Supplementary data

A20^{myelKO} model

A20^{myelKO} mice were generated using the cre-lox system with M lysozyme (LysM) as a promotor to have a specific deletion of A20 in myeloid cells.[5] The mice were bred and housed in specific pathogen-free conditions in accordance with the general recommendations for animal breeding and housing. They were bred in individually ventilated cages and the experiments were conducted in conventional cages.

Culturing of bone marrow-derived macrophages (BMDMs)

BMDMs were cultured for 7 days in 9cm petri dishes (Thermo), using RPMI medium (Gibco BRL) containing 10% fetal calf serum, 10units/mL penicillin, 10mg/mL streptomycin, 2mM GlutaMAX and 40ng/mL macrophage colony-stimulating factor (M-CSF). On day 3 fresh medium was added to maintain a M-CSF concentration of 40ng/mL. The medium was refreshed on day 5. The cells were seeded in 6-well plates (Falcon) at a concentration of 700,000 cells/well on day 7.

Quantitative polymerase chain reaction (qPCR) analysis

Cells were lysed using RLT lysis buffer (Qiagen) and were kept at -80°C until analysis. RNA extraction and cDNA preparation were conducted using the RNeasy mini kit (Qiagen) and the QuantiTect Reverse Transcription Kit (Qiagen), according to the manufacturer's instructions. Each reaction was performed in duplicate. qPCR was performed using a LightCycler 480 system (Roche) and analyzed using qbase+ (Biogazelle). Respectively β-actin and Rp113a or HPRT and Rp113a were used as reference genes.

The following QuantiTect primer assays (Qiagen) were used: CXCL9 (QT00097062), CXCL10 (QT00093436), MX1 (QT01064231), STAT1 (QT01149519), SOCS3 (QT02488990), BCL2L1 (QT00149254) and STAT3 (QT00148750).

The following primers for β-actin, HPRT, Rp113a (Invitrogen) and VEGF (Biolegio) were used : β-actin Fw: ACCCGCGAGCACAGCTTCTTTG; β-actin Rev: GGCCTCGGGAGGGGTTGGTATT; HPRT Fw: TGCTTTCCGGAGCGGTAGCA; HPRT Rev: TCGGCATGACGGGACCGGT; Rp113a Fw: GTGGTCGTACGCTGTGAAGGCATC; Rp113a Rev: GGCCTCGGGAGGGGTTGGTATT; VEGF Fw: ACTCGGATGCCGACACGGGA; VEGF Rev: CCTGGCCTTGCTTGCTCCCC

Western blot analysis

The following primary antibodies were used for Western blot analysis: Phospho-Stat1 (Tyr701) (58D6) Rabbit mAb (Cell Signaling Technology), Stat1 (D1K9Y) Rabbit mAb (Cell Signaling Technology),

Phospho-Stat3 (Tyr705) (3E2) Mouse mAb (Cell Signaling Technology), Stat3 (124H6) Mouse mAb (Cell Signaling Technology) and Anti-Actin Mouse mAb (Bio-Connect)

The following secondary antibodies were used for Western blot analysis: Amersham ECL Mouse IgG, HRP-linked Whole Sheep Ab (GE Healthcare Life Sciences) and Amersham ECL Rabbit IgG, HRP-linked Whole Donkey Ab (GE Healthcare Life Sciences)

The primary antibodies for STAT1 and P-STAT1 can recognize both (P-)STAT1α and (P-)STAT1β isoforms.

Luciferase reporter assays

HEK293T cells were a gift from Dr. M. Hall (Department of Biochemistry, University of Birmingham, UK) as described by Verhelst et al.[16] These cells were seeded in 6-well plates at a concentration of 200,000cells/well and incubated at 37°C with 5% CO_2. After 24h of culture, cells were transfected using $Ca_3(PO4)_2$ co-precipitation with a total of 1µg DNA including a STAT1 or STAT3 luciferase (100ng) reporter plasmid, in the absence or presence of wild-type A20 (200ng) cDNA. A β-galactosidase (100ng) plasmid was transfected to correct for transfection efficiency. After 8h of incubation, the medium was refreshed and the cells were grown overnight. Cells were stimulated for 24h with IL-6 (50ng/mL)[12] or IFN-γ (10ng/mL)[13] or were left untreated. Cells were lysed in luciferase lysis buffer (25mM Tris phosphate pH 7,8; 2mM DTT; 2mM CDTA (1,2 diaminocyclohexane-N.N.N.N-tetraacetic acid); 10% glycerol; 1% Triton X-100) and luciferase activity was determined by adding 50µL of lysate to 30µL luciferase buffer (20mM Tricine; 1,07mM $(MgCO_3)_4Mg(OH)_2.H_2O$; 2,67mM $MgSO_4$; 33,3mM DTT; 0,1mM EDTA; 270µM CoA; 530µM ATP; 470µM Luciferin). Luciferase activity was measured with the GloMax 96 Microplate Luminometer (Promega) for 5 seconds per well. β-galactosidase activity was determined by means of CPRG (Sigma Aldrich) in CPRG substrate buffer (60mM Na_2HPO_4; 10mM KCl; 1mM β-mercaptoethanol).

