Activation of Nuclear Factor kappa B and Mitogen Activated Protein Kinases in psoriatic arthritis before and after etanercept treatment

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Running title: MAPK and NFκB in psoriatic arthritis

Abstract

Objective: To study activation of intracellular pathways depending on Nuclear Factor kappa B (NFκB) and Mitogen Activated Kinases (MAPK) in the synovium of patients with psoriatic arthritis before and after treatment with etanercept.

Methods: Synovial biopsies were obtained by needle arthroscopy of the knee in 9 patients with active psoriatic arthritis before the initiation of etanercept. Follow-up biopsies were taken in the same knee after 6 months. Synovitis was studied by histology. Pathway activation was studied by immunofluorescense for phosphorylated ERK, phosphorylated p38, phosphorylated JNK or phosphorylated inhibitor of kappa B (IκBα) using digital image analysis.

Results: Histological severity scores were significantly reduced after etanercept treatment. Activation of NFκB signaling was found in the lining layer, and in infiltrating and peri-vascular cells in the sublining zone. Activated p38 was present in both lining and sublining layer. In the sublining layer, positive cells were found in inflammatory infiltrates, in perivascular zones and in endothelium. Activated ERK was mainly present in the sublining layer, both in mononuclear cell infiltrates and perivascularly. Occasional positive cells were found in the lining layer. Activation of JNK was recognized in cells of the lining layer, in some of the sublining cell infiltrates and the perivascular compartment.

Conclusions: Etanercept therapy resulted in a significant decrease in NFκB, JNK and ERK, but not in p38 activation. Persistent activation of these pathways, albeit reduced, may trigger positive feedback loops and flares of arthritis after cessation of etanercept.

Mesh: psoriatic arthritis, mitogen activated kinases, tumor necrosis factor.

Introduction

Psoriatic arthritis (PsA) is characterized by a complex process in which cell-cell and ligand-receptor interactions act in concert. The balance between the activation of intracellular signaling cascades that trigger, sustain or amplify the inflammatory reaction and activate tissue-destructive and remodeling processes, and cascades that have anti-inflammatory or tissue homeostatic effects, determines the severity and outcome of the disease. The complex nature of PsA renders it unlikely that treatments with a single target will be sufficient to control or cure arthritis in all patients affected. Despite the impressive clinical improvement seen in patients treated with anti-tumor necrosis factor - drugs (anti-TNF), a number of patients show only partial responses or some may even be refractory to the treatment [1;2;3;4;5]. Moreover, anti-TNF therapy is not curative but disease modifying [4;6]. This suggests that signaling pathways triggering or sustaining the disease processes are only suppressed but not inactivated.

Nuclear factor kappa B (NFκB) transcription factors were identified as critical intracellular messengers in inflammatory and immune responses [7]. Inactive forms of NFκB proteins exist in the cytoplasm in association with Inhibitor of kappa B (IκB) molecules. Signal-induced phosphorylation of IκB releases NFκB from these complexes and allows their nuclear migration. Stimulation or repression of gene transcription herein by NFκBs is mostly dependent on interaction with other cytoplasmic and/or nuclear factors. These often rely on mitogen activated protein kinases (MAPK). MAPKs are a part of complex cascades [7;8]. They are phosphorylated by MAPK kinases (MAP2K) which are in turn activated by MAP2K Kinases (MAP3K). Different stimuli, both

molecular and mechanical, can stimulate distinct MAP3Ks. The 3 major MAPKs have been identified as Extracellular Regulating Kinase (ERK), the c-Jun-N-terminal Kinase (JNK), and the p38 MAPKs [8]. MAPKs have been involved in the induction of pro-inflammatory cytokines and effector enzymes such as matrix metalloproteinases [7;8]. MAPK activation is found both upstream and downstream in TNF signaling and may be of particular importance both in the initiation and sustenance of inflammatory disease.

