



mTOR Blockade by Rapamycin in Spondyloarthritis: Impact on Inflammation and New Bone Formation *in vitro* and *in vivo*

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Introduction: Spondyloarthritis (SpA) is characterized by inflammation, articular bone erosions and pathologic new bone formation. Targeting TNF α or IL-17A with current available therapies reduces inflammation in SpA, however, treatment of the bone pathology in SpA remains an unmet clinical need. Activation of the mammalian target Of rapamycin (mTOR) promotes IL-17A expression and osteogenesis. Therefore, the inhibition of mTOR (with rapamycin) could be a promising therapeutic avenue in SpA.

Objectives: To investigate the effect of blocking mTOR on inflammation, bone erosions and new bone formation in SpA.

Methods: Peripheral blood mononuclear cells (PBMCs) from patients with SpA were stimulated with anti-CD3/CD28 in the presence or absence of rapamycin and the resulting cytokine expression was assessed. Fibroblast-like synoviocytes (FLS) from SpA patients were assessed for osteogenic differentiation potential in conditions with TNF α , IL-17A, or TNF α plus IL-17A, in the presence or absence of rapamycin. HLA-B27/Hu β 2m transgenic rats were immunized with low dose heat-inactivated *Mycobacterium tuberculosis* (*M. tub*), treated with 1.5 mg/kg rapamycin prophylactically or therapeutically and monitored for arthritis and spondylitis. Histology and mRNA analysis were performed after 5 weeks of treatment to assess inflammation and bone pathology.

Results: *In vitro* TNF α and IL-17A protein production by SpA PBMCs was inhibited in the presence of rapamycin. Rapamycin also inhibited osteogenic differentiation of human SpA FLS. *Ex vivo* analysis of SpA synovial biopsies indicated activation of the mTOR pathway in the synovial tissue of SpA patients. *In vivo*, prophylactic treatment of HLA-B27/Hu β 2m transgenic rats with rapamycin significantly inhibited the development and severity of inflammation in peripheral joints and spine (arthritis and spondylitis), with histological evidence of reduced bone erosions and new bone formation

around peripheral joints. In addition, therapeutic treatment with rapamycin significantly decreased severity of arthritis and spondylitis, with peripheral joint histology showing reduced inflammation, bone erosions and new bone formation. *IL-17A* mRNA expression was decreased in the metacarpophalangeal joints after rapamycin treatment.

Conclusion: mTOR blockade inhibits IL-17A and TNF α production by PBMCs, and osteogenic differentiation of FLS from patients with SpA *in vitro*. In the HLA-B27 transgenic rat model of SpA, rapamycin inhibits arthritis and spondylitis development and severity, reduces articular bone erosions, decreases pathologic new bone formation and suppresses IL-17A expression. These results may support efforts to evaluate the efficacy of targeting the mTOR pathway in SpA patients.

Keywords: spondyloarthritis, mTOR, rapamycin, IL-17A, fibroblast-like synoviocytes, small molecule treatment, animal models, HLA-B27 tg rats

INTRODUCTION

Spondyloarthritis (SpA) is the second most prevalent form of chronic inflammatory arthritis. The hallmarks of SpA are joint inflammation, articular bone erosions and pathologic new bone formation (1). Tumor necrosis factor- α (TNF α) and Interleukin-17A (IL-17A) are key disease-modulating cytokines in SpA (1–4). Currently, one third of patients do not respond to available therapy and only 20% of patients achieve remission. Loss of therapeutic efficacy can occur over time and anti-TNF α therapy does not reduce bone formation in the advanced stages of SpA (5–8). Although anti-IL-17A therapy has been demonstrated to reduce bone formation the *M.tub*-induced HLA-B27 transgenic rat model (HLA-B27 tg rats) (9), a beneficial effect of anti-IL-17A therapy on human SpA bone pathology is not well-understood and remains to be formally established (3, 5). Thus, there is an unmet clinical need to find therapies targeting both inflammation and pathologic bone formation in SpA.

The etiology of the new bone formation in SpA remains unclear (1, 10). We and others (9, 11, 12) have previously demonstrated that fibroblast-like synoviocytes (FLS) isolated from the synovial tissue may act as bone precursor cells and differentiate *in vitro* to osteoblast-like-cells. Osteoblasts are the bone-forming cells responsible for bone matrix and bone mineralization (10). It has also been demonstrated that TNF α and IL-17A can accelerate osteogenic differentiation of FLS cells *in vitro* (9, 12).

The mammalian target of rapamycin (mTOR) has been demonstrated to play an important role in inflammation. For instance, mTOR, can be blocked using rapamycin, a small molecular drug that has been applied clinically to prevent graft rejection in kidney transplantation (13–15). mTOR has been demonstrated to activate T cells and regulate ROR γ translocation in murine cells to induce IL-17A expression (16, 17). Blocking mTOR reduces the percentage of Th17 cells in an animal model of colitis (18). RNA sequencing of inflamed synovial tissue from patients with SpA demonstrated the expression of the PI3K-Akt-mTOR pathway (Chen and Ross et al. under review).

In addition to modifying inflammation, mTOR signaling is downstream of bone anabolic pathways and promotes osteoblastic maturation and mineralization (19).

We sought to examine the effect of mTOR blockade with rapamycin on inflammation as well as new bone formation in the pathobiological context of SpA. Since mTOR pathway regulates the expression of the disease modulating cytokine IL-17A and promotes osteogenic differentiation we hypothesized that treatment with rapamycin may modify both inflammation and pathologic bone formation in SpA pathogenesis. Initially, we determined the effect of rapamycin *in vitro* on primary human SpA cells. Specifically, we investigated if rapamycin could inhibit the production of cytokines by SpA peripheral blood mononuclear cells (PBMCs) and if rapamycin could reduce the rate of human SpA FLS to differentiate to osteoblast-like-cells. Next, we confirmed the activation of mTOR pathway in SpA synovitis. In addition, we determined the prophylactic and therapeutic treatment effect of rapamycin in the *M.tub*-induced HLA-B27 transgenic rat model (HLA-B27 tg rats), an experimental model of SpA (20–22). In the HLA-B27 tg rats, we assessed whether rapamycin would reduce the incidence and severity in inflammation of peripheral joints and spine (arthritis and spondylitis), bone erosions and pathologic new bone formation *in vivo*.

