Ras family GTPase signaling contributions to inflammation and joint destruction in rheumatoid arthritis

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Selective involvement of erk and jnk mitogen-activated protein kinases in early rheumatoid arthritis
chapter 5

Selective involvement of ERK and JNK mitogen-activated protein kinases in early rheumatoid arthritis

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Selective involvement of erk and jnk mitogen-activated protein kinases in early rheumatoid arthritis

Abstract

Objectives: To investigate the expression and activation of mitogen-activated protein (MAP) kinases in disease-modifying antirheumatic drug (DMARD) -naïve early arthritis patients.

Methods: 50 DMARD -naïve early arthritis patients (disease duration < one year) were prospectively followed and diagnosed at baseline and after 2 years according to criteria for undifferentiated arthritis (UA), rheumatoid arthritis (RA), or spondyloarthritis (SpA). Synovial biopsies from actively inflamed joints were obtained at baseline by needle arthroscopy and examined by immunohistochemistry for expression and phosphorylation of p38, extracellular signal regulated kinases (ERK) 1/2 and c-Jun N-terminal kinase (JNK) MAP kinases, using computer-assisted image analysis. Results were compared between patients with different diagnoses and different disease outcomes.

Results: Activation of ERK was enhanced at inclusion in patients meeting RA criteria after two years (n = 27) compared to SpA (n = 7) (p < 0.05) and UA (n = 16) (p < 0.005). Similarly, relative JNK activation was significantly higher in RA than in SpA (p < 0.005) and UA (p < 0.01). However, relative p38 activation was similar between diagnostic groups. Logistic regression analysis demonstrated that synovial JNK activation, but not p38 or ERK activation, is a predictor of fulfillment of classification criteria for RA after two years (R² = 0.59, p = 0.02). When comparing patients diagnosed with UA at baseline who fulfilled classification criteria for RA after two years (UA>>RA, n = 8) with those who remained UA (UA>>UA, n = 16), activation of JNK (p < 0.005), but not p38 or ERK was significantly enhanced. Activation of ERK (p < 0.01) and JNK (p < 0.01) at baseline was also enhanced in RA patients with progressive joint destruction as assessed by comparison of X-rays at baseline and after two years. Comparing all early arthritis patients, activation of p38 (p < 0.05), ERK (p < 0.005) and JNK (p < 0.001) was elevated in patients with erosive disease.

Conclusions: In patients with early arthritis, elevated ERK and JNK activity distinguish RA from other forms of arthritis, and JNK activation is already elevated in patients with RA even before classification criteria of RA are met. Activation of MAP kinases is also associated with development of erosive disease, and JNK activation predicts the development of erosive disease in early arthritis patients. Together, our data suggest that strategies targeting ERK and JNK, rather than p38, may be beneficial in treating RA early in the disease process.
Introduction

Mitogen-activated protein (MAP) kinases are ubiquitously expressed intracellular enzymes which occupy critical positions in signal transduction cascades coupling extracellular stimuli to cellular responses. Members of the MAP kinase family, namely p38 kinases (α, β, γ, and δ isoforms), extracellular signal-regulated kinases (ERKs) 1 and 2, and c-Jun N-terminal kinases (JNK) 1-3, serve as substrates of complex protein kinase cascades initiated by diverse cell surface proteins, including antigen receptors, tumor necrosis factor (TNF) family receptors, chemokine and cytokine receptors, and toll-like receptors (TLR) [1,2]. Once phosphorylated, MAP kinases are activated in turn to phosphorylate transcription factors needed for gene transcription.

Members of each MAP kinase family are expressed and activated in the synovial tissue of patients with rheumatoid arthritis (RA) and other forms of inflammatory arthritis [3-5]. Highly selective pharmacological inhibitors of p38 [6-9], ERK [10, 11] and JNK [3, 12, 13] can prevent inflammatory activation of stromal fibroblast-like synoviocytes (FLS) derived from synovial tissue of RA patients, chondrocytes and osteoclasts. Additionally, pharmacological inhibition or genetic deletion of MAP kinase activity reduces inflammation and joint destruction in multiple experimental animal models of RA [6, 8, 10, 12, 14-18]. Together, these data have suggested the possibility that therapeutic strategies aimed at inhibiting MAP kinase activation may be useful in the treatment of RA [1, 2, 19, 20].

