Non-canonical NF-κB signaling in rheumatoid arthritis and beyond

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NF-κB-inducing kinase (NIK) is expressed in synovial endothelial cells in early arthritis patients and correlates with markers of inflammation: a prospective cohort study

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ABSTRACT

Objectives: The NF-κB family of transcription factors is strongly involved in synovial inflammation. We have previously shown that NF-κB-inducing kinase (NIK) is a key regulator of inflammation-induced angiogenesis in rheumatoid arthritis (RA) synovial tissue (ST). Here, we investigated synovial NIK expression in early arthritis patients and in autoantibody-positive individuals at risk for developing RA.

Methods: ST biopsies were obtained via arthroscopy from 154 early arthritis patients (arthritis duration<1 year) with various diagnoses and 54 IgM-RF and/or ACPA-positive individuals, without evidence of arthritis. ST was stained for NIK and endothelial cell (EC) markers. Additionally, measures of disease activity were collected and contrast-enhanced MRI was performed in a subset of these patients.

Results: In early arthritis patients, NIK was predominantly expressed in EC of small blood vessels. Furthermore, NIK expression correlated with ESR (r=0.184; p=0.024), CRP (r=0.194; p=0.017), joint swelling (r=0.297; p<0.001), synovial immune cell markers (lining r=0.585; p<0.001 and sublining macrophages r=0.728; p<0.001, T cells r=0.733; p<0.001 and B cells r=0.264; p=0.040), MRI effusion (r=0.665; p<0.001), MRI synovitis (r=0.632; p<0.001) and MRI total score (r=0.569; p<0.001). In 18.5% of autoantibody-positive individuals ST NIK+EC were present, but this was not predictive of the development of arthritis.

Conclusions: NIK+EC are present in the earliest phase of synovial inflammation and may be indicative of high angiogenic activity in the inflamed ST. Therefore, NIK+EC may play an important role in the persistence of synovitis. Collectively, our data underscore the importance of angiogenesis in synovial inflammation and identify NIK as a potential therapeutic target in arthritis.

Key words
NF-κB, NIK, RA, synovium, early arthritis
INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease characterized by synovial inflammation, which may lead to joint destruction. In RA synovial tissue, angiogenesis can be observed already in the earliest phase of the disease. Angiogenesis is defined as the formation of new blood vessels from the pre-existing vasculature. The number of blood vessels is already significantly increased in patients with early disease, and the vasculature is clearly activated as shown by increased expression of adhesion molecules. Also, dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) in early arthritis patients demonstrate increased vascularity and suggest that angiogenesis plays a key role in the pathogenesis of RA. Interestingly, anti-TNF antibody therapy and other anti-rheumatic therapies result in deactivation of vascular endothelium, providing indirect experimental evidence that targeting vascular endothelium may lead to decreased cell trafficking towards the synovial compartment. This notion is supported by experimental studies in animal models: angiogenesis is involved in the switch from acute to chronic synovial inflammation and specific targeting of neovasculature results in reduced synovial inflammation. Taken together, angiogenesis is considered an important factor in the pathogenesis of RA and may be a good target.

The nuclear factor-κB (NF-κB) family of transcription factors is crucially important in the development and perpetuation of (synovial) inflammation. NF-κB can be activated via two signal transduction pathways that have distinct roles. The canonical NF-κB pathway is activated in response to pro-inflammatory stimuli. In this pathway, inhibitor of κB (IκB) kinase (IKK)β is essential for NF-κB activation, whereas inhibitor of κB kinase α (IKKα) is dispensable. In contrast, the non-canonical pathway is strictly dependent on NF-κB-inducing kinase (NIK) and IKKα homodimers and can be activated via triggering of TNF-receptor superfamily members such as the lymphotoxin β receptor (LTβR), B-cell activating factor belonging to the TNF family (BAFF)-receptor and CD40. Activation of the non-canonical pathway results in stabilization of NIK, the most important activating kinase of this pathway, which results in the activation of IKKα, followed by nuclear translocation of mainly p52-RelB dimers that target specific genes. In RA ST, the non-canonical NF-κB stimuli LTβ and LIGHT (homologous to Lymphotoxins, exhibits inducible expression, and competes with HSV Glycoprotein D for herpes virus entry mediator (HVEM), a receptor expressed by T-lymphocytes), both ligands of the LTβR, and CD40L are widely expressed, mainly by B cells and T cells. We have recently demonstrated that non-canonical NF-κB signaling in endothelial cells (EC) regulates pathological angiogenesis in (pre)clinical models of arthritis, independent of VEGF.