MATERIALS AND METHODS

Human Cells and Tissue

Patient material was obtained from spondyloarthritis (SpA) and rheumatoid arthritis (RA) patients. The SpA patients included in this study fulfilled the Assessment of Spondyloarthritis International Society (ASAS) criteria for peripheral SpA (23). The RA patients were included according to the American College of Rheumatology classification criteria (24). All patients provided written informed consent before enrollment in the study. This study was approved by the Ethics Committee of the Amsterdam University Medical Center, University of Amsterdam, the Netherlands.

In vitro Stimulation of Human PBMCs and SFMCs

Primary human peripheral blood mononuclear cells (PBMCs) were obtained from healthy donors ($n = 3$) and SpA patients ($n = 6$), and synovial fluid mononuclear cells (SFMCs) were obtained from inflamed knee joints from SpA patients ($n = 2$). At the time of inclusion, SpA patients had not taken biologic agents for at least 3 months. PBMCs and SFMCs were isolated by density gradient centrifugation on Lymphoprep (Nycomed).

PBMCs and SFMCs were pre-incubated with vehicle (0.001% DMSO) or rapamycin in IMDM (Lonza) for 30 min and stimulated with anti-CD3 (clone 1XE, 1:1,000, Sanquin) and anti-CD28 (clone 15E8, 2 μ g/ml, Sanquin) for 48 h. Cytokines were measured in supernatants by ELISA (IL-17A and TNF α , eBioscience) according to the manufacturer's recommendations. Counting of viable PBMCs was performed by flow cytometry on an LSR Fortessa X-20 instrument (BD). Exclusion of DAPI (10 nM, 46-Diamidino-2-Phenylindole, Dihydrochloride; Sigma) and PI (3 μ M, Propidium iodide; Sigma) was used to indicate cell viability. Accudrop Beads (BD) were used as the counting standard. Per sample, 10,000 beads were added and PBMCs were counted during the acquisition of 6,000 beads.

In vitro Osteogenic Differentiation of SpA Fibroblast-Like Synoviocytes (FLS)

Primary human SpA FLS ($n = 11$) were obtained from synovial tissue biopsies according to standardized protocol (9, 25). FLS viability was assessed in the presence of vehicle (0.00005%DMSO) or rapamycin (5 nM) with WST-1 assay (Roche) and Trypan Blue 0.4% solution (Gibco) (26). For *in vitro* differentiation, SpA FLS (passage 3–8) were cultured in Xvivo Stempro medium (R&D systems) with 50 μ M ascorbic acid and 10 mM β -glycophosphate, supplemented with TNF α (1 ng/ml), IL-17A (50 ng/ml), or both. Media was refreshed twice weekly. Cells were fixed with 4% formaldehyde at days 7, 14, and 21 of differentiation. Alkaline phosphatase (ALP) staining was performed at days 7 and 14. Alizarin red (2%) staining was performed at day 21 for the osteogenic conditions without cytokines and at day 14 for conditions supplemented with cytokines, consistent with prior published protocols (9). The percentage of ALP staining and alizarin red staining in the wells were scored semi-quantitatively by 2 observers, as described previously (9).

Immunofluorescence

Frozen synovial tissue sections ($n = 20$ from SpA patients and $n = 20$ from RA patients) were fixed and blocked with 10% serum. Staining with isotypes or primary antibodies was performed overnight at 4°C, followed by incubation with Alexa Fluor 488/Alexa Fluor 594-conjugated secondary antibodies for 30 min at room temperature. Antibodies used: monoclonal rabbit IgG anti-human phospho-S6 (pS6, ser235/236, clone D57.2.2E, Cell signaling); monoclonal mouse IgG1 anti-human CD3 (clone UCH-T1; Thermo Scientific Pierce); and monoclonal mouse IgG1 anti-human CD45 (HI30; Biolegend) at a concentration of 5 μ g/ml. Slides were mounted with Prolong Gold with DAPI

(Thermo Fisher). Pictures were taken on an epifluorescence imaging microscope (Leica) and analyzed using ImageJ (1.50i) software. The quantity of pS6 staining was scored on a 3-point semiquantitative scale by two independent observers, who were blinded for the diagnosis of the patients, according to standardized methods (9, 27–29).

Animals

In order to generate *M.tub*-induced HLA-B27/Hu β 2m transgenic rats (HLA-B27 tg rats), the Tg(HLA-B*2705, B2M)21-3Reh and Tg(B2M)283-2Reh Lewis rat lines (30) were bred and housed at the animal research institute of AMC. F1 (21–3 \times 283–2) male and female rats were used for experiments. Animal experiments were approved by the Amsterdam University Medical Center (AUMC) Animal Care and Use Committee.

Male rats were orchietomized to prevent epididymo-orchitis (31), as described previously (22). To synchronize disease onset, 6-week-old, HLA-B27/Hu β 2m transgenic rats were immunized with heat-inactivated *Mycobacterium tuberculosis* (*M. tub*) (Difco, Detroit, MI, USA) in 100 μ l Incomplete Freund's Adjuvant (IFA) (Chondrex, Redmond, WA, USA) as described previously (21, 22).

In vivo Preventive and Therapeutic Treatment With Rapamycin

Rats were treated with 1.5 mg/kg rapamycin or vehicle intra-peritoneally, three times per week for 5 weeks. The prophylactic treatment ($n = 13$ vehicle vs. $n = 11$ rapamycin) started 1 week post-immunization with *M. tub*, which is \sim 1–2 weeks before the onset of arthritis and spondylitis. The therapeutic treatment ($n = 5$ vehicle vs. $n = 4$ rapamycin) started 1 week after 50% of the animals developed arthritis. Vehicle-treated rats were caged separately from rapamycin treated rats.

Clinical Measurement of Arthritis and Spondylitis

The HLA-B27 tg rats were monitored for arthritis and spondylitis incidence and severity, as described previously (9, 21, 22). Clinical measurements were performed by an observer blinded for treatment, and included weight, macroscopic severity scores for arthritis (0–12) and spondylitis (0–3), and hind paw swelling measured by plethysmometry. For the severity analysis, cumulative clinical scores of all limbs were calculated. For plethysmometry, the change in swelling in cm³ was normalized to the measurement on the day before the disease onset as observed clinically (prophylactic experiment) or to the day of treatment start (therapeutic treatment).

