Preclinical studies of spondyloarthritis

Development of two novel disease models and pharmacologic targeting of the IL-17 pathway

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TRANSMEMBRANE TUMOR NECROSIS FACTOR DRIVES
OSTEOPROLIFERATIVE JOINT INFLAMMATION REMINISCENT
OF HUMAN SPONDYLOARTHRITIS

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SUMMARY
The authors show that SpA synovitis comprises of less sTNF and more tmTNF expression compared to RA synovitis associated with less active ADAM17. Moreover, tmTNF overexpression induces SpA-like pathophysiology in mice. tmTNF signaling through TNF-RI induced inflammation, but not osteoproliferation.

ABSTRACT
TNF plays a key role in immune-mediated inflammatory diseases including rheumatoid arthritis (RA) and spondyloarthritis (SpA). It remains incompletely understood how TNF can lead to different disease phenotypes such as destructive peripheral polysynovitis in RA versus axial and peripheral osteoproliferative inflammation in SpA. We observed a marked increase of transmembrane (tm) versus soluble (s) TNF in SpA versus RA together with a decrease in the enzymatic activity of ADAM17. In contrast with the destructive polysynovitis observed in classical TNF overexpression models, mice overexpressing tmTNF developed axial and peripheral joint disease with synovitis, enthesitis, and osteitis. Histological and radiological assessment evidenced marked endochondral new bone formation leading to joint ankylosis over time. SpA-like inflammation, but not osteoproliferation, was dependent on TNF-Receptor I and mediated by stromal tmTNF overexpression. Collectively, these data indicate that TNF can drive distinct inflammatory pathologies. We propose that tmTNF is responsible for the key pathological features of SpA.
**INTRODUCTION**

Spondyloarthritis (SpA) is the second most frequent form of chronic inflammatory arthritis. This progressive and debilitating condition comprises several pathophysiologically related but clinically distinct phenotypes, including ankylosing spondylitis (AS), psoriatic arthritis (PsA), inflammatory bowel disease-related spondyloarthritis (IBD-SpA), reactive arthritis (ReA), and undifferentiated spondyloarthritis (USpA). All these subforms of SpA are characterized by inflammation of the spine and/or peripheral joints, sometimes in association with extra articular manifestations in the skin, gut, or eye. Moreover, SpA patients develop both axial and peripheral structural joint damage which, in contrast to the erosive cartilage and bone damage observed in rheumatoid arthritis (RA), is specifically characterized by formation of pathological new bone ultimately leading to joint ankylosis. Accumulating evidence indicates that this unique and distinctive structural phenotype of SpA is not related to the absence of cartilage and bone destruction, which are both frequently seen in SpA joints, but rather to the marked presence of endochondral new bone formation.

Although the exact pathogenesis of SpA remains incompletely understood, ample evidence indicates a prominent role for the pro-inflammatory cytokine tumor necrosis factor (TNF) in the pathology of this disease. Several single nucleotide polymorphisms in genes encoding molecules of the TNF pathway, such as TNFRSF1A and TRADD, are associated with susceptibility to SpA. Moreover, therapeutic blockade of TNF by either monoclonal antibodies or soluble decoy receptor constructs has a significant beneficial impact on signs and symptoms as well as pathology of the different SpA subforms. Surprisingly, however, experimental models of TNF overexpression do not accurately recapitulate the pathological features of SpA but rather phenocopy human RA. Transgenic overexpression of human TNF results in spontaneous development of severe systemic inflammation and destructive RA-like polysynovitis. Albeit blocking DKK-1, an inhibitor of the Wnt signaling pathway, can reverse the destructive phenotype into a remodeling phenotype characterized by new bone formation in synovial joints, these mice still lack typical SpA features such as spondylitis and enthesitis. In a slightly different model, overexpression of mouse TNF by deletion of the AU-rich elements in the murine TNF locus (TNFARE mice) induces not only destructive polysynovitis, including sacroiliitis, but also inflammatory bowel disease. The association with gut inflammation is of major interest when considering the clinical and genetic overlap between human SpA and Crohn’s disease and ulcerative colitis, but also the TNFARE model fails to recapitulate key pathological features of SpA such as spondylitis and endochondral new bone formation. The exact molecular and cellular mechanisms by which TNF contributes to SpA pathology thus remain poorly understood.

TNF is produced as a homo-trimeric transmembrane-bound cytokine; after enzymatic cleavage by ADAM17 (A disintegrin and metalloproteinase 17, also known as TNF converting enzyme or TACE) the 17 kDa soluble TNF is released extra-cellular. Both transmembrane (tm) and soluble (s) TNF are biologically active and can bind both TNF receptor I (p55) and TNF-RII (p75), albeit the literature about relative affinities of tmTNF and sTNF for each of the TNF receptors is still contradictory. Our observation that sTNF levels in synovial fluid (SF) are significantly decreased in SpA versus RA despite similar levels of overall joint inflammation urged us to revisit the role of the different TNF subforms in spondyloarthritis.
PATIENTS AND METHODS

Patients
This study included 64 patients who fulfilled the European Spondylarthropathy Study Group criteria for SpA and 60 patients who fulfilled the American College of Rheumatology/European League Against Rheumatism 2010 criteria for RA. All patients had active disease with effusion of at least 1 knee joint, and none of the patients was treated with a biologic agent. Synovial biopsy samples were obtained by needle arthroscopy from actively inflamed joints of 20 SpA patients (32% female patients, mean age 40 years, mean C-reactive protein [CRP] level 32 mg/liter, and mean swollen joint count 2.5) and 18 RA patients (58% female patients, mean age 56 years, mean CRP level 39.5 mg/liter and mean swollen joint count 11) for qPCR analyses, and in 19 SpA patients (37% female patients, mean age 43 years, mean CRP level 26 mg/liter, and mean swollen joint count 2.5) and 20 RA patients (75% female patients, mean age 52 years, mean CRP level 36.5 mg/liter and mean swollen joint count 7) for immunohistochemistry. Synovial fluid (SF) was obtained by joint puncture in 25 patients with SpA (39% female patients, mean age 40 years, mean CRP level 22.1 mg/liter, and mean swollen joint count 2.8) and 22 patients with RA (71% female patients, mean age 58 years, mean CRP level 24.6 mg/liter, and mean swollen joint count 8.1). Detailed patient characteristics are available upon request from the corresponding author. Written informed consent was obtained from all patients before inclusion in the study, which was approved by the Local Ethics Committee of the Academic Medical Center at the University of Amsterdam. No samples were excluded from analyses.

