clearer understanding of the molecular events that precede the clinical manifestations of RA could provide new possibilities for interventions, which could prevent or delay the development of the disease in individuals who are predisposed to RA. The role of RATM as prooxidants in regulating the complex interplay in RA is not clear.

In the present set of experiments, we tested the hypothesis that RATM/TLR4-coupled activation in synovial fibroblasts will produce oxidative adaptations that will contribute to RA pathogenesis through dysregulation in AP-1 signalling.

2. Materials and methods

2.1 Reagents and chemicals.

OPTI-MEM™ I reduced serum medium modification of MEM (Eagle's), and Bolt™ 4-12% Bis -Tris Plus, and 12-well plates were purchased from (Gibco®, Invitrogen Life Technologies™; Carlsbad, CA, USA). Anti-JNK1+JNK2+JNK3 antibody [EPR16797-211] was purchased from (Abcam, Cambridge, MA, USA). P38 MAPK (D23E1) XP® Rabbit monoclonal antibody (mAb), P44/42 MAPK (Erk1/2) Rabbit mAb, Phospho-P38 MAPK (Thr180/Tyr182) (D3F9) XP® Rabbit mAb, Phospho-P44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP® Rabbit mAb, Phospho-SAPK/JNK (Thr183/Tyr185) (G9) mouse mAb, c-Fos (9F6), and c-Jun (60A8) Rabbit mAb were purchased from (Cell Signalling Technology Inc., Danvers, MA, USA). Penicillin/Streptomycin combination solution (100 X) was obtained from (Corning, Gibco®- Life Technology, Canada), whereas Phosphate Buffered Saline (PBS) was purchased from (Fisher Scientifics, USA). Pierce ® RIPA buffer and Page Ruler™ Pre-stained Protein ladder was purchased from (Thermo-Scientific; Rockford, IL, USA).

2.2. Cell line and culture conditions

HIG-82 Rabbit synovial fibroblasts cells were purchased from (ATCC® CRL-1832™) at passage #5 and authenticated by ATCC. Cells were grown until 70-80 % confluence in Nutrient Mixture F-12 Ham containing sodium bicarbonate (Sigma, St. Louis, MO) and 10% fetal bovine serum (FBS) for overnight at 37◦ C in 100% humidified incubator. The medium was replaced with fresh warmed Opti-MEM® I Reduced Serum Medium (Invitrogen, San Diego, CA) immediately before treatment with any RATM.

2.3. Preparation of Potassium Peroxychromate (PPC)

Potassium peroxychromate (PPC) is not commercially available. Therefore, it was prepared and characterized in the laboratory according to a published protocol[38].

2.4. Preparation of solutions of redox-active trace metals (RATM).

Ferrous Chloride ($FeCl_2$) (Sigma, St. Louis, MO, cell culture grade) stock solution was prepared fresh just before use in culture grade sterile water then diluted in PBS. Stock solution of cuprous chloride (CuCl) (Sigma, St. Louis, MO, cell culture grade) was prepared in DMSO (culture grade), and then further diluted in PBS to provide 0.01 % DMSO in the final dilution. Ascorbic Acid (AA) (Sigma, St. Louis, MO) was dissolved in PBS to provide 100 µM. CuAA solution was prepared by mixing CuCl and AA solution at 2:1 ratio. RATM concentrations used in the current study were determined previously on the basis of MTT and LDH assays[34].

2.5. TLR4 gene silencing (siRNA TLR4)

2.5.1. Design and Synthesis of siRNA TLR4. Two siRNAs targeting TLR4 (GenBank accession no. AY101394) were Custom Select siRNA (Ambion by Life Technologies). The siRNAs are each 21 nucleotides (nt) in length with a 3′overhang of two thymidines. To avoid cross-silencing of non-target genes, all of the designed siRNA sequences were blasted against the rabbit genome database. A predesigned Silencer Select Negative Control No. 1 siRNA (Cat # 4390843) that does not have any significant similarity with this target gene was used as a negative control (NC) (Table 1).

Table 1 : Predesigned TLR4 siRNAs

	Name	Sequence
5'->3'	siRNA 513	Sense: GAAGUUCACCGGUUGGUUUtt
		Antisense: AAACCAACCGGUGAACUUCta
	siRNA 514	Sense: CACUCAACAACCUCCUUUAtt
		Antisense: UAAAGGAGGUUGUUGAGUGat
	Negative control (NC)	NC No.1 siRNA (Cat # 4390843). As a proprietary product, the sequence was not provided by the manufacturer (Ambion by Life Technologies).

2.5.2 siRNA Transfection. Cells were transfected with siRNA using Lipofectamine® RNAiMax. Briefly, cells were seeded in 6-well plates at a density of 2 $\times 10^5$ cells/well in antibiotic-free medium 12 h prior to transfection. The transfection mixture was prepared by mixing a 1.25-µl aliquot of 20 µM synthetic siRNA and a 1-µl aliquot of Lipofectamine® RNAiMax with two separate aliquots of 50 µl of serum-free OPTI MEM® I and then combining the two solutions at room temperature for 30 min to form a complex. Cells were washed with PBS, and 200 µl of the transfection mixture was added to each well along with 800 µl of serum-free OPTI MEM® I to produce a final concentration of 50 nM siRNA per well. The transfected cells were incubated for 6 or 24 h before being harvested for RNA and protein isolation during siRNA TLR4 optimization. After the initial 24 h incubation period, the medium was replaced with fresh OPTI MEM® I medium, and cells were treated with RATM and incubated for an additional 1h.

2.6. Flow cytometric quantifications of total and phosphorylated ERK1/2, JNK and P38.

Cells were seeded at 5 x 10^5 cells/well in six-well plates and incubated overnight in Nutrient Mixture F-12 Ham with sodium bicarbonate (Sigma) and 10% fetal bovine serum (FBS) at 37 °C, in a 100% humidified incubator. The medium was replaced with fresh warmed Opti-MEM® I Reduced Serum Medium immediately before treatment. Cells were treated in Opti-MEM® I Reduced Serum Medium (control), or $FeCl_2$ (10 µM), CuCl (50 µM), CuAA and PPC (10 nM) for 24 h, after which the washed cells were incubated with Dulbecco's Phosphate Buffered

Saline (DPBS) for 15 min at 37 °C, followed by transfer into 1.5 ml vials for flow cytometry. After fixation in 4 % formaldehyde in PBS for 10 min at room temperature (RT), cells were 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 with for 3 h at 4 °C with any of the following primary antibodies (P38 MAPK Rabbit mAb, Phospho-P38 MAPK (Thr180/Tyr182) Rabbit mAb, P44/42 MAPK (Erk1/2) Rabbit mAb, Phospho-P44/42 MAPK (Erk1/2) (Thr202/Tyr204) Rabbit mAb, Phospho-SAPK/JNK (Thr183/Tyr185) (G9) mouse mAb, and anti-JNK1+JNK2+JNK3(EPR16797-211), while shaking at ratios indicated by each manufacturer. After rinsing in PBS, cells were incubated with FITC-conjugated goat anti-mouse IgG, anti-rat or anti-rabbit for 1 h. Acquisition and analysis of flow cytometry data was conducted on a FACSCantoTM II flow cytometer. The fluorescence intensity corresponding to each antibody was determined using the FITC filter with excitation/emission of 495/519 nm. Unstained cells were used as negative controls. For each parameter investigated, at least 10^4 events (cells) were analysed per sample. The fluorescence intensity data were compared among different treatments.

2.7. Immunoblot analysis.

After the initial 24 h silencing incubation period, the medium was replaced with fresh OPTI MEM® I medium, and cells were treated and incubated for an additional 0, 5, 10, 15, 20, 25, 30 and 1 h. Then cells were washed in iced cold PBS, scraped, and resuspended. Total protein lysate was then centrifuged at 12,000 X g for 10 min at 4 °C. Protein concentrations were determined using BCA Protein Assay Kit. Total lysate of 15-20 µg protein was fractionated on 12% SDS gel and transferred onto PVDF membranes. Membranes were blocked in 5 % non-flat milk or 1% BSA at RT for 1 h followed by overnight incubation at 4°C with primary antibody at (1:1000) or (1:500, mouse monoclonal β actin). Membranes were then incubated with conjugated goat anti- secondary antibodies for 1 h. After subsequent washes with TBST, images were acquired and signals were visualized using Fujifilm LAS-400 imaging system (Fujifilm, Stamford, CT). β-actin signals were used to normalize for potential loading errors.