Histology

Rats were sacrificed after 5 weeks of treatment. Hind paws (peripheral joints) and the tail (axial joints) were isolated and fixed in 10% formalin, decalcified in Osteosoft (Merck) and embedded in paraffin. For hematoxylin and eosin (H&E) or Safranin O/Fast Green staining, 5 μ m sections were stained and scored by two observers blinded to the identities of the treatment groups. Semi-quantitative scoring was performed for

inflammation, bone erosions, periosteal new bone formation and hypertrophic chondrocytes (enchondral new bone formation) as described previously (21, 22). Images were obtained with a light microscope (Leica).

Gene Expression Analysis

Metacarpophalangeal (MCP) joints were homogenized in TRIzol and further processed for RNA isolation on columns (RNAeasy mini columns, Qiagen) according to manufacturer's protocol.

qPCRs were performed with SYBR green primers (Life technologies) for *IL-17A*, *IL-17E*, *IL-22*, *TNF α* , *IL-23*, *RORC*, *IFN γ* , *IL-4*, with *GAPDH* as the reference gene. Data were represented as relative fold-changes [according to the $2^{-\Delta\Delta Ct}$ method (32)] to one reference control sample. *GAPDH* expression was detected in all samples with <22 Ct cycles. When a gene was not detectable, Ct 40 was used for the calculation of the relative fold.

Statistics

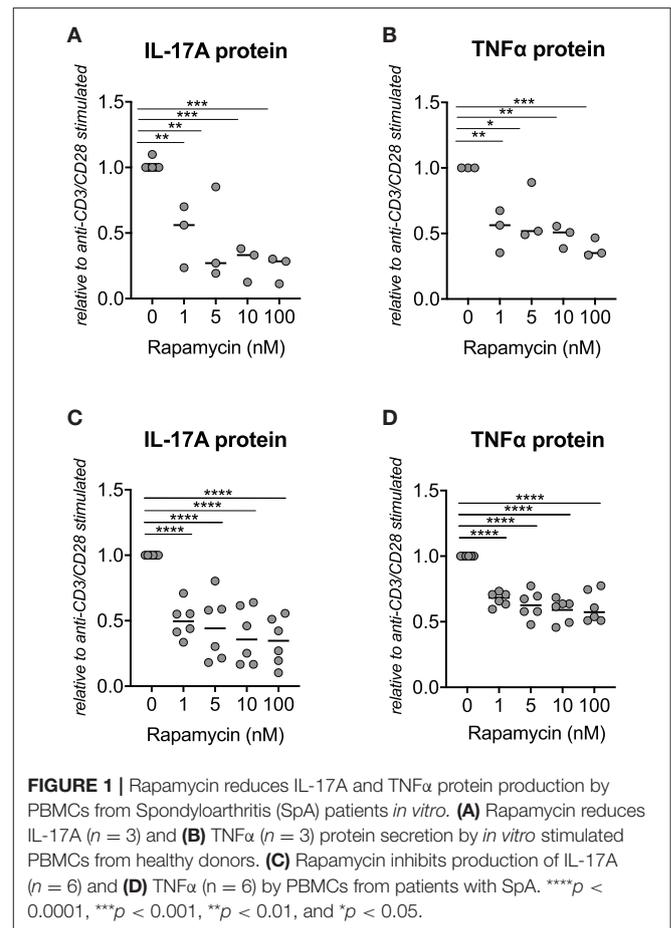
Graphpad Prism 7 was used to perform statistical analysis. For all normally-distributed continuous data, the one-way ANOVA was performed with multiple comparison adjustments according to Bonferroni. Survival curves were analyzed for arthritis and spondylitis incidence and compared with the Log-Rank (Mantel-Cox) test. The Area Under the Curve (AUC) was calculated for clinical scores and hind paw swelling and analyzed with a Mann-Whitney *U*-test. For non-normally distributed data and nominal data, Mann-Whitney *U*-test was used.

RESULTS

Rapamycin Inhibits IL-17A and TNF α Protein Production by Human PBMCs From SpA Patients

As mTOR activation has been demonstrated to induce IL-17A expression in murine T cells (16), we hypothesized that rapamycin treatment would inhibit IL-17A production in human PBMCs. We therefore first examined the effect of rapamycin (0, 1, 5, 10, 100 nM) in healthy donor PBMCs stimulated with anti-CD3/CD28 antibodies. Rapamycin significantly reduced IL-17A and TNF α protein secretion by *in vitro* stimulated healthy donor PBMCs, with a reduction of 51.8% for IL-17A and 47.0% for TNF α at 1 nM, respectively (Figures 1A,B). Rapamycin did not induce cell death over the culture period of 48 h as measured by DAPI and PI staining by flow cytometry (Supplemental Figure 1).

Similarly, rapamycin significantly reduced IL-17A and TNF α production by human SpA PBMC: 1 nM of rapamycin induced a 50.0% reduction of IL-17A and 32.7% reduction of TNF α production (Figures 1C,D). A similar but non-significant trend was also observed when human SpA SFMCs were treated *in vitro* with rapamycin (Supplemental Figures 2A,B).



Rapamycin Reduces Osteogenic Differentiation of Human SpA Fibroblast-Like Synoviocytes (FLS)

In bone precursor cells, the mTOR pathway has also been reported to promote osteogenesis through regulation of bone anabolic pathways (19). To study a potential direct effect of mTOR inhibition by rapamycin on osteoblastic differentiation within the inflammatory context of SpA, we performed *in vitro* osteogenic differentiation assays with human FLS, in the presence and absence of key proinflammatory cytokines IL-17A and TNF α (9). During the differentiation process, we stained the cells for Alkaline phosphatase (ALP) and for mineralization with alizarin red. ALP is expressed early in the osteogenic differentiation process and mineralization is a characteristic of matured osteoblasts.