Enzyme-linked immunosorbant assay
Levels of soluble TNF (Pelipair M9323; Sanquin, Amsterdam, The Netherlands), soluble TNF-RI (BMS203CE; eBioscience, Vienna, Austria), soluble TNF-RII (BMS211CE; eBioscience) and soluble CD163 (DY1607; RnD systems, Abingdon, United Kingdom) were measured by ELISA in synovial fluid according to the instructions of the manufacturer.

Quantitative PCR
Total RNA was extracted from synovial tissue samples according to the protocol of the RNeasy FFPE Kit (Qiagen, Crawley, United Kingdom). The quantity of the RNA was assessed by nanodrop (NanoVue Plus, General Electric, Freiburg, Germany) and 1–2 micrograms of RNA was reverse transcribed into complementary deoxyribonucleic acid (cDNA) using a high-capacity cDNA Archive Kit (Applied Biosystems, Warrington, UK). TaqMan gene expression assays for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (4310884E), TNF (Hs00174128_m1); TNF-RI (Hs01042313_m1) and TNF-RII (Hs00961749_m1); ADAM17 (Hs01041915_m1) were purchased from Applied Biosystems, and gene expression was measured in duplex reactions. Gene expression in mouse samples (GAPDH, ALPL, Col1a1) was measured in duplex reactions using SYBR green primers (sequences are available upon request). The relative expression (represented in arbitrary units, a. u.) was calculated with the “2^(-ddCt) method”, where \( dCt = Ct_{\text{gene}} - Ct_{\text{housekeeping gene}} \), \( ddCt = dCt_{\text{sample}} - dCt_{\text{calibrator}} \). GAPDH was used as a housekeeping gene and one of
the samples as an internal calibrator. The results were calculated using the StepOne Software v 2.1 (Applied Biosystems).

**Immunohistochemistry**

Synovial biopsy samples were snap-frozen in Tissue-Tek OCT (Miles, Elkhart, Indiana, USA) immediately after collection. Cryostat sections (5 µm) were cut and mounted on Starfrost adhesive glass slides (Waldemar Knittel Glasbearbeitungs, Braunschweig, Germany). Frozen sections were acetone fixed (10 minutes) and stained with 1 µg/mL monoclonal antibody directed against TNF (clone S2B83; Hycult Biotech, Uden, The Netherlands) or 5 µg/ml monoclonal antibody directed against ADAM17 (clone ab57484; Abcam, Cambridge, United Kingdom) overnight at 4°C. After rinsing, sections were sequentially incubated with a biotinylated secondary antibody, a streptavidin-horseradish peroxydise link, aminoethylcarbazole substrate as chromogen (LSABII kit, Dako, Heverlee, Belgium) and Gill’s haematoxylin as counterstain. As negative control parallel sections were incubated with a concentration-matched, mouse IgG1 isotype antibody. All samples were stained in a single run to minimize technical biases, and subsequently scored semi quantitatively for cellular infiltration by three independent observers (LMvD, MNvT and DLB) who were blinded to the patients’ diagnoses.

**Immunofluorescence**

Frozen synovial tissue sections were fixed in acetone and blocked with 10% goat serum (Dako, Glostrup, Denmark), followed by incubation with Biotin blocking system (Dako). Stainings with mAb directed against TNF (clone S2B83; Hycult Biotech), CD45 (clone HI30; BioLegend, San Diego, CA), CD55 (clone JS11; BioLegend), CD68 (clone Y1/82A; BioLegend), CD90 (clone 5E10; BioLegend), CD163 (clone GHI/61; BioLegend), and vimentin (clone D21H3; Cell Signaling Technology, Leiden, The Netherlands) were performed overnight at 4°C, followed by incubation with Alexa Fluor 488/Alexa Fluor 555–conjugated goat anti-mouse and goat anti-rabbit secondary antibodies. Slides were mounted with Vectashield containing DAPI (Vector Laboratories) and analyzed on a fluorescence imaging microscope (Leica DMRA) coupled to a CCD camera, with results analyzed using Image-Pro Plus software (Media Cybernetics, Dutch Vision Components).

**ADAM17 activity assay**

Activity of TACE/ADAM-17 in vivo was determined in cell lysates from FLS cultures (SpA cell lines: n=7 and RA cell lines: n=6) using the AnaSpec SensoLyte 520 TACE Activity Assay, following the manufacturer’s instructions (AnaSpec, San Jose, CA).

**Mice**

TgA86 mice (tmTNF transgenic mice) were kindly provided by Prof. Dr. G. Kollias (Institute of Immunology, Biomedical Sciences Research Centre Alexander Fleming, Greece) and the breeding was maintained in the Academic Medical Center animal facility. TgA86 mice were crossed back on a full C57Bl/6 background (CS7Bl/6JOlaHsD; Harlan, The Netherlands). B6.SJL-PtprcPep3/
BoyCrl (CD45.1) congenic mice were obtained from Charles River Laboratories (France). TNF-RI\(^{-/-}\) (Tnfrsf1a\(^{tm1Imx}\)) and TNF-RII\(^{-/-}\) (Tnfrsf1b\(^{tm1Mwm}\)) mice were obtained from the Jackson Laboratory (USA). Experiments were performed in accordance with national legislation and under supervision of the Animal Experimental Committee of the Academic Medical Center and the University of Amsterdam. For our experiments, we did not use any type of randomization as the genotype of the experimental groups differ.

**Clinical scoring**

Mice were scored at least weekly for weight loss, arthritis (swelling and deformation of hind limbs and front paws) and spondylitis (swelling or crinkling of the tail and hunchback formation) until 100 days of age. To measure grip strength, mice were placed on top of the lid of the cage, after which the lid was carefully turned up-side-down, and the time was measured that the mice hold the grid (for a maximum of 20 seconds). No animals were excluded from analyses. Due to the clear phenotype of the tmTNF tg mice, it was not possible to blind the observer during scoring and evaluation of the in vivo experiments.