Despite this wealth of pre-clinical analyses, little is known about the distinct contributions of each MAP kinase to the onset and perpetuation of RA. Clinical parameters and biomarkers have yet to be identified which are associated with synovial MAP kinase activation status, and MAP kinase activation in RA has primarily been examined in patients with destructive end-stage disease [3-5]. In the transgenic human TNF overexpression model of murine arthritis, p38 activation is selectively associated with and required for induction of inflammation and joint destruction [15,21]. Additionally, anti-TNF treatment results in decreased p38 activation in RA patient peripheral blood T cells, and decreased ERK and JNK phosphorylation in the synovial tissue of psoriatic arthritis (PsA) patients [22-24]. These changes in MAP kinase activation following effective treatment indicate a potential role for MAP kinases in promoting arthritis in RA and PsA. Whether these observations can be eventually translated into successful treatment of RA with MAP kinase inhibitors, especially in the early stages of disease, is uncertain however, as clinical trials with p38 inhibitors have not been successful [25, 26].

Recent kinetic analyses of MAP kinase activation status in experimental arthritis have revealed model-specific differences in the degree of activation of p38, ERK and JNK, as well as in the timing of their activation during disease onset and resolution [27]. In this study, to examine if similar differences in MAP kinase involvement might be relevant to the earliest stages of the development of RA, we examined the relationship between MAP kinase expression and activation, and disease diagnosis and outcome in a prospective cohort of disease-modifying antirheumatic drug (DMARD) -naïve early arthritis patients.
Patients and Methods

Patients
50 patients with arthritis of less than 1 year duration, as measured from the first clinical signs of arthritis irrespective of which joint was initially affected and a clinically inflamed knee or ankle joint underwent arthroscopic synovial biopsy. Diagnosis of RA or spondyloarthritis (SpA) was made according to established classification criteria [28,29]. Patients were classified as having undifferentiated arthritis (UA) if no classifying diagnosis for RA, SpA or other forms of arthritis could be made. After 2 years of follow up final diagnosis was made according to classification criteria. All patients were naïve to treatment with disease modifying anti-rheumatic drugs (DMARD) at inclusion. None were taking corticosteroids, but previous corticosteroid use for diagnoses other than inflammatory joint diseases was allowed up to three months before inclusion in the study. At inclusion we assessed disease activity by acquisition of 68 tender and 66 swollen joint score, patients visual analog scale (VAS) of global disease activity (scale 0-100mm), VAS of pain (scale 0-100mm), erythrocyte sedimentation rate (ESR), and c-reactive protein (CRP) levels. Paired serum and arthroscopic synovial tissue biopsy samples were collected at baseline. X-rays were obtained at baseline and after 2 years of follow up; erosion scoring was based on the presence or absence of erosions on X-rays of hands and feet in cases where the modified Sharp-van der Heijde erosion score was ≥ 1 [30]. This study was approved by the institutional review board, performed according to the declaration of Helsinki, and all study patients provided written informed consent.

Measurement of autoantibodies in patient serum
The presence of IgM rheumatoid factor (IgM-RF) and anti-citrullinated protein antibodies (ACPA) in patient serum was measured using IgM-RF (Sanquin, Amsterdam, The Netherlands) and anti-CCP2 (Eurodiagnostica, Arnhem, The Netherlands) ELISA kits, respectively.

Synovial tissue biopsy sampling and immunohistochemistry
All patients underwent arthroscopic synovial tissue biopsy sampling of an ankle, wrist or knee joint [31]. Six synovial tissue biopsies were collected from each patient for immunohistochemistry, as previously described [32]. The synovial biopsy samples were snap-frozen en bloc in TissueTek OCT (Miles, Elkhart, IN) immediately after collection. Prior to staining, cryostat sections of tissue (5 μM) were cut and mounted on adhesive glass slides (Kintetelglas, Braunschweig, Germany) and stored at -80°C until needed for staining. For staining, slides were incubated overnight at 40°C with primary antibody diluted in 1% w/v bovine serum albumin in phosphate-buffered saline (PBS). Primary antibodies used in this study were polyclonal rabbit antibodies specific for p38, ERK, JNK (all from Cell Signaling, Beverly, MA) and murine monoclonal antibodies recognizing phosphorylated (p)-p38, ERK, and JNK (Santa Cruz Biotechnology, Santa Cruz, CA). Sections were washed with PBS and incubated with
goat anti-mouse or swine-anti-rabbit -horseradish peroxidase (HRP)-conjugated antibodies (Dako, Glostrup, Denmark), followed by incubation with biotinylated tyramide and streptavidin-HRP, and development with aminoethylcarbazole (Sigma, St. Louis, MO) [33]. Slides were counterstained with Mayer's hematoxylin and mounted in Kaiser's glycerol gelatin (Merck, Darmstadt, Germany).