A dose-response experiment showed that ≥ 5 nM rapamycin consistently reduced osteogenic differentiation of human SpA FLS *in vitro*, as evidenced by alizarin red staining (data not shown). There was no increase in cell death after rapamycin treatment, as demonstrated by WST-1 assay and by Trypan Blue staining (Supplemental Figure 3). We confirmed this effect in FLS cells from additional SpA patients, finding that rapamycin (5 nM) treatment significantly reduced ALP staining, a marker

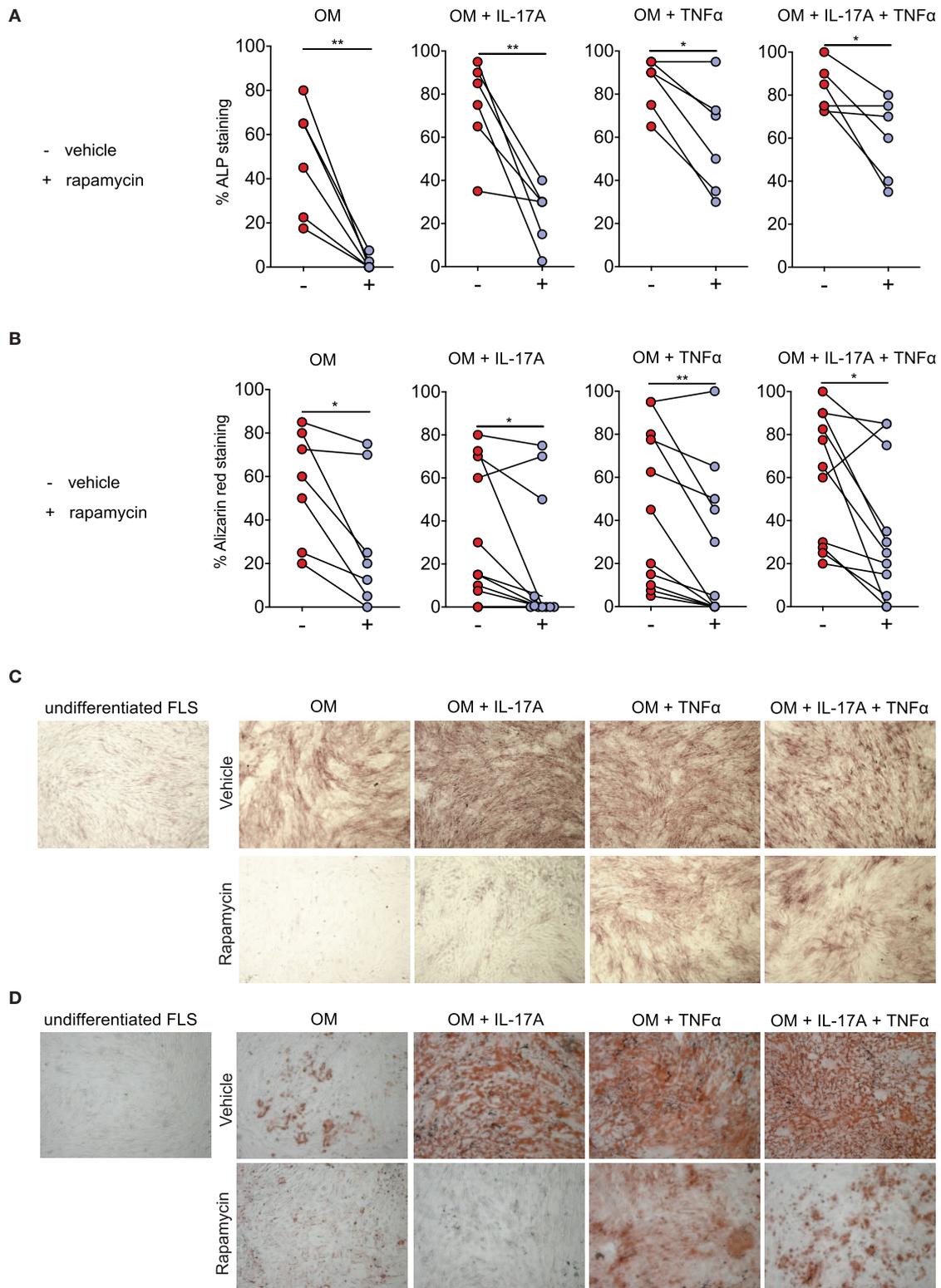


FIGURE 2 | Rapamycin reduces Spondyloarthritis (SpA) fibroblast-like synoviocytes (FLS) osteogenesis. **(A)** Rapamycin reduces osteogenic differentiation as evidenced by percentage of Alkaline phosphatase (ALP) staining at day 14 ($n = 6$), as well as by **(B)** percentage of alizarin red staining at at day 21 for OM ($n = 7$), and day 14 for the conditions with cytokines ($n = 11$). **(C)** Representative pictures of ALP staining and **(D)** alizarin red staining. OM: osteogenic media. $**p < 0.01$ and $*p < 0.05$.