**Sample processing and histologic staining**

Paws, tail, spine, sacroiliac joints, skin, eyes, ileum and ascending colon were harvested and fixed overnight in 4% formalin and sectioned. Joints were decalcified for at least 4 weeks in osteosoft (Merck, Darmstadt, Germany). Samples were embedded in paraffin and cut into 5 μm sections. Sections were deparaftinized and stained for 10 minutes with Mayer’s hematoxylin solution (Sigma-Aldrich, Zwijndrecht, The Netherlands). After rinsing with tap water and 96% ethanol, the sections were stained for 2 minutes with Accustain eosin solution (Sigma-Aldrich), dehydrated, and mounted in entellan (Merck). Alternatively, sections were deparaftinized and stained for 10 minutes with Weigert’s iron hematoxylin solution (Sigma-Aldrich). After rinsing for 10 minutes with tap water, the sections were stained for 5 minutes with Fast Green solution (Sigma-Aldrich), quickly rinsed in 1% acetic acid and stained with 0.1% Saffranin-O for 5 minutes, dehydrated, and mounted in entellan (Merck). Tartrate resistant acid phosphatase (TRAP) staining was performed according manufacturer’s protocol (TRAP staining kit #3871-1KT; Sigma-Aldrich). Stained sections were scored by 2 independent observers (LMvD and MNvT). Based on similar histologic studies of human synovial tissue,\(^{29,69}\) sections were semi-quantitatively graded, according to the degree of lymphocyte infiltration, as normal, mild, moderate, or severe; the same was done for bone- and cartilage destruction and proteoglycan staining. This quantitative analysis was performed on both hind paws and spine and tail sections. The scores were concordant in 84% of the cases. In 16% of the cases, the scores were discordant, and the average of the 2 scores was used. No samples were excluded from analyses.

**Histomorphometric analysis**

Bone histomorphometry was performed using a microscope (Nikon, Japan) equipped with a digital camera and an image analysis system (OsteoMeasure; OsteoMetrics, Decatur, GA,
USA). The following parameters were measured: total size of the proliferation and specificof the hypertrophic chondrocyte part. Sections were scored by 1 independent observer (GS), blinded for the specific mouse strain. No samples were excluded from analyses.

**Radiographic images**

From 3 eight-months old tmTNF tg male mice and 3 non-tg littermates (same age and gender) radiographic images were obtained from hind paws as well as spine and tails. Images were shot with the Senograph Essential (General Electric, Hoevelaken, The Netherlands) at 28 kV and 20 mAs for 10 seconds. No animals were excluded from analyses.

**Calvarial fibroblast cultures**

Primary osteoblasts were isolated from calvariae from tmTNF tg mice or non-tg littermates (n=9-10/group) after aseptic dissection and treatment with Collagenase II (100 U/ml, LS004174; Worthington, Prouvy, France). Cells were digested for 4 hours at 37°C and afterwards cultured in DMEM supplemented with 10% FCS (Biowest, Nuaillé, France), 2 mM L-glutamin (Life Technologies, Bleiswijk, The Netherlands), 0.5 mg/ml penicillin-streptomycin (Life Technologies), 50 ug/ml gentamycin (Life Technologies) and 20 uM beta-mercaptoethanol (Sigma-Aldrich). After expansion of the cells, cells were seeded 30.000 cells/well in a 24 wells plate for Alizarin Red staining or 150.000 cells/well in a 6 wells plate for mRNA analyses. To induce differentiation cells were cultured in StemXvivo osteogenic/adipogenic base medium (RnD Systems) supplemented with 2 mM L-glutamin, 0.5 mg/ml penicillin-streptomycin, 20 uM beta-mercaptoethanol, 50 uM ascorbic acid (Sigma), 10 mM b-glycerophosphatase (Sigma), 50 ng/µl IL-17A (RnD systems). No samples were excluded from analyses.

**Alizarin Red Staining**

Differentiated cells in plates were washed with PBS, fixed with 4% formaldehyde (VWR, Amsterdam, The Netherlands) for 5 minutes, washed again with PBS (three times), stained with 3 mg/ml Alizarin Red (Sigma) for 1 minute, washed several times with tap water and dried by air.

**Bone marrow chimera experiment**

Four week old male tmTNF tg mice and 6 week old male CD45.1 congenic wild type mice received twice a total body irradiation (TBI) with 6 gray in a 24 hrs.-interval. One day after the last TBI, mice were rescued with total bone marrow cells intravenously. Total bone marrow cells were obtained from tibia and femurs of either tmTNF tg or CD45.1 congenic WT mice. Tibia and femurs were flushed with PBS, cell suspensions were washed once with PBS, counted and injected into the recipient mice (10^7 cells / 200 l PBS containing 1% BSA). After BMT, mice were weekly scored for weight, arthritis and spondylitis induction as described above. Four, 8 and 12 weeks after BMT, blood was drawn via a small incision in the tail vein to check BMT engraftment. Sixteen weeks after BMT, all animals were sacrificed. No animals were excluded from analyses.
Sample size selection

For animal in vivo experiments, the program power and precision Pro (http://www.power-analysis.com/) and the 2-sided T test for independent samples was used to calculate the size of the group. We planned our studies of a continuous response variable from independent control and experimental subjects with 1 control(s) per experimental subject. In a previous study the response within each subject group was normally distributed with standard deviation 5. If the true difference in the experimental and control means is 10, we will need to study 5 experimental subjects and 5 control subjects to be able to reject the null hypothesis that the population means of the experimental and control groups are equal with probability (power) 0.8. The Type I error probability associated with this test of this null hypothesis is 0.05. Also for in vitro studies, we calculated the similar sample size.

Statistics

As the data were non-parametrically distributed, a two sided Mann-Whitney test for unpaired data was used. A Log-rank (Mantel-Cox) test was performed for incidence graphs (survival) and multiple group comparison was done by Kruskal-Wallis test. P-values were calculated using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Values of p < 0.05 with a 95% confidence interval were considered significant.

Data availability

All relevant data are available from the authors upon reasonable request.