2.8. Statistical analysis

GraphPad statistical software (version 7.0; GraphPad Software, Inc., La Jolla, CA, USA) was used for data analysis. Data are presented as mean + SEM from at least 3–7 independent experiments and analysed by one- or two-way analysis of variance (ANOVA) followed by Tukey's post hoc tests. P value of ≤ 0.05 was considered significant.

3. Results

3.1. Redox active-trace metal (RATM) oxidants activate total JNK, ERK 1/2, and P38.

We used western blots and flow cytometry to identify the effect of RATM pro-oxidants on total and phospho-JNK. PPC (10 nM) upregulated total JNK protein expression as measured by western blot and FC at 5,15, and 60 min, while $FeCl_2$ showed significant increase at 15 min. Interestingly, CuCl but not CuAA increased JNK expression at 10 and 25 min (**Fig. 1**). For ERK1/2 expression, PPC (10nM) significantly upregulated total ERK1/2 protein expression as measured by western blot and FC at 10 and 60 min., whereas $FeCl_2$ showed significant increase at 5 and 60 min. Interestingly, CuCl and CuAA increased ERK1/2 expression at 25 and 20 min, respectively (**Fig. 2**). For P38 expression, PPC (10 nM) significantly upregulated total P38 protein expression as measured by western blot and FC at 10, 15 and 20 min significantly, while $FeCl_2$ showed the highest significant increase at 25 min. Interestingly again, CuAA but not CuCl increased P38 expression at 10 min (**Fig. 3**).

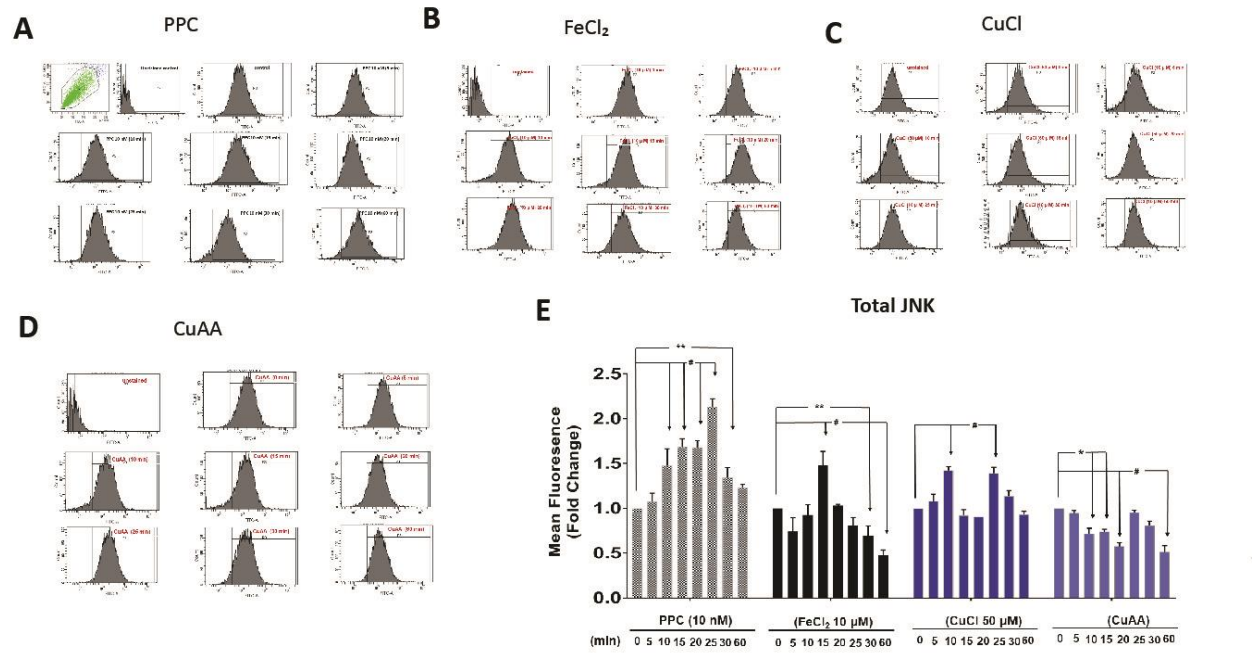

Figure 1. Effect of RATM prooxidants on total JNK protein expression in FLS. HIG-82 cells (synovial fibroblasts) were treated with PPC, $FeCl_2$, and CuCl alone and in combination with Ascorbic acid (CuAA) at different time points. Cells were then further used to run flow cytometric (FC) quantitative analysis of total JNK. **Sub-Figures 1 (A, B, C, and D)** are presentative FC tracings showing changes in fluorescence intensity of total JNK following treatments with vehicle, and RATM. **Sub-Figure 1(E):** Quantitative analyses of FC tracings after each treatment. (n = 3 independent experiments; *p ≤ 0.05, two-way ANOVA with Tukey's multiple comparison tests).

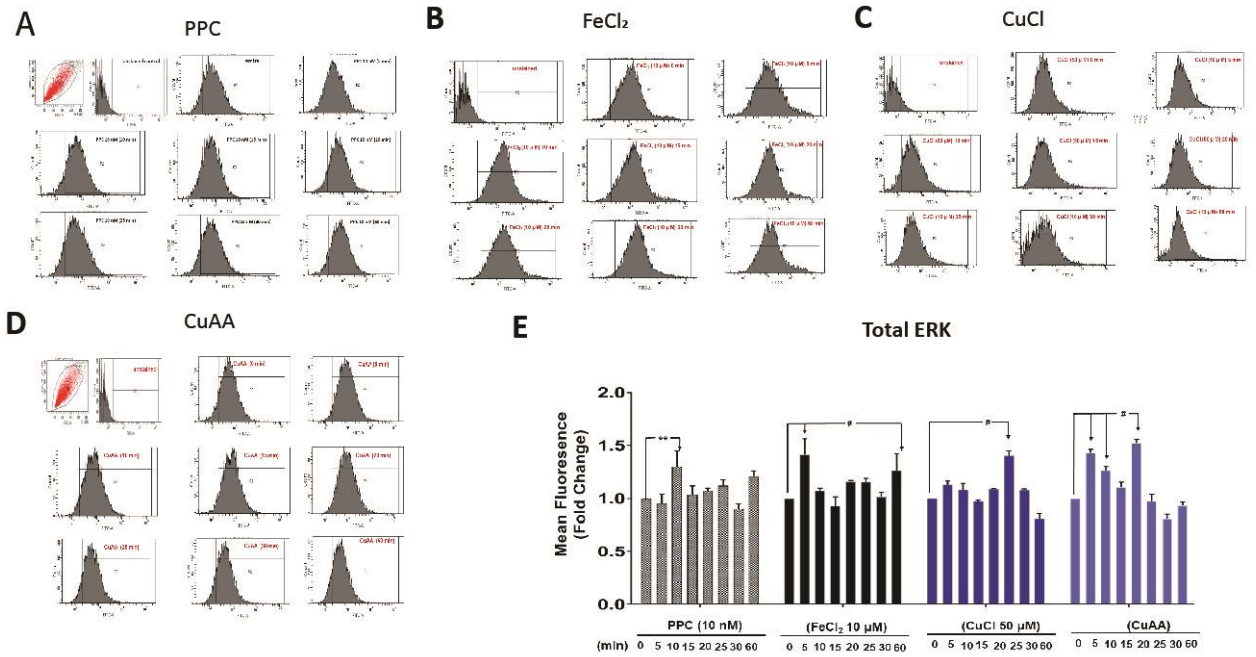

Figure 2. Effect of RATM prooxidants on total ERK1/2 protein expression in FLS. HIG-82 cells (synovial fibroblasts) were treated with PPC, $FeCl_2$, and CuCl alone and in combination with Ascorbic acid (CuAA) at different time points. Cells were then further used to run flow cytometric (FC) quantitative analysis of total ERK1/2. **Sub-Figures** 2 **(A, B, C, and D)** are representative FC tracings showing changes in fluorescence intensity of total JNK following treatments with vehicle, RATM oxidants. **Sub-Figure 2 (E)** Quantitative analyses of FC tracings after each treatment. (n = 3 independent experiments; *p ≤ 0.05, two-way ANOVA with Tukey's multiple comparison tests).