Currently, little is known about the contribution of non-canonical NF-κB signaling to the onset and perpetuation of RA. The function of NIK and downstream non-canonical NF-κB signaling is to a large extent cell type-specific: in synovial fibroblasts, osteoclasts, EC, and B cells non-canonical NF-κB signaling contributes to the inflammatory process, whereas in macrophages, dendritic cells and T cells this pathway probably has a regulatory role. Detailed knowledge on the role of non-canonical NF-κB signaling in angiogenesis and its contribution to chronic inflammation may provide more insight into the pathogenesis of RA.
NIK+ endothelial cells in early arthritis patients

and could lead to the development of novel targeted therapeutic interventions. Therefore, we investigated synovial NIK+EC in the earliest phases of RA compared to other forms of arthritis in a prospective cohort of DMARD naïve patients with early arthritis, as well as in a cohort of autoantibody-positive individuals at risk of developing RA.

METHODS

Study subjects
First, 154 early arthritis patients who were included in the prospective early arthritis cohort ‘Synoviomics’ at the Academic Medical Center (AMC) were enrolled 18. At inclusion, all patients had less than 1 year disease duration, as measured from the first clinical evidence of joint swelling. All patients had an active arthritis of at least a wrist, ankle or knee joint and were DMARD naïve. These patients are collectively referred to as “early arthritis patients”. The second group consisted of 54 individuals from the prospective observational cohort ‘PreSynoviomics’ at the AMC. This cohort included individuals with either arthralgia and/or a positive family history for RA, but without (a history of) arthritis (as determined by an experienced rheumatologist), who were positive for IgM rheumatoid factor (IgM-RF) and/or anti-citrullinated peptide antibodies (ACPA). These individuals can be classified as phase c (systemic autoimmunity associated with RA) with/without phase a (genetic risk factors for RA) and/or phase d (symptoms without clinical arthritis) according to the EULAR Study Group for Risk Factors for Rheumatoid Arthritis 19, and are collectively referred to as “autoantibody-positive individuals”. Both studies were approved by the Medical Ethics Committee of the AMC and performed according to the Declaration of Helsinki. All patients gave written informed consent.

Study design
At baseline, demographics were collected and the following clinical and laboratory parameters were obtained: patient’s visual analogue scale ((VAS) range 0-100 mm) for pain in the biopsied joint; Disease Activity Score in 28 joints (DAS(28)); the severity of swelling of the biopsied joint as assessed by the investigator (range of 0 (no swelling) to 3 (severe swelling)) 19; IgM-RF levels using IgM-RF ELISA (Sanquin, Amsterdam, the Netherlands (upper limit of normal (ULN) 12.5 IU/ml)) until December 2009 and thereafter using IgM-RF ELISA (Hycor Biomedical, Indianapolis, IN (ULN 49 IU/ml)); ACPA using anti-citrullinated cyclic peptide (CCP)2 ELISA CCPlus (Eurodiagnostica, Nijmegen, the Netherlands (ULN 25 kAU/l)); erythrocyte sedimentation rate (ESR); serum levels of C-reactive protein (CRP); and radiographs of hands and feet.

For the early arthritis patients, a diagnosis was made at baseline and after follow-up patients were re-classified based on the 2 year clinical diagnosis (cumulative), based on fulfillment of standard classification criteria for established rheumatic diseases or the absence of these criteria (unclassified arthritis/UA, phase e according to the EULAR Study Group for Risk Factors for RA) 19. Furthermore, UA patients at baseline were categorised
as 'UA-RA' if they converted to RA during this follow-up, or as 'UA-UA' if their arthritis remained unclassified. All patients diagnosed with RA at baseline were categorized as 'RA-RA'. Finally, after 2 years of follow-up, early arthritis patients fulfilling the 2010 ACR/EULAR criteria for RA were classified for arthritis outcome: self-limiting disease, defined as no arthritis on examination and no use of DMARDs or steroids in the preceding three months, persistent non-erosive disease, defined as presence of arthritis in at least 1 joint and/or requirement of DMARDs or steroids in the preceding three months, or erosive disease, defined as presence of joint erosions on radiographs of the hands and/or feet 20.

In the autoantibody-positive individuals, yearly study visits were performed and, for individuals who developed arthritis, an additional visit was performed at which the presence of arthritis was independently assessed by two investigators.