RESULTS

Elevated transmembrane TNF expression in SpA synovitis

We previously demonstrated that synovial fluid sTNF levels were significantly lower in peripheral SpA than RA despite similar degrees of overall inflammation3 and good responsiveness of peripheral SpA to anti-TNF treatment.10 To explore this paradoxical finding, we first assessed in more detail the TNF expression in SpA synovitis using RA as control. qPCR analysis of synovial tissue biopsies revealed similar TNF mRNA levels in both diseases (Figure 1a). Confirming our previous data3 SF levels of sTNF protein were significantly lower in SpA (median: 1.77 pg/ml) versus RA (median: 6.52 pg/ml; p=0.012) (Figure 1d). This difference between both diseases was also found when analyzing rheumatoid factor (RF) negative RA, excluding a bias related to interference of RF with the ELISA (data not shown). The low levels of sTNF in SpA SF were also not related to an increase in the decoy receptors capturing sTNF as the soluble TNF receptor I (p55) (SpA: median 2.45 ng/ml versus RA: median 10.3 ng/ml; p=0.001) and receptor II (p75) (SpA: median 17 ng/ml versus RA: median 31.15 ng/ml; p=0.001) were also significantly decreased in SpA versus RA synovial fluid (Fig 1e-f), despite comparable synovial tissue mRNA levels in both conditions (Fig 1b-c). As the 17 kDa sTNF as well as sTNF-R1 and sTNF-R2 are generated by enzymatic cleavage by ADAM17, we explored if the decrease in sTNF in SpA SF could be related to altered cleavage and a disturbed balance between sTNF and the 26 kDa transmembrane form of TNF (tmTNF) expressed on the surface of various cell subsets22.
FIG. 1. TNF AND TNF RECEPTOR EXPRESSION IN SPA VERSUS RA SYNOVITIS. a-c) Relative TNF, TNF-RI and TNF-RII mRNA expression, respectively, measured by quantitative real-time PCR in synovial biopsies from SpA and RA patients. d-f) soluble TNF, TNF-RI and TNF-RII expression in synovial fluid, respectively, measured by ELISA. g) TNF protein expression measured by immunohistochemistry in synovial biopsies from SpA and RA patients. 200x magnification. h-i) Semi-quantitative score (0-3) of respectively the TNF expression in the lining layer and sublining. Values depicted are medians with interquartile range; n = 19-25 patients/group; P-values < 0.05 are considered statistically significant.

Using an antibody against TNF that specifically binds tmTNF and not TNF captured by either one of the TNF receptors expressed on the cell surface, immunohistochemical analysis revealed higher tmTNF protein expression in the synovial lining layer, but not the synovial sublining layer, of SpA versus RA (SpA median 2 versus RA median 0.667; p<0.001) (Figure 1g-i). Collectively, these data demonstrate a relative overexpression of tmTNF over sTNF in SpA versus RA synovitis.
**Decreased ADAM17 activity in SpA synovitis.**

To address the question why an altered balance in tmTNF versus sTNF expression in SpA synovitis compared to RA synovitis is observed, we investigated the expression and activity of ADAM17. ADAM17 (or TACE) is the only disintegrin and metalloproteinase capable of cleaving TNF. qPCR analysis of synovial tissue biopsies revealed similar ADAM17 mRNA levels in both diseases (Figure 2a). Also protein expression by immunohistochemical analyses revealed similar expression of ADAM17 in SpA and RA synovial tissue (Figure 2b-c). Next, we analyzed the enzymatic activity of ADAM17 in SpA and RA fibroblast-like synoviocyte (FLS) cultures. ADAM17 was less active in SpA FLS compared to RA FLS (SpA median 0.021 versus RA median 1.3437; p=0.0487) (Figure 2d). The same trend towards decreased ADAM17 activity was observed in SpA synovial-fluid-derived-monocytes (SFMCs) compared to RA SFMCs (supplementary Figure 1). To investigate whether ADAM17 enzymatic activity is not only decreased ex vivo in SpA-derived cells but also in vivo in SpA synovitis, we measured the levels of soluble CD163 in SF. CD163 is a scavenger receptor expressed by macrophages and exclusively cleaved by ADAM17. We previously demonstrated a marked and consistent increase in CD163 membrane staining in SpA versus RA synovitis. In line with the in vitro demonstration of decreased ADAM17 activity, we demonstrated that sCD163 levels in SF were decreased in SpA versus RA synovitis (Figure 2e). Together with the lower levels of soluble TNF-RI and TNF-RII, two other substrates of ADAM17, these results indicate that the altered balance in tmTNF versus sTNF in SpA is related to a decrease in enzymatic activity of ADAM17.

**tmTNF overexpressing mice develop a spondyloarthritis phenotype**

To explore the potential functional role of tmTNF in SpA pathogenesis, we studied the phenotype of the TgA86 mouse. This mouse overexpresses systemically a mutant murine TNF gene with a defect in the cleavage site for ADAM17 (muTNF1-12), leading to a specific systemic increase of tmTNF but not sTNF expression. In contrast to other mouse models of TNF overexpression, the tmTNF transgenic (tmTNF tg) mice did not show signs of systemic inflammation such as weight loss or growth defects over a 100 days follow-up period (Figure 3a). In line with previous reports, the mice did, however, develop swelling and deformation of the front and hind paws (Figure 3b), starting already at 30 days of age and reaching a 100% incidence at day 63 (Figure 3c). This peripheral arthritis was associated with a rapid and profound loss of grip strength (Figure 3d). Additionally, the tmTNF tg mice developed clinical spondylitis, characterized by the development of a hunchback and a crinkled tail (Figure 3e), starting at 25 days of age and reaching a 100% incidence at day 45 (Figure 3f). No arthritis or spondylitis was observed in the wildtype littermate controls.

