Inhibition of TGF-β Signaling Suppresses Th17 Differentiation and Promotes Treg Numbers but Does Not Reduce Experimental Arthritis.

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Abstract

Objectives

TGF-β is an important growth factor to promote the differentiation of T helper 17 (Th17) as well as regulatory T cells (Treg). Due to its dual role, the potential of TGF-β as therapeutic target in T cell-mediated diseases like rheumatoid arthritis (RA) is unclear. In this study, we investigated the effect of TGF-β inhibition on murine Th17 differentiation *in vitro*, on human RA synovial explants *ex vivo*, and on the development of experimental arthritis *in vivo*.

Methods

Murine splenocytes were differentiated into Th17 cells, and the effect of the TGF-βRI inhibitor SB-505124 on Th17 differentiation was studied. RA synovial biopsies were cultured for 24h in the presence or absence of SB-505124. Experimental arthritis models were induced in C57Bl6 mice, and were treated daily with SB-505124. FACS analysis was performed to measure different T cell subsets. Histological sections were analysed to determine joint inflammation and destruction.

Results

SB-505124 potently reduced murine Th17 differentiation by decreasing *Il7a* and *Rorc* gene expression and IL-17 protein production. SB-505124 significantly suppressed IL-6 production by RA synovial explants. In the Th17-driven arthritis model, SB-505124 reduced Th17 levels, while increased levels of Tregs were observed. Despite this skewed Th17/Treg balance, SB-505124 treatment did not result in suppression of joint inflammation and destruction in this model.

Conclusions

Blocking TGF-β signalling suppresses Th17 differentiation and improves the Th17/Treg balance. However, SB-505124 treatment does not suppress experimental arthritis, and is therefore not an adequate way to target Th17-driven inflammation.

Key Messages

The potential of TGF-β as target in T cell-mediated diseases like rheumatoid arthritis is unclear.

Blocking TGF-β signalling suppresses Th17 differentiation and improves the Th17/Treg balance.

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease of unknown etiology, that is characterized by chronic joint inflammation leading to destruction of articular cartilage and bone. CD4+ T cells are found in inflammatory infiltrates of the rheumatoid synovium and are known to play a central role in the

development of RA [1–4]. In patients with RA, the T helper 17 (Th17) and regulatory T cells (Treg) balance is skewed in favor of Th17 cells, which contributes to the development of autoimmunity and inflammation [5].

Transforming growth factor beta (TGF-β) is a growth factor that can differentiate CD4+ T cells into Th17 cells in the presence of IL-6 or IL-21 [6]. TGF-β signaling and STAT3 activate and regulate expression of the transcription factor RAR-related orphan receptor C (*RORc*), the master regulator of Th17 cells [7,8]. There are three isoforms of TGF-β: TGF-β1, TGF-β2, and TGF-β3 [9]. Lymphoid cells produce mainly TGF-β1, which has a controversial role in inflammation and autoimmunity as it both suppresses and induces immune reactions [10]. Three canonical TGF-β receptors have been described: TGF-βRI, TGF-βRII, and the co-receptor TGF-βRIII, that together form the TGF-β receptor complex. When TGF-β binds to this receptor-complex, receptor-regulated Smad (R-Smad) proteins are phosphorylated and translocate to the nucleus to modulate target gene expression [11].

In diseases such as cancer and atherosclerosis, the role of TGF-β is well defined, but its role in (experimental) arthritis is still not clear. TGF-β is a regulatory cytokine, having pleiotropic functions on immunity including promoting the expansion of Tregs [12] and regulation of CD4+ T cell polarization [13]. TGF-β promotes the differentiation of Th17 as well as of Treg cells, depending on the cytokine environment [14]. This has resulted in quite some opposing phenotypes in mice transgenic for the TGF-β signaling pathway. On the one hand, mice with T cell-specific deletion of TGF-βRII show uncontrolled T cell activation and massive and fatal multi-organ inflammation [15]. On the other hand, mice with a deletion of the *Tgfb1* gene selectively in CD4+ and CD8+ T cells have a defect in the generation of Th17 cells that protected them from experimental encephalitis (EAE) [16]. In line with that, mice subcutaneously injected with 100 µg anti-TGF-β1,2,3 antibody 1D11 failed to differentiate naïve CD4+ T cells into Th17 cells and were also protected from EAE [17].

Due to its dual role in regulating the immune system, the potential of targeting the TGF-β pathway as therapy in RA is unclear. In this study, we aimed to investigate the effect of inhibition of TGF-β signaling with the TGF-βRI inhibitor SB-505124 on murine Th17 differentiation *in vitro*, on cytokine production by human RA synovial explants *ex vivo*, and to study the effect of local SB-505124 treatment *in vivo* during Th17-driven experimental arthritis.

Materials And Methods

Patient donors

Synovial tissues from eight RA patients were obtained during joint replacement surgery from the orthopedics department of the Sint Maartenskliniek, Nijmegen, The Netherlands. This material was considered surgery surplus material; therefore, its use did not need to be approved by an ethical committee. All patients adhered to the American College of Rheumatology (ACR) criteria and were end-

stage RA. Patients gave written informed consent for the use of their material for research. The patient material was pseudonymized. Procedures were performed in accordance to the code of conduct for responsible use of human tissue in medical research. The presence of a synovial lining was determined on 5 µm cryosections stained with hematoxylin and eosin (H&E) to confirm the synovial origin of the tissue.

Mice

Female C57BL/6N were purchased from Janvier-Elevage (Le Genest Saint Isle, France). Animals were used between 10 and 12 weeks. A standard diet and water were provided ad libitum. All animal procedures were approved by the ethics committee of the Radboud University Nijmegen (permit RU-DEC 2018-0037).

