p38 inhibition and not MK2 inhibition enhances the secretion of chemokines from TNF-α activated rheumatoid arthritis fibroblast-like synoviocytes

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Abstract

Objectives

For many years the p38 MAP kinase (MAPK) has been a major anti-inflammatory target for the development of an oral therapy for rheumatoid arthritis (RA). However, disappointing results from Phase II clinical studies suggest that adaptations may occur, which allow escape from blockade of the p38 pathway. In this study we investigated whether p38 inhibition mediated JNK activation represents such an escape mechanism.

Methods

Interaction between the JNK and p38 pathways was studied in TNF-α stimulated THP-1 monocytes, primary macrophages and fibroblast-like synoviocytes from OA and RA patients using pharmacological inhibitors and siRNAs.

Results

TNF-α induced phosphorylation of JNK and c-Jun was sustained by p38 inhibitors in monocytes, primary macrophages and FLS. Upregulation of Mip1α, Mip1β and IL-8 mRNAs and protein were observed upon p38 inhibition. More importantly, inhibition of MK2, the substrate of p38 did not sustain JNK activation upon TNF-α activation and did not elevate Mip1α, Mip1β and IL-8 chemokines as compared to TNF-α alone. In this study, TNF-α or IL-1β induced JNK activation is sustained by p38 inhibition, resulting in enhanced chemokine secretion.

Conclusion

Based on the suggested role of these chemokines in RA pathogenesis, the upregulation of these chemokines may provide an explanation for the lack of efficacy of p38 inhibitors in Phase II. The absence of any effect of MK2 inhibition in our models on this mechanism, while coming with similar efficacy on blocking p38, provides support for further investigations to reveal the potential of MK2 inhibition as a novel treatment of RA.

Key words

p38, MK2, TNF, synoviocytes, chemokines

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Introduction

The MAPKinase (MAPK) pathways are major targets in the development of novel oral anti-inflammatory drugs to treat rheumatoid arthritis (RA). MAPK are serine-threonine kinases that transmit a variety of intra- and extracellular signals resulting in cell proliferation, differentiation, survival, death and transformation. Three MAPK families have been identified: the extra cellular signal regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38. The ERK signalling pathway is activated by growth factors and mitogens, while the JNK and p38 MAPK pathways can be activated by environmental stress, ultraviolet light, osmotic shock and inflammatory cytokines (1). Ample evidence, both *in vitro* and *in vivo* exists indicating that the p38 MAPK pathway is an essential transducer of pro-inflammatory signals. For example, p38 inhibitors have been shown to decrease the production of prostaglandin E2, interleukin-6 (IL-6), tumour necrosis factor-α TNF-α and interleukin-1β (IL-1β) *in vitro* (2). In addition, p38 inhibitors reduce both paw swelling and joint damage in mouse and rat models of RA (3, 4).

However, attempts to develop p38 inhibitors for treatment of RA failed during clinical evaluation in Phase I/II studies, as a result of unacceptable safety and/ or lack of efficacy, including RO4402257 (5), VX-702 (6), AMG-548 (7), SCIO-469 (8) and TAK715 (9). The failure to demonstrate clinical efficacy suggests that either insufficient trough levels are obtained to fully inhibit the p38 pathway or that other mechanisms compensate for the blockade of the p38 pathway. In fact, data have been reported that indicate that inhibition of p38 results in activation of the JNK pathway. p38 inhibition using SB203580 strongly increased JNK activation in epithelial cells and macrophages (10). Recently, the importance of JNK activation was suggested in primary RA fibroblast-like synoviocytes (11). Several molecular mechanisms have been identified, which explain the p38-inhibition induced JNK activation: activation of upstream kinases MKK4/7, inhibition of dual specificity phosphatase 1 (DUSP1) or growth arrest and DNA damage-inducible gene 45β (10-17). In addition, p38 inhibitors were shown via a feedback control mechanism (TAB1 protein) to result in enhanced activity of TAK1, thereby activating other TAK1 downstream kinases, such as JNK and IκB kinase (18). Based on these and other observations suggestions were made to go upstream in the p38 pathway to obtain efficacy in RA (19). Our own work suggests that systemic inhibition of TAK1 results in a pro-inflammatory phenotype, disqualifying TAK1 as a candidate target in autoimmune diseases (Vink *et al*., submitted).

Alternatively, inhibition of downstream mediators of p38 may come with similar effect on production of pro-inflammatory mediators, potentially without the activation of the JNK pathway. MAPKAP2 kinase forms the direct substrate of p38 kinase, which in its turn phosphorylates the RNA binding protein tristetraprolin (TTP), thereby inducing the stabilisation of AU-rich 3'UTR mRNAs, such as mRNAs encoding TNF-α, IL-6 and other inflammatory cytokines (20, 21). Other MK2 substrates include heat shock protein 27 (Hsp27) and 14-3-3zeta (22). The relevance of p38 downstream mediators MK2 and TTP in arthritic disease was illustrated by observations that showed that $TTP^{-/-}$ mice suffer from severe arthritis (23), MK2 inhibitors show efficacy in *in vitro* and *in vivo* inflammatory models (24) and that $MK2^{-/-}$ mice are resistant to collagen-induced arthritis (25). Finally, with the clinical success of TNF-α targeting agents in mind, the interest in MK2 as a novel therapeutic target in RA is again confirmed by the anti-TNF-α phenotype of MK2 deficient mice (26).

In this report, we reveal that p38 inhibition leads to enhanced chemokine production via activation of JNK. More importantly, we provide data that suggest that inhibition of MK2, the downstream substrate of p38 has a similar effect on the production of pro-inflammatory mediators as p38, while at the same time leaving the JNK pathway untouched. Our data support the notion that targeting MK2 may form a novel strategy to identify small molecule drugs for treatment of RA.

Methods

Materials

Dulbecco's Modified Eagle's Medium (DMEM)-Ham's F-12, RPMI medium, fetal bovine serum (FBS), streptomycin and penicillin were purchased from GIBCO (Auckland, New Zealand). TNF-α and IL-1β were obtained from PeproTech (Hamburg, Germany). Phenylmethanesulfonyl fluoride (PMFS) was obtained from Sigma-Aldrich (Zwijndrecht, Netherlands). Phosphatase I&II and protease inhibitors were from Roche Diagnostics (Mannheim, Germany). Polyclonal antibodies specifically recognising total MAPK/p38, phospho-HSP27, phospho-c-Jun (pS73) and β–actin were purchased from Cell Signaling Technology (Leiden, Netherlands). Polyclonal antibodies against binding phospho-JNK1/2 (pTpY 183/185) and phospho-p38 (pTpY 180/182), cell extraction buffer, sample buffer and SDS-PAGE (NuPAGE) gels were obtained from Invitrogen (Breda, Netherlands). Phosphate Buffered Saline (PBS) was purchased from Hyclone (Etten-Leur, Netherlands). The bicinchoninic acid (BCA) protein assay reagent kit was from Pierce (Rockford, USA). Kinase inhibitors SB203580, SP600125, PH089, BIRB-796 were purchased from Calbiochem (Breda, Netherlands). Vertex-745 and Pamapimod were synthesised in-house with 99.8% and 95.8% purity, respectively and characterised by NMR and MS/MS. Polyvinylidene Difluoride (PVDF) membranes were from Millipore (Badford, USA). Fibroblast-like synoviocytes (FLS) and synoviocyte culture medium were purchased from Tebu-Bio (Heerhugowaard, Netherlands). Mip1α, Mip1β and IL-8 ELISA kits were obtained from R&D systems (Abingdon, UK).