Immunohistochemical analysis
After staining of the slides the sections were analyzed by digital image analysis. All sections were analyzed in random order by readers (DD, GS, MS) who were blinded with regard to the patient’s clinical characteristics. Images were acquired and analyzed using a Syndia algorithm on a Qwin-based analysis system (Leica, Cambridge, UK) as previously described [34]. Expression and/or phosphorylation of proteins was calculated for each section as the median integrated optical density (IOD) per mm² tissue. Relative phosphorylation values were obtained by dividing IOD/mm² phospho-MAP kinase IOD by IOD/mm² total MAP kinase, controlled for cellularity by calculating the number of nucleated cells/mm².

Statistics
Statistical analysis was performed using Windows Graphpad Prism 4 and SPSS V.16.0 (Chicago, IL) software. Comparisons in expression or phosphorylation of markers between cohorts were performed using the Mann-Whitney U test, first using the Kruskall-Wallis test when more than two groups were compared. Univariate logistic regression analysis was used to analyze the relationship between relative MAP kinase expression and development of RA and the development of erosive disease. Results were considered to be of statistical significance if p < 0.05.
JNK en ERK MAP kinases zijn goede biochemische voorspellers van erosiviteit in RA
Results

p38, ERK and JNK are differentially expressed and phosphorylated in early arthritis patients with distinct diagnoses

We performed immunohistochemical staining on synovial biopsy samples from 50 DMARD-naive early arthritis patients, using antibodies which recognized total and phosphorylated (phospho-) p38, ERK, and JNK MAP kinases. Within the cohort, 27 patients were diagnosed with RA at 2 years after enrollment in the study, 7 with SpA, and 16 with UA. Clinical characteristics of each patient group are shown in Table 1. Initial qualitative analysis demonstrated that p38 was readily detected in synovial tissue of patients from all diagnostic groups. Expression of p38 was highest in patients with UA, and significantly different in this group compared to SpA (p < 0.05), but not patients with RA (Figure 1, right top panel). In contrast to p38, phospho-p38 was hardly detectable in early arthritis synovial tissue, although phospho-p38 levels were again higher in patients with UA than those with SpA (p < 0.05) (Figure 1, left top panel). ERK expression was significantly higher in patients with RA than in those with SpA (p < 0.01) and UA (p < 0.01) (Figure 1, right middle panel). Phospho-ERK levels were also highest in the synovial tissue of RA patients, where it was significantly elevated compared to SpA (p < 0.01) and UA (p < 0.01) (Figure 1, left middle panel). No differences in JNK expression were noted between diagnostic groups (Figure 1, right lower panel), but phospho-JNK levels were higher in RA than in SpA (p < 0.005) and UA (p < 0.01) (Figure 1, left lower panel). Together, these initial analyses indicated that MAP kinases are differentially expressed and phosphorylated in early arthritis patients, dependent on patient disease diagnosis.

Table 1 / Characteristics of study patients*

<table>
<thead>
<tr>
<th></th>
<th>UA&gt;&gt;UA</th>
<th>RA</th>
<th>UA&gt;&gt;RA</th>
<th>SpA</th>
</tr>
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<tbody>
<tr>
<td>Age (years)</td>
<td>39 (20-67)</td>
<td>52.5 (22-82)</td>
<td>54 (43-66)</td>
<td>44 (20-56)</td>
</tr>
<tr>
<td>Female (n)</td>
<td>12</td>
<td>10</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>Disease duration (m)</td>
<td>3.75 (1-10)</td>
<td>5 (1-12)</td>
<td>3.5 (1-11)</td>
<td>2 (1-12)</td>
</tr>
<tr>
<td>VAS (0-100)</td>
<td>63 (9-93)</td>
<td>37 (11-99)</td>
<td>48 (32-98)</td>
<td>35 (17-91)</td>
</tr>
<tr>
<td>TJCG8 (n)</td>
<td>3 (0-37)</td>
<td>16 (5-38)</td>
<td>13 (3-25)</td>
<td>4 (0-11)</td>
</tr>
<tr>
<td>SJCG6 (n)</td>
<td>1 (1-19)</td>
<td>8 (4-41)</td>
<td>6 (1-20)</td>
<td>2 (1-5)</td>
</tr>
<tr>
<td>ESR (mm/h)</td>
<td>34 (4-85)</td>
<td>32 (3-91)</td>
<td>30 (14-77)</td>
<td>11 (3-47)</td>
</tr>
<tr>
<td>CRP (mg/l)</td>
<td>11 (2-58)</td>
<td>12 (3-133)</td>
<td>17 (3-42)</td>
<td>7 (1-46)</td>
</tr>
<tr>
<td>IgM-RF pos.</td>
<td>2</td>
<td>13</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>ACPO pos. (n)</td>
<td>2</td>
<td>10</td>
<td>3</td>
<td>0</td>
</tr>
</tbody>
</table>