Murine Th17 differentiation

T lymphocytes from spleens were isolated from naïve C57Bl6 mice as previously described [ref] 500,000 cells/well were cultured in a 24-wells plate for five days at 37°C in an atmosphere of 5% CO2, in X-vivo medium (Lonza, LOT 8MB036) supplemented with 1% Penicillin-Streptomycin (Lonza 09-757F) and 100 mg/ml streptomycin (Gibco). Cells were activated with plate-bound anti-CD3 (5 µg/ml; Biolegend; clone 17A2) and soluble anti-CD28 (2.5 µg/ml; Biolegend; clone 37.51). After 2 hours preincubation with or without 5 µM SB-505124, cells were differentiated into Th17 cells with αIL-2 (10 µg/ml; Biolegend; clone JES6-1A12), IL-1β (10 ng/ml; Biolegend), IL-6 (50 ng/ml; ITK diagnostics), IL-23 (10 ng/ml; ITK diagnostics) and TGF-β1 (1 or 10 ng/ml, Biolegend). After five days of differentiation, the supernatant was collected for cytokine measurement and the cells were processed for RNA isolation.

Luminex

Human and murine cytokines and chemokines (IL-17A, IL-10, TNF-α, GM-CSF, IFN-γ, IL-6 and IL-4) were measured by Luminex using BioPlex kits according to manufacturer's instructions, Bio-Rad Laboratories) and analyzed using BioPlex Manager 4 software.

Flow cytometry

Cells were stimulated for 4 hours with phorbol myristate acetate (PMA; 50 ng/ml; Sigma-Aldrich), ionomycin (1 µg/ml; Sigma-Aldrich), and the Golgi-traffic inhibitor Brefeldin (1 µl/ml: BD Biosciences, Franklin Lakes, NJ, USA). Next, cells were stained with anti-CD3-FITC (145-2C11) (Biolegend), anti-CD4-PerCP/Cy5.5 (RM4-4) (Biolegend), fixable viability dye eFluor 780 (eBioscience), fixed and permeabilized using Cytofix/Cytoperm (BD Biosciences), followed by intra-cellular staining with anti-IL-17-PE (TC11-18H10.1) (Biolegend), anti-FOXp3-Alexa Fluor® 647 (150D) (Biolegend) and anti-RORγt-BV421 (Q31-378) (BD Biosciences). Cells were analyzed on the Cytoflex and subsequent Kaluza software version 2.1.

RNA isolation and quantitative real-time PCR

Cells from *in vitro* studies were collected in TRIreagent for further RNA isolation as previously described [18]. Gene expression levels were determined by QPCR on the StepOnePlus sequence detection system

(Applied Biosystems) using SYBR Green (Applied Biosystems) and 0.2 µM primers (Biolegio). GAPDH was used as reference genes. Relative quantification of the PCR signals was performed by comparing the cycle threshold (Ct) value, of the gene of interest for each sample with the Ct values of the reference gene. Primer sequences were as follows (Table 1):

Table 1
List of murine oligonucleotide primer sequences.

Genes	5'- 3' forward	5'-3' reverse
Il17a	caggacgcgcaaacatga	gcaacagcatcagagacacagat
Rorc	ctgtcctgggctaccctactga	aagggatcacttcaatttgtgttctc
Il22	ggtgcctttcctgaccaaac	cgtcaccgctgatgtgaca
Gapdh	ggcaaattcaacggcaca	gttagtggggtctcgctcctg

Methylated bovine serum albumin/interleukin-1 (mBSA/IL-1) induced-arthritis

Acute inflammatory arthritis was induced as previously described [19] by intra-articular (i.a.) injection with 200 µg of mBSA (Sigma A-1009), followed by daily injections on days 0–2 with 250 ng of human IL-1β (Biolegend). At sacrifice, knee joints were isolated for histological analysis and QPCR. Draining popliteal and inguinal lymph nodes were isolated for FACS analysis. The TGF-βRI inhibitor SB-505124 was applied locally starting two hours before the induction of arthritis, and mice were injected i.a. on four consecutive days with either saline, 20% DMSO as vehicle control, or 75 nmol SB-505124 in 20% DMSO with an injection volume of 6 µl per joint (Sigma). Anti-IL-17a antibodies (BioXCell) were used as positive control, administered intra-peritoneally 50 µg/mouse injected every other day. Mice were sacrificed by cervical dislocation four days after arthritis induction, and knee joints were subsequently isolated for histological analysis.

Streptococcal cell wall (SCW) arthritis induction

Streptococcus pyogenes T12 organisms were cultured overnight in Todd-Hewitt broth. Cell wall fragments were prepared as described previously [20]. Arthritis was subsequently induced by injecting 25 µg SCW fragments into the knee joints of C57BL/6N mice, resulting in local, acute inflammation. Local SB-505124 treatment was applied as described above.

Histology

Histology was processed and scored in line with the 'SMASH' recommendations for standardized microscopic arthritis scoring [21]. In short, isolated joints were fixed for at least four days in 4% formaldehyde, decalcified in 5% formic acid, and subsequently dehydrated and embedded in paraffin. Standard frontal sections of 7 µm were stained with H&E or Safranin O (SO) to study joint pathology. The

severity of arthritis was scored on an arbitrary scale of 0–3, where 0 = no pathology and 3 = maximal pathology, for three different parameters (joint inflammation, cartilage proteoglycan (PG) depletion, and bone erosion), on three semi-serial sections of the joint, spaced 140 μm apart, in a blindfolded manner.