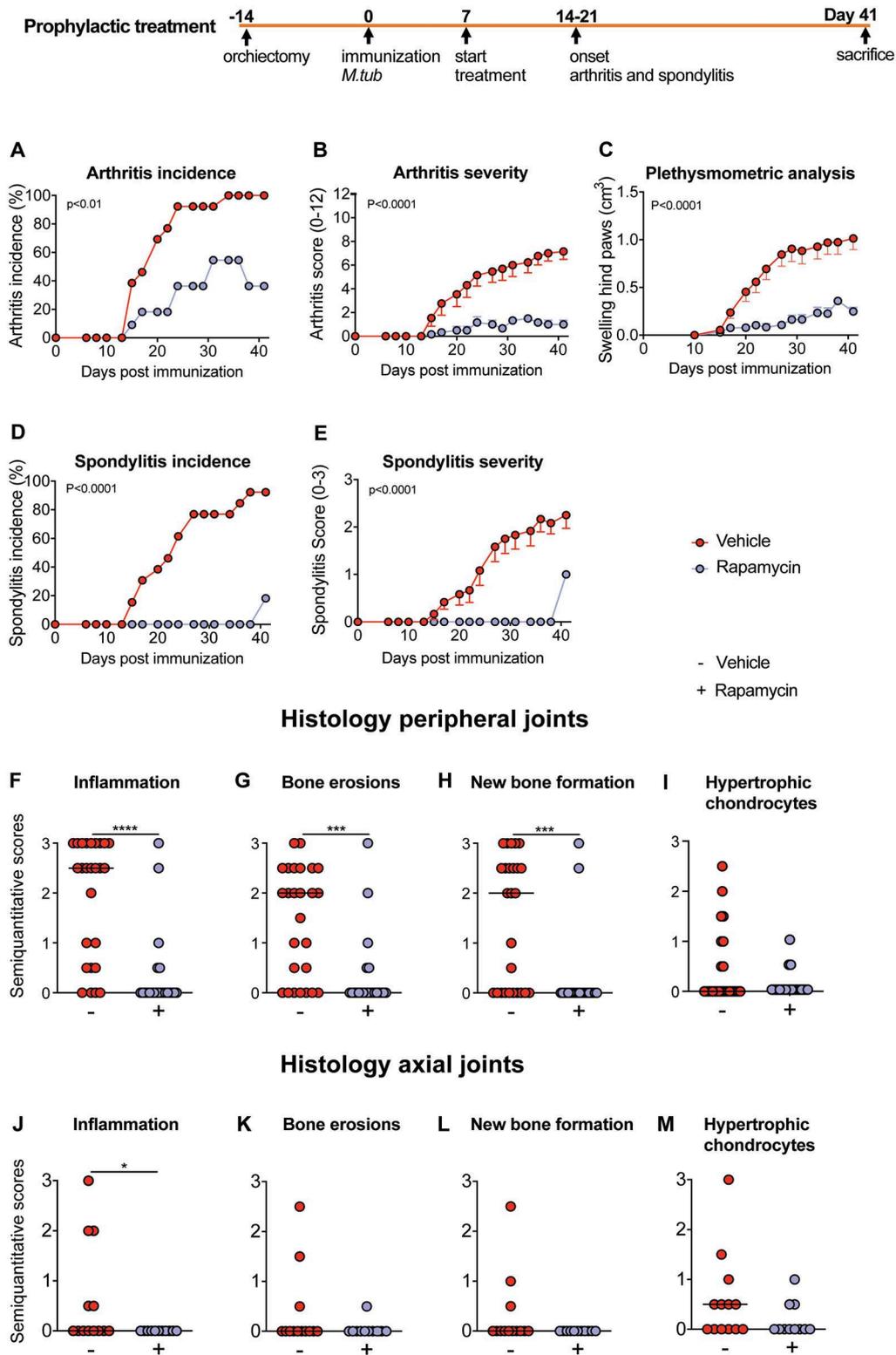
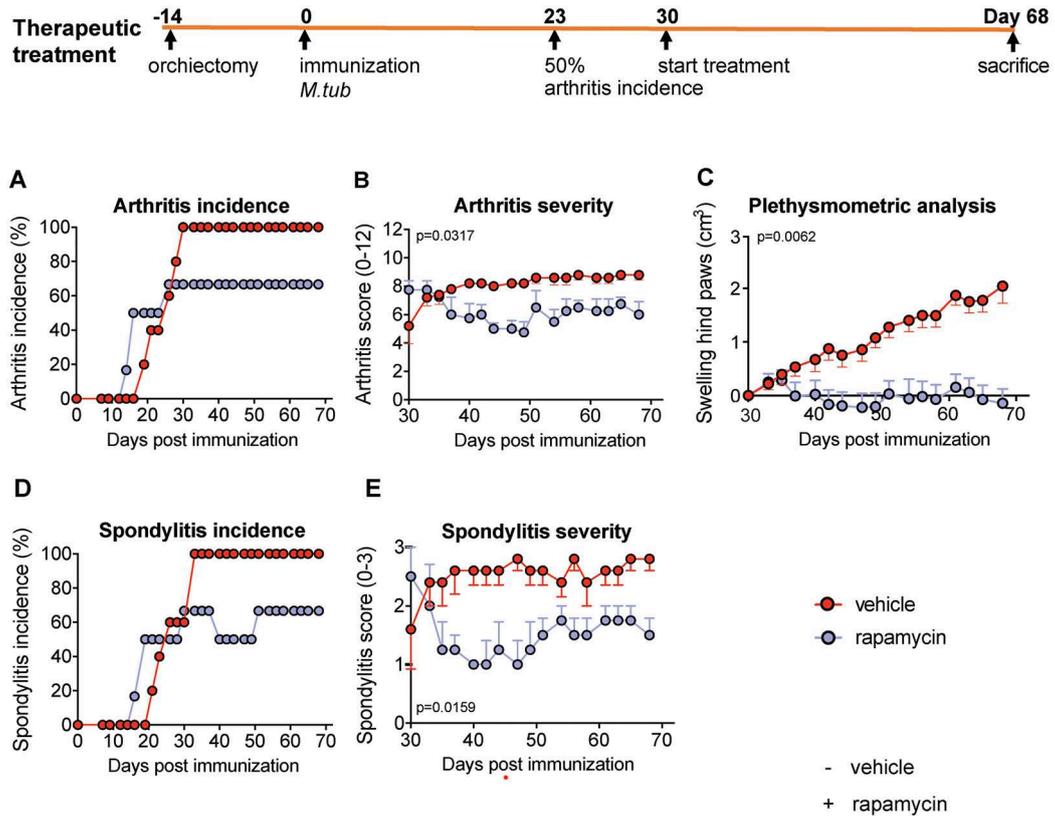
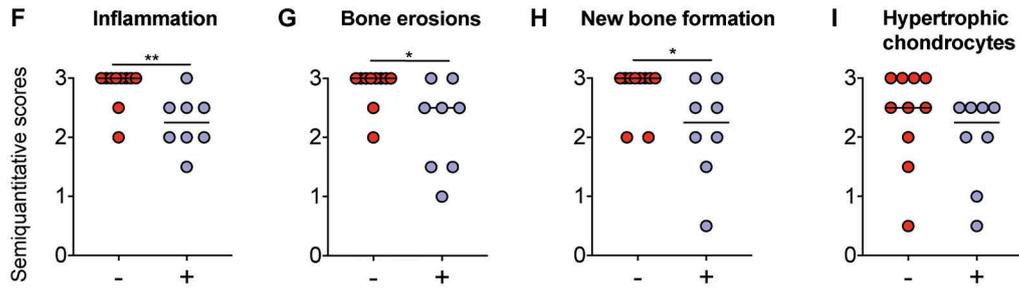


FIGURE 4 | Preventive treatment with rapamycin reduces experimental spondylarthritis *in vivo*. **(A)** Arthritis incidence. **(B)** Arthritis severity. **(C)** Plethysmometric analysis of the hind paws. **(D)** Spondylitis incidence and **(E)** spondylitis severity of vehicle ($n = 13$) and rapamycin treatment group ($n = 11$). **(F–I)** Semiquantitative scoring of inflammation, bone erosions and new bone formation in peripheral joint histology and in **(J–M)** axial histology. Mean \pm SEM were presented in **(B,C,E)**. Median was depicted in **(F–M)**. *M. tub*: *Mycobacterium tuberculosis*. **** $p < 0.0001$, *** $p < 0.001$, and * $p < 0.05$.