To confirm these clinical findings and investigate the pathological processes underlying the arthritic and spondylitic phenotype of the tmTNF tg mice, we assessed histologically ankles and tail sections from tmTNF tg mice and their age- and gender-matched non-tg littermates at 100 days of age. All tmTNF tg mice depicted a marked leukocytic infiltration of the synovial tissue, the enthesis, and the bone marrow (Figure 4a and b) of the peripheral joints, whereas synovitis, enthesitis, and osteitis were not observed in the non-transgenic littermates (Figure 4c and d;
FIG. 2. DECREASED ENZYMATIC ACTIVITY OF ADAM17 IN SPA FLS. a) Relative ADAM17 mRNA expression measured by quantitative real-time PCR in synovial biopsies from SpA and RA patients. b) Semi-quantitative score (0-3) of the ADAM17 protein expression in the synovial lining layer and sublining, respectively, as assessed by immunohistochemistry. c) ADAM17 protein expression measured by immunohistochemistry in synovial biopsies from SpA and RA patients. 200x magnification. d) Enzymatic activity of ADAM17 in total cell lysates of FLS cultures (SpA FLS n=7; RA FLS n=6) e) Soluble CD163 expression in synovial fluid measured by ELISA. Values depicted are medians with interquartile range; n = 17-25 patients/group; P-values < 0.05 are considered statistically significant. RFU = relative fluorescence units.

p<0.001). Likewise, the axial skeleton of the tmTNF tg mice showed an inflammatory infiltration along the ligaments in connective tissue at the border of the intervertebral discs (Figure 4e) as well as lymphoid aggregates in the bone marrow (Figure 4f), whereas no axial pathology was observed in the littermate controls (Figure 4g and h; p<0.001). Extensive histological analysis of extra articular sites revealed no inflammation or other pathology in eyes, skin, colon and intestine of 100 days-old tmTNF tg mice (data not shown). Taken together, these data indicate that
selective overexpression of tmTNF leads to axial and peripheral joint pathology reminiscent of human SpA (synovitis, enthesitis, and osteitis) in the absence of severe systemic disease and extra-articular manifestations.

**tmTNF drives osteoproliferative joint remodeling**

In the commonly used TNF overexpressing mice such as the hTNF mice (Tg197) and TNFARE mice, the inflammatory polysynovitis is characterized by a profound degradation of cartilage and bone, recapitulating the structural phenotype of human RA.\textsuperscript{13,14} Assessing if the SpA-like disease observed in the tmTNF tg mice was associated with a different structural phenotype, histological analysis revealed clear, albeit relatively mild, bone destruction of the peripheral joints with the occasional presence of osteoclasts on TRAP staining (Figure 5a-c). Also the spine of tmTNF tg mice showed...
clear signs of destruction, albeit this was mild and merely restricted to disruption of the cartilage endplate (Figure 5d-f). In sharp contrast with the previously described TNF overexpressing models, however, the tmTNF tg mice displayed clear features of new bone formation. Saffranin-O staining of proteoglycans revealed peri-articular chondro-proliferative lesions in the ankles of tmTNF tg mice but not in non-tg littermates (Figure 5g-i; p=0.0095). This was confirmed by histomorphometric analyses of complete osteophyte proliferation area (data not shown) as well as the specific hypertrophic chondrocyte area (HTC) (Figure 5j). Similarly, hypertrophic chondrocytes were seen in the connective tissue at the edge of the intervertebral disc of the axial skeleton in the tmTNF tg animals but not in their non-tg littermates (Figure 5k-m; p=0.004), which was again confirmed by histomorphometric analyses of complete osteophyte proliferation area (data not shown) as well as the specific hypertrophic chondrocyte area (Figure 5n). To assess if these peri-articular chondro-proliferative lesions lead to endochondral new bone formation, 8 months old tmTNF tg and non-tg littermate animals were subjected to radiographic analysis. Whereas no new bone formation was observed in the non-tg controls (Figure 6a-d), the tmTNF tg mice displayed obvious vertebral fusion in the absence of pronounced bone erosions in the lumbar spine (Figure 6a) and the tail (Figure 6b). By histological analyses, we confirmed the vertebral fusion in these eight months old tmTNF tg mice (Figure 6d). Moreover, these radiographic images also revealed sacroiliitis in the tmTNF tg animals (Figure 6c). Collectively, the histological and radiologic analyses concord to
FIG. 5. STRUCTURAL DAMAGE, IN PARTICULAR ENDOCHONDRAL NEW BONE FORMATION IN TMTNF TG MICE. a) HE staining of an ankle of a tmTNF tg mouse. 200x magnification. b) TRAP positive osteoclasts (in red, arrows) depicted in an ankle joint of a tmTNF tg mouse. 200x magnification. c) Semi-quantitative score (0-3) of destruction in ankle joints of tmTNF tg mice compared to non-tg littermates. d-e) HE staining of axial, tail section of tmTNF tg mice, indicating destruction by disruption of the cartilage endplate (#1) 100x magnification. f) Semi-quantitative score (0-3) of destruction in spine joints of tmTNF tg mice compared to non-tg littermates. g-j) Saffranin-O/fast green staining to detect proteoglycans (square in g) in an ankle joint of a tmTNF tg mouse. Respectively 100x and 200x magnification. j Semi-quantitative score (0-3) of endochondral new bone formation in ankle joints of tmTNF tg mice compared to non-tg littermates. j) Histomorphometric analyses: hypertrophic chondrocyte size. k-n) Saffranin-O/fast green staining to detect proteoglycans (square in K) in a tail joint of a tmTNF tg mouse. Respectively 100x and 200x magnification. m) Semi-quantitative score (0-3) of endochondral new bone formation in axial joints of tmTNF tg mice compared to non-tg littermates. n) Histomorphometric analyses: hypertrophic chondrocyte size. Values are mean +/- SEM; n=7 mice/group; P-values < 0.05 are considered statistical significant.
indicate that the clinical spondyloarthritis induced by selective tmTNF overexpression is associated with a remodeling, rather than destructive, structural phenotype reminiscent of human SpA.

