Neutralization of TGF-β in combination with IL-6 ameliorates septic arthritis by altering RANKL/OPG interaction in murine lymphocytes

Rituparna Ghosh, Biswadev Bishayi*

Abstract

Septic arthritis is an inflammatory joint disease caused by *S. aureus*. Hematogenous entry of the bacteria to the joint space secretes pro-inflammatory cytokines TGF-β and IL-6, which alter the Th17/Treg switch. Inhibition of TGF-β and IL-6 to modulate Th17/Treg homeostasis and RANKL/OPG interaction are not done so far in septic arthritis. Role of lymphocyte-derived TGF-β, IL-10, IL-21 along with OPN, OPG, cellular H_2O_2, SOD and catalase activities, and the expressions of RANKL and MMP2 were studied in total lymphocytes of blood, spleen and synovial tissues of Swiss albino mice treated with antibody against TGF-β along with IL-6 Ab after induction of septic arthritis. Simultaneous neutralization of TGF-β along with IL-6 is effective in shifting the Th17 cell into Treg cell of the arthritic mice and modulates RANKL and MMP2 expression that leads to the down-regulation of osteoclastic activity and reduces the production of OPN. Additionally, such treatment reduces oxidative stress via enhancing the activities SOD and catalase enzymes in lymphocytes. So, simultaneous neutralization of TGF-β and IL-6 reduces *S. aureus* infection-associated inflammatory joint damage by increasing Treg numbers and decreasing the number of pro-inflammatory Th17 cells.

Keywords: Antioxidant enzymes, IL-6, Lymphocytes, OPG**,** RANKL, Septic arthritis, TGF-β, Th17/Treg.

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Introduction

Staphylococcus aureus (*S. aureus*) induces septic arthritis via inflammation of the joints.[1] If *S. aureus* enters the joint highly erosive and rapidly progressive arthritis may evolve.[2] During *S. aureus* infections T cells are essential for regulating immune homeostasis by dampening inflammatory processes.[3] Regulatory T cells (Tregs) are specialized in this vital immune function.[4] Naive $CD4^+$ T cells are developed into Th17 and Treg cells based on the various cytokine environments.[5] Without IL-6, TGF-β may cause the T cell differentiation to shift toward Treg cells.[6] Furthermore, differentiation of the Th17 cell lineage in mice showed that TGF-β, IL-6, IL-21 and IL-23, activation of the JAK/STAT pathway the RORγt had to work in conjunction with each other to produce Th17 cells.[7] Hashizume *et al.* showed that RANK ligand (RANKL), a promoter of osteoclast differentiation, was induced by IL-6 and soluble IL-6 receptor (sIL-6R), but not by IL-6 alone in synoviocytes.[8]

Upon stimulation, activated T lymphocytes synthesize and release functionally active RANKL.[9] Excessive RANKL concentrations are present in local and systemic inflammatory conditions associated with excessive bone resorption. The decoy receptor of RANKL, osteoprotegerin (OPG), negatively regulates osteoclasts. Moreover, Immunosuppressive Treg cells can stop osteoclasts from undergoing transition by lowering the synthesis of RANKL.[10] Treg cells have the capacity to create anti-inflammatory cytokines, such as IL-10, which may result in an indirect immunomodulatory response.[11] By upregulating osteoprotegerin (OPG) and downregulating RANKL expression, IL-10 can prevent T cells from proliferating as well as the differentiation and maturation of osteoclasts.[12] In the inflammatory milieu, as well as by inflammatory cytokines such as TNF-α, IL-1β and IL-6 the RANK/RANKL interaction induces a variety of signaling cascades, such as MAPK, NF-κB, which leads to the activation of various transcription factors specific to osteoclasts which further lowered the signaling cascade.[13-14] MMPs play a role in infectious diseases when the host immune system is challenged by an invading pathogen, favoring the infiltration of leukocytes from the bloodstream after migrating to the site of infection eradicate the pathogen and also modulate the inflammatory response.[15] There is evidence showing that matrix metalloproteinases (MMPs) contribute to extracellular matrix (ECM) degradation in aseptic and septic arthritides.[16-17]. Inflammatory cytokines, hormones and growth factors are among the numerous factors that have been reported to regulate MMP gene expression.[18] However, the effects of simultaneous inhibition of TGF-β and IL-6 on Th17/Treg cell

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ratio and its impact on the interaction of RANKL/OPG/MMP2 and associated changes in cytokines in lymphocytes are not done so far in septic arthritis.

Therefore, we proposed that neutralizing TGF-β and IL-6 by aiming at altered Th17/Treg cells and RANKL/OPG interaction in blood, spleen, and synovium lymphocytes could be helpful in regulating *S. aureus* infection-induced arthritis. This work aimed to identify the molecular mechanism by which the differential lymphocyte response of blood, spleen, and synovial joints may be altered by inhibiting TGF-β and IL-6, individually or in combination, following the induction of arthritis. We investigated the role of various cytokines, including TGF-β, IL-21, IL-10, OPN, and OPG, ROS, antioxidant enzymes, and the expression of RANKL and MMP2. In this study we have shown that simultaneous blocking of TGF-β and IL-6 confers protection from *S. aureus*-induced septic arthritis via promoting the immunosuppressive function of Treg cells. Additionally, we have reported that Treg-derived IL-10 could be able to ameliorate the inflammatory milieu of *S. aureus* infection-induced septic arthritis by modulating RANKL/OPG interaction in lymphocytes.

Materials and Methods

Male Swiss albino mice aged 6 to 8 weeks and weighing 20 ± 4g were utilized for this investigation. Under the supervision of CPCSEA, all animal experiments were approved by the Institutional Animal Ethics Committee (IAEC), Department of Physiology, University of Calcutta, and carried out in accordance with the necessary guidelines (IAEC approval number: IAEC-V/P/BB-7/2019 dated 07.08.2019). All of the mice were kept in a certain setting, fed a regular mouse meal, and given unlimited access to water. Throughout the course of the study, a 23 ± 2°C temperature, 50 ± 5% humidity, and a 12-h light-dark cycle were maintained. Mice were divided into five groups: Control (C), *Staphylococcus aureus*-infected (SA), and post-treated with TGF-β antibody alone (SA+ TGF-β Ab), and *S. aureus*-infected and post-treated with IL-6 antibody alone (SA + IL-6Ab), *S. aureus* infected and post-treated with both TGF-β antibody and IL-6 antibody (SA+ TGF-β Ab+IL-6 Ab). Mice were sacrificed at 3, 9, and 15 days post-infection (DPI). The detailed experimental design was described in Figure 1.

Preparation of bacteria and induction of septic arthritis

The *S. aureus* (*S. aureus* - strain # AG-789) bacterium was utilized to induce septic arthritis. It was maintained in our lab and was originally obtained from Apollo Gleneagles Hospital in Calcutta. The bacteria were cultured until they reached the mid-logarithmic phase of its growth by incubating them overnight in 5 mL of Muller Hinton broth (MHB) at 37°C. Before infection, bacteria were collected, washed in sterile phosphate-buffered saline (PBS), and spectrophotometrically adjusted to the appropriate inoculums (optical density at 620, 0.2 OD = 5 10^7 cells/mL).[19]

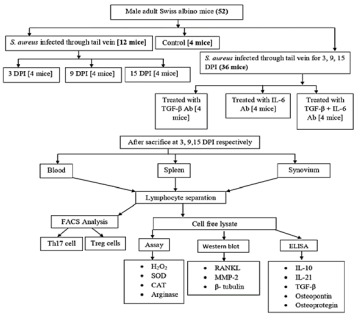

Figure 1: Experimental design

Assessment of septic arthritis

After induction of the arthritic, swelling of the synovial knee joints and paws of the experimental mice were noted regularly with a dial-type Vernier caliper, graduated 0.1 cm increments.[20] Treatment of *S. aureus* infected mice with TGF-β Ab and IL-6 Ab

To neutralize endogenous TGF-β and IL-6, intraperitoneal administration of TGF-β and IL-6 monoclonal antibodies was done intermittently after the induction of septic arthritis.

