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Berberine mitigates IL-21/IL-21R mediated autophagic influx in fibroblast-like synoviocytes and regulates Th17/Treg imbalance in rheumatoid arthritis

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Abstract
In our previous study, we explored the therapeutic effect of berberine (BBR) against IL-21/IL-21R mediated inflammatory proliferation of adjuvant-induced arthritic fibroblast-like synoviocytes (AA-FLS) through the PI3K/Akt pathway. The current study was designed to explore the therapeutic potential of BBR (15–45 μM) against IL-21/IL-21R mediated autophagy in AA-FLS mediated through PI3K/Akt signaling and Th17/Treg imbalance. Upon IL-21 stimulation, AA-FLS expressed elevated levels of autophagy-related 5 (Atg5), Beclin-1 and LC3-phosphatidylethanolamine conjugate 3-II (LC3-II) through the utilization of p62 and inhibition of C/EBP homologous protein (CHOP). BBR (15–45 μM) inhibited autophagy in AA-FLS cells mediated through PI3K/Akt signaling via suppressing autophagic elements, p62 sequestration and induction of CHOP in a dose-dependent manner. Moreover, IL-21 promoted the uncontrolled proliferation of AA-FLS through induction of B cell lymphoma-2 (Bcl-2) and diminished expression of Bcl-2 associated X protein (BAX) via PI3K/Akt signaling. BBR inhibited the proliferation of AA-FLS via promoting apoptosis through increased expression of BAX and diminished Bcl-2 transcription factor levels. Furthermore, T cells stimulated with IL-21 induced $CD4^+$ $CD196^+$ Th17 cells proliferation through RORγt activation mediated in a PI3K/Akt dependent manner. BBR inhibited the proliferation of Th17 cells through downregulation of RORγt in a concentration-dependent manner. BBR also promoted the differentiation of $CD4^+$ $CD25^+$ Treg cells through induction of forkhead box P3 (Foxp3) activation via aryl hydrocarbon receptor (AhR) and upregulation of cytochrome P450 family 1, subfamily A, polypeptide 1 (CYP1A1). Collectively, we conclude that BBR might attenuate AA-FLS proliferation through inhibition of IL-21/IL-21R dependent autophagy and regulates the Th17/Treg imbalance in RA.

Keywords IL-21/IL-21R · p62 · RORγt · Foxp3 · AhR

Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disorder designated with inflammation of the joints resulting in structural abnormalities [1]. Infiltration of various cell subtypes in the joint region mediates a catastrophic change which promotes synovial hyperplasia, cartilage degradation and bone erosion [2]. Fibroblast-like synoviocytes (FLS) are the major population of cells constituting the synovial joint (~70%) mediating several inflammatory processes through tumor-like proliferation resulting in pannus formation [3]. During an inflammatory condition like RA, FLS cells secrete various extracellular cytokines (TNFα, IL-6, and IL-23) and chemokines (RANKL, GM-CSF, and MMPs) that promote migration/differentiation of several cell types of the RA synovium [4, 5]. Several cytokines orbiting around the synovial region promotes the inflammatory proliferation and survival of FLS cells leading to an abnormal joint physiology [6, 7].

Interleukin 21 (IL-21), a double-edged cytokine which is majorly secreted by follicular T helper (Tfh), T helper 17 (Th17) and natural killer (NK) cells promotes aberrant pleomorphic changes to various cell subtypes present in the joint space of RA [8, 9]. In normal physiological conditions, IL-21 promotes NK cell activation, Tfh cell proliferation and B cell maturation [10, 11]. However, in a disease

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condition such as RA, IL-21 invokes several immunomodulatory functions including FLS proliferation, T cell subset differentiation and B cell activation leading to auto-antibody production [12, 13]. Moreover recently, it has also been well established that IL-21 promotes the inflammatory proliferation of FLS and $CD4^+$ T cells through various cellular survival mechanisms [14, 15].