**Synovial tissue biopsy sampling, immunohistochemical/immunofluorescence stainings and quantification**

At baseline, all study subjects underwent arthroscopic ST biopsy sampling as previously described 21-23. In early arthritis patients ST biopsy sampling was performed in either inflamed wrist, ankle, knee or other (metacarpophalangeal or metatarsalphalangeal) joints. Autoantibody-positive individuals underwent ST biopsy sampling from a knee joint 23. ST of all patients was stained using an anti-NIK monoclonal mouse antibody (sc-8417, Santa Cruz Biotechnology, Santa Cruz, CA) and/or a monoclonal anti-von Willebrand factor antibody (vWF; F8/86; DAKO) as described previously 24.

In a random subset of the early arthritis patients (based on availability) sections were stained for CD68 to detect macrophages (n=51), CD3 to detect T cells (n=51), and CD22 to detect B cells (n=61), and analyzed by SQA, as described before 21. In 10 randomly selected early arthritis patients from this subset we performed double-immunofluorescence stainings on NIK (sc-8417, Santa Cruz Biotechnology) with vWF (0082, DAKO). See supplementary methods for staining protocols and quantification.

**Magnetic Resonance Imaging**

At baseline, prior to the arthroscopy, a contrast-enhanced MRI (CE-MRI) of the knee was performed in a subset of both early arthritis patients (n=36) and autoantibody-positive individuals (n=40) 4. Subsets were selected based on presentation with a knee arthritis (for early arthritis patients only), the absence of contraindications for CE-MRI, such as claustrophobia, metal implants or elevated serum creatinine and was only performed during a specific timespan in these studies. Images were acquired on a 1.5 T MRI scanner (GE Signa Horizon Echospeed, LX9.0, General Electric Medical Systems, Milwaukee, WI), as described previously 4. The MRI was scored for effusion in 4 compartments (Lateral, Medial, Central and Suprapatellar), a Bakers cyst was scored as an extra compartment. Synovitis was scored in 4 compartments. For bydrops and synovitis, a score of 0 (normal) to 3 (large volume) for each compartment was given. Edema, cartilage defects and erosions were scored as being present (1) or absent (0) in 6 joint compartments (lateral/medial
femoral surface, lateral/medial tibial plateau, patellar surface and trochlea. A total MRI score was calculated (0–45). Scoring was done by two musculoskeletal radiologists (CvdL and MM), who were blinded to the patients’ diagnoses.

Statistical analysis
Categorical data were depicted as number (%) and differences between study groups analyzed using Chi-square test. Not normally distributed variables were depicted as median (interquartile range, IQR). To compare baseline characteristics and NIK expression between the different classification groups, the Kruskal-Wallis test was used when more than 2 groups were compared; subsequently the Mann-Whitney U test was used to compare differences between two subgroups. Bivariate correlations of not normally distributed variables were analyzed using Spearman’s rank correlation test. All statistical analyses were performed using SPSS v19.0 software (IBM Corp., Armonk, NY). A P-value of <0.05 was considered statistically significant.

RESULTS
Early arthritis patients
Baseline characteristics of the early arthritis patients are shown in Table 1. Of the 154 included early arthritis patients, 64 were classified as RA at baseline, 61 as UA, 11 as crystal arthropathy (CA), 4 as inflammatory osteoarthritis (OA) and 14 as spondyloarthritis (SpA).

Table 1. Baseline characteristics of early arthritis patients.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Early Arthritis patients</th>
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<tbody>
<tr>
<td>Sex, female (n (%))</td>
<td>90 (58)</td>
</tr>
<tr>
<td>Age, years (mean (SD))</td>
<td>49 (14)</td>
</tr>
<tr>
<td>IgM-RF positive (n (%))*</td>
<td>39 (25)</td>
</tr>
<tr>
<td>Anti-CCP positive (n (%)) **</td>
<td>35 (23)</td>
</tr>
<tr>
<td>IgM-RF and anti-CCP both positive (n (%))</td>
<td>28 (18)</td>
</tr>
<tr>
<td>ESR, mm/hr (median (IQR))</td>
<td>25 (11-43)</td>
</tr>
<tr>
<td>CRP, mg/L (median (IQR))</td>
<td>9.3 (3.0-28.3)</td>
</tr>
<tr>
<td>VAS pain biopsied joint, mm (median (IQR))</td>
<td>59 (27-81)</td>
</tr>
<tr>
<td>Swelling biopsied joint, score 0-3 (median (IQR))</td>
<td>2 (1-2)</td>
</tr>
<tr>
<td>DAS28 (median (IQR))</td>
<td>4.4 (3.2-5.4)</td>
</tr>
<tr>
<td>Dis. duration months (median (IQR))</td>
<td>3 (1-8)</td>
</tr>
</tbody>
</table>