Sacrifice of mice

After 3, 9, and 15 DPI, mice from different experimental groups were sacrificed by cervical dislocation after being sedated with ketamine hydrochloride (Sigma, Life Sciences) at a dose of 1-mg/kg body weight. Through heart puncture blood (0.5 mL) was obtained, spleen and synovial tissues were separated.[21]

Isolation of lymphocytes from blood, spleen and synovium

An equal volume of PBS was added to dilute the mouse blood. Then, it was placed in a 2:1 ratio on lymphocyte separation medium for density gradient centrifugation and centrifuged for 20 minutes at 500 g. After that, lymphocytes were retrieved and washed with PBS.[22]

Spleens were separated and homogenized in Alsever's solution in an ice bath. After that, a 2:1 ratio of the homogeneous spleen cell suspension was layered over the lymphocyte separation medium, followed by centrifugation at 500 g for 20 minutes. The bands of cells at the interface were then aspirated. The recovered cells were then rinsed with RPMI to get rid of adherent cells and maintained for an hour at 37°C with 5% CO_2. Lymphocytes were regarded as non-adherent cells.[23]

The hind paws of the experimental animals were treated separately in PBS with collagenase D (2 mg/mL) for 30 min at 37°C for the isolation of lymphocytes from synovial joints. The digestion was then stopped by adding 10 mM EDTA and a further 5 minutes of incubation. The lymphocyte separation medium was then covered with a clear cell solution, and centrifugation was performed for 15 minutes at 3000 rpm. Finally, the aforementioned process resulted in the acquisition of lymphocytes.[24]

Flow Cytometry

Following the recovery of lymphocytes from the various groups' blood, spleen, and synovium, Flow cytometry analysis was carried out to establish the percentage of Th17 and Treg cells. FITC-conjugated CD25 antibody was employed as a general T cell marker that was expressed on both Th17 and Treg cells. RORγt, a Th17-specific marker antibody conjugated with PE and FoxP3, a Treg-specific marker antibody coupled with APC, were used for gating of the $CD25^+$ population. The cells were then washed and put back together in FACS buffer. Utilizing FACS verse from BD Bioscience, an analysis was performed.[25] All of the colored antibodies utilized in FACS staining are raised IgG1s directed either at FoxP3 or mouse RORγt.

Preparation of cell-free lysate

Lymphocytes isolated from mice's blood, spleen, and synovial tissues were then centrifuged separately at 3000 rpm for 5 minutes in a cold centrifuge. After centrifugation, the respective cell pellets were lysed individually with the lysis buffer (containing 0.5 mM PMSF, 1-mM sodium orthovanadate, and 1 µL/mL protease inhibitor cocktail). They were again centrifuged at 10000 rpm for 5 minutes in a cold centrifuge. Then the cell-free lysate was collected and preserved at-80°C for further use.

Hydrogen peroxide (H_2O_2) release assay from the cell-free lysate of lymphocytes isolated from blood, spleen, and synovium

In 40 µL of the medium, 20 µL of Horseradish peroxidase (HRP) (500 µg/mL), and 70 µL of phenol red (500 µg/mL) were added to the 70 µL of lyaste, respectively, and incubated for 2 h at 37°C. After adding 25 µL of 2N sodium hydroxide to halt the process, the absorbance at 620 nm was measured. 40 µL of Hank's balanced salt solutions (HBSS) were given to the control group in place of lysate. A standard H_2O_2 curve was plotted and expressed in µM/10^6 cells to measure H_2O_2 release.[26]

Assay for SOD enzyme activity in the cell-free lysate of lymphocytes isolated from blood, spleen, and synovium

In 100 µL of the lysate was added to 1.5 mL of a Tris-EDTA-HCl buffer (pH 8.5) and 100 µL of 7.2 mmol/l pyrogallol. After that, the mixture was incubated for 10 minutes at 25°C followed by adding 50 µL of 1M HCl to stop the reaction. The absorbance was taken at 420 nm. The activity of the SOD enzyme was expressed as Unit/mg protein, where one unit was considered to be the amount of enzyme that inhibits the oxidation of pyrogallol by 50%.[27]

Assay for the activity of catalase enzyme in the cell-free lysate of lymphocytes isolated from blood, spleen, and synovium

The catalase activity in lysate was measured by quantifying the H_2O_2 concentration in a spectrophotometer at 240 nm.100 µL of the lysate, and 2.89 mL of potassium phosphate buffer (pH 7.4) were taken in a quartz cuvette, followed by the addition of 0.1 mL of 300 mM H_2O. Finally, the absorbance was noted at 240 nm for 5 min at 1 min intervals. The enzyme activity was determined as to nM/min.mg protein.[28]

Assay for arginase enzyme activity in the cell-free lysate of lymphocytes isolated from blood, spleen, and synovium

Lysis of the cells was performed by adding 100 µL PBS that contained 0.1% (vol/vol) Triton X-100. After that, 100 µL of 25 Mm Tris and 100 µL 1 mM MnCl2 were added respectively to the lysate, followed by heating at 55°C for 10 minutes. After cooling, 200 µL of 0.5M arginine was dissolved in PBS (Sigma-Aldrich), added to the sample, and incubated at 37°C for 1-hour. 900 µL of 44.6N H_2PO_4 and 36N H_2SO_4 was added to the sample to stop further reactions. Next, 40 µL of 9% (vol/vol) α-isonitrosopropiophenone in ethanol was mixed in the sample and incubated at 100°C for 30 minutes. Then, absorbance was noted at 550 nm. The assay calculation was done with a standard curve made by using 2-fold serial dilutions of 200 mM urea (200 mM to 3.12 mM).[29] The arginase enzyme activity was expressed by µM urea/mg protein.

Sample preparation and cytokine ELISA from a cell-free lysate of synovial lymphocytes

Before performing the cytokine assay, cell-free lysates were prepared from the synovial lymphocytes only at 9DPI from the respective groups. They were normalized to the protein content after estimation of total proteins in the lysate of respective groups by the Bradford method,[30] and levels of cytokines (TGF-β, IL-21, IL-10, OPG and OPN) were estimated by Sandwich ELISA according to the manufacturer's instruction in a BioRad ELISA Reader. The minimum detectable limit of the cytokines TGF-β, IL-21, IL-10, OPG, and OPN are 3.15, 6, 45, 1, 4 pg/mL, respectively as mentioned in the manual for the individual ELISA kits.