Histology peripheral joints



Histology axial joints

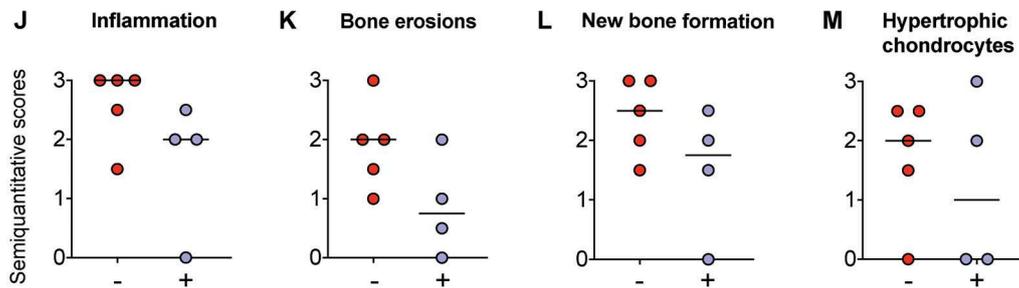
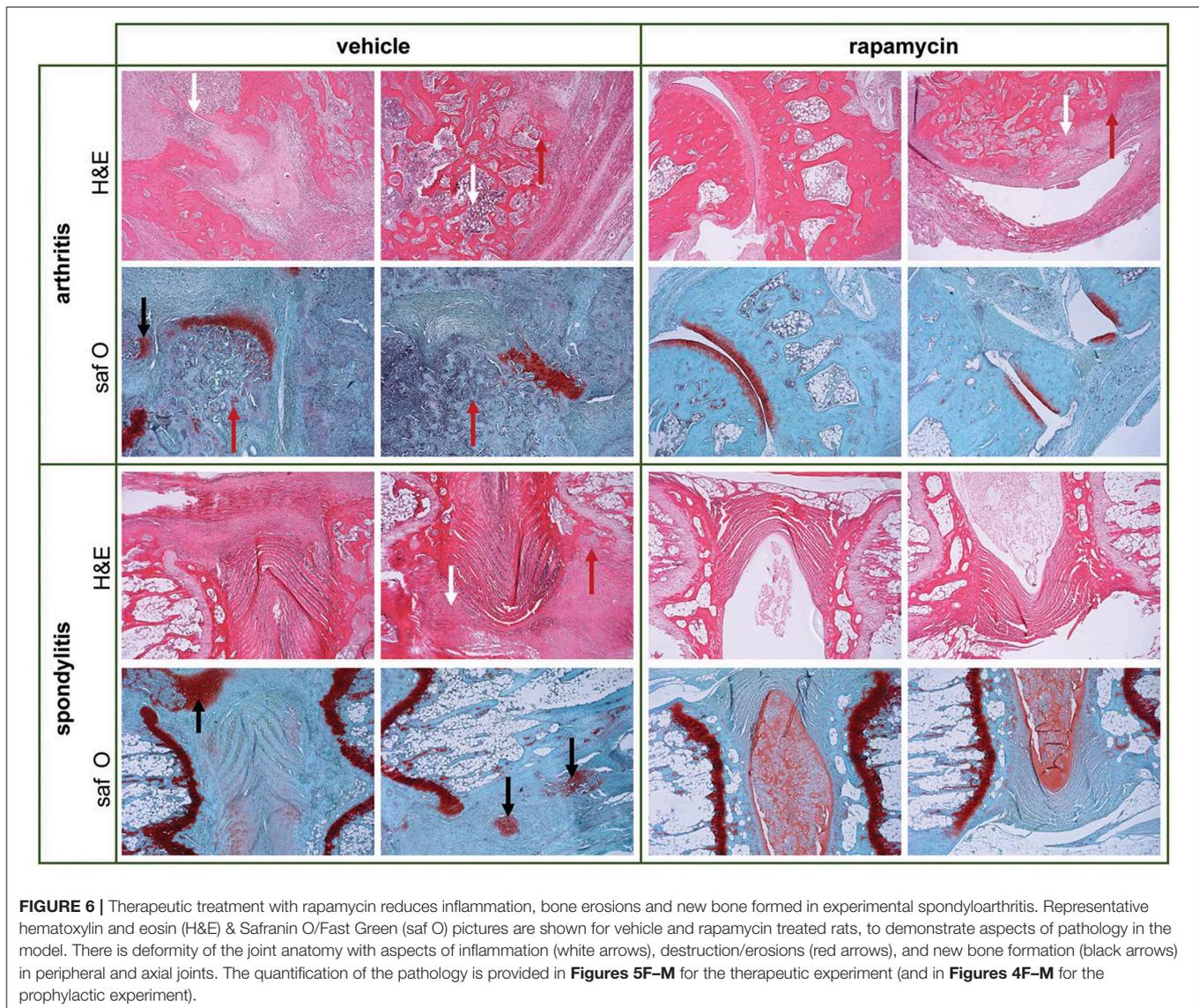


FIGURE 5 | Therapeutic treatment with rapamycin attenuates experimental spondyloarthritis *in vivo*. **(A)** The incidence of arthritis and **(D)** spondylitis are similar in the vehicle ($n = 5$) and rapamycin ($n = 4$) group at treatment initiation. In the diseased animals, treatment with rapamycin **(B)** diminished arthritis severity and **(E)** spondylitis severity. **(C)** Plethysmometric analysis of hindpaw swelling. **(F–I)** Semi-quantitative scoring of inflammation, bone erosions and new bone formation in peripheral joint histology and in **(J–M)** axial histology. Mean \pm SEM were presented in **(B, C, E)**. Median was depicted in **(F–M)**. *M.tub*: *Mycobacterium tuberculosis*. ** $p < 0.01$ and * $p < 0.05$.



of arthritis and spondylitis continued to increase to 100% within 2 weeks after treatment initiation, whereas the incidence of both arthritis and spondylitis completely plateaued once rapamycin treatment was initiated (**Figures 5A,D**). Furthermore, therapeutic treatment with rapamycin significantly diminished arthritis severity (6 vs. 8.8; **Figure 5B**) and spondylitis severity (1.5 vs. 2.8; **Figure 5E**). In agreement with the clinical scores, plethysmometric analysis demonstrated a significant reduction in hind paw swelling in the rapamycin group compared to vehicle controls (**Figure 5C**). Histological analysis of peripheral joint tissues obtained 5 weeks after initiation of treatment confirmed the impact of rapamycin treatment on inflammation (**Figure 5F**), bone erosions (**Figure 5G**), and new periosteal bone formation (**Figure 5H**). A similar, but not significant trend was observed for hypertrophic chondrocytes in peripheral joints (**Figure 5I**). Histological analysis of the spine revealed similar trends (**Figures 5J–M**). Representative H&E and Safranin

O staining of the peripheral joints and spine are shown in **Figure 6**.