Parameters are described as number (n (%)), mean (standard deviation) or median (interquartile range) as appropriate. IgM-RF = immunoglobulin M rheumatoid factor; anti-CCP = anti-cyclic citrullinated peptide; ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; VAS = visual analogue scale (range 0-100 mm); DAS28 = disease activity score in 28 joints; Dis. duration = disease duration in months.* missing for 2 patients ** missing for 3 patients
Overall median (IQR) disease duration at baseline was 3 (1-8) months. Of the 61 patients who were initially classified as UA, 18 fulfilled the 2010 ACR/EULAR criteria after 2 years of follow-up (UA-RA), 31 remained unclassified after follow-up (UA-UA) and 12 were lost to follow-up and therefore excluded from the diagnostic outcome analysis. Of the 82 patients fulfilling the RA criteria after 2 years of follow-up, 12 had self-limiting disease, 38 had persistent non-erosive disease, 11 had erosive disease. For 21 patients the arthritis outcome data were not available and therefore these patients were excluded from these analyses.

NIK is expressed in synovial blood vessel endothelial cells in early arthritis patients

In this exploratory study we found that NIK was almost exclusively expressed in EC of synovial blood vessels of early arthritis patients (Figure 1A, B). This is substantiated by the significant positive correlation between NIK and vWF in these patients ($r=0.628; p<0.001$) (Figure 1C). This is in line with previous results, showing that approximately 70% of ECs in established RA ST were NIK-positive. NIK was expressed in all different diagnostic groups (RA, UA, CA, OA, SpA) and no significant difference was observed in the number of NIK/EC between these different diagnoses at baseline (Figure 2A). When patients were re-classified based on the 2 year clinical diagnosis, the number of NIK/EC at baseline was significantly higher in UA patients compared to RA patients ($p=0.003$) (Figure 2B). In UA patients that were re-classified as RA after 2 years of follow-up the number of NIK/EC at baseline was in the same range as RA patients that fulfilled the 2010 ACR/EULAR criteria for RA already at baseline (Figure 2C).

Interestingly, NIK was significantly differentially expressed in the various biopsied joints ($p<0.001$). NIK expression (median (IQR)) was highest in the knee joint (154.9 (24.6-444.9); n=100), lowest in the wrist joint (0.0 (0.0-18.7); n=19) and intermediate in the ankle joint (4.7 (0.0-56.0); n=33) and in the other joints (32.4 (30.2-34.5); n=2) (Supplementary table 1).

Baseline expression of NIK is not related to arthritis outcome

We next investigated synovial NIK expression in relationship to arthritis outcome (self-limiting, persistent non-erosive or erosive disease) in patients fulfilling the 2010 ACR/EULAR criteria for RA after 2 years of follow-up. However, NIK expression (median (IQR)) did not differ between the different arthritis outcome groups (self-limiting: 14.7 (1.0-69.9), persistent non-erosive disease: 11.6 (0.0-203.9), and erosive disease: 11.9 (0.0-59.3); $p=0.850$).

NIK expression correlates with markers of disease activity in early arthritis patients

NIK expression correlated positively with systemic markers of disease activity, such as ESR ($r=0.184; p=0.024$) and CRP ($r=0.194; p=0.017$), and with local assessment of swelling of the biopsied joint ($r=0.297; p<0.001$) (Figure 3A), but not with the VAS pain for the biopsied joint and DAS28 (data not shown).
NIK expression also correlated positively with cellular markers of inflammation in the ST; NIK correlated highly significantly with CD68+ lining and sublining macrophages (r=0.585; p<0.001, and r=0.728; p<0.001, respectively), CD3+ T cells (r=0.733; p<0.001) and B cells (r=0.264; p=0.040) (Supplementary table 1).