Autophagy is one such cellular homeostatic response essential for restoration of macromolecules during nutrient deprivation, protein aggregation and in repairing damaged cell organelles [16, 17]. However, dysregulated autophagy has been shown to promote disease pathogenesis of various autoimmune disorders including RA [18, 19]. Various factors contribute to this aberrant autophagy in several cell types of RA predominantly in FLS cells. For instance, increased expression of ATG Beclin-1 and reduced expression of C/EBP homologous protein (CHOP) in RA-FLS is correlated with increased autophagy and cellular survival [20]. Moreover, E1 ubiquitin-activating enzyme such as autophagy-related gene 5 (Atg5) plays a key role in autophagy by binding to Atg12, resulting in a dimeric complex [21]. This complex fuses with microtubule-associated proteins 1A/1B light-chain 3 (LC3), which is of two forms namely: unlipidated cytosolic fraction (LC3-I) and lipidated form (LC3-II) resulting in the formation of autophagosome [22]. Furthermore, an important ubiquitin-binding scaffold protein called as sequestosome 1 (SQSTM1/p62) co-localizes with ubiquitinated protein aggregates and thus acts as a substrate for the autophagosome [23]. All these factors put together are vital prognostic markers for autophagy response in FLS cells and are well explored in the recent years. However, cytokine-mediated autophagic response in RA FLS cells are sparse and poorly understood. For instance, Meng et al. recently provided evidence for IL-1β mediated autophagy response in RA-FLS cells resulting in hyperplastic synovium [24]. Moreover, TNFα and IL-17 mediated RA-FLS proliferation through autophagy induction have been witnessed in the present years [25–27]. Furthermore, inflammatory pathways like PI3K/Akt and Stat3 have been recently established to promote FLS cells proliferation through inducing autophagy-related genes [24, 27]. These autophagic pathways have been designated to evade apoptosis through induction of B-cell lymphoma 2 (Bcl-2) transcription factor and diminish the expression of Bcl-2 associated X protein (BAX) in RA disease model [28].

In conjunction with FLS, a subset of $CD4^+$ T cells designated as T helper 17 (Th17) invokes various immunomodulatory effects attenuating the joint pathology in RA. So far studies have reported that Th17 cells secrete various cytokines (TNFα, IL-1β, IL-6, IL-17, and IL-21), chemokines (CCL20 and GM-CSF) and promote B cell activation/maturation [29]. Inflammatory cytokines revolving around the RA synovium including IL-21 and IL-23 majorly helps in Th17 differentiation and proliferation [30, 31]. For instance, a recent report elicited that IL-21 co-stimulated with IL-23 amplifies the proliferation of Th17 cells in the RA synovium [32]. Another report elucidated that IL-21 increased the number of $CD4^+$ Th17 cells in RA patients [33]. Moreover, a recent report revealed that IL-21 has a co-stimulatory effect on Th17 differentiation through inhibition of forkhead box P3 (Foxp3) expression and upregulation of RORγt transcription factor in RA [12]. Another population of $CD4^+$ T cells that are poorly differentiated during arthritic progression designated as regulatory T (Treg) cells is depicted to have a suppressive effect on the disease pathogenesis of RA [34]. The proliferation and survival of Treg cells depend on induction of Foxp3, which is induced through ligand-dependent transcription factor called as aryl hydrocarbon receptor (AhR) and increase in levels of cytochrome P450 family of an enzyme called as cytochrome P450, family 1, subfamily A, polypeptide 1 (CYP1A1) [35]. Current treatment strategies focus on inducing these factors thereby promoting Treg cells differentiation and altering the disease progression of RA.

Currently prescribed medicine used for the treatment of RA marked as anti-rheumatic drugs majorly reduce the level of pain mediators and provide a momentary relief. Moreover, the prescribed drugs post profound comorbidities to individuals with reference to various physiological parameters, which needs an alternative [36]. The better substitute for the currently prescribed medicine is naturally derived compounds from various plant sources. Berberine (BBR), an alkaloid derivative majorly present in Oregon grapes and shoots of barberry acts as a better substitute for currently prescribed drugs as it possesses several medicinal values like anti-microbial, anti-tumorigenic and anti-inflammatory [37, 38]. BBR has been shown to possess anti-arthritic properties witnessed through the inhibition of several inflammatory signaling pathways [39, 40]. Moreover, BBR has been shown to diminish autophagy-mediated survival of mature adipocytes through attenuation of Beclin-1 [41]. Furthermore, BBR has been designated to be an inducer of AhR and in promoting Treg differentiation [42, 43]. Overall, these reports put together makes it as an excellent candidate for disease attenuation of RA through targeting IL-21/IL-21R mediated autophagic response in FLS and modulating the Th17/Treg imbalance.