Activation of MAPK signaling has been described in rheumatoid arthritis synovium [9] and in psoriasis skin lesions [10;11;12]. Little is known about the role of MAPK in psoriatic arthritis, the second most common form of chronic inflammatory arthritis. Danning et al. have described NFκB activation in PsA synovium [13]. Recently, epidermal deletion of Jun-B and c-Jun triggered psoriasis and psoriatic arthritis in mice [14]. In this study we have analyzed MAPK and NFκB activation in PsA arthritis synovium before and after treatment with etanercept, a soluble type TNF-receptor that is efficacious in the treatment of PsA and psoriasis [1;4;15]. Our data indicate that persistent activation of these pathways, albeit sufficiently reduced to prevent clinical signs and symptoms, may trigger flares of arthritis after cessation of etanercept.

Patients and methods

Patients. Nine patients were included in this histomorphological study. They participated in a single-center, open-label, observational trial of etanercept effectiveness in PsA [15]. Patients with PsA and persistent clinical disease activity despite anti-rheumatic drug therapy were included. All patients had psoriasis and ≥ 3 swollen joints at

entry. All patients provided written informed consent. The trial was approved by the local Ethics committee (University Hospitals Leuven, Belgium). Conventional immune-modulating anti-rheumatic drugs (table 1) were stopped at least 4 weeks before the study. Patients received Etanercept (Enbrel®), a soluble TNFα-receptor (Wyeth Pharmaceuticals, Louvain-la-Neuve, Belgium) 25 mg subcutaneously twice weekly. Patients were not allowed to take anti-rheumatic drugs but could take a stable low dose of steroids (≤10 mg prednisolone-equivalent/day) and non-steroidal anti-inflammatory drugs. Needle arthroscopy of the knee with a single entry port and blind biopsies was performed at week 0 and week 26.

Histology and immunofluorescence. For histomorphological assessments, 6 biopsies from different sites were snap-frozen in TissueTek (Sakura, Zoeterwoude, The Netherlands). Cryostat sections were stained with hematoxylin-eosin or used for immunofluorescence. Sections were fixed with acetone for 10 minutes and stained with rabbit-anti-human phosphorylated IκBα (5 μg/ml), goat-anti-human phosphorylated ERK (5 μg/ml), mouse-anti-human phosphorylated p38 (5 μg/ml) or mouse-anti-human phosphorylated JNK (5 μg/ml) (all from Santa Cruz, Santa Cruz, USA). All antibodies in this study have previously been used for specific detection of phosphorylated proteins in both western blots [16;17;18;19] and in immunofluorescence or immunohistochemistry [20;21;22;23]. Cy2-conjugated donkey anti-goat (1/1000), Cy3-conjugated goat anti-rabbit (1/500) or Cy3-conjugated goat anti-mouse antibodies (1/500) (Jackson Immunoresearch, West Grove, USA) were used as secondary antibodies. Negative controls were performed with isotype controls or non-specific IgG.

Histology score. Severity of synovitis was assessed with blinded semi-quantitative scores (0-3) of 4 individual parameters (lining layer thickness, sublining vascularity, inflammatory cell infiltration and presence of lymphoid aggregates) [24] and a composite histology score. For computer assisted digital image analysis in immunofluorescence, we used a modified version of the protocol proposed by Cunnane et al [25]. Six high power fields from 6 separate biopsies were randomly selected by one observer who was unaware of the identity and order of the samples. Images were taken using Spot camera and software (Diagnostic Instruments, Sterling Heights, MI). Further analysis was performed with Image J software (National Institutes of Health, Bethesda, MA). Threshold fluorescence was determined using isotype IgG control antibodies. Fluorescence above the threshold was measured as surface occupied by the positive signal. Values were normalized to cell number by measuring the surface occupied by cell nuclei as shown by 4',6-diamidino-2-phenylindole (DAPI; ICN, Asse-Relegem, Belgium) nuclear staining.

Statistical analysis. For comparisons before and after treatment, non-parametric Wilcoxon signed rank test for paired samples was used. Correlations were tested with non-parametric Spearman correlation test.