Immunohistochemistry

Protein expression of phosphorylated Smad2/3 was evaluated on 7 μm paraffin-embedded sections of murine synovial tissue after co-injection with recombinant 10 ng TGF-β and 75 nmol SB-505124. For immunohistochemistry, endogenous peroxidase activity was blocked with 3% H2O2 (Merck Millipore) in methanol, and antigen retrieval was performed in 10 mM citrate buffer, pH 6.0 at 60°C. Subsequently, sections were stained with primary antibodies: rabbit anti-mouse pSmad2/3 (Cell Signaling #3108) (1:300 for 60 min at RT), mouse or isotype. Subsequently, the primary antibodies were stained with biotinylated anti-mouse IgG H + L (1:100 for 30 min at RT). Next, a biotin-streptavidin detection system was used according to the manufacturer's protocol (PK-6101; Vector Laboratories). Peroxidase was developed with diaminobenzidine (Sigma Aldrich) and counterstained with hematoxylin for 60 sec.

Synovial explant culture

Synovial tissue was obtained during joint replacement surgery and tissue was freshly processed into standardized biopsies using a 3 mm biopsy punch (Stiefel). The synovial explants were subsequently cultured for 24h at 37°C and 5% CO_2 in 200 μl X-vivo serum-free medium in the presence or absence of 5μM SB-505124. After 24 hours, culture medium was centrifuged 10 min, 241x g to remove remaining cells. Before Luminex analysis, culture medium was centrifuged 10 minutes, 10.000 rpm.

Statistical analysis

To determine the level of statistical significance between means of experimental groups, the Student's *t* test or a one- or two-way analysis of variance (ANOVA) was used unless stated otherwise. This depended on the number of experimental groups and normality testing using GraphPad Prism version 5.03 (GraphPad Software). *P*-values < 0.05 were considered significant.

Results

TGFB-RI inhibitor SB-505124 prevents TGF-β-induced expression of Th17 genes and proteins

To investigate the effect of inhibition of TGF-β signaling on murine Th17 differentiation *in vitro*, murine splenocytes were cultured with different concentrations of TGF-β in the presence or absence of 5 μM SB-505124. First, cells cultured with increasing concentrations of TGF-β showed a dose-dependent increase in IL-17 secretion (Fig. 1A) and a dose-dependent decrease in IFNγ production (Fig. 1B). TGF-β also increased the percentage of Th17 cells (defined as CD4 + IL-17 + cells) when dosed 0.05 to 1 ng/ml (Fig. 1C). Remarkably, higher concentrations of 10 ng/ml and 100 ng/ml of TGF-β decreased the percentage of Th17 cells (Fig. 1C) without affecting the IL-17 production (Fig. 1F), demonstrating that IL-17 protein production not always correlates with Th17 differentiation levels.

Addition of SB-505124 to these Th17 differentiation conditions supplemented with 1 ng/ml TGF-β resulted in significantly decreased *Il17a* mRNA expression, and in a trend for suppressed *IL17a* levels in the 10 ng/ml TGF-β group (Fig. 1D). Even more pronounced effects were observed for the mRNA expression of the lineage-specific transcription factor *Rorc*. In line with the data for *Il17a*, TGF-β significantly increased the expression of *Rorc* compared to the plain Th17 cocktail condition, and this TGF-mediated effect could be completely counteracted by SB-505124 (Fig. 1E). Also at protein level, the inhibiting effect of SB-505124 on TGF-β-mediated Th17 differentiation was observed. However, whereas the TGF-β-induced increase in IL-17 production was completely and significantly abolished by SB-505124 in the 1 ng/ml TGF-β group, the SB-505124-mediated suppression of IL-17 production in the 10 ng/ml TGF-β group is only a trend of 30% reduction. The opposite of the TGF-β- and SB-505124-induced effects on *Rorc* and IL-17 expression and production were observed on IFNγ levels: the presence of TGF-β completely blocked IFNγ production, which was reversed by addition of SB-505124 to the Th17 culture (Fig. 1F and G). These results indicate an important role for TGF-β during murine Th17 differentiation and suggest potency for SB-505124 to intervene in this process.

Inhibiting TGF-β signaling using SB-505124 reduces IL-6 production by human RA synovial explants.

In addition to the observed effect of TGF-β blocking by SB-505124 on murine Th17 differentiation, we investigated the potential of SB-505124 to inhibit the spontaneous production of inflammatory cytokines by human RA synovial explants. Interestingly, SB-505124 significantly inhibited the production of IL-6 (Fig. 2A) by the explants from 5,802 pg/ml +/- 848 (mean +/- SEM) in the control group to 2,565 pg/ml +/- 633 in the SB-505124 group. All our nine donor samples were active IL-6 producing explants that showed SB-505124-inhibited IL-6 production of on average 56%, suggesting that active TGF-β signaling contributes to the inflammation in this synovial tissue. The levels of IL-10 were relatively low (Fig. 2B) and remained unaffected by SB-505124 treatment. For TNFα production, not all donors responded similar to the SB-505124 treatment. Five donors showed decreased levels, two donors increased levels and two donors showed no difference by SB-505124 treatment (Fig. 2C). Other cytokines including IFN-γ, IL-4, GM-CSF, and IL-17 were not detectable.

Intra-articular injection of SB-505124 does not induce joint inflammation and cartilage PG depletion in naïve mice

Before inhibiting TGF-β signaling during experimental arthritis, we first aimed to study the potential joint pathology of SB-505124 treatment in naïve mice, as TGF-β is not only important in T cell activation but also in cartilage homeostasis. Repeated intra-articular SB-505124 injections for four consecutive days did not induce synovial inflammation or cartilage proteoglycan depletion in naïve knee joints as observed on histology at day seven. Also the vehicle control 20% DMSO did not result in joint pathology, as these control mice showed a thin synovial lining and healthy cartilage similar to saline-injected knee joints (Fig. 3). Importantly, we demonstrated that our treatment regimen of daily intra-articular injections of SB-505124 can indeed inhibit local TGF-β signaling. By co-injection of recombinant TGF-β1 and the TGF-βRI inhibitor SB-505124 in naïve murine knee joints, we demonstrated that SB-505124 inhibits TGF-β activity

in vivo, as indicated by reduced pSmad2/3 staining by immunohistochemistry in synovium as marker for active TGF-β signaling (Supplementary Fig. 1&2).