Western blot analysis

As our experimental data showed that the pathogenesis of arthritis is most prominent at 9 DPI and dual antibody blocking is also found to be most effective in that time point as depicted from our FACS data, we have performed Western blot analysis from the lymphocytes of blood, spleen and synovium at 9 DPI samples only. Then the lymphocytes isolated from blood, spleen, and synovium were separately lysed with radioimmunoprecipitation assay (RIPA-NP40) buffer, and the protein content was normalized by the Lowry method.[31] Then, Samples containing equal amounts of protein in equal volumes of sample buffer were separated in a denaturing 10% polyacrylamide gel and transferred to a 0.1 mm pore nitrocellulose membrane. Nonspecific binding was blocked with 5% non-fat dry milk. For Western blot analysis, the primary antibodies to RANKL and MMP2 were used and β-tubulin was used to ensure equal amounts of protein loading. The different primary antibodies (RANKL,

MMP2) were diluted in TBST at a ratio of 1/500 to incubate with the blotting membrane overnight at 4°C. However, HRP-conjugated secondary antibody was diluted in 1/5000 ratio to incubate the membrane for 1-hour at room temperature. After that, Blots were washed three times in TBST followed by incubation for 2 h with appropriate horse radish peroxidase (HRP) conjugated secondary antibodies and developed using Super Signal chemiluminescent substrate (Super Signal West Pico Chemiluminescent Substrate; Thermo Scientific). Then exposed to X-Omat BT films (Kodak, Windsor, CO, USA) and Bands were calculated using Image J software.

Statistical Analysis

All results were denoted as mean ± SD (n=4/ group). The estimation of significant differences between group means was executed by one-way analysis of variance (ANOVA). When a significant p-value was found, Scheffe's F-test was performed as a post hoc test for multiple comparisons of the different groups. A ($p < 0.05$) was considered to be a significant level. All the analyses were performed using the Origin Pro 8 software.

RESULTS

Experimental evaluation of arthritis

In (Table 1), at 3 different time points, a significant reduction in arthritis was noted in the arthritic mice that were treated with TGF-β Ab and IL-6Ab either alone or in combination when compared to the Ab non-treated arthritic mice only ($p <0.05$).

The experiments were performed in mice from 5 different experimental groups as mentioned in the methods section. Results were obtained from three independent experiments. Data were expressed as mean ± SD which are significant at the level of $p< 0.05$. '*' denoted significant difference in comparison to control group, '#' indicates significant difference compared to SA group, '@' indicates significant difference in comparison to SA+TGF-βAb group, '&' denoted significant difference compared to SA+ IL-6Ab group.

Percentage of CD25+ RORγt +Th17 cells and CD25+ FoxP3+ Treg cells from the blood, spleen and synovium of different groups of arthritic mice at 9DPI

In (Table 2) at 9DPI, $CD25^+$ $ROR\gamma t^+$Th17 cells and $CD25^+$ $FoxP3^+$ Treg cells collected from blood lymphocytes of the Ab non-treated arthritic mice showed increased and decreased percentages, respectively when compared to the control mice ($p<0.05$). A decrease in $CD25^+$ $ROR\gamma t^+$ Th17 cell count and increase in $CD25^+$ $FoxP3^+$ Treg cell count were noted in arthritic mice treated with TGF-β Ab and IL-6Ab either alone or in combination in comparison to the non-Ab treated arthritic mice respectively ($p<0.05$). The dual Ab recorded minimum CD25+ RORγt+ Th17 and elevated in CD25+ FoxP3+ Treg cell percentage treated (TGF-β + IL-6) arthritic mice when compared only TGF-β Ab treated as well as only IL-6Ab treated arthritic mice at 9DPI ($p<0.05$).

At 9DPI, $CD25^+$ $ROR\gamma t^+$ Th17 cells and in $CD25^+$ $FoxP3^+$ Treg cells isolated from the spleen of the non-Ab treated arthritic mice revealed an increased and decreased percentage, respectively when compared to the control mice ($p<0.05$). A significantly lowered percentage of $CD25^+$ $ROR\gamma t^+$ Th17 cell and elevated in $CD25^+$ $FoxP3^+$ Treg cells were recorded in the arthritic mice treated with TGF-β Ab and IL-6 Ab either alone or in combination when compared to the Ab non-treated arthritic mice ($p<0.05$). A marked increment in the splenic in $CD25^+$ $FoxP3^+$ Treg cell population was observed in the arthritic mice treated with both TGF-β Ab and IL-6 Ab in comparison to the antibody non-treated arthritic mice ($p<0.05$).

At 9DPI, maximum $CD25^+$ $ROR\gamma t^+$ Th17 cell and lowered in $CD25^+$ $FoxP3^+$ Treg cell percentage was shown by the non-Ab treated arthritic mice in comparison to the control mice ($p<0.05$). Conversely, a reduced synovial $CD25^+$ $ROR\gamma t^+$ Th17 cell and elevated in $CD25^+$ $FoxP3^+$ Treg cell population were observed in the arthritic mice treated with TGF-β Ab or IL-6 Ab either alone or in combination when compared to the non-Ab treated arthritic mice respectively ($p<0.05$). Whereas no percentage of the synovial $CD25^+$ $ROR\gamma t^+$ Th17 cells was observed in the dual Ab-treated arthritic mice when compared to either TGF-β Ab or IL-6 Ab treated arthritic mice ($p<0.05$). Maximum $CD25^+$ $ROR\gamma t^+$ Th17 cell percentage was recorded in the synovial lymphocytes of the non Ab treated arthritic mice when compared to blood and splenic lymphocytes, respectively ($p<0.05$). However, elevated in $CD25^+$ $FoxP3^+$ Treg cell percentage was noted in the synovial lymphocytes of the dual Ab-treated arthritic mice in comparison to the blood and splenic lymphocytes, respectively ($p<0.05$).

Data were expressed as mean ± SD which are significant at the level of $p< 0.05$. '*' denoted significant difference in comparison to control group, '#' indicates significant difference compared to SA group, '@' indicates significant difference in comparison to SA+TGF-βAb group, '&' denoted significant difference compared to SA+ IL-6Ab group. '$' indicates the significance difference between Th17and Treg

Table 1: Percentage reduction in septic arthritis compared to SA group

Days post infection	Reduction of arthritis (%) compare to SA group		
	3DPI	9DPI	15DPI
SA + TGF-β Ab	5.00 ± 0.02 #	20.00 ± 0.02 #	16.00 ± 0.01 #
SA + IL-6 Ab	6.20 ± 0.04 #	36.00 ± 0.04 # @	36.00 ± 0.02 # @
SA + TGF-β Ab + IL-6 Ab	6.50 ± 0.02 #	40.00 ± 0.02 # @ &	45.00 ± 0.02 # @ &

Table 2: Percentage of FACS analyzed $CD25^+RORγt^+$ Th17 cells and $CD25^+$ $FoxP3^+$ Treg cells in blood, spleen, and synovium at 9DPI in the 5 different experimental groups post neutralization of TGF-β and IL-6 after *S. aureus* induced septic arthritis

Groups	*Th17 cell %*	*Treg cell %*
Blood lymphocyte		
Control	7.00 ± 0.04	22.00 ±0.41 $^{\$}$
SA	34.00 ± 1.23 *	1.00 ± 0.03*$^{\$}$
SA+ TGF-β Ab	22.00 ± 1.22$^{\#}$	7.00 ± 0.41$^{\# \$}$
SA+ IL-6 Ab	13.00 ±1.11 $^{\#}$	10.00 ± 0.24 $^{\# \$}$
SA+ TGF-β Ab + IL-6 Ab	3.00 ± 0.41 $^{\# @ \&}$	35.00 ± 1.34$^{\# @ \&}$
Splenic lymphocyte		
Control	1.00 ± 0.02	46.00 ± 1.24$^{\$}$
SA	11.00 ± 1.25 *	3.00 ± 0.21*$^{\$}$
SA+ TGF-β Ab	3.00 ± 0.26$^{\#}$	20.00 ± 1.23$^{\#\$}$
SA+ IL-6 Ab	6.00 ± 0.51$^{\#}$	11.00 ± 1.24$^{\# \$}$
SA+ TGF-β Ab + IL-6 Ab	1.00 ± 0.02	46.00 ± 1.24$^{\$}$
Synovial lymphocyte		
Control	1.00 ± 0.02	65.00 ± 1.26$^{\$}$
SA	44.00 ± 2.33*	1.00 ± 0.01*$^{\$}$
SA+ TGF-β Ab	5.50 ± 2.11 $^{\#}$	17.00 ± 1.22 $^{\# \$}$
SA+ IL-6 Ab	21.00 ± 2.34$^{\#}$	12.00 ± 1.32$^{\# \$}$
SA+ TGF-β Ab + IL-6 Ab	1.00 ± 0.02	65.00 ± 1.26$^{\$}$

cells. 'α' indicates a significant difference compared to Blood lymphocytes with splenic and synovial lymphocytes.