Results

Patient characteristics.

Baseline demograpy data from the patients are shown in table 1. Etanercept resulted in significant improvements in Swollen Joint Count, Tender Joint Count, Health

Assesment Questionnaire and Patient disease activity score, Erythrocyte Sedimentation Rate, C-reactive protein ($p < 0.05$ for all parameters) (table 2). Arthroscopy was performed in a clinically affected knee in 8 out of 9 patients at the start of the clinical study . In 1 patient (nr. 5) no clinical signs of arthritis in the knee were present. In 2 out of 9 patients (nrs. 4 and 9), the knee was still affected at week 26 of the study.

Etanercept therapy and synovial histology.

Composite histological severity score was significantly lower after etanercept treatment (median score 4 vs. 1; $p < 0.05$) (figure 1). Lining layer hyperplasia, if present at baseline (6/9 patients) disappeared in all patients (median score 1 vs. 0; $p < 0.05$). Increased sublining vascularity (also present in 6/9 patients) normalized in all patients (median score 1 vs. 0; $p < 0.05$). Cell infiltration, present in 7/9 patients at baseline, decreased in 5/7 and increased in 1/7 (median score 2 vs. 1; $p > 0.05$). Lymphoid aggregates were found in 2/9 patients at baseline in which they disappeared after 26 weeks. In one other patient a lymphoid follicle was found at week 26 (median score 0 vs. 0; $p > 0.05$).

Expression of NFkB and MAPK in PsA.

At baseline, activation of NFκB signaling as revealed by phosphorylation of IκBα [20], was found in the lining layer, as well as in infiltrating and peri-vascular cells in the sublining zone (figure 2 and table 3). Activated p38 was present in both lining and sublining layer. In the sublining layer, positive cells were found in inflammatory infiltrates, in perivascular zones and in the endothelium (figure 2 and table 3). Activated

ERK was mainly present in the sublining layer, both in mononuclear cell infiltrates and perivascularly. Only occasional positive cells were found in the lining layer. Activation of JNK was recognized in cells of the lining layer, in some of the sublining cell infiltrates and the endothelium – perivascular compartment (figure 2 and table 3). Double immunofluorescence did not show an association of any studied signaling pathway with a specific cell type such as macrophages or T cells (data not shown).

Differential regulation of NFkB and MAPK activation.

Treatment with etanercept resulted in differences in the activation pattern of these signaling pathways. These changes were mainly found in the sublining layer. However, as mentioned above, etanercept therapy also resulted in a reduction in inflammatory infiltrates in the sublining zone. Activation of both NFκB and MAPK signaling was still found in the lining layer and the perivascular compartment (table 3). To better evaluate the effect of etanercept therapy quantitatively rather than qualitatively, we used digital image analysis. Etanercept therapy resulted in a significant decrease in NFκB (median normalized fluorescent area (mfa) 0.84 vs. 0.69; $p < 0.03$), ERK (mfa 0.096 vs. 0.04; $p < 0.03$) and JNK (mfa 0.91 vs. 0.34; $p < 0.03$) but not in p38 activation (mfa 0.04 vs. 0.07; $p > 0.07$) (figure 3). NFκB and ERK activation were decreased in 7/9 patients and showed an increase in 2/9 patients. JNK activation showed a decrease in 8/9 patients and an increase in 1/9 patients. P38 activation decreased in only 2/9 patients, remained stable in 1/9 and increased in 6/9 patients.

Activation of signaling pathways and clinical response.

No correlation was found between pre- and posttreatment measurements of NFκB and MAPK activation and disease activity parameters (Swollen Joint Count, Tender Joint Count, Health Assesment Questionnaire and Patient disease activity score, Erythrocyte Sedimentation Rate, C-reactive protein) ($r_s < 0,78$ Spearman Rank Correlation, $p > 0,05$).