Intra-articular injection of SB-505124 enhances Tregs and suppresses Th17 cell levels in the draining lymph nodes of arthritic mice

To study the effect of inhibition of TGF-β signaling on Th17-driven experimental arthritis, we treated mice during IL-1/mBSA arthritis by daily intra-articular injections with SB-505124. Interestingly, in the draining lymph nodes of SB-505124-treated mice, we observed a significant reduction in CD3 + CD4 + T cells at day 4 (Fig. 4A). Moreover, we observed that inhibition of TGF-β signaling resulted in increased numbers of FOXp3 + CD3 + CD4 + T cells (Tregs) (Fig. 4B) and less RORγt + CD3 + CD4 + T cells (Fig. 4C), although IL-17 + CD3 + CD4 + cells were not altered by SB-505124 treatment (Fig. 4D). This indicates that local treatment with SB-505124 inhibits an important role for TGF-β signaling during T cell proliferation and Th17/Treg differentiation in this experimental arthritis model.

TGF-β signaling inhibition with SB-505124 does not decrease arthritis joint pathology

By subsequent histological analysis, the effect of local TGF-β signaling inhibition on arthritis severity was studied in more detail. Despite the skewed Th17/Treg balance in the draining lymph nodes of these arthritic mice, no differences were observed on inflammation and PG depletion on day four (Fig. 5A) and day seven (Fig. 5B) of arthritis between the SB-505124 group and its vehicle control. As expected, the positive control treatment using anti-IL-17 antibodies significantly reduced arthritis pathology (Fig. 5B). Additionally, no effects were observed in T cell-independent SCW arthritis after local SB-505124 treatment (supplementary Fig. 3).

Discussion

RA patients may benefit from the inhibition of Th17 cells, either by neutralizing their main effector cytokine IL-17 [22], or by targeting further upstream via its transcription factor RORγt [23] or Th17-inducing cytokines [24,25]. In this study, we explored the potential of targeting TGF-β signaling to inhibit Th17 cells, this in view of the importance of this growth factor in the differentiation of these cells.

Previous studies in experimental arthritis models showed contradictory results with pro- or anti-inflammatory roles for TGF-β. For instance, recombinant TGF-β1 injection in naïve rat and mouse joints induced joint inflammation with synovial infiltration of T lymphocytes and neutrophils [26–28]. Treatment of arthritic rats with anti-TGF-β antibodies inhibited inflammation and bone resorption [29]. Additionally, intraperitoneal (IP) injected HTS466284 (TGF-β type 1 receptor kinase inhibitor) prevented experimental arthritis in mice [30]. IP treatment with the specific TGF-β blocking peptide p17 reduced severity of CIA, however these effects were not statistically significant [31].

In contrast, other studies showed that IP administration of TGF-β1 reduced the incidence and severity of CIA, especially when injected in late disease [32,33]. In rats with SCW-induced arthritis, IP [34] and

intramuscular injection [35] of TGF-β at peak of inflammation suppressed the development of arthritis, whereas anti-TGF-β in CIA mice increased pro-inflammatory cytokines and arthritis severity [36,37]. Furthermore, an increase in TGF-β during the remission phase of CIA suggests an important role for TGF-β in regulating the disease [38]. These opposing findings indicate a differential role for TGF-β, probably depending on the type of animal model, route of administration, disease stage, and site of inflammation.

In our *in vitro* studies, we observed that blocking TGF-β during Th17 differentiation efficiently and significantly reduced *Rorc* mRNA expression, but not IL-17 protein production. TGF-β is important in T cell differentiation, but also in dampening of the immune response of various T and B lymphocytes [39]. TGF-β inhibits differentiation and activation of specific T helper subsets by suppressing their lineage-specific transcription factors such as T-Bet and GATA-3, which are critical for Th1 and Th2 responses, respectively [40] The importance of TGF-β in regulating T cell responses *in vivo* has been strengthened by the observation that mice lacking TGF-βRII specifically on T cells develop lethal multi-organ inflammation [15]. Also TGF-β1−/− mice develop multi-organ inflammation due to an impaired control of T cell activation and differentiation [41]. The phenotype of the TGF-β1−/− mice is completely rescued if mice are crossed to an MHCII knockout background, highlighting a crucial role for TGF-β in regulating pathological CD4 + T cell responses [42]. Furthermore, subsequent studies using mice with T cell-specific deletions of Smad2 and Smad3 showed that intracellular signaling via Smad2/3 is essential for the TGF-β-mediated inhibition of effector T cells [43]. In our *in vivo* experiment, intra-articular SB-505124 treatment during experimental arthritis caused a clear reduction in Th17 levels,. However, this did not result in suppression of arthritis, suggesting that (1) the Th17 pathway is not that important in this model, (2) the remaining (Th17) cells are highly active due to loss of TGF-β-mediated dampening, or (3) that the treatment with SB-505124 had a too limited duration in time on TGF- β signaling to suppress the arthritis measured at end stage of our *in vivo* experiment. Unfortunately, our study design did not enable us to investigate the activation of the local Th17 cells and other immune cells by checking cytokine levels in the joints. However, this would be in line with our *in vitro* studies where we observed that blocking TGF-β during Th17 differentiation efficiently and significantly reduced the *Rorc* mRNA expression but not the IL-17 production. From the potent effects of the anti-IL-17 treatment group as reference control, we can conclude that IL-17 is an important cytokine to block during this phase of this arthritis model.