**tmTNF-induced SpA-like inflammation requires TNF-RI signaling**

To delineate the cellular and molecular mechanisms by which selective tmTNF overexpression leads to SpA-like pathology, tmTNF tg mice were crossed to either TNF-RI knock out or TNF-RII knock out mice and analyzed clinically and histologically at the age of 100-120 days. Peripheral synovitis, osteitis and enthesitis were observed in 100% of the tmTNF<sup>+/WT</sup> (20/20) as well as in 100% of the tmTNF<sup>+/WT</sup>xTNF-RII<sup>−/−</sup> mice (7/7) but not in the tmTNF<sup>+/WT</sup>xTNF-RI<sup>−/−</sup> mice (0/7) (n=20; n=7 and n=9 respectively) (Figure 7a), confirming the findings of Alexopoulou and colleagues that tmTNF mediated synovitis induction requires the presence of the TNF-RI<sup>33</sup>. Cellular infiltrates at the edge

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**FIG. 6. JOINT FUSION OF VERTEBRA AND SACROILIITIS IN 8 MONTHS OLD TMTNF TG MICE.** a-c) Radiographic images of 1 n8-months old on-tg littermate and 2 tmTNF tg mice. a) Radiographic images of the lumbar spine. b) Radiographic images of the tail. c) Radiographic images of the sacroiliac joints. d) HE staining of ankylosis in the tail. 40x magnification.
of the intervertebral unit in the connective tissue as characteristic for spondylitis was also only observed in all tmTNF<sup>+/WT</sup> tg mice as well as all tmTNF<sup>+/WT</sup>xTNF-RI<sup>−/−</sup> mice, and not in the tmTNF<sup>+/WT</sup>xTNF-RI<sup>+/−</sup> mice (Figure 7b). Similarly, hunch back formation and crinkled tails were observed in the tmTNF<sup>+/WT</sup> and the tmTNF<sup>+/WT</sup>xTNF-RII<sup>−/−</sup> mice but not in the tmTNF<sup>+/WT</sup>xTNF-RI<sup>−/−</sup> mice (Figure 7c). Interestingly, although tmTNF<sup>+/WT</sup>xTNF-RII<sup>−/−</sup> mice did have the inflammatory characteristics of SpA, we were not able to find endochondral new bone formation in these mice at all (0/7) (Figure 7d). These results suggest that SpA-like inflammation by selective

**FIG. 7.** TMNF-INDUCED SPA-LIKE INFLAMMATION REQUIRES TNF-RI SIGNALING. a) HE stainings of an ankle joint of a 100-day old tmTNF<sup>+/WT</sup>tg mouse, depicting inflammation and destruction; an ankle joint of a 120-day old tmTNF<sup>+/WT</sup>xTNF-RI<sup>−/−</sup> mouse without cellular infiltrate and an ankle joint of a 115-day old tmTNF<sup>+/WT</sup>xTNF-RII<sup>−/−</sup> mouse. Inflammation is observed in the bone (osteitis) as well as in the enthesis (enthesitis). 80x magnification. b) HE stainings of a spine joint of a 100-day old tmTNF<sup>+/WT</sup>tg mouse, depicting cellular infiltrate at the border of the intervertebral disc in the connective tissue; a spine joint of a 120-day old tmTNF<sup>+/WT</sup>xTNF-RI<sup>−/−</sup> mouse without cellular infiltrate and a spine joint of a 115-day old tmTNF<sup>+/WT</sup>xTNF-RII<sup>−/−</sup> mouse. Again, inflammation is observed at the edge of the intervertebral disc in the connective tissue. 100x magnification. c) Photo of a 100-day old tmTNF<sup>+/WT</sup>tg mouse, depicting hunchback formation and a crinkled tail; photo of a 120-day old tmTNF<sup>+/WT</sup>xTNF-RI<sup>−/−</sup> mouse without clinical symptoms and a picture of a 115-day old tmTNF<sup>+/WT</sup>xTNF-RII<sup>−/−</sup> mouse with hunchback formation and a crinkled tail. d) Overview of pathological features of spondyloarthritis observed in the three lines. tmTNF<sup>+/WT</sup>tg : n=20; old tmTNF<sup>+/WT</sup>xTNF-RI<sup>−/−</sup> : n=9; tmTNF<sup>+/WT</sup>xTNF-RII<sup>−/−</sup> : n=7.
tmTNF overexpression requires TNF-RI signaling, although TNF-RII signaling is needed for osteoproliferation.

Expression of tmTNF by stromal cells is required and sufficient for the induction of SpA-like disease

Based on the identification of a specific stromal signature in SpA synovitis, we have recently postulated that stromal cells may play a crucial role in SpA immunopathology. To explore this hypothesis in the context of tmTNF overexpression, we investigated first whether tmTNF overexpression on calvarial fibroblasts leads to enhanced differentiation towards osteoblasts. Fibroblasts derived from skulls from either tmTNF tg mice or non-tg littermates were cultured and differentiated with osteogenic medium with or without IL-17A, as additional pro-inflammatory cytokine, for a maximum of 27 days. Seven days after differentiation, mRNA levels were measured for genes regulating osteogenesis. Alkaline phosphatase expression was significantly increased in the tmTNF tg mice in osteogenic medium with additional IL-17A, which was not the case in the non-tg littermates (Figure 8a). Collagen type I revealed a similar trend towards more expression in the tmTNF tg cells in osteogenic medium with additional IL-17A, whereas there was no increase observed in Collagen type I expression in non-tg cells (Figure 8b). Mineralization was also analyzed at day 21 (data not shown) and day 28 (Figure 8c) with an Alizarin Red staining. More mineralization was measured in tmTNF overexpressing cells compared to non-tg cells.

To test the role of tmTNF overexpression on stromal cells in vivo, we made bone marrow chimeric mice overexpressing tmTNF either only on hematopoietic cells or only on radio-resistant stromal cells. Non-treated tmTNF tg mice and lethally irradiated tmTNF tg mice reconstituted with their own tmTNF tg bone marrow were used as controls. Bone marrow reconstitution was between 70-90% and 90-95%, respectively 4 and 8 weeks after bone marrow transplantation (BMT) (data not shown). In line with our previous experiments, all non-treated tmTNF tg animals developed arthritis as well as spondylitis at the mean age of 9 and 6 weeks, respectively (Figure 9a-b). tmTNF tg mice that were irradiated and rescued with tmTNF tg BMT ('treated control' group) also developed arthritis and spondylitis with similar time of onset and incidence (Figure 9a-b), albeit the severity of the peripheral arthritis as assessed by number of affected paws (Figure 9c) and grip strength (Figure 9d) was decreased compared to the untreated control group. Interestingly, animals that only overexpress tmTNF on stromal cells (irradiated tmTNF tg + WT BMT) developed arthritis as well as spondylitis with the same disease onset-rate as both control groups (Figure 9a-b) and similar peripheral disease severity as the treated control group. In sharp contrast, mice with selective overexpression of tmTNF on hematopoietic cells were completely protected from peripheral joint disease (Figure 9a, c-d; p < 0.05 at least in all cases) and displayed a lower incidence (66.7% vs 100%, p<0.001) and delayed onset (87.5 days) of spondylitis in comparison with the treated control group (12 days; p =0.001) (Figure 9b). Collectively, these data indicate that tmTNF overexpression on stromal cells is required and sufficient for the full articular SpA phenotype in this model, whereas hematopoietic tmTNF expression can induce some spondylitis but no peripheral arthritis within the 16 weeks the animals were followed for disease development. To explore whether stromal
tmTNF expression may also be relevant to human SpA, we performed double immunofluorescence stainings on SpA synovial tissue sections. tmTNF expression was observed on CD45+ hematopoietic cells, CD68+ and CD163+ macrophages (Figure 9e-g) but also on vimentin-positive stromal cells in the synovial lining layer (Figure 9j). tmTNF did not co-localize with either CD55 or CD90 stromal markers (Figure 9h-i).