Discussion

Etanercept therapy not only improves clinical symptoms but also histological severity of disease in the synovium of patients with psoriatic arthritis. Digital image analysis demonstrated significant effects on the activation of NFκB, ERK and JNK MAPK signaling, but not on p38. Persistent activation of intracellular signaling even in the absence of clinically apparent symptoms provides an explanation for the recurrence of symptoms after interruption of anti-TNF therapy.

Changes in PsA synovial histology have been demonstrated for different treatment options, including conventional anti-rheumatic drugs [26] as well as biologicals [24;27;28;29;30;31]. In patients that were successfully treated with low-dose methotrexate, T cells, macrophages, adhesion molecules and MMP-3 were significantly reduced. However, synovial inflammation was not abolished, T cell infiltration had not disappeared and no effect on synovial hypervascularity was seen [26]. Goedkoop et al. demonstrated a reduction in T cells and macrophages in skin and synovium after short term treatment with infliximab [30]. In a group of patients with Spondyloarthritis, including 4 with PsA, lining layer thickness, vascularity, neutrophils and macrophages were decreased but overall infiltration remained similar, probably due to an increase in B

cells and plasma cells. Only $CD4^+$ T cells showed a decrease, no change was seen in total number of T cells, in $CD8^+$ and in $CD45RO^+$ cells [24]. In similar studies with infliximab, short-term effects on macrophages, vascularisation, angiogenic factors and endothelial activation were seen [28;29]. Treatment with etanercept in spondyloarthritis patients with peripheral joint involvement resulted in a reduction in cell infiltration, in T lymphocytes and in macrophages, but not in B cells. An effect was seen on lining layer thickness and vascularity but not on the presence of lymphoid aggregates [31]. Treatment with alefacept, an inhibitor of T cell activation, demonstrated a reduction in synovial T cells and macrophages [27]. Our observations after 6 months of etanercept treatment are in line with these studies. Although current therapies, in particular biologicals, may lead to clinical remission in a number of patients, and affect synovial histology in most patients, no remission at the molecular level or restoration of synovial and hence joint homeostasis is seen.

Etanercept therapy had a different effect on distinct MAPK enzymes. The absence of effect on p38 activation can be explained in different ways. First, the current analysis does not distinguish between disease stages. Different patterns of intracellular signaling pathway activation in distinct stages of the disease have not been studied. The existence of different subtypes of synovial inflammation and organization, e.g. presence or absence of lymphoid follicles, may explain the lack of response in a few patients. Secondly, different isoforms of the enzyme exist that differ in tissue distribution and downstream targets [32]. Alternatively, activation of MAPKs is not limited to TNFα and other pro-inflammatory cytokines but can be triggered by different ligands and stress signals. For instance, Bone Morphogenetic Proteins and other molecules from the Tranforming

Growth Factor-β superfamily may trigger MAPK in the synovium [33;34;35;36]. Increasing evidence suggests that these pathway are important in joint homeostasis and remodeling [37].

The decrease in JNK activation after etanercept treatment may be of particular importance. Epidermal deletion of reciprocal antagonists c-Jun and JunB in mice leads to psoriasis-like skin lesions and arthritis [14]. C-Jun is strongly upregulated in psoriatic skin and epidermal downregulation of JunB seems involved in human psoriasis. In TNF-receptor $I^{-/-}$/JunB/c-Jun conditional KO mice skin lesions were reduced and arthritis almost absent [14]. Taken together these data suggest a role for JNK in different feedback loops in psoriasis and PsA and therefore modulation of JNK may be a specific therapeutic target.

The variable pattern of MAPK activation in PsA reflects the complexity of molecular signaling in chronic arthritis. Their well-known roles in positive feedback loops in inflammatory cascades suggest that MAPKs contribute to the chronicity of disease. Their roles are not likely to be limited to inflammation but may include effects on tissue remodeling. Further studies in particular in different animal models of joint destruction and remodeling, may indicate whether inhibition of MAPK signaling is a complementary approach to current therapeutic strategies in PsA.