Figure 1. Baseline synovial NF-κB-inducing kinase (NIK) expression in early arthritis patients. In A, representative immunohistochemical staining of a NIK positive early arthritis patient (left) and of a NIK negative early arthritis patient (right). Magnification 400x, scale bar 50µm. In B, immunofluorescence staining of NIK (red) with vWF (green) and nuclei (blue) in a NIK positive early arthritis patient. Magnification 250x, scale bar 100µm. In C, the correlation between NIK expression and vWF (n=52) (left) as analyzed using Spearman’s rank correlation test and a representative immunohistochemical staining of a vWF positive early arthritis patient (right), scale bar 200µm.
NIK expression also correlated significantly with MRI effusion ($r=0.665; p<0.001$), MRI synovitis ($r=0.632; p<0.001$) and MRI total score ($r=0.569; p<0.001$) in a subset of the early arthritis patients ($n=36$) (Figure 3B-D). NIK expression did not significantly correlate with MRI edema ($r=-0.012; p=0.946$), MRI cartilage damage ($r=-0.032; p=0.855$), and MRI erosion scores ($r=-0.026; p=0.882$).

Figure 2. Synovial tissue (ST) NIK expression in relation to different classification groups. A, NIK ST expression of early arthritis patients diagnosed at baseline with rheumatoid arthritis (RA; $n=64$), inflammatory osteoarthritis (OA; $n=4$), crystal arthropathy (CA; $n=11$), unclassified arthritis (UA; $n=61$) and spondyloarthritis (SpA; $n=14$). B, NIK ST expression after re-classifying patients based on the 2 year clinical diagnosis with RA ($n=82$), OA ($n=4$), CA ($n=11$), UA ($n=31$) and SpA ($n=14$). C, UA patients at baseline were categorized as 'UA-RA' ($n=18$) if they converted to RA during follow-up, or as 'UA-UA' ($n=31$) if their arthritis remained unclassified. RA patients at baseline were categorized as 'RA-RA' ($n=64$). The Kruskal-Wallis test was used and data are presented as box plots (25th to 75th percentiles), lines within the box mark the median value, lines outside the boxes denote the 10th and 90th percentiles. Lines connecting data sets indicate statistically significant differences between groups. 12 UA patients were lost to follow-up after 2 years. *$p<0.01$. 

NIK expression also correlated significantly with MRI effusion ($r=0.665; p<0.001$), MRI synovitis ($r=0.632; p<0.001$) and MRI total score ($r=0.569; p<0.001$) in a subset of the early arthritis patients ($n=36$) (Figure 3B-D). NIK expression did not significantly correlate with MRI edema ($r=-0.012; p=0.946$), MRI cartilage damage ($r=-0.032; p=0.855$), and MRI erosion scores ($r=-0.026; p=0.882$).
Figure 3. Correlations between baseline synovial NIK expression and local markers for disease activity in early arthritis patients. In A, comparison of NIK expression and the amount of swelling of the biopsied joint as assessed by the doctor (score of 0 (no swelling) – 3 (severe swelling))(n=145). The MRI (n=36) was scored for effusion in 5 compartments, for synovitis in 4 compartments and for edema, cartilage degeneration and erosions in 6 compartments. A total MRI score was calculated (0-45). In B, the correlation between NIK expression and MRI effusion. In C, the correlation between NIK expression and MRI synovitis. In D, the correlation between NIK expression and total MRI score. Spearman’s rank correlation test was used to analyze all correlations.

Subjects at risk of developing RA

Baseline characteristics of autoantibody-positive individuals are depicted in Table 2. Twenty individuals were solely IgM-RF positive, 22 were solely ACPA-positive and 12 were positive for both autoantibodies. Thirteen of the 54 individuals (24%) developed arthritis during follow-up after a median duration of 18 (IQR 6-40) months. Of the 13 patients who developed arthritis, 9 fulfilled the 2010 ACR/EULAR criteria for RA at arthritis onset and 3 were initially diagnosed as having UA but fulfilled the RA classification criteria later on. One patient fulfilled the ACR classification criteria for osteoarthritis of the hand, but not for RA.
The median follow-up time of the 41 (76%) individuals who did not develop an arthritis was 42 (IQR 18-59) months. In the individuals that developed arthritis after follow-up, significantly more individuals were both IgM-RF and ACPA positive.

**NIK is expressed in synovial EC in certain autoantibody-positive individuals, but this is not predictive of the development of arthritis**

In autoantibody-positive individuals NIK expression was very low in general and NIK+EC were only present in 10 out of the 54 autoantibody-positive individuals (Figure 4 and Supplementary table 2).

Synovial NIK expression in the 13 patients who developed arthritis was in the same range as in the autoantibody-positive individuals, who did not develop arthritis during follow-up (Supplementary table 2). Therefore, the presence of NIK+EC is not predictive of the development of arthritis in autoantibody-positive individuals.