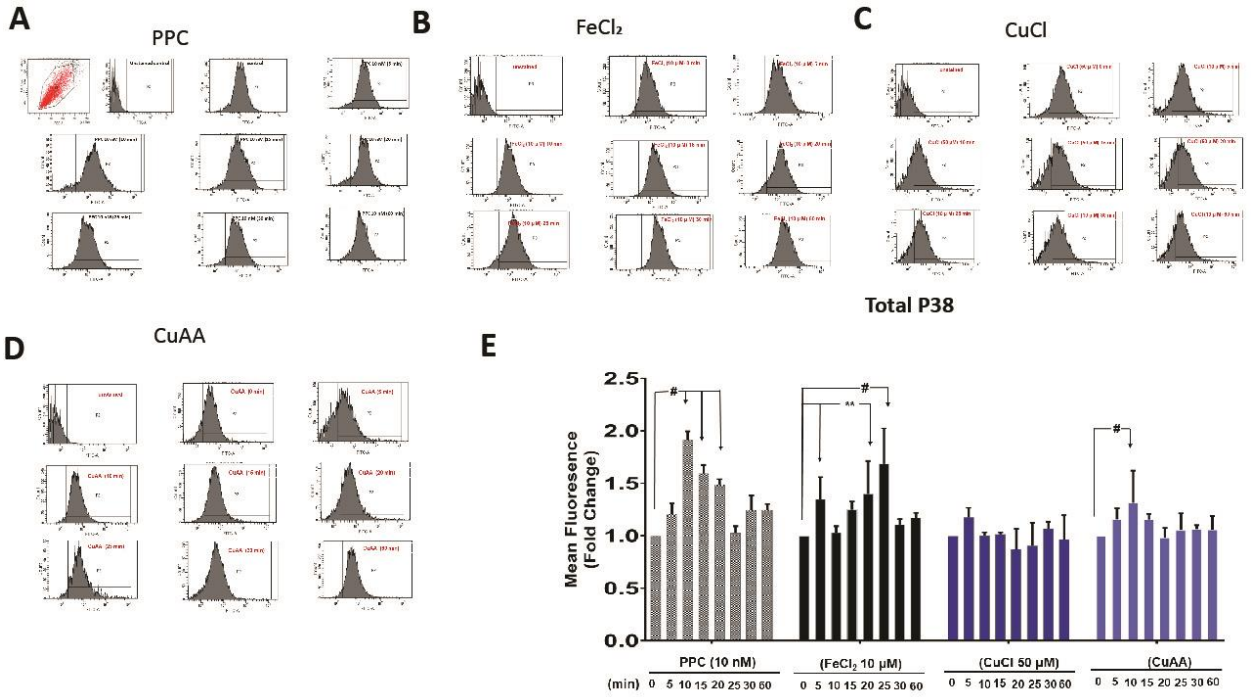

Figure 3. Effect of RATM prooxidants on total P38 protein expression in FLS. HIG-82 cells (synovial fibroblasts) were treated with PPC, $FeCl_2$, and CuCl alone and in combination with Ascorbic acid (CuAA) at different time points. Cells were then further used to run flow cytometric (FC) quantitative analysis of total P38. **Sub-Figures** 3 **(A, B, C, and D)** are representative FC tracings showing changes in fluorescence intensity of total JNK following treatments with vehicle and RATM oxidants. **Sub-Figure 3 (E)** Quantitative analyses of FC tracings after each treatment. (n = 3 independent experiments; *p ≤ 0.05, two-way ANOVA with Tukey's multiple comparison tests).

3.2. RATM oxidants activate phospho JNK, ERK 1/2, and P38. To validate the biological significance of the up-regulation of total signalling proteins, we used flow cytometry and western blot to determine the extent of the phosphorylated signalling proteins post treatment. Our results show significant upregulation of both the phosphorylated ERK ½ and P38 proteins post RATM treatments (**Fig. 4**). We further confirmed the effect of RATM oxidants on MAPK and to show that the persistence of theses activated signals was due to the induction of the activity of the kinase enzymes rather than only upregulation of protein expressions. FLS cells were treated with PPC, $FeCl_2$, CuCl, and CuAA, then MAPK pathways were assayed by phospho – specific antibodies for JNK, ERK1/2 and P38 by western blot and FC at 0, 5, 10, 15, 20, 25, 30 and 60 min.

Our data clearly show that PPC (10 nM) or $FeCl_2$ (10 µM) treatment upregulated phospho ERK1/2 and P38 and P-JNK. Furthermore, treatment with CuCl (50 µM) was shown to upregulate P- JNK, ERK1/2 while the increase in phosphorylated P38 was not significant. In addition, treatment with CuAA significantly upregulated JNK at 20 and 60 min along with upregulation of P-ERK1/2 at 5 and 20 min, while the upregulation for P38 was only at 5min.

Over all the data suggests that RATM oxidants readily activate phosphorylation in the three main MAPK modules with a potential implication in the molecular role of these RATM oxidants in clinical initiation and maintenance of RA.

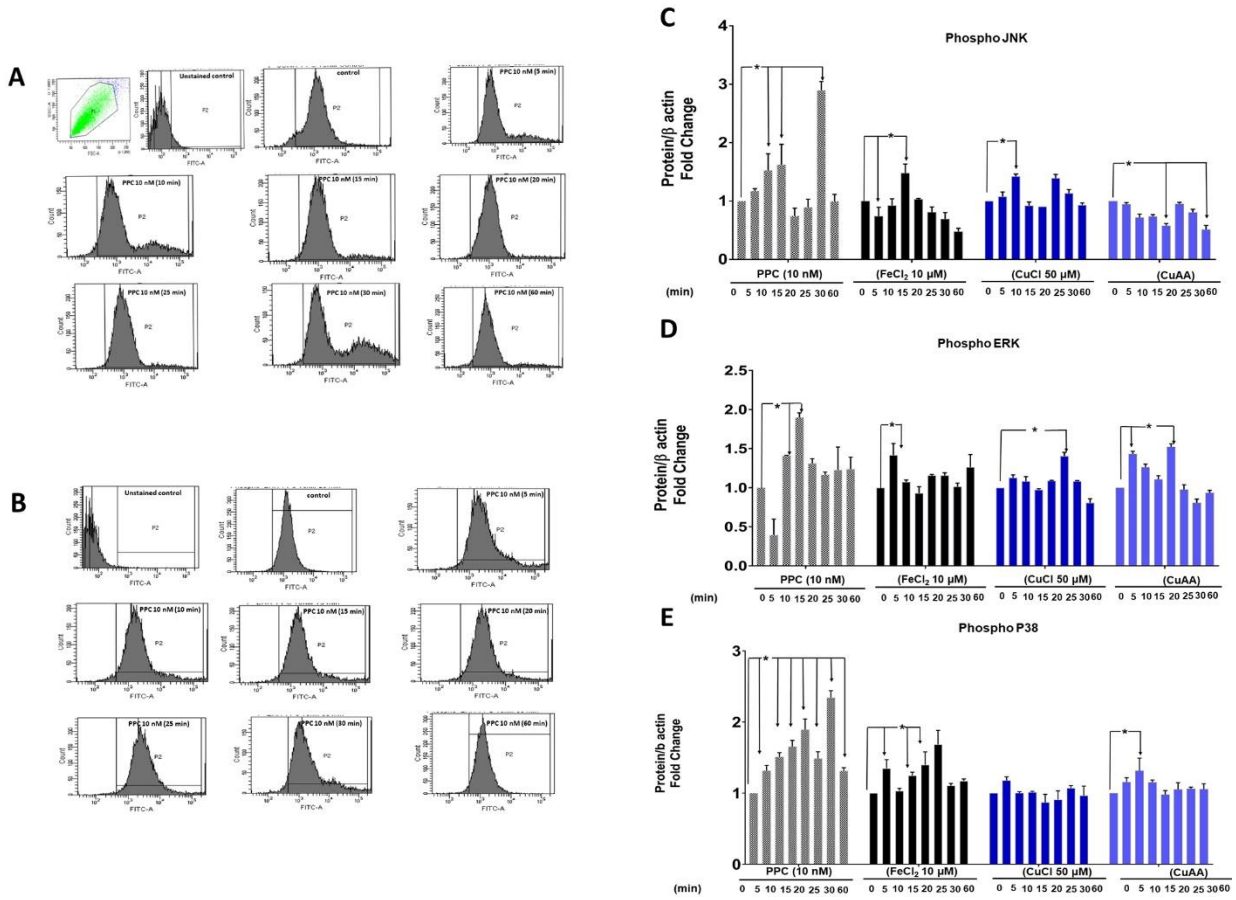

Figure 4. Effect of RATM treatment on phosphorylated MAPKs expression in FLS cells.
HIG-82 cells (synovial fibroblasts) were treated with PPC, $FeCl_2$, and CuCl alone and in combination with Ascorbic acid (CuAA) at different time points. Cells were then further used to run flow cytometric quantitative analysis of phosphorylated MAPKs. Figures represent **(A)** FC tracings showing changes in fluorescence intensity of p-JNK, **(B)** FC tracings showing changes in fluorescence intensity of p-ERK, **(C)** Quantitative analyses of FC tracings of p-JNK after each treatment, **(D)** Quantitative analyses of FC tracings of pERK and **(E)** Quantitative analyses of FC tracings of pP38, following treatments with vehicle, RATM oxidants. ($n = 3$ independent experiments; $*p \leq 0.05$, # $p < 0.0001$, two-way ANOVA with Tukey's multiple comparison tests).