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Table 1: Patient demographic characteristics

Patient number	Age (years)	Sex	disease duration (years)	Prior use of anti-rheumatic drugs*
1	30	male	12	Methotrexate, Cyclosporin
2	35	male	2	Methotrexate, Leflunomide
3	48	male	16	Methotrexate, Cyclosporin, Sulfasalazine, Azathioprin
4	45	male	6	Methotrexate, Cyclosporing, Sulfasalazine
5	49	male	7	Methotrexate, Sulfasalazine
6	53	female	2	Methotrexate
7	28	male	7	Methotrexate, Sulfasalazine
8	45	male	29	Methotrexate
9	32	male	1	Methotrexate, Sulfasalazine

* All conventional anti-rheumatic drugs were stopped at least 4 weeks before the start of etanercept therapy.

Table 2: Disease activity before and after etanercept therapy (weeks 0 and 26)

patient number	TJC[1]		SJC[2]		HAQ[3]		VAS activity[4]		ESR[5] (mm/h)		CRP[6] (mg/l)	
	w0	w26	w0	w26	w0	w26	w0	w26	w0	w26	w0	w24
1	10	0	15	0	0.625	0	51	8	24	2	47	14
2	10	1	4	0	0.5	0	56	1	12	2	7	3
3	2	1	12	0	1.5	0.375	82	66	41	12	23	4
4	1	0	15	1	0.5	0	59	7	36	2	12	3
5	12	0	8	0	1	0	32	13	22	3	15	3
6	25	0	11	8	2	0.5	36	14	87	35	34	3
7	2	2	4	1	1.125	0.25	36	3	15	2	32	3
8	13	1	7	4	1.125	0.5	33	5	22	2	22	4
9	28	27	27	20	1.75	1.875	69	17	18	14	18	10

[1] TJC, Tender Joint Count, [2] SJC, Swollen Joint Count, [3] Health Assesment Questionnaire, [4] VAS, Visual Analogue Scale of patient reported disease activity, [5] ESR, Erythrocyte Sedimentation Rate, [5] CRP, C-reactive Protein.

Table 3: Activation of signaling pathways in different synovial compartments

	Before etanercept therapy (week 0)			After etanercept therapy (week 26)		
	lining layer	sublining zone	peri-vascular	lining layer	sublining zone	peri-vascular
p-IκBα	8/9	7/9	9/9	6/9	4/9	8/9
p-ERK	8/9*	8/9	1/9	4/9*	5/9	2/9
p-JNK	8/9	6/9	8/9	7/9	3/9	7/9
p-p38	9/9	5/9	6/9	8/9	3/9	6/9

* lining layer cell positivity for p-ERK was discrete and limited to a few cells in all samples tested.

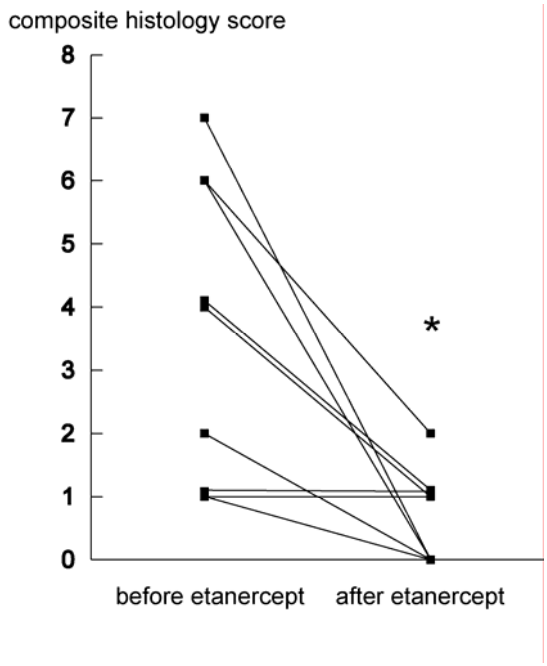
Figure legends

Figure 1. Composite histology severity score before and after 26 weeks of etanercept therapy. Four parameters (lining layer thickness, sublining vascularity, inflammatory cell infiltration and presence of lymphoid aggregates) are scored semi-quantitatively (0-3). (* $p < 0.05$ Wilcoxon paired samples test).