pACTbgal (LMBP4341), which contains a β-galactosidase gene after the β-actin promoter, was from Dr. J. Inoue (Institute of Medical Sciences, Tokyo, Japan) as described by Verhelst et al.[16]

pCAGGSEhA20 (LMBP3778) was previously described by Verhelst et al.[16]

Ex vivo assessment of JAK inhibition

$A20^{myelKO}$ mice with marked clinical inflammation (total clinical score ≥ 1 and < 6) were included in the study. They were allocated to treatment based on stratified randomization with baseline clinical score and gender as covariates. Mice were treated twice a day with tofacitinib citrate (50 mg/kg body weight, dissolved in 0.5% methyl cellulose; 2% Tween80 in H_2O) or vehicle without active compound (n=4 per group). Mice were treated for 14 days, since previous experiments showed that there was already a marked effect of tofacitinib citrate after 14 days of treatment. On day 14 the mice were sacrificed and cells were isolated from the bone marrow and the spleen.

Single cell suspensions from the spleen were resuspended in PBS containing 1mM EDTA and 0.5% BSA and layered over Ficoll gradients. Bone marrow cells were flushed from the femur and tibia, treated with ACK lysis buffer and resuspended in PBS containing 1mM EDTA and 0.5% BSA. $CD11b^{+}$ cells were positively selected using mouse CD11b Microbeads (Miltenyi Biotech) and MS-MACS columns (Miltenyi Biotech).

Surface staining was performed in the dark for 30 minutes at 4°C in staining buffer containing 0.5µg FcR Block (Miltenyi Biotech). Cells were then washed twice with staining buffer and were sorted on FACS Aria II (BD Biosciences). The following antibodies were used: CD19-eFlour-450 (eBioID3), CD11c-PE-Cy7 (N418), NK1.1-PE (PK136), CD11b-APC (M1/70), CD4-V500 (RM4-5), CD8a-V500 (53-6.7), Ly6c-Alexa Flour-488 (HK 1.1) and Ly6G (Gr-1)-PE-Cyanine5.5 (RB6-8C5) (all from eBioscience). After exclusion of debris, doublets, nonviable cells and lineage markers (CD4, CD8, CD19, NK1.1, $CD11c^{Hi}$), monocytes and macrophages were sorted as a $CD11b^{+}$ $Ly6C^{Lo\text{-}neg}Ly6G^{-}$ SSC^{Lo} population.

Clinical scoring and histology:

$A20^{myelKO}$ mice were clinically scored three times a week to evaluate the degree of inflammation in the paws. Clinical scoring was performed by a blinded assessor. Each paw was scored individually and received a score ranging from 0 (normal) to 3, resulting in a maximum score of 12 for each mouse. Paws received a score of 1 if the ankle/wrist region was inflamed, they received an extra point if the metatarsal/metacarpal region was inflamed and another 0.5 or 1 point if respectively one or more fingers/toes were inflamed. In case of doubt between 2 scores, the mean value was given.

After sacrificing the $A20^{myelKO}$ mice, the hind paws were isolated and fixed in 4% formaldehyde for 24 hours. Ankle joints were decalcified in 5% formic acid for 7 days and embedded in paraffin. Paraffin sections of the ankle were stained with hematoxylin and eosin (H&E) to evaluate inflammation of the synovio-entheseal complex (SEC). Histology was scored by two blinded assessors; scores ranged from 0 (normal) to 3. A score of 1 was given for mild inflammation of the SEC, a score of 2 was given for moderate inflammation of the SEC and a score of 3 was given for severe inflammation of the SEC. In case of doubt between 2 scores, the mean value was given.

Statistical analysis

To compare the evolution of clinical scores between two groups, delta values were calculated by subtracting the clinical score at the end of the experiment from the score at day 0. A two-tailed Mann-Whitney U-Test was conducted on these delta values.

During the in vivo tofacitinib experiments 1 placebo-treated and 1 tofacitinib-treated mouse died; the clinical scores of these mice were excluded and no histology was performed on these mice. 1 $A20^{myelKO}$ mouse died during the time-dependent assessment; the clinical scores obtained before the mouse died were used in the incidence calculations.