TGF-β is often considered as an immunosuppressive cytokine, inhibiting for instance TNF-α, IFN-γ and IL-1β [10]. Interestingly, when inhibiting the TGF-β signaling pathway by SB-505124 on human RA synovium explants, IL-6 protein production by RA synovium was significantly reduced, suggesting that TGF-β is an important inducer of IL-6 in arthritic synovium. In line with this observation, we have shown that TGF-β stimulates the production of IL-6 by primary articular chondrocytes [44] and by the chondrocyte G6 cell line [44]. TGF-β also stimulates IL-6 production in PBMCs [45]. The other way around, TGF-β down-regulates IL-6 signaling in intestinal epithelial cells [46], showing that TGF-β regulation of IL-6 signaling is cell type, tissue and context dependent. In our *ex vivo* studies, synovial explants are originating from end-stage RA patients undergoing total knee or hip replacement, and thereby less active in secreting cytokines than

early RA tissue. However, even with this less inflamed tissue, we observe an interesting suppression of the proinflammatory cytokine IL-6 in all donors after inhibition of TGF-β signaling by SB-505124.

In our approach we chose to use the small molecule inhibitor SB-505124 to inhibit TGF-β signaling, which has an advantage over antibodies in tissue and cell penetration [47,48]. Disadvantage is the short half-life [49], therefore SB-505124 must be administered frequently. With daily intra-articular injections, we observed that this compound highly efficiently blocked TGF-β-mediated Smad2/3 phosphorylation (supplementary Figs. 1 and 2).

In our proof-of-concept study to demonstrate the potential of controlling Th17 cells by targeting TGF-β signalling, we included the positive control anti-IL17 treatment, since the arthritis model we used is known to be dependent on CD4 + T cells and IL-17 [19,50]. Depletion of CD4 + T lymphocytes in mBSA/IL-1-induced arthritis led to dose-dependent T cell proliferation in draining lymph nodes in response to mBSA. Anti–IL17 antibody administered during our mBSA/IL-1-induced arthritis markedly reduced disease, confirming that the model is indeed IL-17 dependent [19,50]. However, our blocking of TGF-β signalling apparently did not sufficiently suppress the formation or activity of Th17 cells, as arthritis pathology was not affected by SB-505124 treatment, showing that our strategy to target the Th17 pathway upstream is not as effective as blocking the main effector cytokine IL-17 itself.

Conclusions

We revealed suppressive effects of SB-505124 on Th17 differentiation *in vitro* and on the Th17/Treg balance in arthritic mice. However, SB-505124 did not suppress joint inflammation and destruction during experimental arthritis. This indicates that, despite the importance of TGF-β in Th17 differentiation, TGF-β signalling is not an easy target to suppress the arthritis process.

Abbreviations

EAE: experimental encephalitis; H&E: hematoxylin and eosin; PG: cartilage proteoglycan; RA: rheumatoid arthritis; RORc: RAR-related orphan receptor C; SCW: Streptococcal cell wall; Th17: T helper 17 cell; T reg: Regulatory T cell; TGF-β: Transforming growth factor beta.

Declarations

Authors' contributions

J.A. designed the research, performed the experiments, analyzed data, drafted wrote, and edited the paper. R.M., M.H., B.W., E.V., performed the experiments. A.C., P.K., M.K, analyzed data and edited the manuscript. F.L., P.L., edited the manuscript. The authors read and approved the final manuscript.

Availability of data and materials

All data generated or analyzed during this study are included in this published article [and its supplementary information files].

Ethics approval and consent to participate

Synovial tissue was obtained as remnant material from RA patients (n=8) undergoing joint replacement surgery. Written informed consent was obtained from all patients. The study was approved by the local Ethics Review Board (SMK Nijmegen, The Netherlands). Tissue was processed into standardized 6mm biopsies as described previously [51]. Procedures were performed in accordance to the code of conduct for responsible use of human tissue in medical research.

Consent for publication

All authors read the manuscript and gave their consent for publication.

Competing interests

The authors declare that they have no competing interests.

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DISCLOSURES

The authors have nothing to disclose.