Figure 2. Immunofluorescent staining for intracellular signaling pathway activation in PsA synovium. **(A)** Activation of NFκB signaling demonstrated by detection of phosphorylated IκBα. Positive cells are found in the lining layer (arrowhead), cell infiltrates in the sublining (arrow) and the perivascular compartment (detail in right-sided image). **(B)** Activation of p38 signaling demonstrated by detection of phosphorylated p38. Positive cells are found in the lining layer (arrowhead), the sublining zone (arrow) and the perivascular zone (detail in right-sided image). **(C)** Activation of ERK signaling demonstrated by detection of phosphorylated ERK. Positive cells are rare in the lining layer. Positive staining is restricted to the sublining zone (arrow) and the perivascular zone. The detail on the right shows the presence of phosphorylated ERK in inflammatory infiltrates. **(D)** Activation of JNK signaling demonstrated by detection of phosphorylated JNK. Positive cells are found in the lining layer (arrowhead), the sublining zone (arrow) and the perivascular zone (detail in right-sided image). (Bar = 200 μm and 50 μm in left and right side images respectively).

Figure 3. Digital image analysis of intracellular signaling pathway activation before and after 26 weeks of etanercept therapy. **(A)** IκBα **(B)** ERK **(C)** JNK **(D)** p38 phosphorylation. (* $p < 0.05$, Wilcoxon paired samples test).

Figure 1


composite histology score
8
7
6
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4
3
2
1
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before etanercept
after etanercept