**NIK expression is not associated with markers of inflammation in autoantibody-positive individuals**

The autoantibody-positive individuals had normal inflammatory parameters (median (IQR)), such as ESR (8 (2-15) and CRP (2.1 (1.0-7.5). Synovial NIK expression was not associated with these systemic markers of inflammation (ESR (p=0.544) and CRP (p=0.227)), or with the VAS pain of the biopsied joint (p=0.526). Previously, we have demonstrated that the ST in these autoantibody-positive individuals contains very low numbers of immune cells, comparable to healthy controls 27. In line with this, we did not observe a correlation between NIK+EC and CD68+ macrophages, CD3+ T cells, or B cells in these tissues (data not shown). Furthermore, the presence of NIK+EC also did not correlate with the MRI data (MRI effusion (p=0.881), MRI synovitis (p=0.475), MRI cartilage damage (p=0.293), MRI edema (p=0.415) and MRI erosion scores (p=0.804)) in autoantibody-positive individuals (data not shown).

![Figure 4. Baseline synovial NIK expression in auto-antibody positive individuals.](image-url)
DISCUSSION

We demonstrated that NIK is highly expressed in synovial blood vessels of patients with various forms of early arthritis. Additionally, we showed that synovial NIK expression is associated both with systemic and local markers of disease activity in early arthritis patients. Interestingly, NIK+EC could also be found in some individuals at risk of developing RA. Taken together, we demonstrate that NIK is expressed in synovial blood vessels already in the earliest phases of inflammatory joint disease.

NIK was not differentially expressed between the different diagnoses at baseline. Nevertheless, re-classification of patients based on the 2 year clinical diagnosis showed that ST from UA patients contained significantly more NIK+EC at baseline compared to the other types of arthritis. Although this could be an intrinsic feature of UA, another perhaps more likely explanation for this is the fact that the mean swelling of the biopsied joint was also higher in this group (data not shown). Also, the variation in NIK expression was high, which makes it unsuitable in clinical practice as a prognostic marker for individual UA patients. Therefore, we do not advocate the routine use of synovial biopsy to establish the final diagnosis in UA patients, except in specific cases to rule out infectious causes, crystal induced arthritis or neoplasms, or in early drug development studies 28.

We also observed differential NIK expression in the various joint types. Although this could be a specific characteristic of the individual joints, a more likely explanation is again the increased mean swelling and cellular inflammation scores in the joints that contained the highest NIK expression irrespective of the joint type (Supplementary table 1). Nevertheless, we cannot completely rule out that joint specific features, such as positional gene expression patterns, can affect synovial NIK expression or predispose joints to development of arthritis 29. The presence of NIK+EC may be indicative of high angiogenic activity in the inflamed ST, which is in line with previous work from our group and others demonstrating that NIK is a key regulator of pathological angiogenesis and is requisite for pathology in animal models of arthritis 16,30. In light of the importance of angiogenesis in the progression from acute to chronic inflammation and the fact that NIK also regulates endothelial expression of CXCL12, an important chemokine in the attraction of immune cells, NIK+EC may contribute to the persistence of synovial inflammation 7,31.

Of interest, we also showed that NIK+EC are present in ST in 18.5% of autoantibody-positive individuals. However, NIK expression was very low compared to the expression in early arthritis patients and did not correlate with cellular or other local and systemic markers for (subclinical) disease activity. Also, the presence of NIK+EC at baseline did not predict development of arthritis in this relatively small cohort. Previously, we have demonstrated that features of the synovium are similar between autoantibody-positive individuals and healthy controls, all showing very low scores for phenotypic cellular markers, adhesion molecules and vascularity 27. This may explain the lack of an association between synovial NIK+EC and (subclinical) inflammation or development of arthritis. Nonetheless, NIK+EC are sometimes present already before the onset of clinical arthritis and therefore targeting these cells in the earliest phases of the disease may be beneficial.
With respect to arthritis outcome (self-limiting, persistent non-erosive or erosive disease) we did not find a correlation with synovial NIK expression. This may seem surprising given the important role of NIK and the downstream NF-κB subunit RelB in osteoclast biology and the bone-destructive components of inflammatory arthritis. In a human study, genetic association analysis showed that single nucleotide polymorphisms in MAP3K14, the gene encoding for NIK, affect bone mineral density and bone turnover. However, we did not study the synovial tissue-bone interphase (pannus), but regular ST biopsies in which NIK was predominantly expressed in EC. Therefore, we cannot exclude the possibility that any potential expression of NIK in osteoclasts in pannus tissue may be predictive of development of erosive disease, but this was outside the scope of the present study.