Cell cultures

THP-1 monocytic cell-line was obtained from ATCC and grown in DMEM-F12 supplemented with 7.5% FBS and 20 μg/mL streptomycin and 20 U/mL penicillin at 37°C in a humidified 5% CO_2 atmosphere. Cells were seeded in a 96-wells plate ($2x10^4$ cells per well) and pre-treated for 30 minutes (min) with MAPK kinase inhibitor. Cells were stimulated with 20 ng/mL TNF-α. At the indicated time points, medium was collected, cells were washed with cold PBS and cell-lysates were obtained for western blot analysis or RNA was isolated for gene array analysis.

Fibroblast-like synoviocytes (FLS) were obtained, with informed consent, from rheumatoid arthritis (RA) and osteoarthritis (OA) patients in which synovial tissue was minced, digested with collagenase, filtered and cultured overnight with synoviocyte medium (Tebu-Bio, Heerhugowaard, The Netherlands). Non-adherent cells were removed and adherent cells were cultured further. Cells were characterised morphologically and phenotypically using flow cytometry and RT-PCR as described previously (27). FLS, ($1x10^5$ cells per well) were cultured in a 96-wells plate with or without MAPK inhibitor. FLS were incubated for 24 hours with various concentrations TNF-α or IL-1β (data not shown). The TNF-α and IL-1β concentrations which showed the most optimal JNK activation and is known to be present in inflammed synovial tissue, were used for the experiments. Therefore, FLS were incubated in the presence or absence of 20 ng/mL TNF-α or 2 ng/mL IL-1β. After 24 hours, medium was collected and stored at -20°C and cells were washed with PBS and lysed for western blot analysis. Mip1α, Mip1β and IL-8 levels in supernatants were determined by ELISA according to the manufacturer's instructions.

Preparation of cell extracts and western blot analysis

Cells were lysed for 30 minutes at 4°C with cell extraction buffer supplemented with 10 μl/mL PMFS, 10 μl/ml phosphatase I&II and 20 μl/ml protease inhibitor. Extracts from lysed cells were cleared by centrifugation for 5 minutes and supernatants were stored at -20°C. Protein concentration was determined using a BCA protein assay kit according to the manufacturer's instructions. Lysates were subjected to SDS-PAGE using a 4%-12% NuPAGE gradient gel and transferred to PVDF membranes. Membranes were blocked with 1% BSA in TBST buffer (10 mM Tris [pH=7.6], 150 mM NaCl, 0.1% Tween 20) at RT for 1 h and incubated with primary antibodies against MAPK's overnight at 4°C. After three washes with TBST, the membranes were incubated with secondary antibodies (Biosource) at room temperature for 1 h and followed by five washes with TBST. The reacting bands were detected using enhanced chemiluminescence (Pierce). Membranes were then reprobed with anti-actin antibody for sample loading control.

RNA interference

One day prior to electroporation, THP-1 cells were diluted to a cell-density of $0.5x10^6$ cells per mL and cultured overnight. Next, cells were centrifuged and the pellet was resuspended in RPMI medium at a density of $0,25x10^6$ cell/μL. For siRNA duplex electroporation, $1x10^7$ THP-1 cells were mixed with p38 or MK2 duplex in a Gene Pulser Cuvette (0.4 cm electrode gap, Bio Rad no. 165-2088) and electroporation was performed (250 Volt, 975 μFarad and ∞ Ohm) in a Genepulser Xcell (Bio Rad). After electroporation, cells were transferred in a T175 flask in 20 ml RPMI medium and incubated overnight. Transfected THP-1 cells were stimulated with or without 20 ng/mL TNF-α for 15 minutes or 2 hours. Supernatants were harvested at indicated timepoints. To monitor knockdown efficiency $1x10^6$ cells were harvested, pelleted and resuspended in cell extraction buffer supplemented with 1 mM PMSF and protease inhibitor. After 30 min incubation on ice, lysates were cleared by centrifugation and stored at -20°C for western blot.

Results

p38 inhibitors SB203580 and Vertex-745 but not the MK2 inhibitor PHO89 induce activation of JNK in monocytic THP-1 cells and primary macrophages

Recent data indicate that p38 MAPK inhibition leads to phosphorylation of JNK, which may form an explanation for the observed lack of clinical efficacy observed in Phase II clinical evaluation of p38 inhibitiors in RA. In this study we set-out to reveal the consequences of the p38-inhibition induced