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Figures

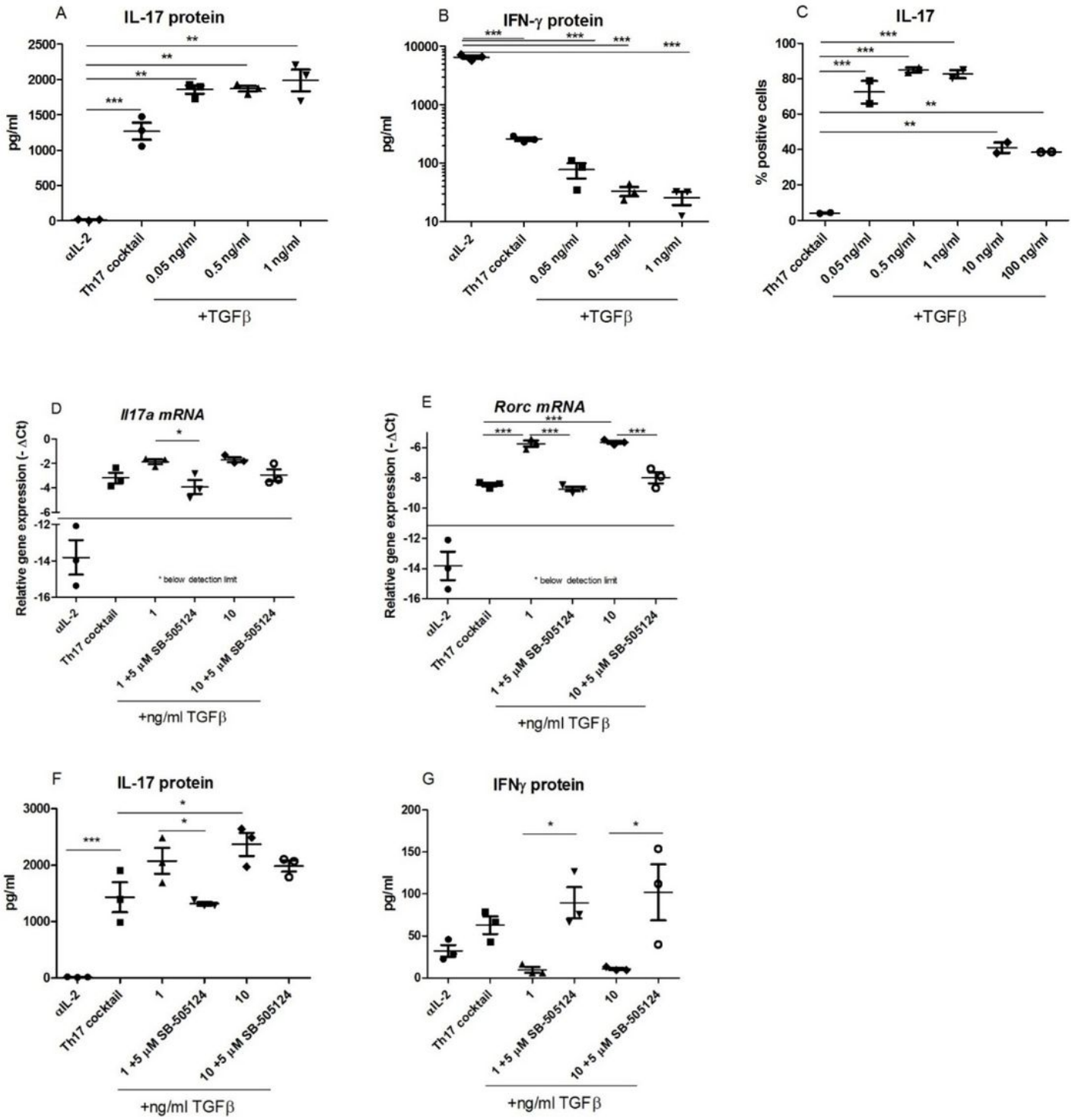

Figure 1

TGFB-RI inhibitor SB-505124 prevents TGF-β-induced expression of Th17 genes and proteins. Splenocytes were differentiated into Th17 cells in the presence of αCD3 and αCD28 with either αIL-2 alone, Th17 cocktail (IL-1, IL-6, IL-23) with or without the addition of TGF-β (1 or 10 ng/ml) and SB-505124 (5 μM) for five days of culture. Cytokine levels in supernatant were measured using Luminex (A, B, F, G). CD4+IL-17+ T cells were determined by flowcytometry (C). Gene expression was determined by

QPCR (D, E). N=3/group, values are mean ± SEM. * P < 0.05, ** P < 0.01, *** P < 0.001 calculated using one-way ANOVA followed by Bonferroni post-test.

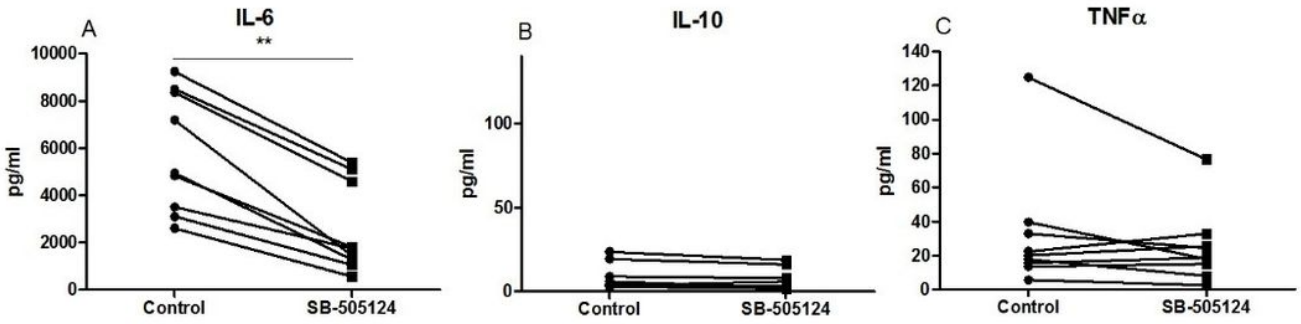

Figure 2

Inhibiting TGF-β signaling using SB-505124 significantly reduced IL-6 production by human RA synovial explants. Synovium biopsy punches were collected from synovium collected during joint replacement surgery. Explants were cultured for 24hr in the presence or absence of 5μM SB-505124 and cytokine levels in the supernatants were measured by Luminex. Graphs show results for IL-6 (A), IL-10 (B), and TNFα (C). N=9 donors, mean of 2-6 biopsies per donor per condition. **P < 0.01, as determined by paired Student's t-test.