Effect of neutralization of TGF-β and IL-6 either alone or in combination on H_2O_2, production in lymphocytes isolated from blood, spleen and synovium of the different groups of arthritic mice

In (Figure 2), at 3DPI, elevated production of H_2O_2 was recorded in the lymphocytes recovered from blood (Figure 2A), spleen (Figure 2B), and synovium (Figure 2C) of the non-Ab treated arthritic mice when compared to the control mice ($p<0.05$). In contrast, reduced H_2O_2 production was exhibited in the blood lymphocytes of dual Ab-treated arthritic mice compared to the non-Ab-treated arthritic mice at 3DPI ($p<0.05$).

At 9DPI and 15DPI, increased production of H_2O_2 was observed in the lymphocytes isolated from blood, spleen, and synovium of the non-Ab treated arthritic mice in comparison to the control mice ($p<0.05$). In different tissues, a diminished H_2O_2 production was revealed by the arthritic mice treated with either IL-6Ab alone or both TGF-β Ab and IL-6 Ab in combination compared to the non-Ab treated arthritic mice, respectively ($p<0.05$). In blood, spleen, and synovium of dual Ab (TGF-β Ab and IL-6Ab) treated arthritic mice showed decreased H_2O_2 production in comparison to the arthritic mice treated with either TGF- βAb or IL-6Ab alone respectively at 9 and 15DPI ($p<0.05$).

Application of exogenous TGF-β and IL-6 antibodies together after the onset of septic arthritis SOD and Catalase enzyme activity in lymphocytes that recovered from blood, spleen, and synovium

At all the three different time intervals, lymphocytes recovered from blood (Figure 2D), spleen (Figure 2E) and synovium (Figure 2F) of the non-Ab treated arthritic mice showed reduced SOD activity in comparison to the control mice respectively ($p<0.05$). Elevated activity of the SOD enzyme were observed in the blood lymphocytes of the arthritic mice treated with either IL-6 Ab alone or both TGF-β Ab and IL-6 Ab in combination when compared to the non-antibody treated arthritic mice at 3DPI ($p<0.05$).

At 9DPI, the blood lymphocytes (Figure 2D) of the arthritic mice treated with TGF-β Ab and IL-6 Ab either alone or in combination exhibit a higher SOD enzyme activity when compared to the non-antibody treated arthritic mice, respectively ($p<0.05$). Lymphocytes recovered from the spleen (Figure 2E),and synovium (Figure 2F),of the arthritic mice treated with either IL-6Ab or both TGF-β Ab and IL-6 Ab revealed an elevated SOD enzyme activity compared to the non-Ab treated arthritic mice, respectively ($p<0.05$). Increased SOD enzyme activity was noted in the lymphocytes that were isolated from different tissues of the dual Ab-treated arthritic mice than single Ab-treated arthritic mice ($p<0.05$). At 15DPI, lymphocytes isolated from blood, spleen, and synovium of the different experimental groups followed the same pattern of SOD enzyme activity at 9DPI ($p<0.05$).

A significant decrement in catalase enzyme activity was recorded in the lymphocytes isolated from blood (Figure 2G), spleen (Figure 2H) and synovium (Figure 2I), of the non-Ab, treated arthritic mice when compared to the control, respectively, at a different time interval ($p<0.05$). Lymphocytes recovered from the spleen and synovium of the arthritic mice treated with either IL-6Ab or both TGF-β Ab and IL-6Ab showed increased Catalase enzyme activity compared to the non-Ab treated arthritic mice at 3DPI, respectively ($p<0.05$). At 9 and 15DPI, lymphocytes isolated from blood spleen and synovium of different experimental groups resemble an identical pattern in catalase enzyme activity at 3DPI ($p<0.05$).

Assessments of arginase enzyme activity in S. aureus infected mice that were treated with TGF-β Ab and IL-6 Ab either alone or in combination in lymphocytes isolated from blood, spleen, and synovium

In (Table 3) at 3DPI, a significant diminished in arginase enzyme activity was recorded in the lymphocytes recovered from the blood, spleen and synovium of the non-antibody arthritic mice when compared to the control ($p<0.05$). Blood lymphocytes of dual Ab-treated arthritic mice showed an elevated arginase activity than the non-Ab-treated arthritic mice ($p<0.05$).

At 9 DPI, a decreased arginase activity was recorded in the lymphocytes of the different tissues of non-Ab-treated

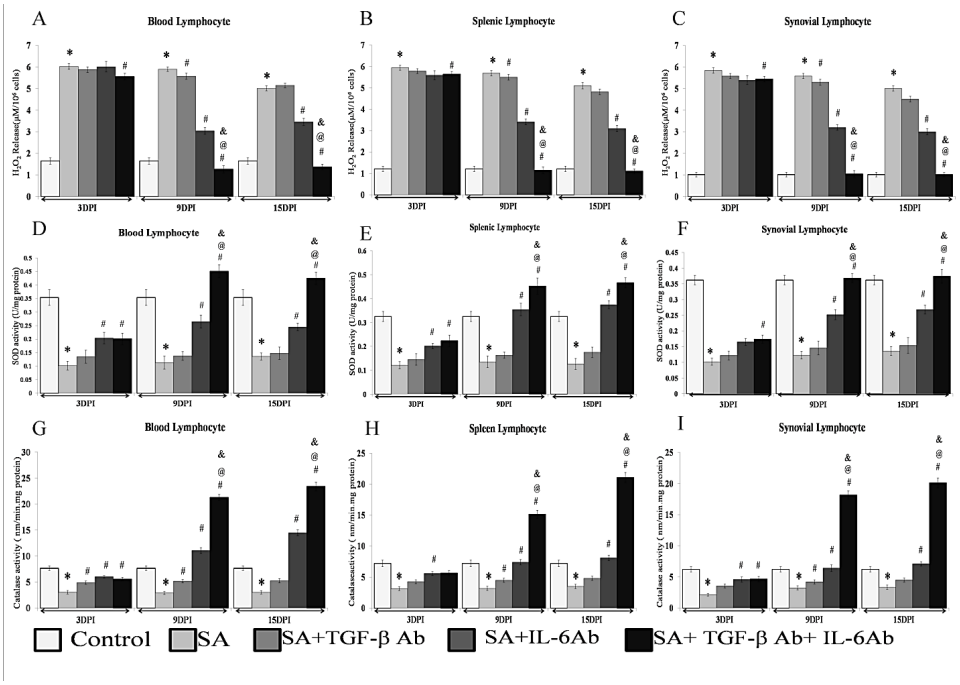

Figure 2: Effects of Neutralization TGF-β and IL-6 either alone or in combination after the induction of septic arthritis on H_2O_2 production (A-blood, B-spleen, C-synovium), SOD (D-blood, E-spleen, F-synovium), and catalase enzyme activities (G-blood, H-spleen, I-synovium): Data were expressed as mean ± SD which are significant at the level of p< 0.05. '*' denoted significant difference in comparison to control group, '#' indicates significant difference compared to SA group, '@' indicates significant difference in comparison to SA+TGF-βAb group, '&' denoted significant difference compared to SA+ IL-6Ab group.