Materials and methods

Reagents

Berberine and TRIzol were purchased from Sigma chemicals co. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), trypsin,

antibiotics (penicillin and streptomycin) were obtained from HIMEDIA (Mumbai, India). Recombinant IL-21, TGF-β, IL-6, and IL-10 were purchased from PeproTech (NJ, USA). PMA cocktail containing ionomycin and brefeldin A was obtained from BioLegend (SanDiego, CA, USA). The PI3K inhibitor LY294002 was obtained from MedChem Express (NJ, USA). High capacity cDNA reverse transcriptase kit was purchased from Applied Biosystems (Foster City, NY, USA) and EvaGreen mastermix was procured from G-Biosciences (St. Louis, MO, USA). Rat specific primers for AhR, CYP1A1, Atg5, Beclin-1, Bcl-2, BAX, CD196, CD25, Foxp3, RORγt and β-actin were purchased from Sigma Aldrich (St. Louis, MO, USA). Primary antibodies against Atg5, Beclin-1, CHOP, LC3, p62, Bcl-2, and BAX were obtained from ABclonal technology (Woburn, Massachusetts, USA). Antibody against β-actin was purchased from Bioss antibodies (Woburn, Massachusetts, USA). FITC tagged monoclonal antibodies against CD90.2 and CD55 were obtained from Santa Cruz (CA, USA). Secondary horseradish peroxidase (HRP) conjugated antibody was purchased from Cell Signaling Technology (Danvers, MA, USA). EasySep^{TM} rat specific T cell isolation kit was obtained from STEMCELL technologies (Vancouver, Canada). Fluorescent-tagged antibodies against CD3, CD4, Foxp3, CD196, CD25, and RORγt were procured from BioLegend (SanDiego, CA, USA). Small interfering RNA (siRNA) targeted against aryl hydrocarbon receptor (AhR) and negative controls were purchased from RiboBio (Guangzhou, China).

Animals

Wistar albino rats of either sex (120–150 g) were procured from Animal House, Vellore Institute of Technology, Vellore, India. The rats were provided with ad libitum access to food (rodent pellet diet) and water. They were acclimatized in light and temperature controlled room with a 12 h dark–light cycle. The animals were treated and cared in accordance with the guidelines recommended by the Committee for the Purpose of Control and Supervision of Experiments on animals (CPCSEA), Government of India. The experimental procedure was carried out in accordance with the guidelines of the Institutional Animal Ethical Committee (IAEC), Vellore Institute of Technology, Vellore, India.

Isolation and culture of adjuvant-induced arthritic fibroblast-like synoviocytes (AA-FLS)

Induction of arthritis was carried out by intra-dermal injection of (100 µl) Freund's complete adjuvant (FCA) (Sigma Aldrich, St. Louis, USA) into the right hind paw of the rats. AA-FLS was isolated as previously described with certain modifications [44]. In brief, synovial tissues were excised from adjuvant-induced arthritic rat knees under sterile conditions on the 19th day after arthritis induction and were chopped into small pieces followed by incubation in complete DMEM medium containing 0.4% type II collagenase for 3 h at 37 °C. Non-adherent tissues were further broken down in serum-starved DMEM medium containing 0.25% trypsin for 30 min. The tissue suspension was then traversed through a sterile nylon mesh filter (200 mm^2) and centrifuged at 1500 rpm for 10 min. The acquired cells were washed and cultured in DMEM medium supplemented with 10% FBS and 1× antibiotic solution at 37 °C in a humidified atmosphere of 5% CO_2. After overnight incubation, the debris and non-adherent cells were removed and the adherent cells were cultured under the similar condition for a week. At confluency of 80–90%, the adherent cells were trypsinized, passaged and sub-cultured under similar conditions. A homogenous population of AA-FLS cells was used in all experiments. Control FLS cells were isolated using the similar procedure from normal rats.

Purity and phenotype analysis of AA-FLS

Purity and phenotype of isolated AA-FLS cells were performed by flow cytometry analysis as described previously with minor changes [44]. In brief, 5×10^3 cells were initially washed with ice-cold PBS (1×) to remove any debris. After washing, the cells were suspended in FACS buffer (500 µl) containing FITC-tagged monoclonal CD90.2 and CD55 antibodies (Biolegend, San Diego, CA, USA) and incubated for 30 min at 4 °C. Cell fluorescence was estimated by flow cytometry analysis (FACS Calibur, BD Biosciences, San Jose, CA, USA) and data were analyzed using the CellQuest 3.3 software (Becton–Dickinson, New Jersey, USA). Experiments were performed in triplicates.

Real-time cell migration assay (xCELLigence)

Real-time cell migration was detected as per the manufacturer's protocol (xCELLigence, OMNI Life Science, Germany). Briefly, IL-21 stimulated AA-FLS cells (5×10^3) were seeded on CIM-Plate (16 wells) with the xCELLigence DP system. After stimulation, cells were treated with varying concentration of BBR (15–45 µM) and cell impedance was calculated. Impedance was calculated as a dimensionless parameter cell index (CI) measured every 15 min for a time point of 48 h. Curve analysis was indicated by the normalized cell index. Experiments were performed in triplicates.