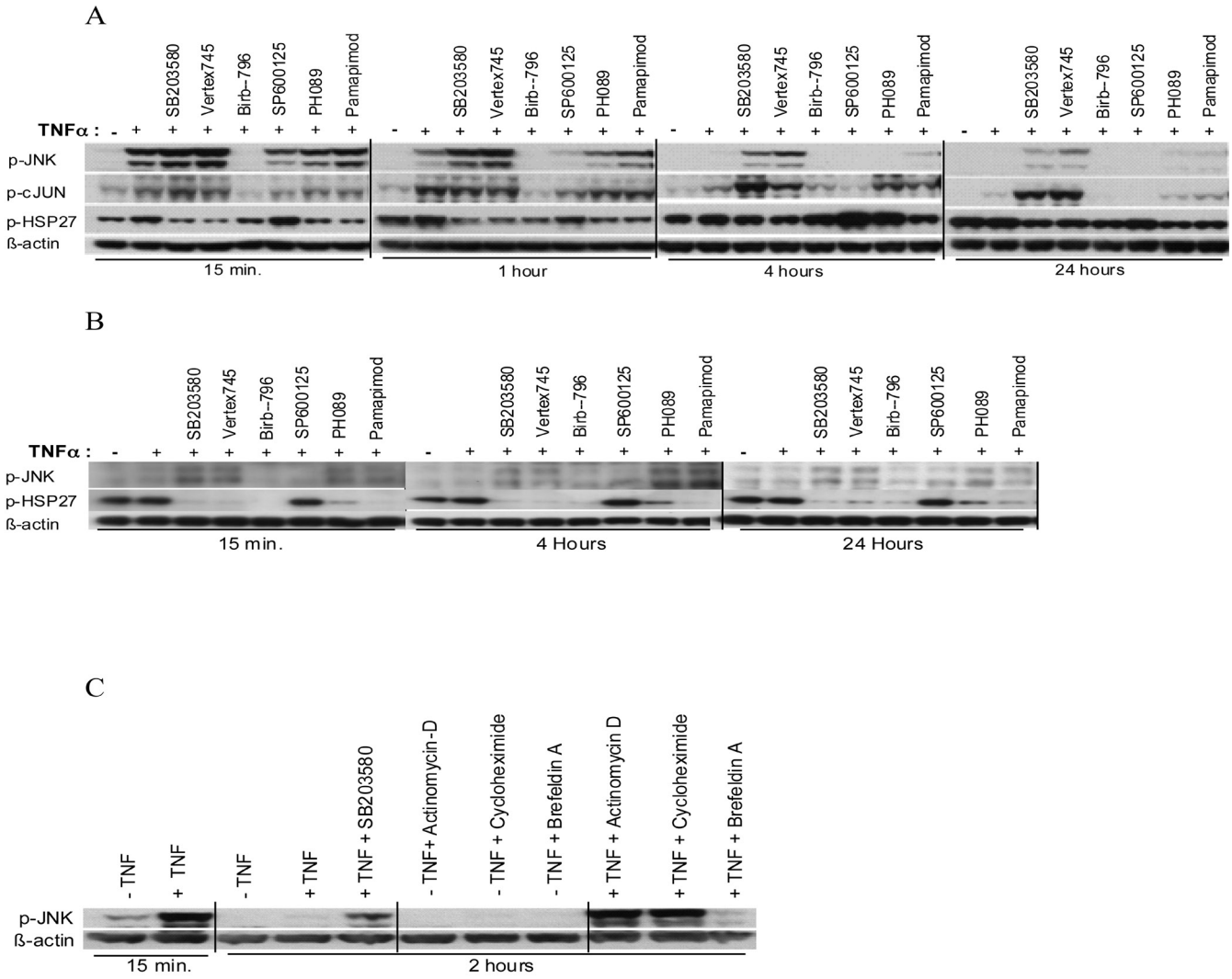

Fig. 1. p38 inhibition sustains JNK activation in TNF-α-stimulated THP-1 and primary macrophages; activation of JNK by TNF-α is regulated by transcription and translation. A, THP-1 cells or B, GM-CSF differentiated $CD14^+$ macrophages were pre-treated for 30 minutes (min.) with or without 20 ng/mL TNF-α) and subsequently treated with 10 μM of the p38 MAPK inhibitors SB203580 and Vertex-745, p38/JNK inhibitors BIRB-796 and pamapimod, pan-JNK inhibitor SP600125 and MK2 inhibitor PH089 for the indicated time. Total cell lysates were prepared and western blot analysis of phopho (p-) JNK, p-c-Jun and p-HSP27 was performed. β-actin was used as a protein loading control. C, THP-1 cells were stimulated with TNF-α (20 ng/mL) for 15 min or 2 hours in absence or presence of transcription inhibitor actinomycin-D, translation inhibitor cycloheximide, golgi transport inhibitor brefeldinA or SB203580 (10 μM) and subsequently stimulated with TNF-α for 2 hours. Controls include cells without treatment (-TNF-α) or treated with actinomycin-D, cycloheximide or brefeldinA alone for 2 hours. Total cell lysates were made and used for western blotting. β–actin was used as a protein loading control.

JNK activation and to identify the effect of targeting downstream substrates of p38 in relation to JNK activation.

First, we evaluated whether p38 inhibition resulted in phosphorylation of JNK in THP-1 monocytes and primary macrophages in presence of TNF-α. As shown in Figure 1A, JNK was rapidly (≤15 min.) phosphorylated upon TNF-α stimulation. p38 inhibitors, SB203580 and vertex-745 sustained JNK activation. Sustained JNK phosphorylation by p38 inhibitors gradually decreases but remains detectable for at least 24 hours, while in the absence of p38 inhibitors JNK phosphorylation is rapidly (1-4 hours) diminished. No sustained JNK phosphorylation was observed with the p38/JNK inhibitor, BIRB-796 and the pan-JNK inhibitor, SP600125 when compared to controls (±TNF-α). Surprisingly, the p38 inhibitor pamapimod showed no or weak sustained JNK activation, which could be due to the more p38/JNK rather than p38 biochemical profile of this compound (data not shown). Surprisingly, PH089-mediated inhibition of MK2, a downstream substrate of p38, did not sustain JNK phosphorylation.

In accordance with the current knowledge of the JNK pathway, p38-selective inhibitors, SB203580 and vertex-745, enhanced the phosphorylation of the JNK downstream substrate, c-Jun (Fig. 1A), while BIRB-796 and SP600125 suppressed c-Jun phosphorylation, which reflects the JNK activity of these inhibitors. Again, PH089 has no effect on c-Jun phosphorylation, which is in line with the absence of an effect on JNK phosphorylation upon MK2 inhibition. As controls, the efficacy of the p38, p38/JNK and MK2 inhibitors was verified using HSP27 phosphorylation