UA>>UA = undifferentiated arthritis at baseline and at two years follow-up; RA = rheumatoid arthritis at baseline; UA>>RA = classified as UA at baseline and diagnosed as RA at two years follow-up; SpA = spondyloarthritis; VAS = visual analog scale of global disease activity; TJCG8 = 68 tender joint count; SJCG6 = 66 swollen joint count; ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; IgM-RF pos. = immunoglobulin M rheumatoid factor positive; ACPO pos. = anti-citrullinated protein antibody positive.
Selective involvement of erk and jnk mitogen-activated protein kinases in early rheumatoid arthritis

Figure 1 / Quantitative comparison of mitogen-activated protein kinase (MAPK) phosphorylation (phospho-MAPK) and expression (total MAPK) in the synovial tissue of patients with early arthritis. Tissue sections from patients diagnosed with rheumatoid arthritis (RA), spondyloarthritis (SpA), and undifferentiated arthritis (UA) after two years follow-up were stained with antibodies against phospho- and total p38, extracellular-signal regulated kinase (ERK), and c-Jun N-terminal kinase (JNK). Stainings were developed with biotin tyramide enhancement, horseradish peroxidase and aminoethylcarbazole, followed by counterstaining with Mayer's hematoxylin, and evaluated by digital imaging analysis. Values indicated are the integrated optical density (IOD)/mm² of stainings with the indicated antibodies. Data is presented as box plots, where the boxes represent the 25th to 75th percentiles, the lines within the box mark the median value, and lines outside the boxes denote the 10th and 90th percentiles. Lines connecting data sets indicate statistically significant differences between groups. * p < 0.05. ** p < 0.01. *** p < 0.005.
Relative phosphorylation of ERK and JNK is enhanced in patients with early RA, even before fulfillment of classification criteria for RA.

To gain more insight into the relative degree of engagement of each MAP kinase by inflammatory stimuli in synovial tissue, we calculated the relative phosphorylation of p38, ERK, and JNK proteins for each patient (IOD phospho-MAP kinase/IOD total MAP kinase, arbitrary units), and compared these values between diagnostic groups. Relative phosphorylation of p38 was similar in all diagnostic groups (Figure 2, left panel). In contrast, relative ERK phosphorylation in RA (mean ± SEM, arbitrary units, 1.51 ± 0.82) was significantly higher than in SpA (0.49 ± 0.45, p < 0.05) and UA (0.010 ± 0.005, p < 0.01) (Figure 2, middle panel). Striking differences were also observed for relative JNK phosphorylation levels between diagnostic groups (Figure 2, right panel). Relative JNK phosphorylation was highly elevated in patients with RA (1.77 ± 0.58) compared to UA (0.41 ± 0.09, p < 0.01) and SpA (0.06 ± 0.0003, p < 0.005).

**Figure 2** / Quantitative comparison of relative MAPK phosphorylation in the synovial tissue of patients with early arthritis.

Relative phosphorylation levels (ratio of IOD phosphorylated protein to IOD of total protein, arbitrary units) of p38, ERK, and JNK were calculated for patients diagnosed with RA, SpA, and UA. Data is presented as box plots, where the boxes represent the 25th to 75th percentiles, the lines within the box mark the median value, and lines outside the boxes denote the 10th and 90th percentiles. Lines connecting data sets indicate statistically significant differences between groups. * p < 0.05. ** p < 0.01. *** p < 0.005.
Given the observed elevation in ERK and JNK phosphorylation of patients with RA compared to other diagnostic groups, we examined if the MAP kinases might already be preferentially activated in patients diagnosed with UA at baseline who were classified as RA after two years (see Table 1 for patient characteristics). We observed no differences in relative p38 phosphorylation between patients diagnosed with UA who remained classified as UA (UA>>UA) (n=16) and UA patients who later met ACR criteria for RA (UA>>RA) (n=8) (Figure 3, left panel). ERK phosphorylation was also similar in the two diagnostic groups (Figure 3, middle panel). In contrast, JNK phosphorylation levels were significantly elevated in UA>>RA patients (Figure 3, right panel) compared to UA>>UA patients (p < 0.005). Univariate logistic regression analysis showed that relative JNK activation at baseline was significantly related to fulfillment of classification criteria for RA after follow up with an explained variance of 59% (R² = 0.59, p = 0.02). However relative ERK and p38 expression were not related to fulfillment of classification criteria for RA after follow up. Thus, in patients with early arthritis, elevated ERK and JNK activity distinguish RA from other forms of arthritis, and JNK activation is already elevated in patients with RA even before classification criteria of RA are met.