Figure 2

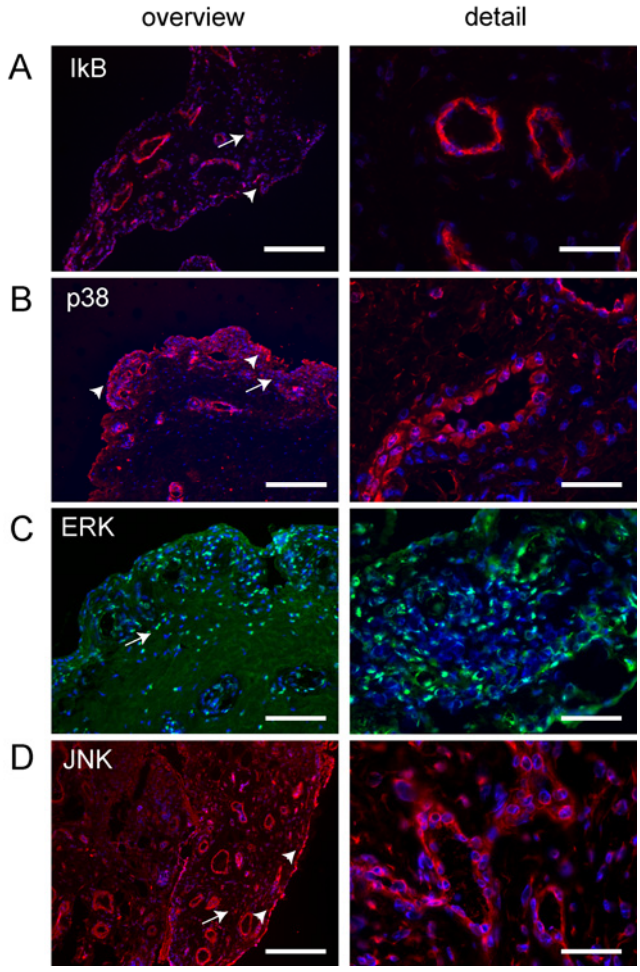
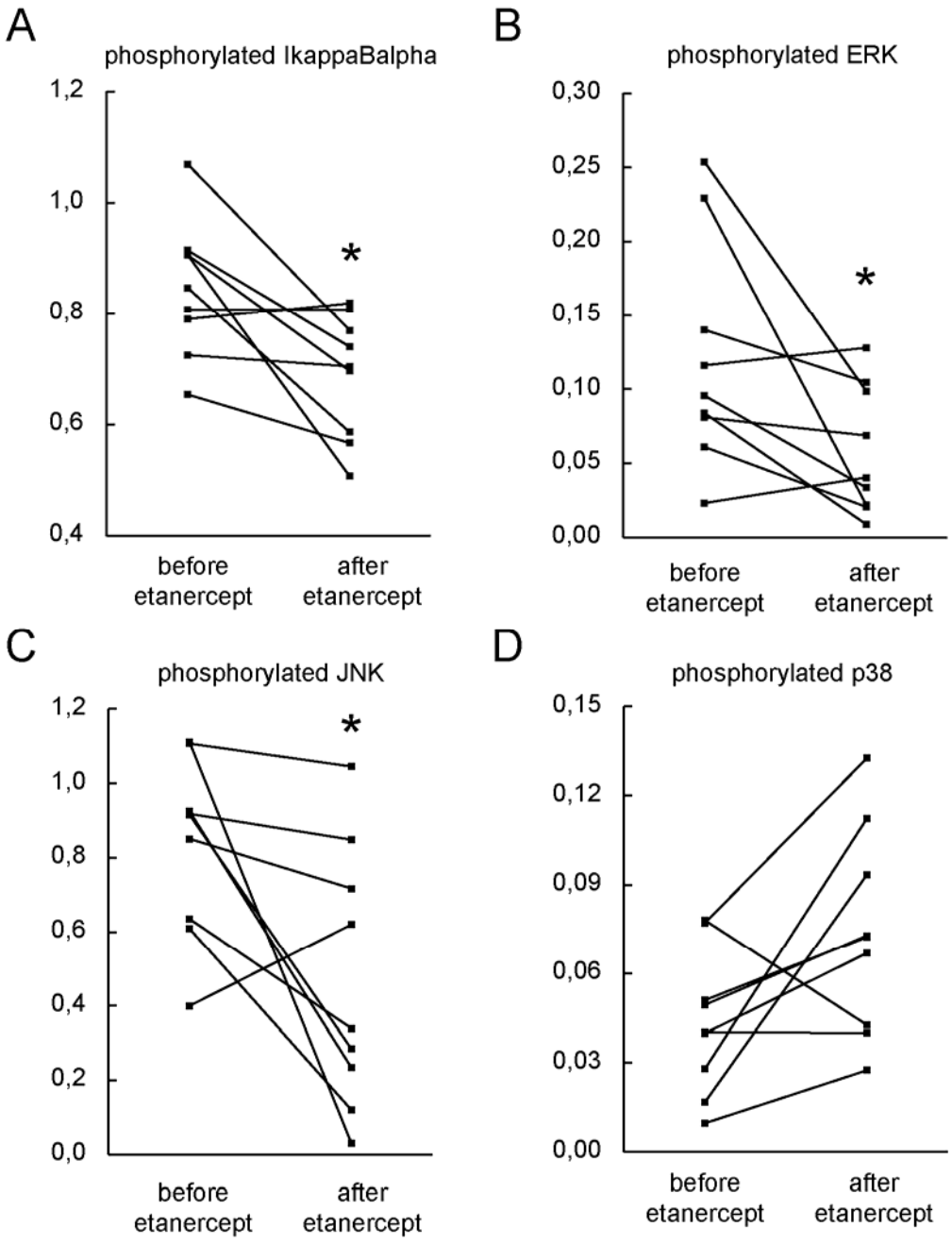


Figure 3


A
phosphorylated IkappaBalpha
1,2
1,0
0,8
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before etanercept
after etanercept
B
phosphorylated ERK
0,30
0,25
0,20
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before etanercept
after etanercept
C
phosphorylated JNK
1,2
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0,8
0,6
0,4
0,2
0,0
*
before etanercept
after etanercept
D
phosphorylated p38
0,15
0,12
0,09
0,06
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before etanercept
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