Rapamycin Treatment Reduced IL-17A Expression in Inflamed Joints

We demonstrated that rapamycin inhibits IL-17A and TNF α production by human SpA PBMCs *in vitro*. To test the *in vivo* efficacy of rapamycin in the HLA-B27 tg rat model, we assessed the mRNA expression of key inflammatory cytokines such as IL-17A and TNF α in metacarpophalangeal (MCP) joints after therapeutic treatment with rapamycin. mRNA expression of *IL-17A*, *IL-17F*, and *IL-22* was significantly reduced in the MCP joints from the rapamycin vs. vehicle treatment group (**Figure 7**). This was paralleled by a trend toward reduction of the IL-17 master transcription factor *RORC*, but not the upstream Th17 differentiation cytokine *IL-23*. Also *IFN γ* mRNA expression was significantly reduced. There was a decreasing trend observed

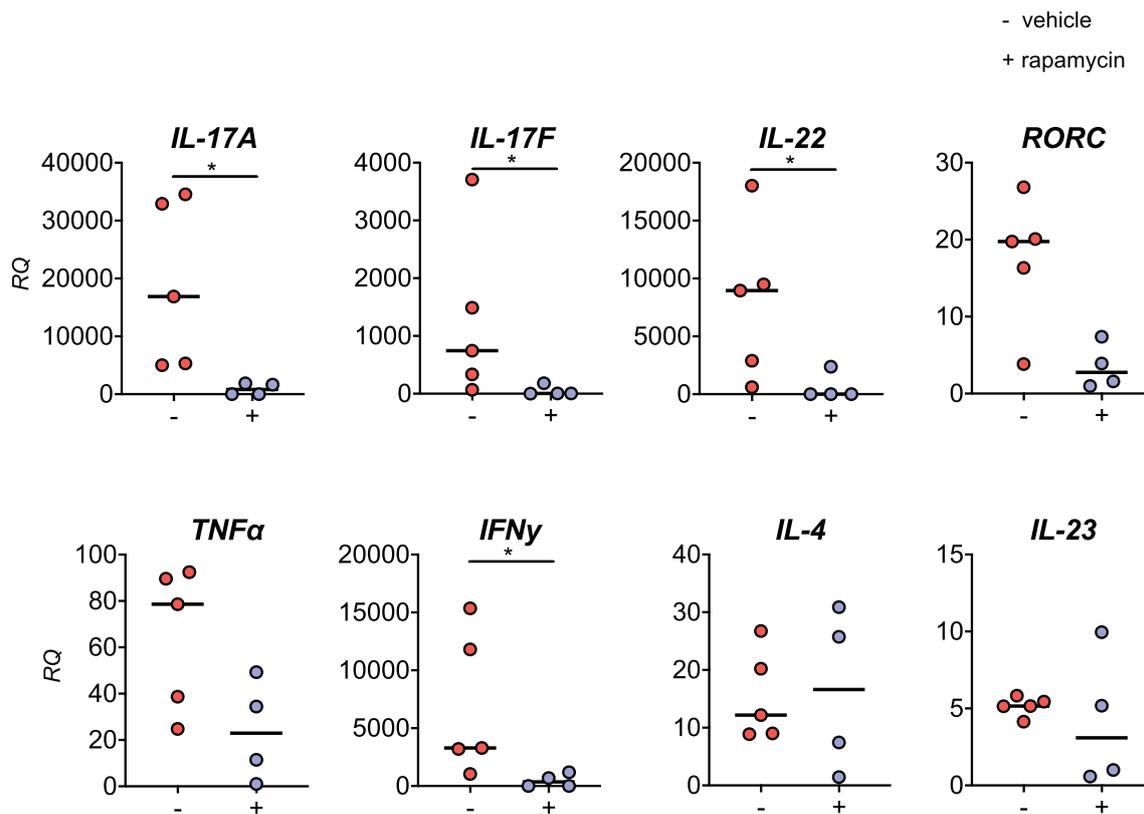


FIGURE 7 | Rapamycin treatment reduces IL-17A expression in inflamed joints. In the metacarpophalangeal (MCP) joints from HLA-B27 tg rats after the therapeutic treatment, mRNA expression of *IL-17A*, *IL-17F*, *IL-22*, and *IFNγ* are reduced in the rapamycin group. RQ, relative quantity. * $p < 0.05$.

for *TNFα*, whereas the prototypical Th2 cytokine *IL-4* was not modified.

DISCUSSION

We demonstrate here for the first time that targeting mTOR by rapamycin, prophylactically and therapeutically, reduces the incidence and severity of arthritis and spondylitis. Histology confirmed reduced inflammation, bone erosions and new periosteal bone formation in the peripheral joints with similar trends observed in the spine. These findings were supported by data we obtained *in vitro* in human SpA cells and from human synovial tissue *ex vivo*: rapamycin attenuated inflammatory cytokine production by human SpA PBMCs; rapamycin reduced human SpA fibroblast-like synoviocytes (FLS) osteogenesis rate, both in the presence and absence of *TNFα* and *IL-17A*; and the mTOR pathway is activated in human SpA synovitis.

Taken together, these data suggest that mTOR blockade by rapamycin attenuates inflammation, bone remodeling and new periosteal bone formation, which are all hallmarks of SpA pathogenesis. mTOR might be a promising therapeutic target in SpA patients, especially considering the efficacy of rapamycin in reducing new bone formation and bone erosions *in vivo*. This would address an important unmet clinical need in SpA patients to target bone pathology (1, 5).