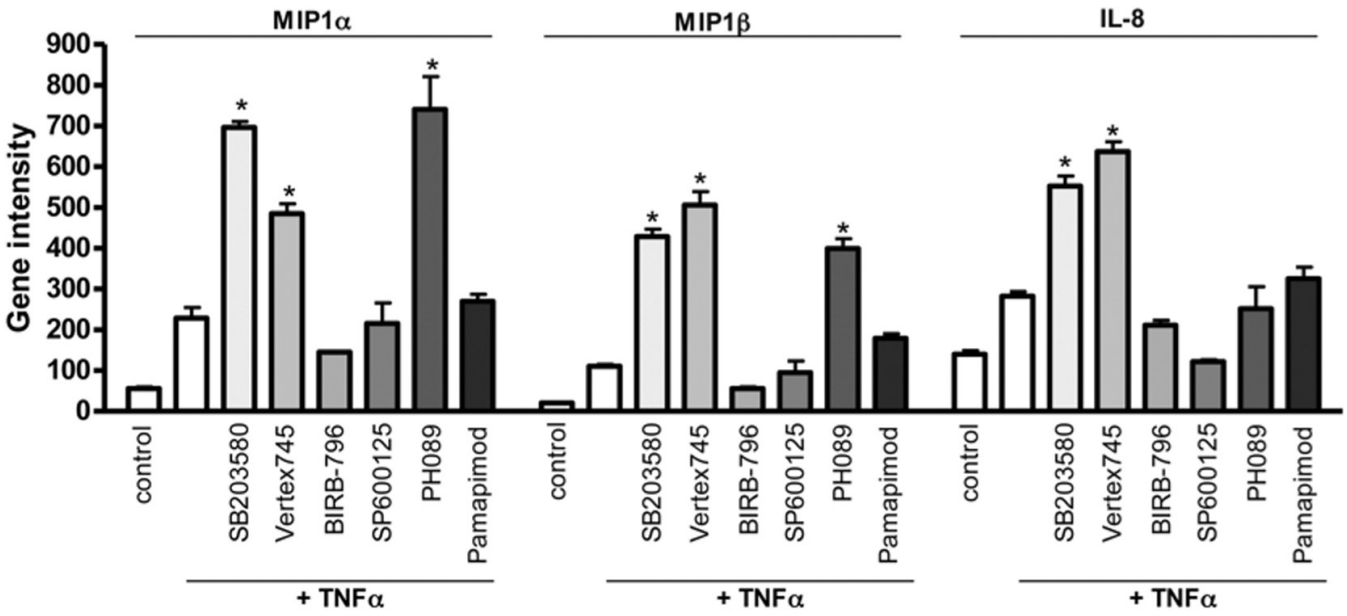

Fig. 2. Increased TNF-α-induced Mip1α, Mip1β and IL-8 mRNA expression and protein following SB203580 and vertex-745 p38 inhibition. A, THP-1 cells were incubated in the presence or absence (-) of TNF-α (20 ng/ml) or cells were incubated with TNF-α following treatment with 10 μM inhibitor. RNA samples were obtained in triplicates per condition, 8 hours after TNF-α stimulation, cDNA was made, hybridised on the human genome HGU133 plus 2.0 array (Affymetrix) and scanned. Normalisation and statistical analysis was performed with Limma. Intensity of the significantly (multiple testing corrected *p*-value<0.05) regulated genes is depicted. Affymetrix files were analysed with Bioconductor (www.bioconductor.org) for the statistical software R (www.r-project.org). The data were normalized using gcrma. Probe sets were discarded for which the expression value was 20 or less after normalisation in all or all but one of the 30 arrays in the experiment. For the remaining 22,722 probe sets mean expression values the Benjamini-Hochberg method was used to correct for multiple testing. Sets of differentially regulated genes were determined for TNF-α stimulation *versus* no stimulation, for each of the compound treatments *versus* TNF-α stimulation, and for each of the pairwise compound comparisons. Gene selection was based on a minimum fold-change of 2 and adjusted p-value <0.05.

THP-1 cells were treated with or without 10 μM SB203580, Vertex-745, BIRB-796, SP600125, PH089 or Pamapimod. Next, cells were stimulated in presence or absence of TNF-α. Culture supernatants were obtained and B, Mip1α, C, Mip1β and IL-8 protein (data not shown) were measured with ELISA or Luminex at 4 and 24 hours after TNF-α stimulation. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ where TNF-α is compared with TNF-α and SB203580, vertex-745, BIRB-796, SP600125, PH089 or pamapimod using Students *t*-test (unpaired, assuming unequal variances).

as readout. Treatment with SB203580, Vertex-745, BIRB-796 and PH089 reduced HSP27 phosphorylation, which confirms the p38 or MK2 selective inhibition of these drugs. As expected, no suppression of HSP27 phosphorylation was observed using the JNK inhibitor SP600125.

To determine whether the effect of p38 inhibition on sustained JNK phosphorylation was also observed in primary cells, $CD14^{+}$ monocytes were differentiated with GM-CSF, stimulated with TNF-α following incubation with inhibitors. Although less prominent, the effect of p38 inhibition on sustained JNK phosphorylation in primary macrophages could be confirmed (Figure 1B). Again, phosphorylation of HSP-27 was suppressed by p38, p38/JNK or MK2 inhibition, while SP600125 did not have any effect of HSP27 phosphorylation. These results indicate a critical role for p38, but not for MK2 in sustaining JNK phosphorylation and thereby inducing c-Jun activation under inflammatory conditions.

TNF-α induces JNK phosphorylation, which is rapidly inactivated. To determine whether dephosphorylation of JNK, which is inhibited by p38 inhibitors, is mediated by a protein that is *de novo* synthesised or already present, THP-1 cells were stimulated with TNF-α in the presence or absence of actinomycin-D, cycloheximide or BrefeldinA, respectively. As shown above, SB203580 sustained the activation of JNK in TNF-α stimulated THP-1 cells (Fig. 1C). Pre-treatment with either Actinomycin-D or cycloheximide, but not with the Golgi inhibitor BrefeldinA, increased the TNF-α-induced JNK phosphorylation in THP-1 cells. Treatment with actinomycin-D, cycloheximide or brefeldinA in the absence of TNF-α as controls showed no regulation of JNK phosphorylation. These results suggest that TNF-α-induced sustained JNK phophorylation is mediated via inhibition of a *de novo* synthesised, yet unknown factor.

SB203580 and vertex-745 enhances TNF-α-induced Mip1α, Mip1β and IL-8 gene expression and protein in THP-1 cells

To identify the *de novo* factor "x" that mediates the p38-inhibition sustained JNK activation and the consequences of the sustained p38-inhibition sustained JNK phosphorylation, gene-array studies were performed. Inhibitors of p38, p38/JNK, JNK and MK2 were used to profile effects on gene expression in TNF-α-stimulated THP1 cells. A total of 1,314 Affymetrix probe sets were regulated upon TNF-α stimulation. Relative to TNF-α stimulation alone, 344 probe sets were regulated by p38 SB203580, 252 by Vertex-745, 66 were regulated by p38/JNK Pamapimod, 97 were regulated by p38/JNK (BIRB-796), 84 were regulated by pan-JNK (SP600125) and 326 were