3.3 Optimization conditions for TLR4 siRNA knockdown transfection. Our previous work had shown that RATM as exogenous oxidants induced oxidative stress and activated TLR4 [34]. To further investigate the potential function of TLR4, we used TLR4 siRNA technique to knockdown the endogenous TLR4 gene expression and to study the AP-1 downstream signalling of TLR 4 activation.

The best transfection conditions were established to ensure that siRNA could efficiently be transfected into HIG-82 cells using Lipofectamine RNAiMax. TLR4 mRNA and protein were detected by RT-PCR and by western blot analysis subsequent to transfection of cells. Negative control TLR4 siRNA was transfected into cells with Lipofectamine RNAiMax. Subsequent to 24 h of transfection, the transfection efficiency was determined using RT-PCR. The ratio of TLR4 siRNA to Lipofectamine RNAiMax that resulted in the highest transfection efficiency was (1:1.25). In total, two custom designed siRNAs were transfected into FLS cells, with medium alone (blank), negative TLR4 siRNA or Lipofectamine RNAiMax alone as controls. Subsequent to 24 h, TLR4-siRNA-514 effectively reduced TLR4 m RNA expression in transfected cells (**Fig. 5**). There was a significant difference between the expression of TLR4 in TLR4-siRNA-514 group and blank control group. The TLR4 mRNA expression in the TLR4-siRNA-513, negative sequence transfection and RNAiMax alone groups was not significantly different from the expression.

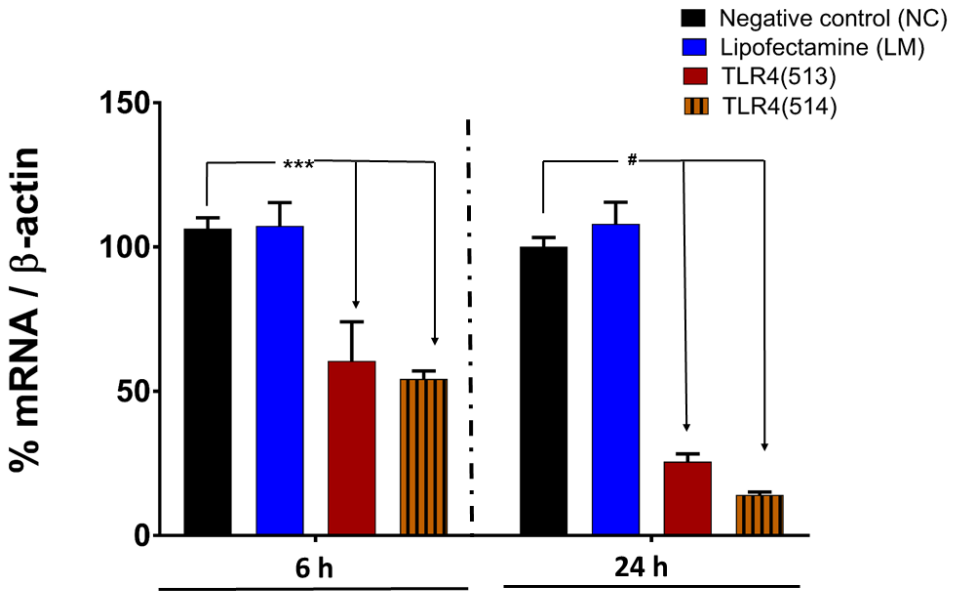

Figure 5. Role of TLR4-siRNA in TLR4 gene expression in FLS. Cells were transfected with siRNA-513, siRNA-514 or NC at two different time points (6 and 24 h). Relative TLR4 mRNA expression was quantified at the two time points after treatment using RT-qPCR and normalized against β- actin. The data represent 3 independent experiments # $P < 0.0001$, one-way ANOVA with Tukey's multiple comparison tests.

At 6 and 24 h post-silencing, mRNA expression levels of TLR4 in FLS were determined by RT-PCR. All samples were analyzed in duplicates in three separate experiments. The mRNA expression level of TLR4 in FLS from the siRNA-514 showed the highest level of efficiency at 24 h compared with 6 h. Therefore, 24 h was chosen as an experimental time for treatment. The results were further confirmed by WB. As shown in (**Fig. 6**), expression levels of TLR4 in FLS decreased significantly at 24 h post silencing, while no obvious changes in TLR4 levels occurred in the negative TLR4 siRNA group. The result showed that interference in TLR4 gene expression can persist for a long time. Therefore, the present study demonstrated a specific siRNA-directed knockdown of the TLR4 gene in this rabbit fibroblast-like synovial cell line. The data confirmed that TLR4-siRNA-514 provided a more effective silencing of TLR4 gene expression than TLR4-siRNA-513. On this basis, we used TLR4-siRNA-514 in all subsequent experiments. This suggests that cells transfected with TLR4-siRNA-514 using Lipofectamine RANiMax are viable and effective, thereby providing a reliable tool to study RATM oxidant /TLR4 coupled signalling in FLS.

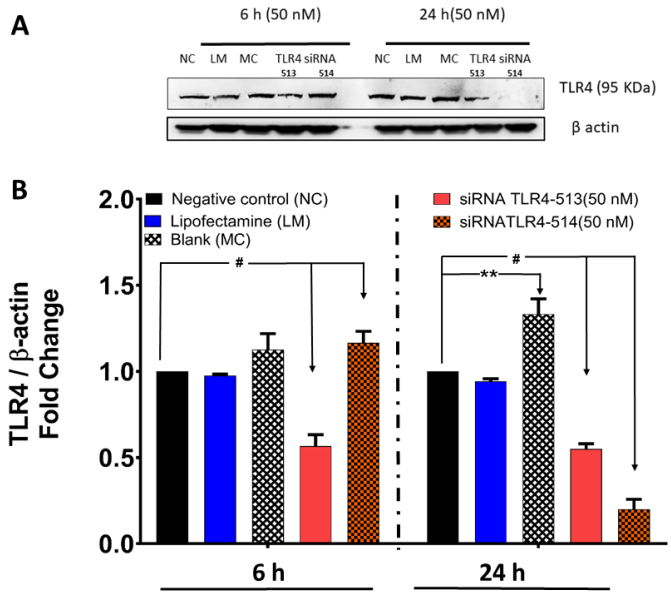

Figure 6. Role of TLR4-siRNA in TLR4 protein expression in FLS following transfections with TLR4-siRNA. Cells were transfected with siRNA-513, siRNA-514, NC or vehicle control at two different time points (6 and 24 h). Cells were treated with vehicle control. **(A)** TLR4 protein expression levels were determined by Western blots. The histogram **(B)** represents the optical density (OD) ratios of TLR4 immunoblot signals normalized to those of β-actin from the same tests. The data represent 3 independent experiments [n=3, # P < 0.001, two-way ANOVA with Tukey's multiple comparison tests].

3.4. Phospho- ERK1/2, JNK, and P 38 protein expression after knockdown of TLR4 gene using siRNA. To investigate the potential function of TLR4 in ERK1/2, JNK, and P38, TLR4-siRNA-514 was used to knockdown the endogenous TLR4 gene expression. WB data following PPC (10 nM) treatment after TLR4-siRNA-514 transfection showed dysregulation in phosphorylated ERK1/2, JNK and P38 expression (**Fig. 7**).