Two potential explanations for the inhibitory effect of rapamycin on new bone formation in the HLA-B27 tg model are:

it may either be the result of rapamycin reducing expression of the cytokine *IL-17A*, or a direct inhibitory effect of rapamycin on bone precursor cells. We have previously demonstrated that *IL-17A* promotes pathologic bone processes in the HLA-B27 tg rats and shown that *IL-17A* accelerates osteogenic differentiation of human SpA FLS *in vitro* (9). In line with these findings, others have reported that *IL-17A* accelerates osteogenic differentiation of FLS cells from rheumatoid arthritis (RA) and osteoarthritis (OA) patients (12). We now demonstrate that FLS osteogenesis can be inhibited by rapamycin treatment, independently of *IL-17A* and *TNFα* cytokines. Rapamycin treatment in SpA PBMCs *in vitro* also inhibits production of *IL-17A* and *TNFα*. In addition to *TNFα* (1) and *IL-17A* (2, 3), *IL-17F* has recently been demonstrated to play an essential proinflammatory role in SpA pathogenesis, similarly to *IL-17A* (36). *IL-17A* and *IL-17F* mRNA expression were significantly reduced *in vivo* after rapamycin treatment.

Although we did not directly test this, rapamycin may suppress inflammation by promoting autophagy of misfolded HLA-B27. HLA-B27 heavy chain misfolding has been postulated to play a role in SpA pathogenesis (37, 38). In a HLA-B27 transgenic rat model (33-3 rats), rapamycin promoted autophagy-mediated degradation of misfolded B27 heavy chains *in vitro* and could thereby suppress the *IL-23/IL-17* pathway (39-41). The presence of HLA-B27 misfolding and the effect of rapamycin treatment on autophagy remains to be tested in our model (21-3 × 283-2 rats).

Rapamycin may also have a direct effect on bone differentiation via mTOR's interaction with several anabolic bone pathways (19, 42, 43). mTOR is a kinase that forms mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (19), and these complexes integrate signals from a multitude of signaling pathways, including Wnt, PI3K-Akt, IGF, Notch, BMP, and mechanical stress (19, 44–52). Prolonged treatment with rapamycin has been demonstrated to inhibit both mTORC1 and mTORC2 (53), and likely alters bone pathway signaling. The exact mechanism of new bone formation in SpA remains to be elucidated (10, 54). In another model of ectopic bone formation, heterotopic ossification (HO), rapamycin treatment reduced ectopic bone by 50% (55). How the pathways involved in heterotopic ossification (HO) compare to those of SpA may be of interest.

In addition to bone precursor cells and osteoblasts, osteoclasts are also important cellular players in bone remodeling. We did not address osteoclasts in the present study, as the effect of rapamycin on osteoclasts has been studied previously. Inhibition of mTOR has been demonstrated to halt osteoclastogenesis (56, 57) and to improve joint erosions in a TNF-transgenic mice model of rheumatoid arthritis (33). These findings are in line with the attenuated bone erosions we observe in the HLA-B27 tg rats after rapamycin treatment, which could be explained by the inhibitory effect of rapamycin on IL-17A and TNF α production, as both these cytokines promote osteoclastogenesis (58–61).

Given that there is currently limited therapy in SpA, these results may support efforts to evaluate the efficacy of rapamycin treatment for SpA patients. Rapamycin has also been found to reduce inflammation in animal models of psoriasis (62) and colitis (18, 63). These disease manifestations can co-occur with SpA and have overlapping disease mechanisms with SpA (1). The side-effects of rapamycin have been characterized and include hyperlipidemia and osteonecrosis (64). Recently, low dose of rapamycin has been demonstrated to be efficacious in reducing musculoskeletal manifestations in mildly active SLE patients without serious side-effects (65). It is also promising that short-term side-effects such as dyslipidemia may subside after long-term mTOR blocking therapy (64). Moreover, there are new generation small molecules that target the mTOR pathway with potentially fewer side effects. The upstream PI3K/Akt/mTOR pathway may also present an additional set of potential targets for modulating mTOR activity in SpA pathology.

CONCLUSIONS

We provide a rationale for targeting the mTOR pathway in spondyloarthritis by demonstrating that mTOR blockade with rapamycin inhibits IL-17A and TNF α production by SpA PBMCs and osteoblastic differentiation of human SpA FLS *in vitro*. In the HLA-B27 transgenic rat model of SpA, mTOR blockade reduces arthritis and spondylitis development and severity with decreased inflammation and bone defects with suppression of IL-17A. These results may support efforts to evaluate the efficacy of targeting the mTOR pathway in SpA patients.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the Amsterdam University Medical Center, University of Amsterdam, the Netherlands. The patients/participants provided their written informed consent to participate in this study. Animal experiments were approved by the Amsterdam University Medical Center (AUMC) Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

SC, LD, and DB contributed to study design, data collection, analysis, interpretation, and wrote the manuscript. MT, VK, LK, LB, and DP contributed to data collection, analysis, and interpretation and revised the manuscript. JT, EG, and MS contributed to analysis, interpretation of the data and critically revised the manuscript. All authors read and approved the submitted version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2019.02344/full#supplementary-material>

Supplemental Figure 1 | Viability of PBMCs ($n = 3$) in the presence of vehicle (DMSO) and rapamycin. **(A)** FACs gating strategy for DAPI and PI staining. **(B,C)** The percentage of viable cells are shown, normalized to (untreated) control conditions after 48 h of culture (mean \pm SD, $n = 3$).

Supplemental Figure 2 | The effect of rapamycin on IL-17A and TNF α protein production by synovial mononuclear cells (SFMCs) from SpA patients ($n = 2$) *in vitro*. **(A)** IL-17A and **(B)** TNF α protein concentrations were measured in the supernatant.

Supplemental Figure 3 | Viability of fibroblast-like synoviocytes (FLS) in the presence of vehicle (DMSO) and rapamycin (5 nM). **(A)** Measurements by WST-1 assay after 30 min and **(B)** after 2.5 h (mean \pm SD, $n = 4$). **(C)** The percentage of Trypan Blue-negative cells after 48 h of culture (mean \pm SD, $n = 3$). ns, not significant.

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Conflict of Interest: DB is an employee of UCB.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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