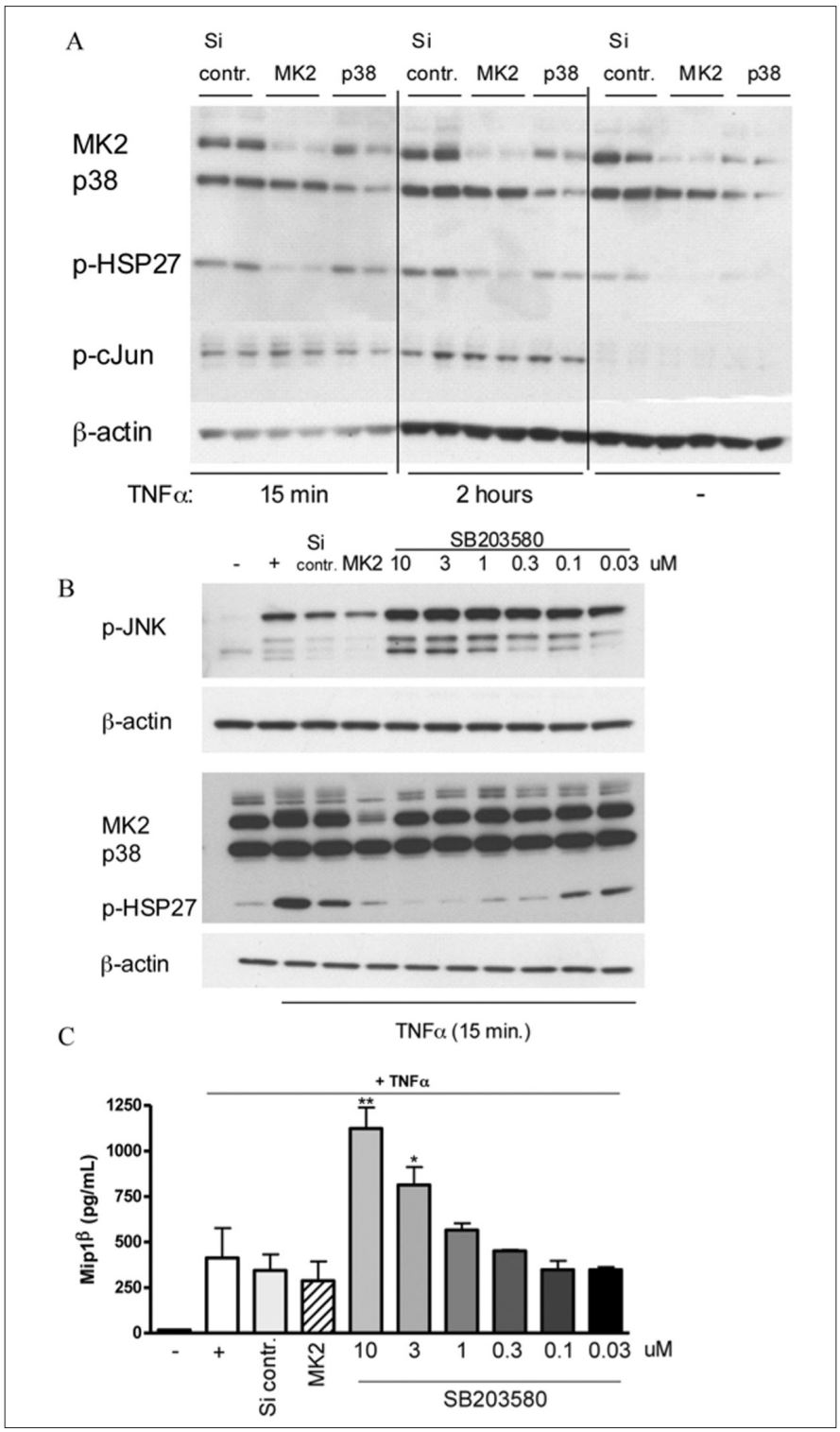

Fig. 3. No modulation of JNK activation and Mip1β production by MK2 siRNA knockdown. A, THP-1 cells were electroporated in duplo with non-specific siRNA control (Si contr.) and MK2 specific siRNA (MK2) and were stimulated in the presence or absence (-) of 20 ng/ml TNF-α. After 15 minutes (min.) or 2 hours total cell lysates were made and analysed for total MK2, total p38 and phosphorylated HSP27 and c-Jun. B, THP-1 cells were electroporated with non-specific siRNA (Si contr.) or MK2 siRNA and treated with or without (-) the p38 inhibitor SB203580 (0.03, 0.1, 0.3, 1, 3 and 10μM). Subsequently, the cells were stimulated with TNF-α and total lysates were made and analysed for p-JNK, MK2 p38 and p-HSP27 after 15 min. using western blot. C, Supernatant was collected after 24 hours and Mip1β production was measured with Luminex (average ± stdev is depicted). $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ where Si control plus TNF-α is compared with TNF-α and SB203580 dose-response using Students *t*-test (unpaired, assuming unequal variances).

regulated by MK2 PH089 inhibition. Regulation is defined as a minimum fold-change of 2 and with *p*-value <0.05 (corrected for multiple testing). Surprisingly, only a limited number of genes (14 in total) were significantly differentially regulated by the p38 inhibitors (SB203580 and Vertex-745) when compared to the p38/JNK inhibitors BIRB-796 and Pamapimod. For only 10 of the 14 genes the effect of TNF-α on gene expression, compared to unstimulated, is statistically significant (data not shown). The chemokines CCL3 (Mip1α), CCL4 (Mip1β) and IL-8 (Figure 2A), chemokines which are known to attract immune cells to the site of inflammation are among those genes with the strongest regulation by p38 inhibition as compared to p38/JNK inhibitors. The MK2 inhibitor PH089 increased Mip1α and Mip1β gene transcription, while no regulation of IL-8 mRNA was observed as compared to p38 inhibition. In addition, 62 genes were significantly regulated by the p38 inhibitors (SB203580 and Vertex-745) but not with the MK2 inhibitor PH089 (data not shown).

We next investigated whether the p38–inhibition mediated upregulation of Mip1α, Mip1β and IL-8 mRNAs is resulting in the induction of Mip1α, Mip1β and IL-8 protein. TNF-α induced Mip1α (Fig. 2B), Mip1β (Fig. 2C) and IL-8 (data not shown) production in THP-1 cells. p38 inhibition by SB203580 and Vertex-745 significantly increased Mip1α, Mip1β and IL-8 protein in the supernatants of THP-1 cells, which was already observed after 4 hours and sustained for 24 hours (and 48 hours, data not shown). In contrast, p38/JNK or JNK inhibitors (BIRB-796, SP600125 and Pamapimod) decreased Mip1α, Mip1β and IL-8, which likely reflects the JNK activity of these kinase inhibitors. In contrast to the mRNA data, no enhanced levels of Mip1α, Mip1β and IL-8 protein was observed upon MK2 inhibition. Unfortunately, no regulation of Mip1α, Mip1β and IL-8 protein by p38 inhibitors was observed in macrophages, suggesting that no translation from THP-1 monocytic cells to primary macrophages could be obtained (data not shown).