Figure 3 | Quantitative comparison of relative MAPK phosphorylation in the synovial tissue of patients classified as UA which remained UA after two years (UA>>UA), and UA which was diagnosed as RA after two years (UA>>RA).
Relative phosphorylation levels (ratio of IOD phosphorylated protein to IOD of total protein, arbitrary units) of p38, ERK, and JNK were calculated for patients diagnosed with UA>>UA and UA>>RA. Data is presented as box plots, where the boxes represent the 25th to 75th percentiles, the lines within the box mark the median value, and lines outside the boxes denote the 10th and 90th percentiles. Lines connecting data sets indicate statistically significant differences between groups. * p < 0.05. ** p < 0.01. *** p < 0.005.
Relative ERK and JNK activation is elevated in RA patients developing erosive disease

We next examined the relationship between MAP kinase activation and disease outcome in patients with RA. All patients with RA (RAxRA and UAxRA) were pooled and then grouped based on the development of erosive disease. Patients were defined as having erosive disease based on the presence of erosions observed on X-rays of hands or feet at 2 year follow up where the modified Sharp-van der Heijde erosion score was ≥ 1 [35]. Clinical characteristics of these patients are described in Table 2. Of all the early arthritis patients, only one had erosive disease at baseline.

When MAP kinase engagement was assessed in this manner, no differences were observed in the relative phosphorylation of p38 between patients with non-erosive (n=15) and erosive disease (n=12) (Figure 4A, left panel). In contrast, relative phosphorylation of ERK (p < 0.01) (Figure 4A, middle panel) and JNK (p < 0.01) (Figure 4A, right panel) was significantly higher in patients who developed erosive disease. When we compared the relationship of disease progression with MAP kinase activation in all early arthritis patients, regardless of diagnosis (see Table 3 for patient characteristics), we found that activation of each of the MAP kinases was elevated in patients with erosive disease (Figure 4B). Comparing all early arthritis patients, activation of p38 (p < 0.05), ERK (p < 0.005) and JNK (p < 0.001) was elevated in patients with erosive disease, and JNK activation predicted development of erosive disease (R² = 0.16, p < 0.05).

Table 2 / Characteristics of RA patients with erosive and non-erosive disease*

<table>
<thead>
<tr>
<th></th>
<th>RA erosive n=12</th>
<th>RA non-erosive n=15</th>
<th>p-value</th>
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<tbody>
<tr>
<td>Age (years)</td>
<td>53 (24-82)</td>
<td>54 (22-58)</td>
<td>0.88</td>
</tr>
<tr>
<td>Disease duration (months)</td>
<td>5 (1-10)</td>
<td>4 (1-12)</td>
<td>0.86</td>
</tr>
<tr>
<td>DAS 28</td>
<td>5.0 (2.9-6.6)</td>
<td>5.9 (3.3-6.7)</td>
<td>0.13</td>
</tr>
<tr>
<td>ESR (mm/h)</td>
<td>37 (3-91)</td>
<td>27 (7-76)</td>
<td>0.67</td>
</tr>
<tr>
<td>CRP (mg/l)</td>
<td>9 (3-114)</td>
<td>16 (3-133)</td>
<td>0.62</td>
</tr>
<tr>
<td>VAS (0-100)</td>
<td>27 (11-76)</td>
<td>50 (25-98)</td>
<td>0.12</td>
</tr>
<tr>
<td>IgM–RF pos. (%)</td>
<td>66</td>
<td>43</td>
<td>0.27</td>
</tr>
<tr>
<td>ACPA pos (%)</td>
<td>44</td>
<td>50</td>
<td>0.80</td>
</tr>
</tbody>
</table>

RA = rheumatoid arthritis at two years follow-up; DAS28 = disease activity 28; ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; VAS = visual analog scale of global disease activity; IgM–RF pos. = immunoglobulin M rheumatoid factor positive; ACPA pos. = anti-citrullinated protein antibody positive.
Selective involvement of erk and jnk mitogen-activated protein kinases in early rheumatoid arthritis