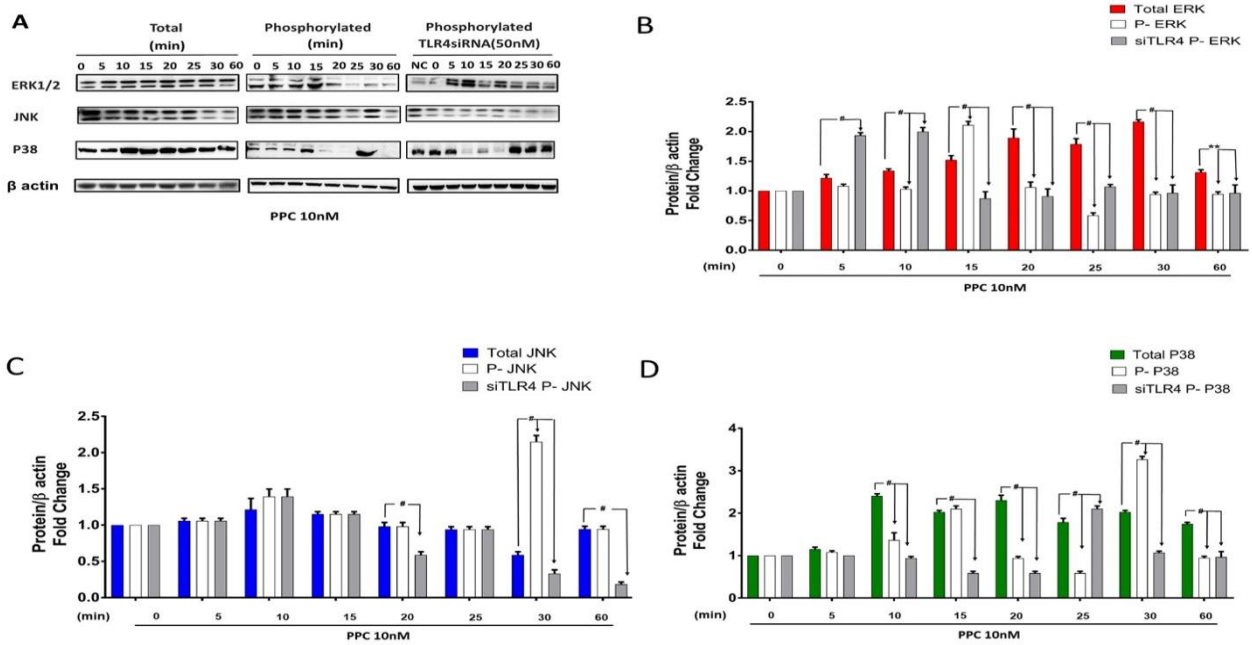

Figure 7. Role of TLR4-siRNA-514 in ERK1/2, JNK and P38 protein levels expressed in FLS following PPC (10 nM) treatment.. At different time points, cells were treated with vehicle control or PPC. **(A)** TLR4 protein expression levels were determined by Western blots. The histograms represent the optical density ratios of **(B)** ERK immunoblot signals, **(C)** OD ratios of JNK immunoblots and **(D)** OD ratios of P38, all normalized to β-actin from same blots. The data represent 3 independent experiments [n=3, # P < 0.001, two-way ANOVA with Tukey's multiple comparison tests]

3.5. c- Fos and c- Jun (AP-1) protein levels after knockdown of the TLR4 gene using siRNA. To investigate the potential function of TLR4 c-Fos and c-Jun proteins, we again used TLR4-siRNA-514 to knockdown endogenous TLR4 gene expression. Treatment with PPC (10 nM) significantly upregulated c-Jun at 5 min, and c-Fos at 10 and 20 min. However, treatment with PPC post siRNA transfection showed dysregulation in c Jun and c Fos expression (**Fig. 8**).

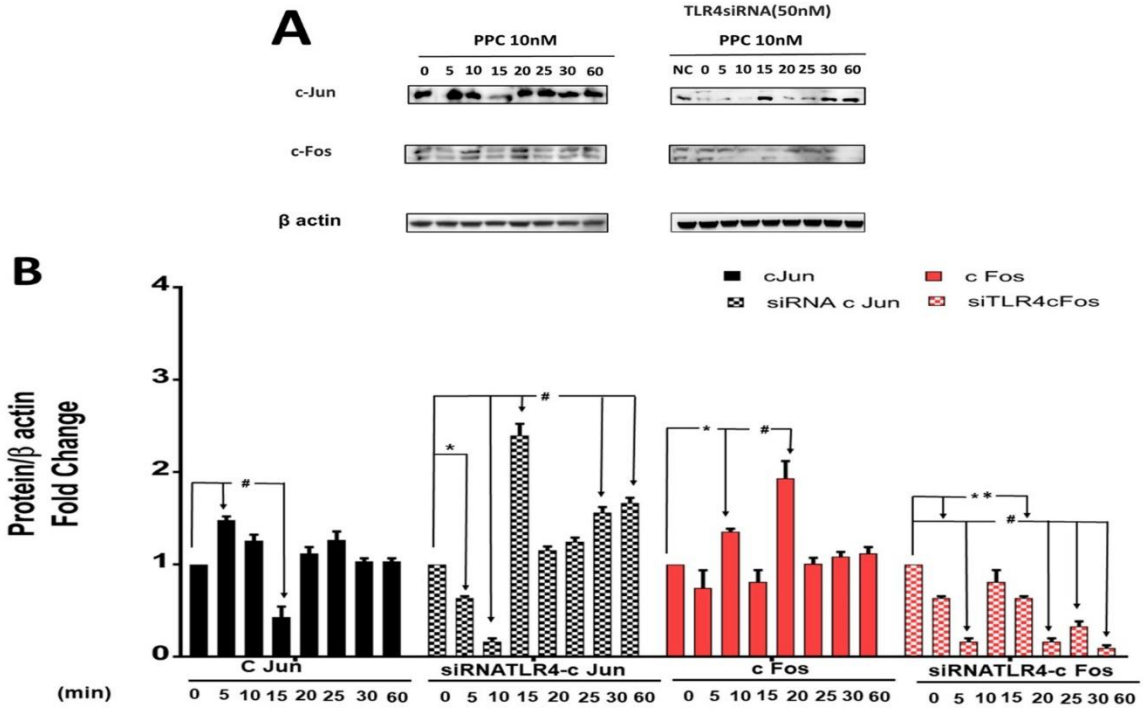

Figure 8. Role of TLR4-siRNA-514 in ERK1/2, JNK, and P38 proteins expression in FLS following PPC (10 nM) treatment. At different time points, cells were treated with vehicle control or PPC. **(A)** The protein expression levels were determined by Western blots. The histograms represent **(B)** the optical density (OD) ratios of c Fos and c Jun immunoblot signals normalized to β-actin from same tests. The data represent 3 independent experiments [n=3, # $P < 0.001$, two-way ANOVA with Tukey's multiple comparison tests]

