ONLINE SUPPLEMENTARY DATA

MATERIALS AND 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 ^{o}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 decribed 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 mm^2) 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 (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 (11).

In 10 randomly selected early arthritis patients from the previously mentioned subset we performed double-immunofluorecence 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 (Brunschwig 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).

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SUPPLEMENTARY TABLE 1. NIK expression, mean swelling and cellular inflammation scores in the different joints. Values are median (interquartile range).

Joint	NIK Expression	Swelling Biopsied Joint	CD68 Lining	CD68 Sublining	CD3+ T Cells	CD22+ B Cells
Knee, n = 100	154.9 (24.6-444.9)	2 (1-2)	464.1 (174.5-652.2)	1595.5 (526.7-1789.3)	392.9 (188.9-989.8)	305.5 (118.6-812.4)
Ankle, n = 33	4.7 (0.0-56.0)	1 (1-2)	56.8 (12.2-226.7)	126.9 (38.0-396.4)	88.5 (33.7-273.2)	252.9 (25.6-607.9)
Wrist, n = 19	0.0 (0.0-18.7)	1 (1-1)	50.0 (13.7-132.2)	30.1 (8.7-135.0)	41.1 (6.7-109.4)	190.1 (87.0-301.8)
Other, n = 2	32.4 (30.2-32.4)	3 (2-3)	179.0 (108.4-179.0)	432.9 (295.1-432.9)	165.8 (79.0-165.8)	374.8 (34.9-374.8)

SUPPLEMENTARY TABLE 2. Development of arthritis in autoantibody-positive individuals in relation to baseline synovial NIK expression. For the frequencies of NIK-negative individuals and NIK-positive individuals in the 2 outcome groups, Pearson chi-square test was used (p = 0.739). Values are n (%).

Characteristic	Autoantibody-positive Individuals		
	No Arthritis Developed, n = 41	Arthritis Developed, n = 13	Total
NIK-negative individuals	33 (80.5)	11 (84.6)	44 (81.5)
NIK-positive individuals	8 (19.5)	2 (15.4)	10 (18.5)