**DISCUSSION**

TNF is one of the best studied and most important pro-inflammatory cytokines. It plays a major role in many chronic inflammatory diseases, including RA and inflammatory bowel disease, as
FIG. 9. STROMAL OVEREXPRESSION OF TMTNF IS SUFFICIENT FOR BOTH ARTHRITIS AND SPONDYLITIS INDUCTION. a) Percentage with arthritis in the 4 groups over time. P<0.001 for hematopoietic tmTNF expression group compared to all other groups. b) Percentage of spondylitis in the 4 groups. P<0.01 for hematopoietic tmTNF expression group compared to all other groups. c) Number of affected paws per animal per group. P<0.01 for hematopoietic tmTNF expression group compared to all other groups. d) Grip strength in seconds over time is depicted. P<0.05 for hematopoietic tmTNF expression group compared to all other groups. TBI/BMT is total body irradiation/bone marrow transplantation control group. Values are mean +/- SEM; n=6 per group; P-values < 0.05 are considered statistical significant. e-j) Representative photographs of double immunofluorescence staining of TNF (green) and CD45, CD68, CD163, CD55, CD90 and vimentin in red (respectively). Nucleus staining is performed with DAPI (blue). 40x magnification; n=3 SpA patients analyzed.
evidenced by experimental overexpression of TNF in different rodent models as well as by targeted TNF blockade in patients. The cellular and molecular mechanisms contributing to TNF-driven chronic tissue inflammation have been well delineated. Moreover, the mechanisms contributing to tissue destruction in TNF-mediated inflammation are also fairly well understood. In chronic arthritis, TNF directly contributes to cartilage and bone damage by stimulating the production of destructive enzymes, by triggering the activation of osteoclasts, and by the same time inhibiting bone and cartilage repair pathways.

What is not yet fully understood, however, is by which mechanisms the same cytokine could contribute to inflammatory disorders with completely different phenotypes, pathologies, and structural damage. A prototypical example is spondyloarthritis, a highly TNF blockade responsive form of chronic inflammatory arthritis which displays a set of unique characteristics such as spinal involvement, tendinitis, osteitis, and extensive new bone formation which are not recapitulated in the commonly used TNF overexpression models. Whereas this paradox may be partially explained by the interaction of TNF with other pro-inflammatory cytokines in each specific disease setting, such as IL-6 in RA versus IL-17A in SpA, the present study proposes a new concept that the exact expression form of a single cytokine, in this case soluble versus transmembrane TNF, may also contribute to determine the phenotype and pathology of a disease.

In sharp contrast with the destructive polysynovitis observed in the TNF overexpression models with high sTNF production, we show here that selective overexpression of tmTNF induces a distinct phenotype with a 100% incidence of spinal as well as peripheral joint inflammation in the absence of signs of severe systemic inflammation. Detailed histopathology confirmed the previously reported synovial inflammation in these mice, but additionally revealed marked enthesitis and osteitis, which are both key pathological features of human SpA. Whereas synovial inflammation in proximity of the enthesis has been reported in the TNFARE mice, the tmTNF tg mice showed marked infiltration of the enthesis itself with leukocytes. In addition to the peripheral pathology, histological analysis confirmed spinal enthesitis and osteitis, again features that are reminiscent of human SpA and have not been observed in the sTNF overexpressing models. Most strikingly, however, selective tmTNF overexpression did not only mimic the pathology of SpA (synovitis as well as peripheral and axial enthesitis and osteitis) but also the structural phenotype of this disease. Both peripheral joints and spine showed cartilage and bone destruction, which appears relatively mild in comparison with sTNF overexpression with a few observed osteoclasts which do not visibly excavate bone. This observation is in line with a previous publication that tmTNF actually diminishes osteoclastogenesis in contrast to sTNF. More notable is the marked endochondral new bone formation ultimately leading to complete joint ankylosis over time. In line with previous observations, the pathological new bone formation was not merely a repair mechanism occurring after resolution of inflammation as we observed foci of hypertrophic chondrocytes only in close proximity of articular tissue inflammation. Collectively, the clinical, pathological and radiological assessment of the model indicates that selective overexpression of tmTNF recapitulates the key articular features of human SpA. Interestingly, tmTNF tg mice did not spontaneously develop clinical or subclinical extra-articular disease of gut, eyes, or skin. One potential explanation is that tmTNF overexpression may confer increased susceptibility to extra-
articular inflammation but additional triggers are needed to induce full-blown colitis, uveitis, and/or psoriasis. Supporting a role for tmTNF in extra-articular manifestations, recent studies in human Crohn’s disease indicated that tmTNF expression in the gut is a good predictor of response to TNF inhibition\(^{47}\) and that binding of anti-TNF antibodies to tmTNF activates a macrophage-mediated immunoregulatory response which is crucial for the therapeutic effects in IBD.\(^{58,60}\) Alternatively, however, either sTNF or other cytokines may be more important than tmTNF for these extra-articular manifestations of SpA.