Our study had some limitations; there was a limited number of UA patients that progressed to RA (n=18), and multiple subsets were used for various analysis, which was largely based on the availability of ST sections. Nevertheless, we provide clear evidence that NIK+ EC correlate positively with markers of local inflammation, such as ST B and T cells and MRI scores. Given the important role of angiogenesis in the perpetuation of arthritis, targeting this process has been proven to be beneficial in (pre-)clinical disease models. Importantly, targeting angiogenesis will probably not result in the severe immune suppression that is induced by treatment with biologics that target cytokines or specific immune cells, and may cause infectious complications. We have recently established that NIK is only involved in pathological angiogenesis in a preclinical model of arthritis and not in normal developmental angiogenesis. Therefore, selective targeting of NIK in EC may have several advantages over targeting other well-known pro-angiogenic pathways such as VEGF, which also blocks physiological angiogenesis and can have adverse effects like hypertension and thrombo-embolic events.

Collectively, our combined data identify NIK as a potential therapeutic target in arthritis. To target NIK selectively in EC a multi-modular recombinant protein that specifically binds to cytokine-activated endothelium under inflammatory conditions, including arthritis, could be employed. Since we demonstrate that NIK+EC can already be observed in the earliest phase of the disease, targeting NIK in patients with early arthritis may block pathological angiogenesis and prevent the switch from acute to chronic inflammation. This could be done via local intra-articular treatment (e.g. via gene therapy) or using small molecule inhibitors. Recently, the crystal structure of the catalytic domain NIK has been identified, which may facilitate the development of new potent NIK inhibitors. This may lead to new treatment strategies that could be beneficial in RA, and in other types of arthritis and other inflammatory diseases.

ACKNOWLEDGEMENTS

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SUPPLEMENTARY METHODS

Synovial tissue biopsy sampling, immunohistochemical/immunofluorescence stainings and quantification

At baseline, all study subjects underwent arthroscopic synovial tissue (ST) biopsy sampling as previously described 1-3. In early arthritis patients ST biopsy sampling was performed in an inflamed wrist, ankle, knee or other (metacarpophalangeal or metatarsalphalangeal) joints. Autoantibody-positive individuals underwent ST biopsy sampling from a knee joint 3. No major complications of the arthroscopy were reported. At least six specimens were collected for immunohistochemistry, as described before 4, to correct for sampling error. The ST biopsy samples were snap-frozen en bloc in Tissue-Tek OCT (Sakura Finetek Europe B.V., Alphen aan de Rijn, the Netherlands) immediately after collection. Cryostat sections were cut (5 μm each) and mounted on Star Frost adhesive glass slides (Knittelglass, Braunschweig, Germany). Sealed slides were stored at -80 °C until further use.

ST sections were stained in two sessions for the early arthritis patients and in one session for the autoantibody-positive individuals. The sections were fixed with acetone, and endogenous peroxidase activity was blocked by immersion in 0.3% hydrogen peroxide and 0.1% sodium azide in phosphate-buffered saline (PBS) for 20 minutes. Slides were incubated overnight at 4°C with primary antibody diluted in 1% bovine serum albumin/ PBS. The primary antibody used in this study was monoclonal mouse antibody specific for NIK (sc-8417, Santa Cruz Biotechnology, Santa Cruz, CA). Sections were washed with PBS and incubated with goat anti-mouse antibodies (p0447, DAKO, Glostrup, Denmark), followed by incubation with biotinylated tyramide and streptavidin-HRP. Biotinylated tyramide was used for amplification, as previously described 5, and development with the AEC peroxidase substrate kit (SK-4200, Vector Laboratories, Burlingame, CA). In a subset of the early arthritis patients (n=52), depending on the availability of the tissue, ST sections were stained in one session using a monoclonal anti-von Willebrand factor (vWF; F8/86; DAKO) antibody for blood vessels 6. A three-step immunoperoxidase protocol was used to detect specific staining for vWF, as described previously 7. Slides were counterstained with Mayer's hematoxylin (Merck, Darmstadt, Germany) and mounted in Kaisers glycerol gelatin (Merck). As a negative control, isotype-matched immunoglobulins were applied to the sections instead of the primary antibody.