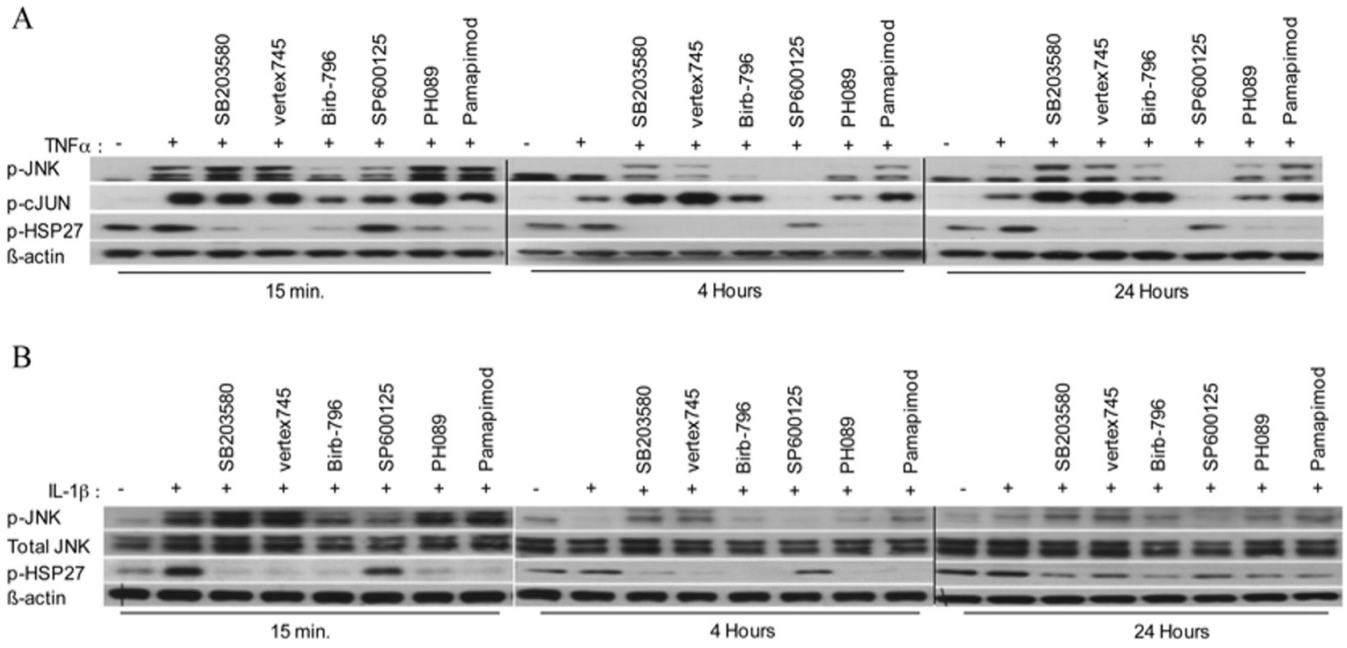

Fig. 4. SB203580 and vertex-745 increased JNK and c-Jun activation in TNF-α stimulated RA fibroblast-like synoviocytes. FLS from RA patients were pre-incubated (30 min.) in the presence or absence (-) of 10 μM SB203580, vertex-704, BIRB-796, SP600125, PH089 and Pamapimod. A, FLS were stimulated with 20 ng/ml TNF-α or B, IL-1β for another 15 min., 4 hours and 24 hours. Cell lysates were prepared and western blot analysis of phopho (p-) JNK, p-c-Jun, p-HSP-27 and total JNK was assessed. β-actin was used as a protein loading control.

No enhanced JNK activation and Mip1β production by MK2 siRNA knockdown

To confirm that the lack of effect of the MK2 inhibitor PH089 on sustaining JNK phosphorylation is mediated via MK2 and is not due to compound a-selectivity, MK2 siRNA knockdown was established to study the effect on JNK phosphorylation and Mip1β production in THP-1. As shown in Figure 3A, knockdown of MK2 almost completely (>90%) reduced MK2 protein levels in unstimulated and TNF-α-stimulated THP-1 cells. Interestingly, knockdown of p38 using siRNA partly reduced both p38 and MK2 protein levels. In accordance to our findings with MK2 and p38 compounds, inhibition of MK2 or p38 using siRNA inhibited phosphorylation of HSP27 upon TNF-α stimulation. As a negative control, no inhibition of c-Jun by MK2- or p38 siRNA was observed.

Next, the effect of MK2 siRNA on JNK phosphorylation and Mip1β production was studied. MK2 siRNAs-treated cells showed no effect on TNF-α-induced JNK phosphorylation (Fig. 3B) after 15 minutes and Mip1β production after 24 hours (Fig. 3C). As a control, THP-1 cells were treated with increasing concentrations of SB203580 and stimulated with TNF-α. As shown above, SB203580 dose-dependently enhanced JNK phosphorylation (Fig. 3B) and Mip1β production (Fig. 3C).

Taken together, both MK2 small molecule inhibitors and MK2 siRNA demonstrate that targeting MK2, while inhibiting TNF-α induced downstream signal transduction of the p38 pathway, does not result in the activation of the JNK pathway.

SB203580 and vertex-745 activate JNK in TNF-α and IL-1β stimulated fibroblast-like synoviocytes

To assess the effect of p38-inhibition on fibroblast-like synoviocytes (FLS), FLS from RA and OA patients were stimulated with TNF-α presence or absence of MAPK inhibitors. As shown in Figure 4A, TNF-α stimulation of RA FLS resulted in increased phosphorylation of JNK after 15 min. Similar results were obtained using FLS from OA patients (data not shown). Treatment with SB203580 and Vertex-745 sustained both MKK4 (data not shown) and JNK phosphorylation up to 24 hours, which is in agreement with the data obtained using THP-1 cells. The effect of p38 inhibitors on JNK phosphorylation translated in phosphorylation of its substrate c-Jun after 4-24 hours (Fig. 4A). Again, the MK2 inhibitor PHO89 did not show an effect on JNK phosphorylation. As a control, all MAPK compounds, except SP600125 inhibited phophorylation of HSP27 as expected. Similar effects were observed when FLS were stimulated with IL-1β, another key cytokine in RA (Fig. 4B).

Next, we investigated of the effect of p38, p38/JNK and MK2 compounds on TNF-α (Fig. 5A and 5C) or IL-1β (Fig. 5B and D) induced Mip1β. Indeed, in both RA and OA FLS TNF-α- and IL-1β-induced Mip1β was dose-dependently enhanced by the p38 inhibitors SB203580 and Vertex-745 when compared to TNF-α alone (Fig. 5). In agreement with its strong JNK inhibition, SP600125 decreased Mip1β levels. More importantly, the MK2 inhibitor PH089 showed no induction of Mip1β production. Bell-shaped inhibition curves were observed with the p38/JNK inhibitors BIRB-796 and pamapimod, likely reflecting p38 inhibitory effect at lower compound concentration and JNK inhibitory effect at higher concentrations (≥1 μM). Similar effects as described for Mip1β were found for Mip1α (data not shown). In conclusion, inhibition of p38 inhibi-