arthritic mice compared to the control (p<0.05). Lymphocytes recovered from blood of dual Ab-treated arthritic mice revealed an elevated arginase enzyme activity compared to the single Ab-treated and non-Ab-treated arthritic mice, respectively (p<0.05). Splenic and synovial lymphocytes of the IL-6Ab treated as well as dual Ab treated arthritic mice exhibit higher arginase enzyme activity compared to the non-Ab treated arthritic mice at 9DPI (p<0.05). Lymphocytes isolated from different tissues of the arthritic mice treated with both TGF-β Ab and IL-6Ab showed more arginase enzyme activity than single Ab-treated arthritic mice (p<0.05).

At 15 DPI, Lymphocytes isolated from blood, spleen, and synovium of the non-Ab treated arthritic mice showed a decreased arginase enzyme activity compared to the control (p<0.05). Lymphocytes of the different tissues of dual Ab-treated arthritic mice followed a alike pattern with 9DPI in comparison to the non-Ab treated as well s single Ab-treated arthritic mice (p<0.05).

Table 3: Effects of neutralization of TGF-β and IL-6 either alone or in combination after the induction of septic arthritis on the arginase enzyme activity in blood, spleen and synovial lymphocytes: Data were expressed as mean ± SD which are significant at the level of p< 0.05. '*' denoted significant difference in comparison to control group, '#' indicates significant difference compared to SA group, '@' indicates significant difference in comparison to SA+TGF-βAb group, '&' denoted significant difference compared to SA+ IL-6Ab group.

Quantification of cytokines TGF-β, IL-21, IL-10, OPG and OPN from lymphocytes recovered from the synovium of the TGF-β Ab and IL-6 Ab post-treated septic arthritic mice at 9DPI

Lymphocytes recovered from the synovium of the arthritic mice at 9DPI exhibited increased content of TGF-β (Figure 3A), IL-21 (Figure 3B) in comparison with the control group, respectively (p<0.05).

At 9DPI, lymphocytes isolated from the synovium of the non-Ab treated arthritic mice showed decreased production of IL-10 (Figure 3C) and OPG (Figure 3D) when compared to the control (p <0.05). On the contrary, increased production of IL-10 and OPG were recorded in the synovial lymphocytes of the arthritic mice that were treated with TGF-β Ab and IL-6 Ab either alone or in combination in comparison to the non-Ab-treated arthritic mice only. The elevated level of IL-10 and OPG were more significant in the synovial lymphocytes of the arthritic mice treated with both TGF-β Ab and IL-6 Ab than in the infected mice treated with either TGF-β Ab or IL-6 Ab alone at 9DPI (p<0.05).

A marked reduction in OPN (Figure 3E) level was recorded in the arthritic mice with TGF-β Ab and IL-6 Ab either alone or in combination when compared to the non-Ab treated arthritic mice (p<0.05). However, this diminishes in the OPN content was higher in the arthritic mice treated with both TGF-β Ab and IL-6 Ab than only TGF-β Ab or IL-6Ab in the synovial lymphocytes at 9DPI (p<0.05).

Table 3: Effects of neutralization of TGF-β and IL-6 either alone or in combination after the induction of septic arthritis on arginase enzyme activity [μM urea/mg protein] in blood, spleen and synovial lymphocytes

	3DPI	*9DPI*	*15DPI*
Blood lymphocyte			
Control	0.42 ± 0.02	0.42 ± 0.02	0.42 ± 0.02
SA	0.04 ± 0.01*	0.10 ±0.03*	0.14 ± 0.03
SA+ TGF-β Ab	0.08 ± 0.02#	0.11 ± 0.02#	0.10 ± 0.02
SA+ IL-6 Ab	0.10 ± 0.03 #	0.20 ± 0.03#	0.24 ± 0.02#
SA+ TGF-β Ab + IL-6 Ab	0.13 ± 0.03#@&	0.58 ± 0.04#@&	0.53 ± 0.03#@&
Splenic lymphocyte			
Control	0.32 ± 0.01	0.32 ± 0.01	0.32 ± 0.01
SA	0.08 ± 0.01*	0.04 ± 0.02 *	0.06 ± 0.01
SA+ TGF-β Ab	0.11 ± 0.02 #	0.05 ± 0.02 #	0.09 ± 0.02
SA+ IL-6 Ab	0.10 ± 0.02 #	0.22 ± 0.02 #	0.25 ± 0.04 #
SA+ TGF-β Ab + IL-6 Ab	0.09 ± 0.02#@&	0.48 ± 0.04 #@&	0.46 ± 0.04 #@&
Synovial lymphocyte			
Control	0.28 ± 0.02	0.28 ± 0.02	0.28 ± 0.02
SA	0.10 ± 0.03*	0.06 ± 0.01*	0.03 ± 0.01*
SA+ TGF-β Ab	0.05 ± 0.02 #	0.06 ± 0.01 #	0.03 ± 0.01 #
SA+ IL-6 Ab	0.05 ± 0.02 #	0.16 ± 0.02#$	0.22 ± 0.03 #
SA+ TGF-β Ab + IL-6 Ab	0.10 ± 0.02 #@&	0.41 ± 0.02#@&	0.45 ± 0.03#@&

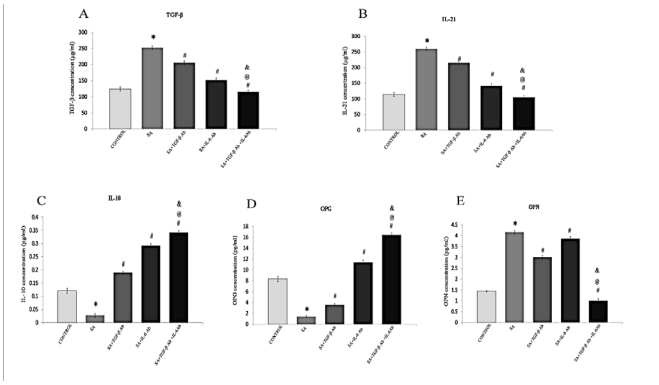

Figure 3: Effects of neutralization TGF-β and IL-6 either alone or in combination on the level of TGF-β (A), IL-21 (B), IL-10 (C), OPG (D), OPN (E) in synovial lymphocytes at 9 DPI of arthritic mice: Results were obtained from three independent experiments. Data were expressed as mean ± SD which are significant at the level of p< 0.05. '*' denoted significant difference in comparison to control group, '#' indicates significant difference compared to SA group, '@' indicates significant difference in comparison to SA+TGF-βAb group, '&' denoted significant difference compared to SA+ IL-6Ab group.