ST was only further used for analysis if the quality of the tissue sections were sufficient according to the strict quality control system based on the presence of an intimal lining layer. In the early arthritis cohort the expression of synovial NIK and vWF was quantified by digital image analysis within one week after staining, as previously described 8. For each slide 18 representative high power fields (2.2 mm²) were analyzed. To correct for between-session variation, the factor correction program was used 9. In the autoantibody-positive individuals the expression of synovial NIK was much lower and therefore analyzed by semi-quantitative analysis (SQA) by two independent observers (KIM and KvZ), as previously
described\textsuperscript{10}. Minor differences in assessment between the two observers were resolved by mutual agreement. The expression of synovial NIK was scored as either positive or negative.

In a random subset of the early arthritis patients, sections were stained for CD68 to detect macrophages (n=51), CD3 to detect T cells (n=51), and CD22 to detect B cells (n=61), and analyzed by SQA, as described before\textsuperscript{11}.

In 10 randomly selected early arthritis patients from the previously mentioned subset we performed double-immunofluorescence stainings on NIK and vWF using the same mouse monoclonal anti-NIK antibody (sc-8417, Santa Cruz Biotechnology) and a polyclonal rabbit anti-vWF antibody (0082, DAKO). After incubation with goat anti-mouse-HRP (p0447, DAKO), the slides were incubated with streptavidine-Alexa-594 (S-32356, Molecular Probes Europe, Leiden, the Netherlands) and Alexa-488-conjugated goat anti-rabbit (A-11008, Molecular Probes Europe). The slides were mounted with Vectashield containing DAPI (Brunschnig VC-H-1500, Amsterdam, the Netherlands). As a negative control, sections were incubated with isotype controls. The slides were analyzed using a Leica DMRA fluorescence microscope (Leica, Wetzlar, Germany) coupled to a CCD camera and Image-Pro Plus software (Dutch Vision Components, Breda, the Netherlands).
REFERENCES


Supplementary table 1. Baseline characteristics of auto-antibody positive individuals.

<table>
<thead>
<tr>
<th>Joint</th>
<th>NIK expression median (IQR)</th>
<th>Swelling biopsied joint median (IQR)</th>
<th>CD68 lining median (IQR)</th>
<th>CD68 sublining median (IQR)</th>
<th>CD3+ T cells median (IQR)</th>
<th>CD22+ B cells median (IQR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Knee</td>
<td>154.9 (24.6-444.9)</td>
<td>2 (1-2)</td>
<td>464.1 (174.5-652.2)</td>
<td>1595.5 (526.7-1789.3)</td>
<td>392.9 (188.9-989.8)</td>
<td>305.5 (118.6-812.4)</td>
</tr>
<tr>
<td>Ankle</td>
<td>4.7 (0.0-56.0)</td>
<td>1 (1-2)</td>
<td>56.8 (12.2-226.7)</td>
<td>126.9 (38.0-396.4)</td>
<td>88.5 (33.7-273.2)</td>
<td>252.9 (25.6-607.9)</td>
</tr>
<tr>
<td>Wrist</td>
<td>0.0 (0.0-18.7)</td>
<td>1 (1-1)</td>
<td>50.0 (13.7-132.2)</td>
<td>30.1 (8.7-135.0)</td>
<td>41.1 (6.7-109.4)</td>
<td>190.1 (87.0-301.8)</td>
</tr>
<tr>
<td>Other</td>
<td>32.4 (30.2-32.4)</td>
<td>3 (2-3)</td>
<td>179.0 (108.4-179.0)</td>
<td>432.9 (295.1-432.9)</td>
<td>165.8 (79.0-165.8)</td>
<td>374.8 (34.9-374.8)</td>
</tr>
</tbody>
</table>

Parameters are described as number (n (%)) or median (interquartile range) as appropriate. IgM-RF = immunoglobulin M rheumatoid factor; anti-CCP = anti-cyclic citrullinated peptide; ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; VAS = visual analogue scale (range 0-100 mm. * missing for 8 individuals * missing for 4 individuals

Supplementary table 2. Development of arthritis in autoantibody individuals in relation to baseline NIK expression.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Autoantibody-positive individuals</th>
<th>Autoantibody-positive individuals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No arthritis developed N= 41</td>
<td>Arthritis developed N= 13</td>
</tr>
<tr>
<td>NIK negative individuals</td>
<td>33 (80,5)</td>
<td>11 (84,6)</td>
</tr>
<tr>
<td>NIK positive individuals</td>
<td>8 (19,5)</td>
<td>2 (15,4)</td>
</tr>
</tbody>
</table>

Parameters are described as number (n (%)). For the frequencies of NIK negative individuals and NIK positive individuals in the two outcome groups, Pearson χ² test was used (P=0.739).