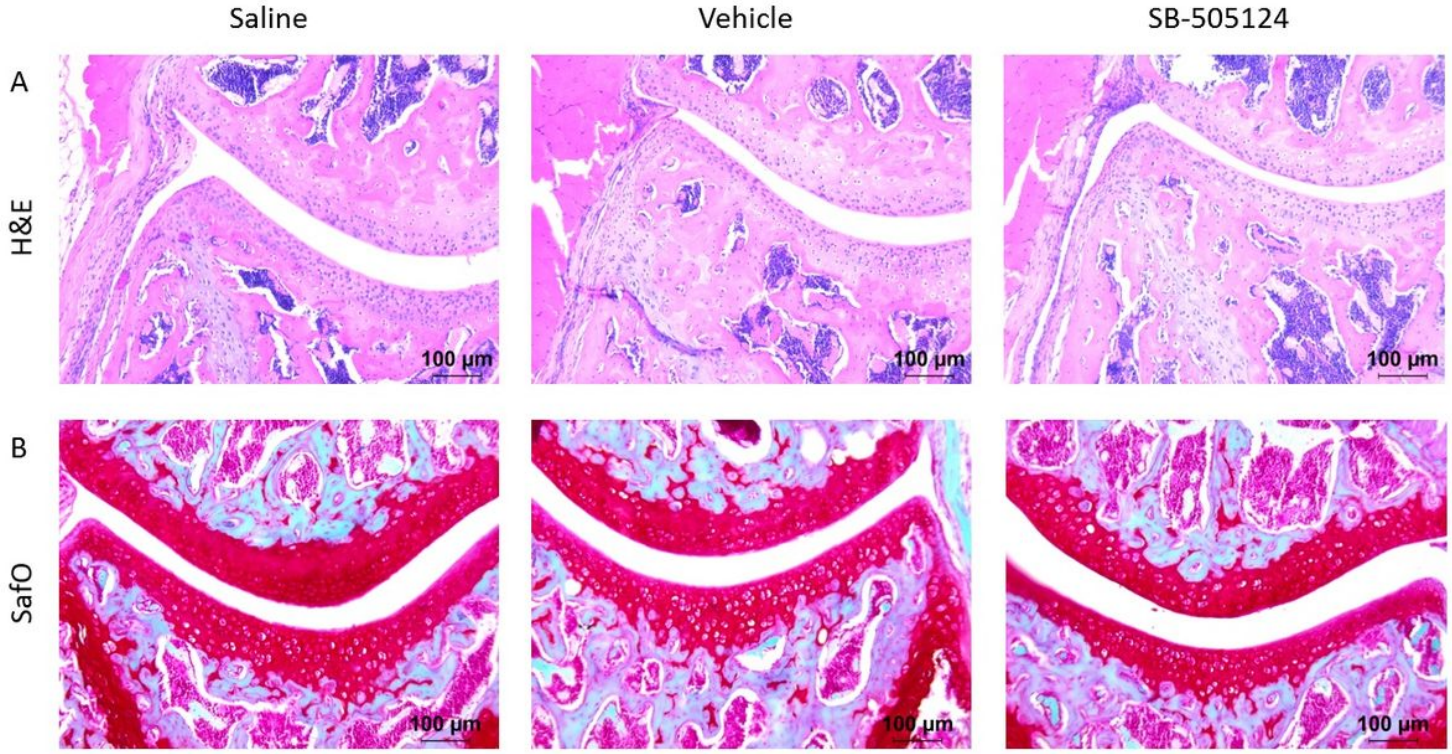

Figure 3

Intra-articular injection of SB-505124 does not induce joint inflammation and cartilage PG depletion. Mice were daily injected with 75 nmol SB-505124 or 20% DMSO as vehicle control from day 0-4, and sacrificed after 7 days. Knee joints were subsequently isolated for histological analysis of inflammation (HE stain, original magnification 100x) (B) and cartilage PG depletion (SafO stain, original magnification 100x) (B) Representative pictures of n=4 joints per group.