Table 3 / Characteristics of early arthritis patients with erosive and non-erosive disease*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Erosive (n=17)</th>
<th>Non-erosive (n=33)</th>
<th>p-value</th>
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<tr>
<td>Age (years)</td>
<td>54 (24-81)</td>
<td>46 (20-75)</td>
<td>0.92</td>
</tr>
<tr>
<td>Disease duration (months)</td>
<td>5.8 (1-11)</td>
<td>4.9 (1-12)</td>
<td>0.39</td>
</tr>
<tr>
<td>DAS28</td>
<td>3.7 (1.1-5.7)</td>
<td>2.4 (0.6-6.3)</td>
<td>0.012</td>
</tr>
<tr>
<td>ESR (mm/h)</td>
<td>36 (3-91)</td>
<td>32 (9-93)</td>
<td>0.72</td>
</tr>
<tr>
<td>CRP (mg/l)</td>
<td>14 (1-114)</td>
<td>10 (1-133)</td>
<td>0.68</td>
</tr>
<tr>
<td>VAS (0-100)</td>
<td>42 (11-81)</td>
<td>38 (3-98)</td>
<td>0.88</td>
</tr>
<tr>
<td>IgM-RF pos. (%)</td>
<td>35</td>
<td>21</td>
<td>0.32</td>
</tr>
<tr>
<td>ACPA pos (%)</td>
<td>24</td>
<td>27</td>
<td>0.88</td>
</tr>
</tbody>
</table>

DAS28 = disease activity score 28; ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; VAS = visual analog scale of global disease activity; IgM-RF pos. = immunoglobulin M rheumatoid factor positive; ACPA pos. = anti-citrullinated protein antibody positive.

Figure 4 / Quantitative comparison of relative MAPK phosphorylation in the synovial tissue of early arthritis patients with persistent erosive and persistent non-erosive disease.

Relative phosphorylation levels (ratio of IOD phosphorylated protein to IOD of total protein, arbitrary units) of p38, ERK, and JNK were calculated for RA (a) and all early arthritis patients (b) diagnosed with erosive (Sharp-van der Heijde erosion score ≥ at two year follow up) and non-erosive persistent disease. Data is presented as box plots, where the boxes represent the 25th to 75th percentiles, the lines within the box mark the median value, and lines outside the boxes denote the 10th and 90th percentiles. * p < 0.05. ** p < 0.01. *** p < 0.005.
Relative ERK and JNK activation is enhanced in early arthritis patients seropositive for RF and ACPA

Lastly, we examined if differences in the relative activation of MAP kinases might be associated with the presence of RF and ACPA, humoral biomarkers predictive in the development of RA, as well as disease course and prognosis [36, 37]. RA and UA+RA patients were grouped and then classified based on the absence or presence of serum RF or ACPA at baseline (see Tables 4 and 5 for clinical characteristics of patients). When comparing RF+ (n=14) to RF- patients (n=13), the relative activation of ERK (p < 0.0005) and JNK (p < 0.005), but not p38, was significantly higher in RF+ patients (Figure 5, left panels). Similarly, ACPA+ patients (n = 13) also displayed enhanced relative ERK (p < 0.001) and JNK (p < 0.005) activation compared to ACPA- patients (n = 14) (Figure 5, right panels).

Table 4 / Characteristics of RF seropositive and seronegative RA patients*

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<tr>
<td>Age (years)</td>
<td>53.3 (30-75)</td>
<td>57.1 (27-71)</td>
<td>0.53</td>
</tr>
<tr>
<td>Disease duration (months)</td>
<td>7.3 (1-12)</td>
<td>5.4 (1-11)</td>
<td>0.23</td>
</tr>
<tr>
<td>VAS (0-100)</td>
<td>36 (20-75)</td>
<td>23 (3-43)</td>
<td>0.23</td>
</tr>
<tr>
<td>TJC68 (n)</td>
<td>12 (5-38)</td>
<td>4 (3-14)</td>
<td>0.07</td>
</tr>
<tr>
<td>SJC66 (n)</td>
<td>7 (1-41)</td>
<td>3 (1-17)</td>
<td>0.07</td>
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<tr>
<td>DAS 28</td>
<td>3.7 (1.1-6.3)</td>
<td>2.7 (1.3-3.4)</td>
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</tr>
<tr>
<td>ESR (mm/h)</td>
<td>31 (14-91)</td>
<td>30 (3-77)</td>
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</tr>
<tr>
<td>CRP (mg/l)</td>
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<td>20 (3-42)</td>
<td>0.96</td>
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RF+ = rheumatoid factor seropositive; RF- = rheumatoid factor seronegative; RA = rheumatoid arthritis diagnosis at two years of follow-up; VAS = visual analog scale of global disease activity; TJC68 = 68 tender joint count; SJC66 = 66 swollen joint count; DAS28 = disease activity score 28; ESR = erythrocyte sedimentation rate; CRP = C-reactive protein.