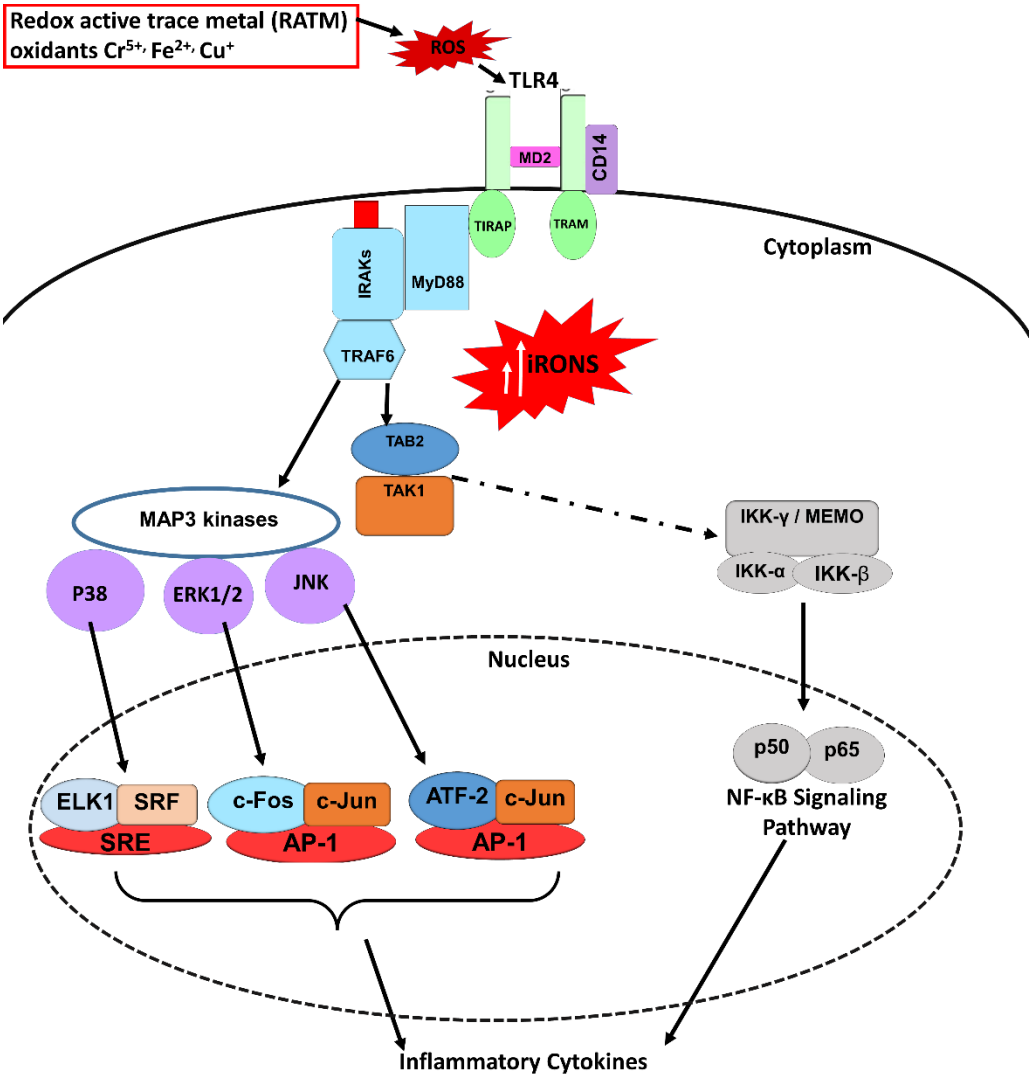

Figure 9. A graphical abstract representation of **AP-1 signaling pathway** in a putative mechanism of inflammation induced by exogenous RATM-coupled activation of TLR4. Abbreviations: CD14: cluster of differentiation 14, MD-2: Myeloid differentiation protein 2, TLR: toll-like receptor, MyD88: myeloid differentiating primary response gene 88, IRAK: IL-1 receptor associated kinase, TRAF: tumor-necrosis-factor receptor associated factor 6, TAK: transforming growth factor β activated kinase, TAB: TAK1-binding protein, AP-1: activator protein 1.

4. Discussion

We have shown that RATM can induce oxidative stress through TLR4 activation, which results in the release of pro-inflammatory cytokines and high mobility group box 1 protein. Total expression levels of mitogen-activated protein kinase (MAPK) and the time-course formation of phosphorylated moieties of MAPK were increased in the rabbit fibroblast-like synovial (FLS) cells. TLR4 siRNA treatment attenuated the role of TLR4 in activator protein -1 (AP-1) signaling activity with a corresponding decrease in the time-course phosphorylation levels of AP-1-activation-related proteins as well.

Given the nature of RA and its prevalence in the general population, our data presents another molecular dimension in understanding of the disease process, which further reveals the complexities of the disease pathogenesis. There is a critical need to understand the molecular biology that may underlie the development, propagation, and maintenance of RA in other to develop an effective treatment, which is presently elusive. Previously, we showed that RATM as exogenous oxidants can induce oxidative stress, which may contribute to the pathogenesis of RA through the ROS/TLR4-coupled activation[34]. We also have shown that RATM-mediated increase in intracellular ROS (iROS), which protected FSL cells against apoptosis by induction of autophagy, but also increased proliferation of these cells[39]. To understand the role of RATM in the pathogenesis of

RA, it is crucial to clarify their potential role in TLR4 signalling in FLS under oxidant stress.

Whereas TLR mediates immune inflammatory responses, different studies have proposed an association between TLR activation, uncontrolled inflammation and tumour development[20,39,40]. TLR4 and other TLRs have been detected in throat, breast, colorectal, gastric, prostate, and lung cancer cell lines[40]. Silencing of TLR4 signalling in cancer cells has been shown to reduce the risk of tumour formation [41,42]. LPS-induced TLR4 signalling in cancer cells promoted cell survival and proliferation in hepatocellular carcinoma (HCC) [43]. This suggests that the role of TLR4 in different cell types is distinctive. Although TLR4 was hypothesized to play an important role in the initiation and progression of HCC, little is known about the interaction between TLR4 and the progression of many diseases.

In the present study, we investigated the biological effects of TLR4 on AP-1 signalling pathway. We determined the best transfection conditions to ensure that siRNA could efficiently transfect into HIG-82 cells using Lipofectamine RNAiMax. TLR4 mRNA and protein were detected by RT-PCR and by western blot analysis subsequent to transfection of cells. Our data confirmed that TLR4-siRNA-514 was a more efficient siRNA for silencing TLR4 than TLR4-siRNA-513. This confirmed that cells transfected with TLR4-siRNA-514 were both viable and effective in providing a reliable tool to study the biological effects of RATM oxidant / TLR4-coupled signalling in FLS.

The signal transduction pathways that are activated in RA in response to elevated levels of proinflammatory cytokines include JNK, ERK1/2, P38 kinase and JAK/STAT[44, 45], which are activated by myeloid-related protein (MRP) complex[46]. JNK, p38 and ERKs are constitutively expressed in RA and osteoarthritis (OA) FLS, while p38 and ERK1/2 are readily phosphorylated in both RA and OA FLS after interleukin-1 (IL-1) stimulation. JNK was phosphorylated in RA FLS but not in OA FLS after IL-1 stimulation [47]. JNKs are known to be activated by oxidative stress and thus, are likely to be involved in RATM activation of cells. This is consistent with our data where RATM oxidants elevated the levels of proinflammatory cytokines[34] and induced the activity of JNK, ERK1/2, p38 kinase. More importantly *in vitro* MRP8/14-mediated gene expression upregulation by macrophages was associated with activation of several protein kinase-mediated pathways including c-JNK, ERK1/2 and JAK/STAT as well as NF-κB activation[46]. This again provides another possible rationale for our results, where our data as well has shown RATM-induced upregulation in MRP8/14 expression.

MAPKs are highly activated in the rheumatoid synovium and contribute to the inflammatory and destructive mechanisms. MAPKs are known to be involved in regulating COX-2 expression [48], and we have also shown that TNF-α up-regulates COX-2 expression with increased prostaglandin E2 (PGE_2) production in the FLS cells leading to increased activation of MMP-9 expression and enhanced HMGB1 release to potentially propagate and maintain RA pathogenesis[49]. Therefore, it is anticipated that RATM oxidants by upregulating MAPK and TNF-α expression also upregulates COX-2 expression to contribute to the inflammatory process. JNKs had been shown to play an important function in cytokine production and extracellular matrix (ECM) degradation by regulating matrix metalloproteinase (MMP) in FLS and animal models of RA[24, 47]. In particular, JNK plays an essential role in cytokine-mediated AP-1 induction and MMP gene expression in FLS[26, 47].

Three isoforms of JNK have been characterized, namely JNK1, 2 and 3. JNK1 and 2 are ubiquitous while JNK3 is primarily restricted to neurologic tissue[50]. JNK2 deficiency has only modest progenitors that do not mature into bone-resorbing osteoclasts[51]. These data suggest that JNK participates in synovial inflammation and joint destruction associated with RA, and could potentially be targeted in RA therapeutics. While JNKs are attractive targets, they regulate many normal cell functions, especially in matrix remodeling and host defense[52,53]. Thus, blocking all JNK activity, or even a specific JNK1 activity, may potentially affect host defenses or matrix homeostasis. An alternative strategy, would be targeting a distinct upstream kinase like MKK4 or MKK7. This could permit some normal JNK functions while interfering with a subset that is pathogenic in synovitis. MKK4 and MKK7, two JNK upstream kinases, exhibit some different properties although they can synergistically activate JNKs. TNFα and IL-1β mainly activate MKK7 in murine embryonic fibroblasts, while ultraviolet radiation, anisomycin, heat and osmotic shock activate both MKK4 and MKK7[54]. These data suggest that MKK4 and MKK7 contribute separately to the activation of JNKs in response to environmental stress or inflammatory cytokines.

The ERK signalling pathway is involved in the regulation of cell survival and cell death. ERK-1, -2 signallings are important regulators of the biological activity of hemopoietic progenitor cells in

the RA synovium [55]. The suppression of ERK activation by IL-1, has been used to reduce inflammation and downgrade joint destruction in a rat model of adjuvant-induced arthritis[23]. The impact of inhibiting ERK signalling in arthritis appears to center on the fact that ERK is a key mediator involved in joint destruction. For example, the production of the protease stromelysin (MMP3) by cultured rabbit synovial fibroblasts has been shown to be dependent on IL-1α-induced activation of ERK [56]; comparable results were obtained as well in human synovial fibroblasts[57].

Interestingly, the inhibition of P38 MAPK α using the orally–administered P38 inhibitor, SCIO-469, to patients with active RA failed to show efficacy in adult RA clinical trials[32]. In view of these disappointing results, further attention should be focused on ERK1/2 and JNK signalling in synoviocytes.

Our data has shown that PPC as RATM induces c- Jun, which regulates a program of pro- and anti-inflammatory gene expression during macrophage activation and thereby influences the severity of arthritis. Thus c-Jun acts as a checkpoint during macrophage activation and promotes arthritis via differentially regulating COX-2 and arginase-1 levels and indeed may represent an interesting therapeutic target in RA[58].