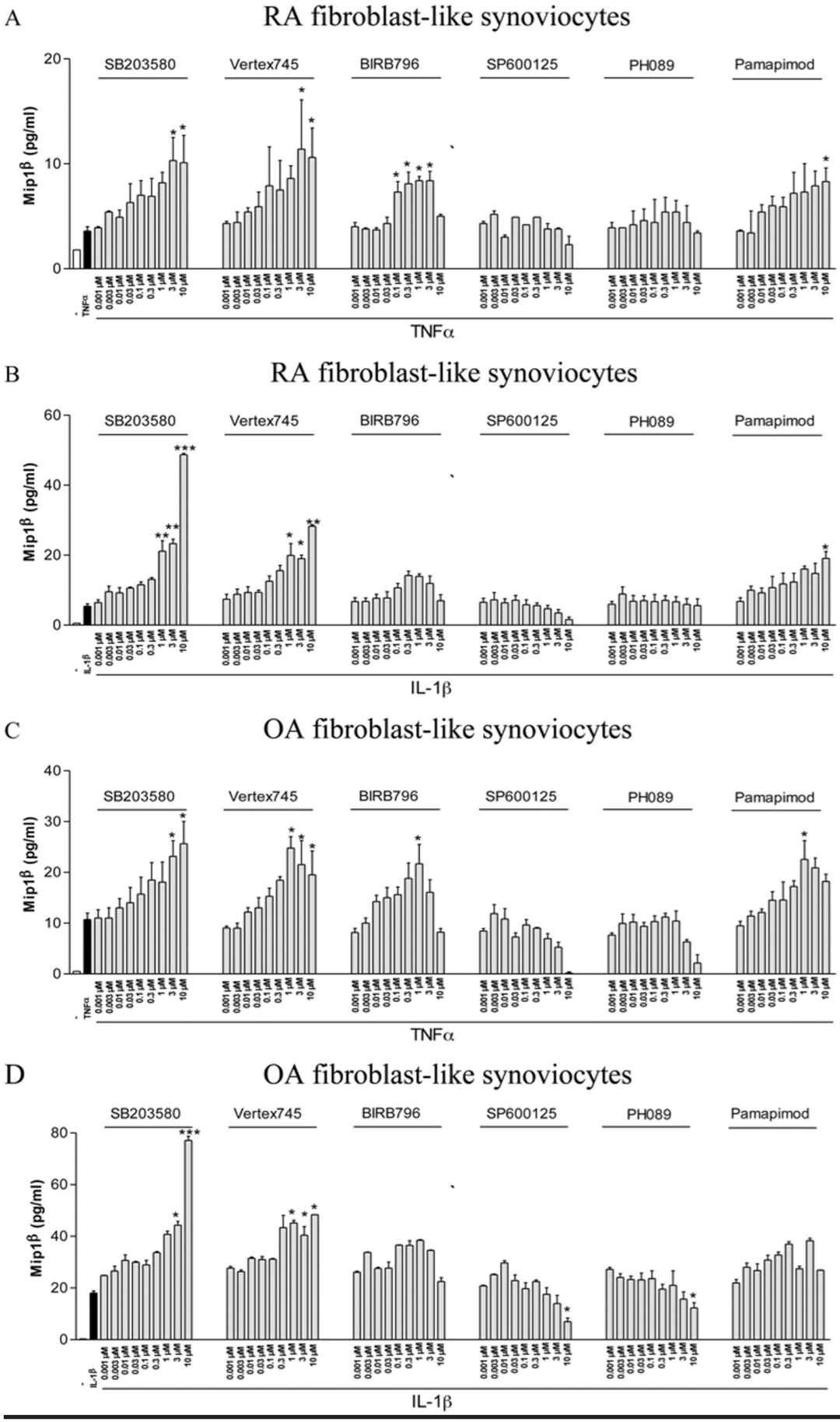

Fig. 5. Enhanced Mip1β production by p38 inhibition in fibroblast-like synoviocytes is concentration-dependent. Fibroblast-like synoviocytes (FLS) from RA (**A** and **B**) and OA (**C** and **D**), patients were stimulated with or without (-) 20 ng/ml TNF-α (A, C) or 2 ng/ml IL-1β (B, D) and treated with the MAPK inhibitors SB2103580 (p38), vertex-704 (p38), BIRB-796 (p38/JNK), SP600125 (pan-JNK), PH089 (MK2) and pamapimod (p38) at the indicated dosages. Mip1β release was measured with Luminex after 24 hours. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ where TNF-α is compared with TNF-α and SB203580, vertex-745, BIRB-796, SP600125, PH089 or pamapimod using Students *t*-test (unpaired, assuming unequal variances).

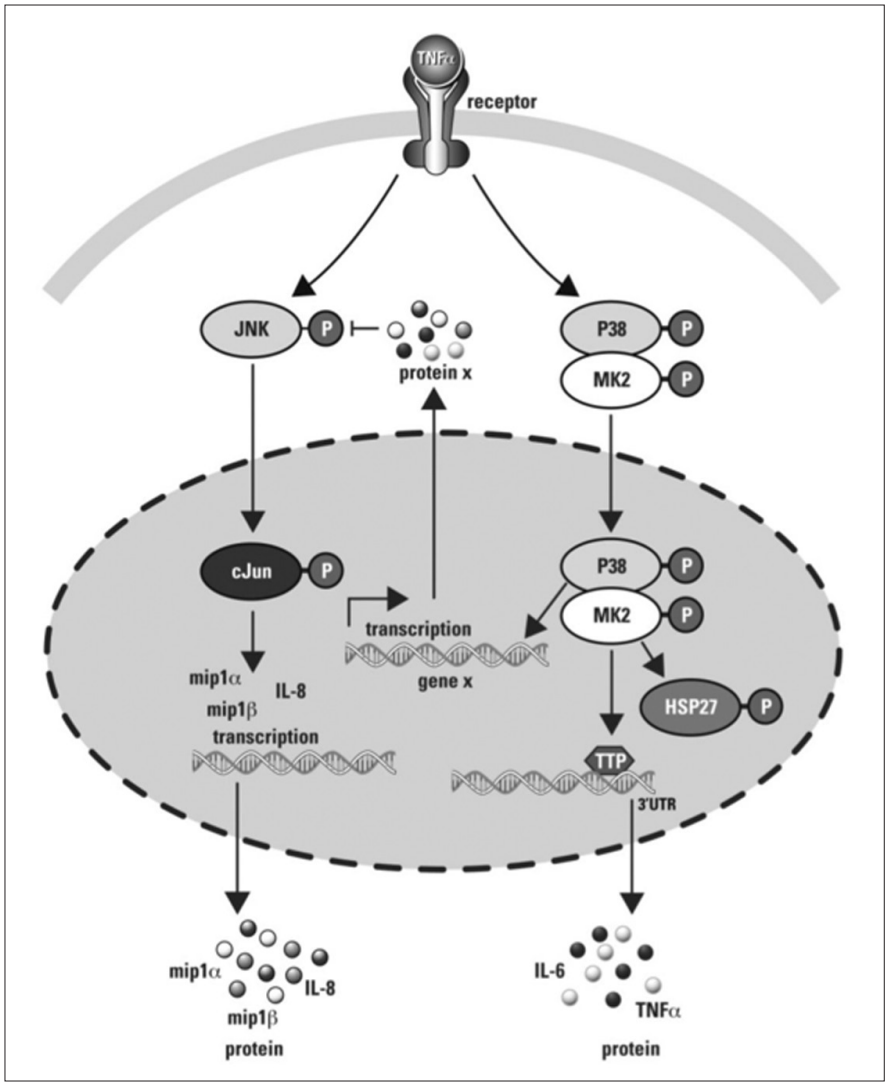

Fig. 6. Summary of the role of p38 and MK2 on Mip1α, Mip1β and IL-8 regulation in fibroblast-like synoviocytes of RA patients. A, c-Jun N-terminal kinase (JNK) and p38 signalling cascade which is activated by TNF is illustrated. p38 inhibitors sustained TNF-α-induced JNK activation via transcription/ translation of a yet unknown gene/protein x which results in enhanced Mip1α, Mip1β and IL-8 secretion. MK2 inhibition lacks the effect on enhancing Mip1α, Mip1β and IL-8 while the overlapping response between p38 and MK2 by inhibiting TTP mediated TNF-α and IL-6 production remains.

tion, but not MK2 of TNF-α- or IL-1β stimulated monocytes and FLS sustains JNK phosphorylation resulting in enhanced chemokine production.