Table 5 / Characteristics of ACPA seropositive and seronegative RA patients*

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<th>p-value</th>
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<tr>
<td>Age (years)</td>
<td>53.7 (40-75)</td>
<td>56 (30-71)</td>
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</tr>
<tr>
<td>Disease duration (months)</td>
<td>7.5 (1-12)</td>
<td>5.6 (1-11)</td>
<td>0.23</td>
</tr>
<tr>
<td>VAS (0-100)</td>
<td>35 (0-75)</td>
<td>22 (3-43)</td>
<td>0.11</td>
</tr>
<tr>
<td>TJC68 (n)</td>
<td>11 (5-38)</td>
<td>7 (3-17)</td>
<td>0.44</td>
</tr>
<tr>
<td>SJC66 (n)</td>
<td>6 (2-41)</td>
<td>3 (1-17)</td>
<td>0.43</td>
</tr>
<tr>
<td>DAS 28</td>
<td>3.6 (1.1-6.3)</td>
<td>3.2 (1.2-6.2)</td>
<td>0.72</td>
</tr>
<tr>
<td>ESR (mm/h)</td>
<td>31 (14-91)</td>
<td>30 (3-77)</td>
<td>0.86</td>
</tr>
<tr>
<td>CRP (mg/l)</td>
<td>23 (3-133)</td>
<td>16 (3-42)</td>
<td>0.49</td>
</tr>
</tbody>
</table>

ACPA+ = anti-citrullinated protein antibody seropositive; ACPA- = anti-citrullinated protein antibody seronegative; RA = rheumatoid arthritis diagnosis at two years of follow-up; VAS = visual analog scale of global disease activity; TJC68 = 68 tender joint count; SJC66 = 66 swollen joint count; DAS28 = disease activity score 28; ESR = erythrocyte sedimentation rate; CRP = C-reactive protein.
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Figure 5 /Relationship between relative MAPK phosphorylation in the synovial tissue of early arthritis patients and the presence of autoantibodies in patient serum.
RA patients were grouped based on the presence (+) or absence (-) of rheumatoid factor (RF, left panels) or anticitrullinated protein antibodies (ACPA, right panels) and relative MAPK phosphorylation levels (ratio of IOD phosphorylated protein to IOD of total protein, arbitrary units) of p38, ERK, and JNK were calculated for each patient. Data is presented as box plots, where the boxes represent the 25th to 75th percentiles, the lines within the box mark the median value, and lines outside the boxes denote the 10th and 90th percentiles. P values (p) of statistically significant differences between patient groups are indicated.
Discussion

The MAP kinases p38, ERK, and JNK are each detected in their activated form in the synovial tissue of patients with various forms of inflammatory arthritis [3,4]. Moreover, a requisite role for each of these MAP kinases in the initiation and perpetuation of inflammation, as well as joint destruction, has been demonstrated in multiple animal models of arthritis, using both gene-silencing techniques and increasingly specific pharmacological inhibitors [1-3]. These findings have cumulatively suggested that MAP kinases might represent attractive therapeutic targets in the treatment of RA and other forms of arthritis. However, the clinical inefficacy of p38 inhibitors displayed in early trials with RA patients has indicated a need for a greater understanding of the contributions of MAP kinase activation to synovitis and joint destruction [25, 26, 38]. In this study, we provide the first analysis of MAP kinase activation in relationship to disease development and prognosis in a prospective study of DMARD-naive early arthritis patients. We find that activation of ERK and JNK, but not p38, is significantly elevated in patients with RA compared to patients with other diagnoses, and is closely associated with patient production of RF and ACPA auto-antibodies, considered important biomarkers for development of RA and disease course [39, 40]. Activation of ERK and JNK is also highly elevated in the synovium of patients who develop erosive RA. Perhaps important to the etiology of RA, JNK activation is elevated in patients with RA even before classification criteria of RA are met. When we assessed all early arthritis patients, regardless of disease diagnosis, we found that each of the MAP kinases was more highly activated in patients with erosive disease, and here, JNK activation predicted the development of erosive disease.