In RA synovial tissues, constitutive expression of c-fos gene has been reported[59]. c-Fos protein is also expressed in fibroblast-like cells in RA synovium. The pathological importance of c-fos in RA was supported by the finding of destructive arthritis in c-fos transgenic mice. Therefore, c-fos may be considered an appropriate target gene in the treatment/management of RA as well.

In summary, our studies suggest that RATM oxidants play a role in RA pathogenesis by enhancing the activity of MAPK and upregulating AP-1 transcription in FLS. This is again in agreement with the pathophysiological changes that occur in active RA, which thus validates our simple *in vitro* model. Our findings may serve as a basis for individualized RA therapy considering the underlying mechanism of the pathogenesis. Possible interventions involve not only the classical cytokine blockades of TNFα, IL-6, or IL1β, but a number of additional targets, including TLRs, chemokines, and chemokine receptors – such as IL-8 and its receptors – and possibly more. Importantly, though, this would allow such agents to be efficient mainly in the phase of disease development, which precedes the initiation of joint inflammation; an inflammation which involves a broad spectrum of inflammatory mechanisms that are very difficult to completely eradicate even with broad and powerful interventions such as those caused by TNF-α and IL-6 blockade. A positive correlation observed between oxidant stress damage and RA severity suggests that inclusion of antioxidants in the pharmacotherapy of RA is worthy of serious consideration.

Conclusions:

Whereas RATM increased intracellular ROS that can induce oxidative stress, they also induced MAPK kinases to upregulate the expression of AP-1 proteins in synovial cells. Our results show that RATM/TLR4-coupled activation potentially contribute to the pathogenesis of RA. Therefore, the signaling pathway by which inflammatory responses and their destructive sequence of events may occur in RA through synovial cells underlies the need for developing therapeutic agents for individualized RA therapy with crucial consideration for the underlying mechanism(s) of its pathogenesis.

Acknowledgements

#The project described was supported by Award Number DE021888 from the National Institute of Dental & Craniofacial Research (OJI). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Dental & Craniofacial Research or the National Institutes of Health.

Author Contributions

This work was done by AA in partial fulfilment of the Interdisciplinary PhD degree at UMKC School of Graduate Studies under the direction and guidance of Dr. Igwe.

Conflict of Interest:

Authors have no Conflict of Interest.

REFERENCES

1. Lajas C, Abasolo L, Bellajdel B, Hernandez-Garcia C, Carmona L, Vargas E, Lazaro P, Jover JA. Costs and predictors of costs in rheumatoid arthritis: a prevalence-based study. *Arthritis Rheum*. 2003;49(1):64-70.
2. Helmick CG, Felson DT, Lawrence RC, Gabriel S, Hirsch R, Kwoh CK, Liang MH, Kremers HM, Mayes MD, Merkel PA, Pillemer SR, Reveille JD, Stone JH. Estimates of the prevalence of arthritis and other rheumatic conditions in the United States. Part I. *Arthritis Rheum*. 2008;58(1):15-25.
3. Gibofsky A. Overview of epidemiology, pathophysiology, and diagnosis of rheumatoid arthritis. *The American J Managed Care*. 2012;18(13 Suppl):S295-302.
4. McInnes IB, Schett G. The pathogenesis of rheumatoid arthritis. *N Engl J Med*. 2011;365(23):2205-2219.
5. Noss EH, Brenner MB. The role and therapeutic implications of fibroblast-like synoviocytes in inflammation and cartilage erosion in rheumatoid arthritis. *Immunological reviews*. 2008;223:252-270.
6. Datta S, Kundu S, Ghosh P, De S, Ghosh A, Chatterjee M. Correlation of oxidant status with oxidative tissue damage in patients with rheumatoid arthritis. *Clinical rheumatology*. 2014;33(11):1557-1564.
7. Kundu S, Ghosh P, Datta S, Ghosh A, Chattopadhyay S, Chatterjee M. Oxidative stress as a potential biomarker for determining disease activity in patients with rheumatoid arthritis. *Free Radic Res*. 2012;46(12):1482-1489.
8. Schonthaler HB, Guinea-Viniegra J, Wagner EF. Targeting inflammation by modulating the Jun/AP-1 pathway. *Ann Rheum Dis*. 2011;70 Suppl 1:i109-112.
9. Okamoto H, Cujec TP, Yamanaka H, Kamatani N. Molecular aspects of rheumatoid arthritis: role of transcription factors. *The FEBS journal*. 2008;275(18):4463-4470.
10. Guha M, Mackman N. LPS induction of gene expression in human monocytes. *Cellular signalling*. 2001;13(2):85-94.
11. Zenz R, Eferl R, Scheinecker C, Redlich K, Smolen J, Schonthaler HB, Kenner L, Tschachler E, Wagner EF. Activator protein 1 (Fos/Jun) functions in inflammatory bone and skin disease. *Arthritis research & therapy*. 2008;10(1):201.
12. Zenz R, Wagner EF. Jun signalling in the epidermis: From developmental defects to psoriasis and skin tumors. *The international journal of biochemistry & cell biology*. 2006;38(7):1043-1049.
13. Chang L, Karin M. Mammalian MAP kinase signalling cascades. *Nature*. 2001;410(6824):37-40.
14. Kyriakis JM, Avruch J. Mammalian MAPK signal transduction pathways activated by stress and inflammation: a 10-year update. *Physiological reviews*. 2012;92(2):689-737.
15. Harnett MM, Katz E, Ford CA. Differential signalling during B-cell maturation. *Immunology letters*. 2005;98(1):33-44.
16. Liu JO. The yins of T cell activation. *Science's STKE : signal transduction knowledge environment*. 2005;2005(265):re1.
17. Kolls JK, Linden A. Interleukin-17 family members and inflammation. *Immunity*. 2004;21(4):467-476.
18. Moens U, Kostenko S, Sveinbjornsson B. The Role of Mitogen-Activated Protein Kinase-Activated Protein Kinases (MAPKAPKs) in Inflammation. *Genes (Basel)*. 2013;4(2):101-133.
19. Pearson G, Robinson F, Beers Gibson T, Xu BE, Karandikar M, Berman K, Cobb MH. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. *Endocr Rev*. 2001;22(2):153-183.
20. Goodridge HS, Harnett W, Liew FY, Harnett MM. Differential regulation of interleukin-12 p40 and p35 induction via Erk mitogen-activated protein kinase-dependent and -independent mechanisms and the implications for bioactive IL-12 and IL-23 responses. *Immunology*. 2003;109(3):415-425.
21. Nah SS, Won HJ, Ha E, Kang I, Cho HY, Hur SJ, Lee SH, Baik HH. Epidermal growth factor increases prostaglandin E2 production via ERK1/2 MAPK and NF-kappaB pathway in fibroblast like synoviocytes from patients with rheumatoid arthritis. *Rheumatology international*. 2010;30(4):443-449.
22. Barchowsky A, Frleta D, Vincenti MP. Integration of the NF-kappaB and mitogen-activated protein kinase/AP-1 pathways at the collagenase-1 promoter: divergence of IL-1 and TNF-dependent signal transduction in rabbit primary synovial fibroblasts. *Cytokine*. 2000;12(10):1469-1479.
23. Yamamoto A, Fukuda A, Seto H, Miyazaki T, Kadono Y, Sawada Y, Nakamura I, Katagiri H, Asano T, Tanaka Y, Oda H, Nakamura K, Tanaka S. Suppression of arthritic bone destruction by adenovirus-mediated dominant-negative Ras gene transfer to synoviocytes and osteoclasts. *Arthritis Rheum*. 2003;48(9):2682-2692.
24. Han Z, Boyle DL, Chang L, Bennett B, Karin M, Yang L, Manning AM, Firestein GS. c-Jun N-

terminal kinase is required for metalloproteinase expression and joint destruction in inflammatory arthritis. *J Clin Invest*. 2001;108(1):73-81.

25. Liacini A, Sylvester J, Qing Li W, Huang W, Dehnade F, Ahmad M, Zafarullah M. Induction of matrix metalloproteinase-13 gene expression by TNF-α is mediated by MAP kinases, AP-1, and NF-κB transcription factors in articular chondrocytes. *Exp Cell Res*. 2003;288(1):208-217.

26. Liacini A, Sylvester J, Li WQ, Zafarullah M. Inhibition of interleukin-1-stimulated MAP kinases, activating protein-1 (AP-1) and nuclear factor kappa B (NF-kappa B) transcription factors down-regulates matrix metalloproteinase gene expression in articular chondrocytes. *Matrix biology : journal of the International Society for Matrix Biology*. 2002;21(3):251-262.