Discussion

Developing inhibitors of p38 MAPK has been a main focus in pharmaceutical industry, mostly based on the effect of p38 inhibitors on inflammation, cartilage and bone destruction in rodent models of RA (2-4). However, clinical development of these inhibitors in RA patients was stalled by either drug-related toxicity or lack of efficacy. With the crucial function of the p38 pathway, adaptations that allow escape from the blockade of the p38 pathway are suggested as one of the explanations for the disappointing efficacy in Phase II studies in RA patients.

In this report, we studied a molecular mechanism, which is activated upon blockade of the p38 pathway (summarised in Figure 6). We showed that inhibition of the p38 pathway in monocytes, macrophages and synoviocytes results in sustained JNK phosphorylation. These findings are in line with Muniyappa and coworkers, who demonstrated sustained JNK phosphorylation in A549 epithelial cells, HUVEC endothelial cells and MCF-7 breast cancer cells upon p38 inhibition using SB202190 and SB203580 (17). In addition, sustained JNK phosphorylation was reported in TNF-α stimulated insulin-producing TC6 cells and IL-1β activated human kidney cells upon p38 inhibition (28, 29).

Previously, JNK activation has been linked to the pathogenesis of inflammatory arthritis. JNK has been reported to be important for matrix metalloproteinase (MMP) expression in RA FLS, *e.g.* JNK1 or JNK2 knockout mice have defective MMP-3 production (30). JNK1 but not JNK2 is crucial for joint swelling and destruction in a serum transfer mouse model of arthritis and in mouse antigen-induced arthritis (31, 32). In addition, the panJNK inhibitor SP600125 suppressed joint destruction in rat adjuvant arthritis (30).

The exact mechanism how JNK phosphorylation is sustained by p38 inhibition is currently unknown. p38 may sustain JNK phosphorylation via inhibition of MKP-1 (DUSP-1), involved in dephosphorylation of JNK (16, 33). Alternatively, inhibition of p38 may frustrate the negative feedback control of TAK1 kinase, resulting in sustained JNK activation (29). Upregulation of the expression of Gadd45β, which has recently been shown to act as a negative regulator of the JNK pathway forms another mechanism to explain the sustained JNK phosphorylation (11). Our microarray data indicate that another candidate for the *de novo* factor is Gadd45α. The gene encoding Gadd45α is differentially expressed between p38 inhibitor *versus* MK2 inhibitor treated cells upon TNF-α stimulation (data not shown). In addition, Song and coworkers demonstrated that Gadd45α overexpression induced both MKK4 and JNK phosphorylation (34). However, in limited studies, we could not demonstrate modulation of Gadd45α or DUSP-1 protein levels upon p38 *versus* MK2 inhibition and more extensive studies would have to reveal whether Gadd45α or DUSP-1 is the yet unknown factor 'x' (data not shown).

In our study, we demonstrated that inhibition of p38 activates the JNK pathway, probably via activation of

upstream kinase MKK4. Inoue and coworkers suggested that MKK3 and MKK6 are regulated by p38 in RA FLS (14), while JNK upstream kinases MKK4/MMK7 have been described to be activated in epithelial and endothelial cells upon p38 inhibition (13, 17). In line with our observations, Song and colleagues reported that JNK activation is MKK4 dependent (34). Furthermore, MLK3, MSK1 or MEK1 could induce JNK activation upstream from MKK4 (17, 33, 35). Further studies are necessary to determine the exact upstream kinases involved in p38 medicated enhancement of the MKK4-JNK-c-Jun signalling cascade.

Up to now the functional relevance of the crosstalk between the p38 and JNK pathway was unknown. We demonstrated that different p38 inhibitors dose-dependently enhanced the production of pro-inflammatory chemokines Mip1α, Mip1β and IL-8. Mip1α and Mip1β levels are increased upon activation of RA FLS with TNF-α or IL-1β (36,37). In addition, Mip1α and Mip1β levels are elevated in serum and synovial fluid of RA and OA patients (36, 37). These chemokines are suggested to be secreted by fibroblasts and T-cells in the synovial tissue and subsequently migrate into the blood by a concentration gradient, thus resulting in enhanced chemokine levels in the blood (37). As such, these chemokines may cause continued inflammatory cell migration into the synovium due and neovascularisation of the synovial tissue (38). Therefore, increased Mip1α and Mip1β secretion by p38 inhibition could contribute to the sustained pro-inflammatory status of the synovium.

In our study we found that MK2 inhibition, in contrast to p38 inhibition does not result in sustained JNK activation and elevation of chemokines, while as efficiently blocking the signal transduction of the p38 pathway. In light of these results, MK2 may form a more promising target than p38 (39). MK2 transduces the signal from p38 to the RNA binding protein tristetraprolin (TTP), which results in the stabilisation of the mRNAs of among others two RA key cytokines: TNF-α and IL-6 (20,21). The relevance of TTP as a substrate of the p38 pathway was provided by TTP knockout mice, which suffering from severe arthritis (23). In addition, genetic depletion of MK2 results in the protection of the incidence as well as arthritis severity in mice (25). Small molecule inhibitors of MK2 reduced LPS-induced TNF-α production in the rat (40), as well as next generation MK2 inhibitors, which showed efficacy in *in vitro* and *in vivo* inflammatory models (24). Alternatively, orally active p38 inhibitors are in development, which discriminate between p38-MK2 and p38-JNK-ATF2 inhibition (41).

We showed for the first time that p38 inhibitors sustained TNF-α and IL-1β-induced JNK activation resulting in enhanced chemokine secretion (schematically depicted in Figure 6). Based on the suggested role of these chemokines in RA pathogenesis, the upregulation of these chemokines may provide an explanation for the lack of efficacy of p38 inhibitors in Phase II clinical trial. The lack of effect of MK2 inhibition on this mechanism, while coming with similar efficacy on blocking the p38 pathway would strongly support further investigations to reveal the full potential of MK2 inhibitors as novel therapeutics in RA. Recent clinical studies demonstrated efficacy of a p38 inhibitor (PH-797804) in clinically stable chronic obstructive pulmonary disease (COPD) on Transitional Dyspnoea Index as well as secondary endpoints, including inspiratory capacity and C-reactive protein levels (42). Apparently, p38 kinase plays a more crucial role in the COPD pathology than in RA, which can be explained by tissue and/or cell-type specific routing of the p38 pathway. In this context and in the light of our data it will be highly interesting to study the activation status of JNK upon p38 inhibition, the effect of MK2 inhibition and the consequences on chemokine expression in tissues and cells isolated from COPD patients.

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