Initial characterizations of MAP kinase activation status in patients with RA were confined to patients undergoing joint surgery [4, 41]. Although relative activation levels of each MAP kinase were not described, the percentages of cells in which phosphorylated p38, ERK, and JNK could be detected in RA synovial tissue were similar [4]. We have recently reported similar findings in studies of synovial tissue from patients with active RA and PsA (de Launay et al, submitted). However, we also noted that the relative activation of total p38 and ERK protein was elevated in RA compared to PsA, indicating that in different forms of arthritis, active engagement of each MAP kinase may vary and make specific contributions to pathology. Intriguingly, recent kinetic analyses of MAP kinase activation in multiple murine models of RA have also indicated differential involvement of p38, ERK and JNK in distinct phases of initiation, perpetuation, and resolution of disease in inflammatory arthritis [27]. This latter observation prompted us to examine MAP kinase expression and phosphorylation in the earliest stages of human arthritis.
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In this study, we find no evidence supporting a specific role for p38 in the onset of RA or eventual joint destruction in arthritis. Phosphorylated p38 is readily detected in the synovial tissue of patients with active and end-stage destructive RA, and observations that p38, along with ERK, is highly activated in the human TNF-transgenic murine model of RA were also consistent with the idea that p38 contributed to inflammation and joint destruction [3, 4, 21]. Indeed, pharmacological inhibition of p38 blocks disease onset in this animal model of RA [15]. Despite this, initial clinical trials using highly selective p38 inhibitors to treat RA patients have reported little clinical efficacy, while noting a small and transient, albeit reproducible decrease in systemic inflammation [25, 26]. This has raised the possibility that p38 may also make significant contributions to negative feedback mechanisms which dampen inflammatory responses, or even, primarily participate in cellular attempts to resolve inflammation or repair tissue in established RA. Several experimental and clinical observations support these possibilities. At the cellular level, p38 activity can both promote and suppress the stability of mRNA encoding pro-inflammatory gene products, initiate negative feedback loops suppressing the activity of ERK and JNK, and is required for IL-10 production [42]. In murine collagen-induced arthritis (CIA), p38 phosphorylation levels are only modestly increased over baseline until late in the disease (day 40-50), when clinical parameters and cytokine biomarkers of disease activity are receding [27]. In the human TNF-transgenic murine model of RA, anti-TNF therapy resolves disease in arthritic mice but fails to diminish synovial p38 phosphorylation to levels observed in healthy mice [21]. Parallel observations are emerging in the clinic, as successful treatment of PsA patients with etanercept decreases phosphorylation of synovial ERK and JNK, while leaving the phosphorylation status of p38 unaffected [43]. Possibly, our failure to detect significant levels of p38 phosphorylation in patients with early arthritis may reflect that repair mechanisms have not yet been initiated in this stage of the disease. This can be formally tested in the future as follow-up synovial samples are acquired from patients enrolled in early arthritis studies.

We observe a clear and significant elevation of ERK phosphorylation in early arthritis patients diagnosed with RA. Strikingly, ERK phosphorylation is also elevated in those RA patients who develop erosive disease. Previous reports have readily detected ERK phosphorylation in the synovial tissue of RA patients undergoing joint replacement [4]. ERK also participates in the pathology of murine models of RA, although the kinetics and degree of ERK activation vary between specific models. In the passive K/BxN serum transfer model of RA, ERK activation is detected early during disease initiation, peaks at the height of clinical disease activity, and then subsides in parallel with disease resolution [27]. In contrast, the same authors were unable to detect ERK phosphorylation throughout the disease course of CIA. This may suggest a negligible role for ERK in CIA, but other investigators have detected a sharp transient increase in ERK activation between days 28–35 of the CIA model [10]. Indeed, a generalized role for ERK in inflammatory arthritis is suggested by findings that either genetic deletion of scaffolding proteins needed for ERK activation, or pharmacological inhibition of the upstream kinases responsible for ERK activation, MEK-1 and MEK-2, are protective in passive serum transfer arthritis and CIA [10,
However, in vitro studies in RA FLS with MEK/ERK inhibitors have revealed that many secreted products routinely assessed as TNF-dependent activation markers relevant to RA, including IL-6, IL-8, MMP-1, MMP-3 and PGE2, are relatively insensitive to ERK activity status [44]. Thus, the mechanisms by which ERK contribute to the initiation of inflammation and disease progression, in RA and animal models of RA, remain to be established.

We find that JNK phosphorylation is also significantly elevated in early RA patient synovial tissue, even before classification criteria are met. Synovial JNK phosphorylation has previously been detected in patients with longstanding RA, especially in FLS of the intimal lining layer [4, 45]. In terms of kinetics of activation, JNK participation in RA appears to be most similar to the K/BxN, rather than CIA, animal model [27]. Similarly to CIA, synovial JNK phosphorylation is not prominent in transgenic human TNF-induced arthritis, and JNK1 is dispensable for disease onset in this model [46]. In murine FLS, both JNK1 and JNK2 contribute to IL-1β-induced collagenase expression, and an absolute requirement for JNK2 activity in passive murine CIA has been demonstrated [12, 18]. Additionally, pharmacological inhibition of JNK signaling is protective in rat adjuvant-induced arthritis, particularly in regard to suppressing collagenase-3 expression and bone damage [12].

Our studies suggest that pharmacological targeting of ERK and JNK may be of particular benefit in limiting inflammation and joint destruction early in the development of RA. Continued research will be needed to determine the exact mechanisms by which ERK and JNK contribute to these phases of RA, and profiling of ERK and JNK–dependent gene expression in early arthritis patients will likely accelerate accomplishment of this goal.
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