Western blot analysis of RANKL and MMP2 in blood, splenic and synovial lymphocytes of the TGF-β and IL-6 neutralized arthritic mice at 9DPI

In Figure 4, at 9DPI, elevated expression of RANKL and MMP2 were shown in the lymphocytes isolated from the blood (Figure 4A-B), spleen (Figure 4C-D) and synovium (Figure 4E-F) of the non-Ab treated arthritic mice when compared to the control mice respectively (p<0.05). At the same time, significantly lowered RANKL, and MMP2 expressions were recorded in the arthritic mice treated with TGF-β Ab and

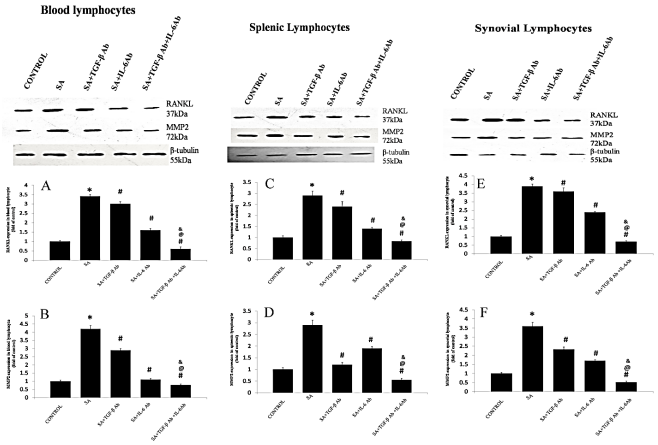

Figure: 4 Western blot analysis of RANKL and MMP2 in the lymphocytes isolated at 9DPI from the blood (A-B), spleen (C-D) and synovium (E-F) of *S.aureus* infected mice treated with TGF-β Ab or IL-6 Ab either alone or in combination : Results were obtained from three independent experiments. Data were expressed as mean ± SD which are significant at the level of p< 0.05. '*' denoted significant difference in comparison to control group, '#' indicates significant difference compared to SA group, '@' indicates significant difference in comparison to SA+TGF-βAb group, '&' denoted significant difference compared to SA+ IL-6Ab group.

IL-6Ab either alone or in combination compared to the non-Ab treated arthritic mice. Nevertheless, the reduced expression of RANKL, and MMP2 was more prominent in the blood lymphocytes of the arthritic mice treated with dual antibody (TGF-β Ab and IL-6Ab) than treated with either TGF-β Ab or IL-6Ab after the induction of septic arthritis (p<0.05).

DISCUSSION

Septic arthritis (SA) is an orthopedic emergency initiated by the contagious bacteria *S. aureus*, responsible for acute joint destruction, leading to enhanced mortality and morbidity. After entering the joint space, *S. aureus* initiates an array of inflammatory responses. This includes the activation of T lymphocytes and several pro-inflammatory cytokines TGF-β, IL-6 and increasing the levels of ROS. $CD4^+$ T cell is a common precursor for the Th17 and Treg cells. A diminished Treg cell function is correlated with an increment in the severity of the disease. Additionally, Treg cell has been found to down-regulate the inflammatory response and acts as reciprocal to Th17 cells. Thus alteration in the equilibrium between Th17/Treg cells could permit the regulation of septic arthritis. Our previous study reported the effects of simultaneous neutralization of IL-17A and Supplementation of IL-2 on the Th17/Treg cell population in *S. aureus* infected septic arthritis. Simultaneously increased expression of TNFR2 in accordance with the decreased expression of TLR2 and TNFR1 in Treg cells of the IL-17A treated arthritic mice

resulted in rapid GSH accumulation that decreased GSSG level by reducing the expression of GP_x, which finally reduces the oxidative damage.[32] But the Th17/Treg cell ratio shifting in response to pro-inflammatory cytokines like TGF-β and IL-6 in septic arthritis is not studied yet. This has prompted us to address whether simultaneous neutralization of TGF-β and IL-6 could be beneficial in ameliorating *S. aureus* infection-induced arthritis by modulating Th17/Treg homeostasis, the interaction of RANKL/OPG/MMP2 and the associated cytokines in the lymphocytes of blood, spleen, and synovium.

So in this study, we are interested to find out the role of neutralization of TGF-β and IL-6, either alone or in combination, could able to shift the Th17/Treg cell ratio in the lymphocytes isolated from blood, spleen, and synovium after the induction of septic arthritis at 3, 9, 15DPI respectively. Additionally, we also quantified the production of H_2O_2 along with the estimation of activities of SOD and Catalase enzymes in lymphocytes of different experimental groups. We also focused on the cytokine profile, including TGF-β, IL-21, IL-10, OPG and OPN in synovial lymphocytes of the arthritic mice at 9DPI. The expressions of several arthritic markers like RANKL and MMP2 were further analyzed from the blood, spleen, and synovial lymphocytes to have an indication on arthritic bone destruction as well as inflammation in this experimental set up.

In this present study, we have reported that neutralization of TGF-β alone after the onset of septic arthritis cannot exhibit any significant changes compared to only IL-6 neutralization. But the optimum effect and shift in Th17/Treg cell ratio and downregulation of other pro-inflammatory cytokines are observed when TGF-β and IL-6 are simultaneously neutralized at 9DPI. Thus, we can say that neutralization of both TGF-β and IL-6 after the onset of septic arthritis was found to be more potent than that of single neutralization of either TGF-β or IL-6 in the shifting of Th17 cells to the Treg type.

Similarly, in our in vivo experiment, dual antibody treatment during Staphylococcal arthritis caused a clear reduction in Th17 cells and simultaneous induction of Treg cell type. However, this did not define the cell population co-expressing foxP3 and RORγt, are of whether Treg or Th17, suggesting that (i) further characterization is important in this model, along with estimation of IL-6, TGF-β, IL-21 and responsible for such interconversion (ii) further studies after sorting of such cells co-expressing foxP3 and RORγt, even if they are either Treg or Th17 cells from different treatment groups isolated from blood, spleen and synovium and their signature cytokine profile, (iii) the remaining (Th17) cells in the blood, spleen or in the synovium are highly active due to loss of TGF-β1-mediated dampening, or (iv) that the dual treatment with antibody had a too limited duration in time on IL-6/TGF-β1 signaling to suppress the arthritis measured at different stages of our in vivo experiment. Unfortunately, our study design did not enable us to characterize and investigate the potential function of this population and to study the differences compared with foxP3 + Treg and RORγt + Th17 and other immune cells by checking several signature cytokine levels in the joints.

Moreover, recently it has been shown that regulatory T cells also showed characteristics of T helper-17 (Th17) cells in mice inflammatory disease model.[33] Whereas, TGF-β suppresses Th1 and Th2 cell differentiation, this cytokine promotes Th9, Th17, and $FoxP3^+$ regulatory T cells depending upon the existence of other cytokines. IL-6 promotes Th17, but suppresses regulatory T cell differentiation. Moreover, natural but not TGF-β-induced regulatory T cells convert into Th17 cells in the inflammatory milieu. So at this moment, it is difficult to define whether cell population co-expressing foxP3 and RORγt is Treg or Th17.

Our study reveals that neutralizing both TGF-β and IL-6 reduces oxidative stress at 9DPI in synovium and other tissues and enhances the activities of antioxidant enzymes. The mechanisms behind the simultaneous shifting of the Th17/ Treg cells ratio concerning reduced oxidative stress are still unclear, so it needs further investigation. According to previous studies, the presence of pro-inflammatory cytokine TGF-β in the systemic circulation enhances the production of H_2O_2 by diminishing mitochondrial function, which further induces the activity of NADPH oxidases (NOXs).[34] In addition, TGF-β also reduces the activity of SOD and catalase enzymes which further initiates the fibrogenic effects of TGF-β. Gabriel *et al.*, 2021 suggested that TGF-β increases mitochondrial oxygen consumption and ROS production via the activation of the mammalian target of the rapamycin (mTOR) pathway.[35] However, we have not tested the mTOR expression in our experimental groups. At the cellular level, IL-6 in association with TGF-β shifts Th17/Treg cell ratio towards Th17 cells. Our experiment speculated that neutralizing TGF-β or IL-6 alone is insufficient to reduce the devastating effect of septic arthritis. In contrast, dual neutralization of TGF-β and IL-6 can reduce oxidative stress.