The striking difference in pheno- and pathotype between the tmTNF tg mice and other TNF overexpression models with high sTNF levels raises the question how these two forms of TNF can induce distinct biological responses. Previous in vitro and in vivo work convincingly demonstrated that the function of sTNF and tmTNF are not completely overlapping. Amongst other examples, sTNF but not tmTNF strongly promotes osteoclastogenesis in vitro\(^{43}\) whereas tmTNF but not sTNF actively suppresses autoimmune inflammation in EAE.\(^{50}\) One potential explanation for these differential effects is the difference in relative affinity of sTNF versus tmTNF for the TNF receptors: whereas both sTNF and tmTNF trigger TNF-RI, tmTNF was reported to have a higher affinity for TNF-RII.\(^{22,23}\) As TNF-RI has been shown to be crucial for RA-like polysynovitis in other TNF overexpression models,\(^{14,17}\) we tested whether TNF-RII may be more important than TNF-RI in the SpA-like phenotype of tmTNF tg mice. In line with a previous study showing that tmTNF-mediated peripheral synovitis requires the presence of TNF-RI,\(^{33}\) we did not observe any arthritis or spondylitis in tmTNF\(^{+/WT}\)xTNF-RI\(^{-/-}\) mice. In contrast, tmTNF\(^{+/WT}\)xTNF-RII\(^{-/-}\) mice developed clinical spondylitis as marked by hunchback formation and a crinkled tail and subclinical arthritis with synovitis, enthesitis, and osteitis in the ankle joints. Interestingly, the tmTNF\(^{+/WT}\)xTNF-RII\(^{-/-}\) mice examined in these pilot experiments did not display endochondral new bone formation at the age of 100-120 days. In agreement with the fact that peripheral arthritis is delayed in the absence of the TNF-RII,\(^{33}\) these data suggest a complex interplay between TNF-RI and TNF-RII in the pathology of tmTNF tg mice. More extensive in vivo and in vitro studies are currently ongoing to assess the exact role of TNF-RII in the osteoproliferative aspects of the disease in tmTNF tg mice.

Besides differential activation of TNF-RI versus TNF-RII, a second possible explanation for the distinct phenotype of tmTNF tg mice is the expression by specific cell types, especially since tmTNF has also been suggested to be involved in outside-in signaling.\(^{31,32}\) In CNS inflammation, for example, pathology was shown to be mediated by astrocyte-specific, but not neuron-specific, tmTNF expression.\(^{33}\) As stromal cells have been recently proposed to play a major role in human and experimental SpA,\(^{18,35}\) we aimed to investigate the effect of tmTNF overexpression on stromal cells. In the current study we show that tmTNF overexpression on mouse calvarial cells leads to enhanced alkaline phosphatase and collagen type I expression and an increased mineralization compared to wildtype calvarial cells, suggesting a direct effect of tmTNF on new bone formation. To study the effect of stromal tmTNF overexpression in vivo, we made bone marrow chimeric mice overexpressing tmTNF either on hematopoietic cells or on radio-resistant stromal cells. Mice overexpressing tmTNF on hematopoietic cells were completely protected from peripheral joint disease and displayed a lower incidence and delayed onset of spondylitis, whereas mice overexpressing tmTNF on stromal cells developed both spondylitis and arthritis with the same
onset and severity as the control treated group. Interestingly, the mice overexpressing tmTNF on hematopoietic cells and developing delayed spondylitis showed a destructive rather than remodeling phenotype on radiographic examination and histology (data not shown). These data indicate that tmTNF overexpression on stromal cells plays an essential role in this model in general and in the new bone formation in particular. The underlying mechanisms, including reverse signaling to stromal cells, are currently under investigation.

A third key question raised by the observations in the tmTNF tg mice is to what extent stromal tmTNF expression is also relevant to human SpA. Making use of synovial tissue biopsies, which is the only reasonably accessible target tissue in human SpA, we demonstrated a relative overexpression of tmTNF over sTNF in active SpA versus active RA as control at similar TNF mRNA expression levels and RNA stability (data not shown). Moreover, tmTNF expression in human SpA synovitis was not restricted to infiltrating myeloid cells but was also present on vimentin-positive stromal tissue cells. The fact that not only sTNF but also sTNF-RI, sTNF-RII and sCD163 were decreased in SpA versus RA SF indicates that impaired ADAM17 (or TACE) expression and/or activity might be implicated in the relative overexpression of tmTNF over sTNF in SpA synovial lining layer. Although, we did not observe differences in mRNA and protein levels of ADAM17 in SpA versus RA synovitis, we were able to show a clear decrease in enzymatic activity of ADAM17 in SpA FLS compared to RA FLS, suggesting indeed impaired ADAM17 activity. At this point, the reason for decreased ADAM17 activity in SpA synovitis needs to be further investigated. Protein expression and function of ADAM17 is complex and regulated at several steps, including control of endoplasmic reticulum exit by iRhom1/2, proteolytic maturation by removal of the ADAM17 pro-domain in the trans-Golgi network by furin, stimulation of ADAM17 surface translocation by mitogen-activated protein kinases, and activation of ADAM17 at the cell surface through conformational alterations. Dysfunction of any of these processes could lead to an impaired function of ADAM17 and need to be studied in more detail in the context of tissue inflammation and new bone formation in SpA.

Collectively, the human and animal data of the present study concord to indicate that TNF can drive strictly distinct inflammatory pathologies depending on its specific expression form. We propose that the transmembrane form of TNF rather than soluble TNF contributes to key pathological features of SpA, including new bone formation. Further elucidation of the mechanisms underlying the increased tmTNF expression as well as the tmTNF-induced pathology, will not only help to understand how a single cytokine can drive distinct pathologies but may also have direct therapeutic implications for the treatment of SpA. Despite the success of TNF inhibition in this disease, this therapeutic approach still fails to induce full inflammatory remission in a majority of patients and, even in those with inflammatory remission, the progression of structural damage in form of new bone formation is not halted. The current findings raise the hypothesis that current anti-TNF drugs may efficiently neutralize sTNF without sufficiently blocking tmTNF, especially in poorly vascularized stromal compartments such as the enthesis. If this hypothesis is correct, alternative strategies to target tmTNF expression, TNF-R signaling, and/or downstream effector mechanisms may open new avenues for effective treatment of inflammation and structural progression in SpA.
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SUPPLEMENTARY DATA

SUPPL. FIG. 1. ENZYMATIC ACTIVITY OF ADAM17 IN SYNOVIAL-FLUID DERIVED MONOCYTES FROM SPA AND RA PATIENTS. Values depicted are medians with interquartile range; n = 9-13 patients/group; P-values < 0.05 are considered statistically significant. RFU = relative fluorescence units.