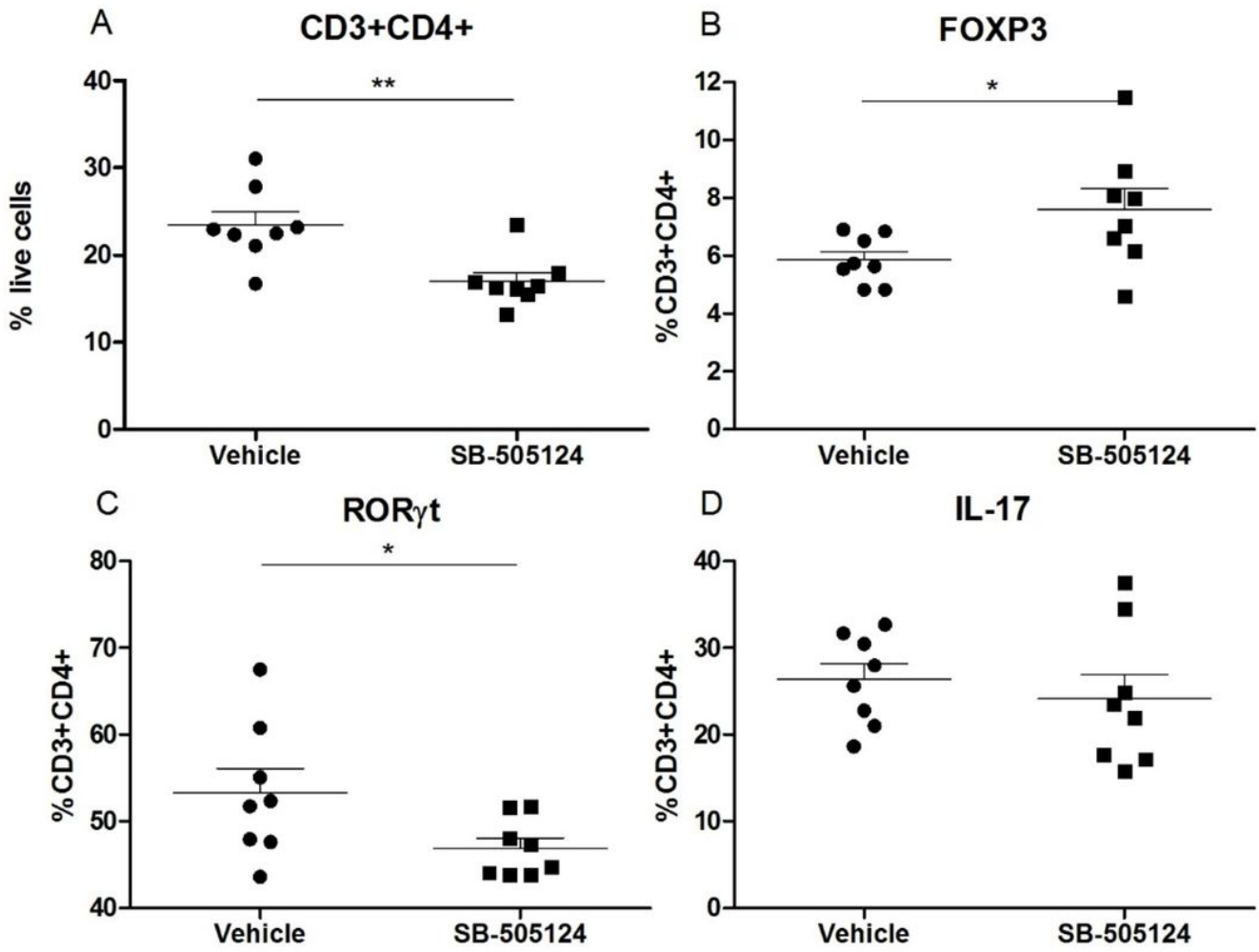

Figure 4

Intra-articular injection of SB-505124 enhances Tregs and suppresses Th17 cell levels in arthritic mice. Cells from draining lymph nodes were stimulated for 4h with PMA/ionomycin and analyzed by flow cytometry. The percentage of CD3+CD4+ T cells was decreased in the SB-505124 injected mice (A). Levels of Th17 cells were decreased by SB-505124 treatment (B), whereas FOXP3+ T cells (Tregs) were increased. (B). Levels of IL-17+ T cells were similar in vehicle and SB-505124 injected mice. ** P<0.01, * P<0.05 by Student's t-test. Values are mean +SEM.

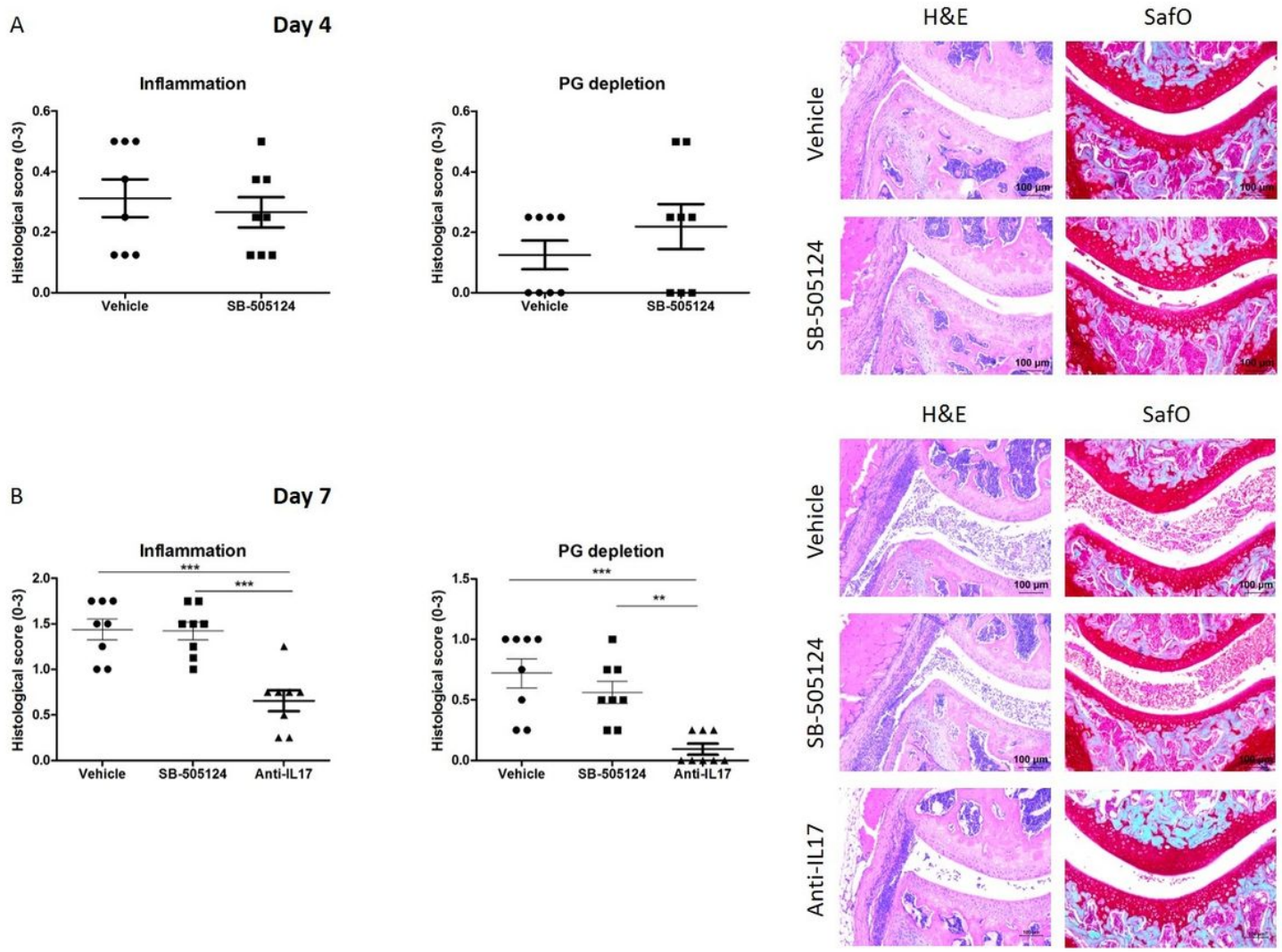

Figure 5

Intra-articular injections of SB-505124 do not decrease joint inflammation and PG depletion during experimental arthritis. Mice were daily injected i.a. with vehicle or SB-505124 for four days. Total knee joints were isolated for histopathologic analysis (n=8 mice/group). Joint inflammation (H&E stain, original magnification 100x) and PG depletion (Safranin O staining, original magnification 100x) on day four (A) and day seven (B) were analyzed on histological slides. Values are mean ±SEM. ** P<0.01, *** P<0.001 by Student's t test (A) or one-way ANOVA and Bonferroni's multiple comparison test (B) for all treatment groups.

Supplementary Files

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