An increased SOD and catalase enzyme activity were observed in the lymphocytes of the dual antibody-treated arthritic mice, which signify the decrease in ROS production. Additionally, superoxide dismutase shows immunomodulatory effects via inhibiting T-cell differentiation. But the mechanisms are still unknown.

Decreased arginase enzyme activity is noted in the lymphocytes of the blood, spleen, and synovium of the dual antibody treated arthritic mice at 9DPI, which signifies that it might play a role in decreasing the production of nitric oxide (NO). *In-vitro*, studies[36] suggest that it has some T cell suppressive ability, though its role in Th17/Treg cell regulation is unknown.

First and foremost, Treg cells directly sense the inflamed environment, i.e., the presence of TGF-β, IL-6, IL-21 within the synovium favoring differentiation of the Th17 cells. These elevated levels of cytokines further activate STAT3, which ensures Treg cell-mediated suppression of Th17 cells. Secondly, the Treg cell with the help of the anti-inflammatory cytokine IL-10 amplifies its adverse regulatory effects on Th17

cells.[37] In an ongoing experiment, we are addressing whether TGF-β and IL-6 plus IL-23/IL-21 are critical in regulating *S. aureus* activated TH17/Treg-induced inflammatory responses through STAT1/STAT3 signaling.

To maintain the synovial homeostasis during the infectious condition, the inflammatory Th17 cells must be restrained by Treg cell-mediated different types of intrinsic and extrinsic mechanisms. IL-10 is produced by the effecter T cells and acts as a potent negative regulator that suppresses infection-induced inflammation by targeting a broad range of hematopoietic cells. Our study indicates that upon neutralizing TGF-β and IL-6 after the onset of septic arthritis, IL-10 level drastically increases in the synovial lymphocytes, further reducing bone destruction. To explain these facts, two pathways of Treg cell-mediated inhibition of Th17 cell response can be considered. Additionally, IL-10 sufficiently down-regulates the production of the aforementioned pro-inflammatory cytokines. Previous reports suggested that the combination of TGF-β and IL-6 can induce osteoclast-like cells, indicating bone resorption activity. Thus neutralization of TGF-β and IL-6 implies that IL-10 plays a crucial role in bone formation. IL-10 down-regulates the expression of osteoclast precursors, namely RANKL, and upregulates OPG expression, thereby inhibiting RANKL-mediated osteoclastogenesis in septic arthritis.

Synovial lymphocytes of the *S. aureus-infected* mice showed higher OPG production when treated with both TGF-β Ab and IL-6 Ab. Receptor activators of nuclear factor-κB ligand (RANKL) and OPG are the two major factors that regulate osteoclasts' maturation and differentiation followed by bone resorption. The balance between RANKL and OPG activity is vital in maintaining bone metabolism homeostasis. RANKL regulates osteoclast development via RANK activation, while OPG suppresses the process by inhibiting the binding of RANKL to its receptor.[38] At 9DPI, a low level of OPG and higher RANKL expression in the synovium of the septic arthritic mice indicates the disrupted OPG/RANKL balance that enhances bone resorption. According to the researchers, OPG is expressed more highly under normal physiological conditions than RANKL, which ensures stabilization of the osteoclast and osteoblast cell formation in the synovial joints. In septic arthritis, as the disease progresses, the RANKL expression increases more than that of OPG, resulting in synovial tissue destruction. Additionally, OPG can inhibit the differentiation of osteoclast and bone resorption by inducing apoptosis of the mature osteoclast cells. Conversely, RANKL activates the RANK receptor on the membrane of the osteoclast precursor cells, accelerating the formation of osteoclast cells. On the other hand, Th17 cell-derived cytokine IL-17 triggers RANKL production by upregulating the RANK expression and thereby maintaining the T lymphocyte and bone cell interaction. Thus, the alterations between the RANKL/OPG/RANK axis can shift the formation of the osteoclast towards the osteoblast. Understanding how RANKL and OPG identify RANK and accelerate the downstream pathways could be a new therapeutic approach for treating septic arthritis.

OPN is secreted by the activated T lymphocytes and found at the site of inflammation and in the ECM of the synovial tissue. In our study, we observed that upon neutralization of TGF-β and IL-6, the OPN level was decreased. Still, the maximum effect was noted in the dual neutralization after the disease induction. Though the crosstalk between OPN and other inflammatory cytokines is not well known, various prior studies suggested that OPN can suppress the Treg cell activity and thereby enhance the production of Th17 cells, leading to further destruction in joints. Furthermore, OPN is abundant in bone and enables the attachment of the osteoclast cells to the bone matrix and facilitates cell-cell interaction.[39]

The current status of IL-6-targeting therapies in the clinic has been reviewed, suggesting IL-6 also acts as an essential factor in bone homeostasis as opposed to RANKL, an inducer of osteoclast differentiation.[40] Whether IL-6 could affect the immune microenvironment of septic arthritis by changing the ratio of Th17/Treg cells has not been studied. A recent report demonstrated that chronic inflammatory disease could be counteracted by inhibiting the IL-6 signalling pathwasys[41]. Supporting our study with IL-6 antibody treatment could regulate the Th17/Treg balance of arthritic mice to ameliorate the progression of arthritic disease. Moreover, anti–IL-6R antibody–induced increase in Tregs has been shown to be associated with a significant reduction in pro-inflammatory Th1 and Th17 cells.[42] We examined whether anti–IL-6 antibody administration affected the number of Th17 cells, which earlier have been increased due to synovial inflammation**.** Our studies also demonstrated that blockade of IL-6 signaling was associated with a significant reduction in these cells in the blood, spleen, and synovium.

Recently it has been shown that blocking TGF-β1 signaling suppresses Th17 differentiation and improves the Th17/ Treg balance in experimental arthritis, showing that TGF-β1 regulation of IL-6 signaling is cell type, tissue and context-dependent.[43] Treg cells inhibit the differentiation of osteoclasts in vivo and in vitro, while Th17 cells promote the differentiation of osteoclasts.**[5]** Therefore, upon dual antibody treatment and understanding the balance between Treg cells and Th17 cells is also expected to provide a link towards the development of novel treatments for bacterial arthritis. Therefore, blockade of IL-6 signaling and simultaneous neutralization of TGF-β, reduces *S. aureus* infection-associated inflammatory damages by increasing Treg numbers and decreasing the number of pro-inflammatory Th17 cells. This serves to establish the direct link in favour of the beneficial effect of dual antibody treatment with the change of Th17/ Treg balance which might be helpful to recalibrate the effector and regulatory arms of the immune system and thereby mitigate the severity of septic arthritis.