27. Pulverer BJ, Kyriakis JM, Avruch J, Nikolakaki E, Woodgett JR. Phosphorylation of c-jun mediated by MAP kinases. *Nature*. 1991;353(6345):670-674.

28. Han J, Lee JD, Bibbs L, Ulevitch RJ. A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. *Science* (New York, NY). 1994;265(5173):808-811.

29. Lee JC, Laydon JT, McDonnell PC, Gallagher TF, Kumar S, Green D, McNulty D, Blumenthal MJ, Heys JR, Landvatter SW, et al. A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature*. 1994;372(6508):739-746.

30. Herskowitz I. MAP kinase pathways in yeast: for mating and more. *Cell*. 1995;80(2):187-197.

31. Duch A, de Nadal E, Posas F. The p38 and Hog1 SAPKs control cell cycle progression in response to environmental stresses. *FEBS Lett*. 2012;586(18):2925-2931.

32. Malemud CJ. Targeted drug development for arthritis. *Future Medicinal Chemistry*. 2012;4(6):701-703.

33. Karki R, Igwe OJ. Toll-like receptor 4-mediated nuclear factor kappa B activation is essential for sensing exogenous oxidants to propagate and maintain oxidative/nitrosative cellular stress. *PLoS ONE*. 2013;8(9):e73840.

34. Alsousi AA, Igwe OJ. Redox-active trace metal-induced release of high mobility group box 1(HMGB1) and inflammatory cytokines in fibroblast-like synovial cells is Toll-like receptor 4 (TLR4) dependent. *Biochimica et Biophysica Acta (BBA) - Mol Basis of Dis*. 2018(11) 3847-3858.

35. Andersson U, Erlandsson-Harris H. HMGB1 is a potent trigger of arthritis. *Journal internal medicine*. 2004;255(3):344-350.

36. Kokkola R, Sundberg E, Ulfgren AK, Palmblad K, Li J, Wang H, Ulloa L, Yang H, Yan XJ, Furie R, Chiorazzi N, Tracey KJ, Andersson U, Harris HE. High mobility group box chromosomal protein 1: a novel proinflammatory mediator in synovitis. *Arthritis Rheum*. 2002;46(10):2598-2603.

37. Kokkola R, Li J, Sundberg E, Aveberger AC, Palmblad K, Yang H, Tracey KJ, Andersson U, Harris HE. Successful treatment of collagen-induced arthritis in mice and rats by targeting extracellular high mobility group box chromosomal protein 1 activity. *Arthritis Rheum*. 2003;48(7):2052-2058.

38. Miesel R, Kroger H, Kurpisz M, Weser U. Induction of arthritis in mice and rats by potassium peroxochromate and assessment of disease activity by whole blood chemiluminescence and 99mpertechnetate-imaging. *Free Radic Res*. 1995;23(3):213-227.

39. Alsousi AA, Igwe OJ. Autophagy protects against redox active trace metal (RATM) – induced cell death in rabbit synovial fibroblasts. *Exp Cell Res*. 2018; 374 (1):19-28.

40. Gu J, Liu Y, Xie B, Ye P, Huang J, Lu Z. Roles of toll-like receptors: From inflammation to lung cancer progression. *Biomedical reports*. 2018;8(2):126-132.

41. Liu Y, Li T, Xu Y, Xu E, Zhou M, Wang B, Shen J. Effects of TLR4 gene silencing on the proliferation and apotosis of hepatocarcinoma HEPG2 cells. *Oncology letters*. 2016;11(5):3054-3060.

42. Ahmed A, Redmond HP, Wang JH. Links between Toll-like receptor 4 and breast cancer. *Oncoimmunology*. 2013;2(2):e22945.

43. Wang L, Zhu R, Huang Z, Li H, Zhu H. Lipopolysaccharide-induced toll-like receptor 4 signaling in cancer cells promotes cell survival and proliferation in hepatocellular carcinoma. *Dig Dis Sci*. 2013;58(8):2223-2236.

44. Malemud CJ. Dysfunctional Immune-Mediated Inflammation in Rheumatoid Arthritis Dictates that Development of Anti-Rheumatic Disease Drugs Target Multiple Intracellular Signaling Pathways. *Anti-inflammatory & anti-allergy agents in medicinal chemistry*. 2011;10(2):78-84.

45. Malemud CJ. Intracellular Signaling Pathways in Rheumatoid Arthritis. *J Clin & Cellular Immunol*. 2013;4:160.

46. Youssef P, Roth J, Frosch M, Costello P, Fitzgerald O, Sorg C, Bresnihan B. Expression of myeloid related proteins (MRP) 8 and 14 and the MRP8/14 heterodimer in rheumatoid arthritis synovial membrane. *J Rheumatol*. 1999;26(12):2523-2528.

47. Han Z, Boyle DL, Aupperle KR, Bennett B, Manning AM, Firestein GS. Jun N-terminal kinase in rheumatoid arthritis. *J Pharmacol Exp Ther*. 1999;291(1):124-130.

48. Dean JL, Sarsfield SJ, Tsounakou E, Saklatvala J. p38 Mitogen-activated protein kinase stabilizes mRNAs that contain cyclooxygenase-2 and tumor necrosis factor AU-rich elements by inhibiting deadenylation. *J Biol Chem*. 2003;278(41):39470-39476.

49. Alsousi AA , Siddiqui S, Igwe OJ. Cytokine-mediated Differential Regulation of Cyclooxygenase-2, High Mobility Group Box 1 Protein and Matrix Metalloproteinase-9 Expression in Fibroblast-like Synovial Cells. *J Clin & Exp Pharmacol*. 2017;7(4).

50. Davis RJ. Signal transduction by the JNK group of MAP kinases. *Cell*. 2000;103(2):239-252.

51. David JP, Sabapathy K, Hoffmann O, Idarraga MH, Wagner EF. JNK1 modulates osteoclastogenesis through both c-Jun phosphorylation-dependent and -independent mechanisms. *J Cell Sci*. 2002;115(Pt 22):4317-4325.

52. Nishina H, Fischer KD, Radvanyi L, Shahinian A, Hakem R, Rubie EA, Bernstein A, Mak TW, Woodgett JR, Penninger JM. Stress-signalling kinase Sek1 protects thymocytes from apoptosis mediated by CD95 and CD3. *Nature*. 1997;385(6614):350-353.

53. Sabapathy K, Jochum W, Hochedlinger K, Chang L, Karin M, Wagner EF. Defective neural tube morphogenesis and altered apoptosis in the absence of both JNK1 and JNK2. *Mech Dev*. 1999;89(1-2):115-124.

54. Tournier C, Dong C, Turner TK, Jones SN, Flavell RA, Davis RJ. MKK7 is an essential component of the JNK signal transduction pathway activated by proinflammatory cytokines. *Genes & Development*. 2001;15(11):1419-1426.

55. Varfolomeev E, Goncharov T, Maecker H, Zobel K, Komuves LG, Deshayes K, Vucic D. Cellular inhibitors of apoptosis are global regulators of NF-kappaB and MAPK activation by members of the TNF family of receptors. *Sci Signal*. 2012;5(216):ra22.

56. Thiel MJ, Schaefer CJ, Lesch ME, Mobley JL, Dudley DT, Tecle H, Barrett SD, Schrier DJ, Flory CM. Central role of the MEK/ERK MAP kinase pathway in a mouse model of rheumatoid arthritis: potential proinflammatory mechanisms. *Arthritis Rheum*. 2007;56(10):3347-3357.

57. Tagoe CE, Marjanovic N, Park JY, Chan ES, Abeles AM, Attur M, Abramson SB, Pillinger MH. Annexin-1 mediates TNF-alpha-stimulated matrix metalloproteinase secretion from rheumatoid arthritis synovial fibroblasts. *J Immunol*. 2008;181(4):2813-2820.

58. Hannemann N, Jordan J, Paul S, Reid S, Baenkler HW, Sonnewald S, Bäuerle T, Vera J, Schett G, Bozec A. The AP-1 Transcription Factor c-Jun Promotes Arthritis by Regulating Cyclooxygenase-2 and Arginase-1 Expression in Macrophages. *J Immunol*. 2017;198(9):3605-3614.

59. Dooley S, Herlitzka I, Hanselmann R, Ermis A, Henn W, Remberger K, Hopf T, Welter C. Constitutive expression of c-fos and c-jun, overexpression of ets-2, and reduced expression of metastasis suppressor gene nm23-H1 in rheumatoid arthritis. *Ann Rheum Dis*. 1996;55(5):298-304.