Elevated expression of RANKL in lymphocytes of the blood, spleen, and synovium of the arthritic mice leads to synovial tissue damage that further increases inflammatory

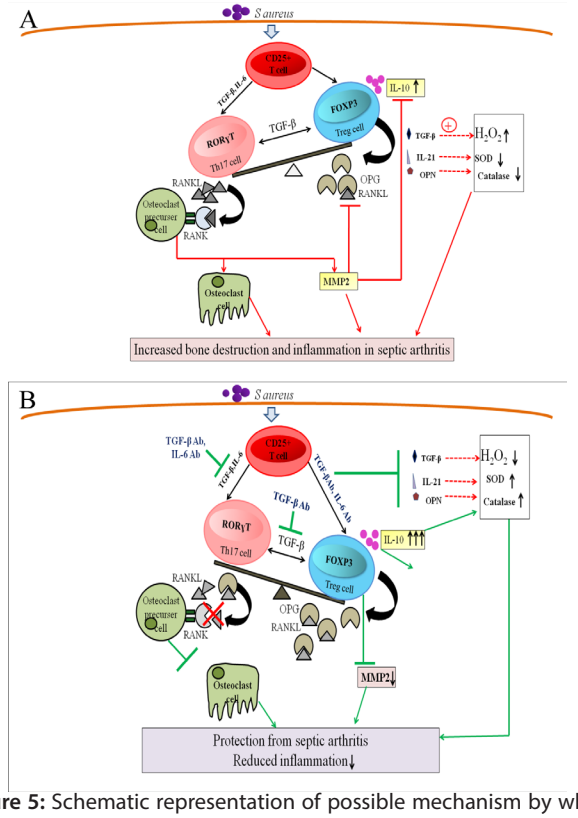

Figure 5: Schematic representation of possible mechanism by which TGF-β and IL-6 neutralization ensure protection from septic arthritis via attenuating osteoclastic activity

Upper panel: *S. aureus* enters the host via systemic circulation and is subsequently identified by the antigen-presenting cells (APCs) to the naïve T cells.Th0 cells differentiated into either Th17 or Treg cell and influenced inflammatory cytokines like TGF-β and IL-6. Elevated amount of Th17 cells increase the production of pro-inflammatory cytokines like TNF-α, IL-21. This further stimulates RANKL, which naturally interacts with receptor RANK on osteoclast precursor cells. These pro-inflammatory cytokines suppress the OPG production and the competitive interaction between OPG and RANKL can be halted by the increased expression of MMP2. RANK-RANKL interaction stimulates NF-κB. Excessive production of ROS led to higher lipid peroxidation within the cell. Simultaneously, the decreased activity of SOD, catalase, cannot neutralize the excess amount of free radicals, leading to the acute inflammation indicated by higher serum CRP level.

Lower panel: Dual blocking of TGF-β, IL-6 by neutralizing antibodies may able to inhibit the differentiation of Th17 cell population. The immunosuppressive Treg cells plays a dominant role in controlling the cytokines. IL-10 produces by Treg cells suppresses TGF-β, IL-21, and OPN. IL-10 promotes OPG concentration and stimulates the RANKL-OPG binding via blocking RANK-RANKL interaction. Lower expression of MMP2 also facilitates the RANKL-OPG binding, which may reduce the activation of NF-κB, further reducing bone destruction.

events. Conversely, treatment with TGF-β Ab and IL-6Ab either alone or in combination after *S. aureus* infection reduces the degradative effects of RANKL. In septic arthritis, activated RANKL plays a crucial role in forming osteoclast cells, as mentioned earlier. Interaction of RANKL with specific receptor RANK initiates NF-κB signaling in osteoclast cells and T lymphocytes that secretes several osteoclastic factors that help in resorption of the bones.[44] Additionally, RANK interacts with TRAF6, which further activates MAPK and JNK signaling pathways in response to the infection.[45] However, a conceivable explanation for the reduced severity of the disease after the treatment with both TGF-β Ab and IL-6Ab could be that a reduced level of RANKL might be the result of the competitive binding of circulating OPG with RANKL that inhibits the RANK/RANKL interaction which in turn down-regulates the inflammatory signaling pathways.

Therefore, from the entire study (Figure 5), it was found that the pathogenic *S. aureus* could get entry into the host through systemic circulation and subsequently recognized by the professional antigen-presenting cells (APCs) through their cell surface TLR2 (Figure 5A: upper panel). They could be presented to the naive T cells (Th0) upon recognition. During this presentation on the influence of several inflammatory cytokines like TGF-β, IL-6, IL-1β, IL-2, etc., Th0 cells can be differentiated into either Th17 or Treg population. The FACS analysis data confirmed that the Th17 population ruled over the Treg counterpart during *S. aureus-induced* septic arthritis, particularly at 9DPI. These excess amounts of Th17 cells secreted IL-17, accompanied by the increased production of pro-inflammatory cytokines like TNF-α and IL-21. They could stimulate the RANKL production, which is an interacting partner of its natural receptor RANK present on osteoclast precursor cells. Another crucial factor for developing septic arthritis is the relative abundance of osteopontin (OPN) and osteoprotegerin (OPG). The amount of OPN was observed to be higher than OPG during *S. aureus* infection. It can be speculated that IL-17 might suppress OPG production, and the competitive interaction between OPG and RANKL can be halted by the increased expression of MMP2, as evident from our data.

Additionally, the RANK-RANKL interaction could stimulate NF-κB pathway, possibly through TRAF6. Thus NF-κB translocation to the nucleus promoted osteoclastogenesis leading to arthritic bone destruction. When focusing on the inflammatory reactions, excessive production of ROS (O2.-, H_2O_2) and nitric oxide (NO) led to higher lipid peroxidation within the cell. Simultaneously, the lower activity of SOD and catalase would not be able to neutralize the excess amount of free radicals, ultimately leading to the profuse inflammation indicated by higher synovial tissue C-reactive protein (CRP) level.

During dual blocking of TGF-β, IL-6 by neutralizing antibodies could restrict the differentiation of the Th17 cell population (Figure 5B: lower panel). The immunosuppressive Treg cells now dominate in controlling the cytokine-mediated network to control arthritic inflammation. The increased production of anti-inflammatory IL-10 from Treg cells came into action via suppressing TGF-β and IL-21, and OPN. IL-10 directly promoted the OPG concentration, thereby stimulating the RANKL-OPG binding via blocking RANK-RANKL interaction. Moreover, lower expression of MMP2 also facilitates the RANKL-OPG binding, which may hinder the activation of NF-κB. Due to the lack of osteoclastogenic

factors, osteoclastic activity reduces and results in lower bone resorption. IL-10 possesses multi-factorial effects by directly blocking the Th17 differentiation and the down-regulation of CXCL8. On the other hand, it could exert its anti-inflammatory effects by reducing NO production, possibly by stimulating arginase activity. The Treg population also recovers the cellular antioxidant mechanisms via boosting SOD, and catalase activity. Therefore, it can be concluded that dual blocking of TGF-β and IL-6 confers protection from *S. aureus-induced* septic arthritis via promoting the immunosuppressive function of Treg cells. This is to be further added that Treg-derived IL-10 could be able to ameliorate the inflammatory consequences of *S. aureus-induced* septic arthritis via influencing RANKL/OPG interaction.

CONCLUSION

Here we may conclude that simultaneous neutralization of both TGF-β and IL-6 after the onset of septic arthritis is supposed to be more potent than that of individual inhibition of either TGF-β or IL-6 in the shifting of Th17 cells to the Treg type. Moreover, it may be suggested that dual neutralization of TGF-β and IL-6 may be chosen as an alternative therapeutic treatment for improving the severity of septic arthritis via inducing Treg-derived IL-10 that could ameliorate the inflammatory sequels of septic arthritis by influencing RANKL/OPG interaction in lymphocytes. However, a detailed understanding of different cytokine signaling pathways and their transcriptional regulation (STAT3, STAT4) might help us to address the regulatory crosstalking of Th17/Treg lymphocytes in septic arthritis.

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Conflict of interest

The authors declare no conflicts of interest in this work.

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