Quantitative RT-PCR analysis

Total RNA was extracted using TRIzol reagent (Sigma Chemicals co., St. Louis, MO, USA) and was reversed transcribed into cDNA using a high capacity cDNA reverse

transcription kit (Applied Biosystems, CA, USA) according to manufacturer's protocol. Gene-specific primers were designed manually using online NCBI primer-BLAST tool and were purchased from Sigma Aldrich (St. Louis, MO, USA) (Table 1). The gene expressions of AhR, CYP1A1, Atg5, Beclin-1, Bcl-2, BAX, CD196, CD25, Foxp3, RORγt and β-actin respectively were quantified using EvaGreen PCR mastermix (G-Biosciences, St. Louis, MO, USA) following the manufacturer's instruction. Thermal cycling conditions were as follows: denaturation at 94 °C for 15 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s. The fold change in gene expression levels of target genes was calculated with normalization to β-actin values using the 2-ΔΔCt comparative cycle threshold method.

Protein isolation and western blot analysis

Western blot analysis was performed with certain modifications [45]. In brief, whole cell lysates were acquired by homogenization in RIPA buffer (Ice cold) with protease inhibitor cocktail and centrifuged at 14,000 rpm for 15 min at 4 °C. The protein concentration in the cell lysates was estimated using the Bradford method (Bio-Rad, Hercules, CA, USA). The cell lysates (30 µg/ml) were separated on 12% SDS-PAGE and electro-transferred onto PVDF membrane (Amersham Pharmacia Biotech, Uppsala, Sweden). The transfer was confirmed using Ponceau S staining method. After destaining, the membranes were blocked with 5% (w/v) BSA overnight at 4 °C. Subsequently, the membranes were incubated with rabbit polyclonal antibody against Atg5, BAX, Bcl-2, CHOP, p62, Beclin-1, LC3-I/II and β-actin followed by washing and probing for 2 h with horseradish peroxidase (HRP) conjugated secondary antibody. Protein bands were visualized using the enhanced chemiluminescence detection system (Bio-Rad Laboratories, Mississauga, Canada). The blots were stripped and reprobed with β-actin to confirm equal protein being loaded. Each protein blot is representative of three similar independent experiments.

Immunofluorescence analysis

Immunofluorescence was performed as previously reported with minor modifications [55]. Briefly, AA-FLS cells were seeded on gelatin-coated round glass coverslips and treated with/without BBR (15, 30 and 45 µM) or LY294002 (20 µM) for 24 h after IL-21 (20 ng/ml) stimulation for 24 h. Subsequently, the media was discarded and the cells were washed with 1 × PBS. Immediately the cells were then fixed with 4% formaldehyde for 15 min at 37 °C. Thereafter the cells were rinsed with 1 × PBS and were subjected to permeabilize with 0.1% Triton X–100 in 1 × PBS for 5 min. The coverslips were stained with rabbit polyclonal antibodies of p62 (1:250) and LC3-II (1:250) overnight at 4 °C. Subsequently, the cells were incubated with secondary goat-anti-rabbit antibody labeled with Alexa Fluor 488 (Cell Signaling Technology, Beverly, MA) at 37 °C for 2 h in the dark. The nuclei were counterstained with DAPI (4′6-diamindino-2-phenylindole, 1 µg/ml; Sigma Aldrich, St Louis, MO, USA) for 5 min at 37 °C and examined using Olympus Confocal microscope (Olympus America, Melville, NY, USA). Mean fluorescence intensity was measured using ImageJ software as described previously [55].

Cell migration assay and annexin V/propidium iodide (PI) staining

For cell migration assay, FLS/AA-FLS cells were seeded on six well plates at a confluence of 70–80% and serum starved overnight. A linear wound was initiated using a sterile 1 ml micropipette and washed to remove unattached cells. Cells were stimulated with IL-21 (20 ng/ml) with/without BBR (15–45 µM) or LY294002 (20 µM) treatment. Microscopic images were taken after stimulation with/without treatment and migrated cells were counted using ImageJ software. The number of migrated cells were normalized to control and represented as migration index.

Table 1 Primer sequences used for quantitative real-time PCR analysis

Gene	Forward	Reverse
AhR	5′-CTGCGTGTGAGTCGATGCTC-3′	5′-TAAACCCCGCCAAAGACCAG-3′
Beclin-1	5′-CACCCACTGTGTGAGGAATG-3′	5′-TCCTCCAAGGCCAACTCCTT-3′
Atg5	5′-GTAAAGAGCGCACCCGGAG-3′	5′-CTTCGGACTCGAGGCTACAT-3′
CYP1A1	5′-CCGAAGAGTAGCATGTCAGGG-3′	5′-GCCCCTAAGGACCTCTACCTT-3′
BAX	5′-CAAGGCCCTGTGCACTAAAG-3′	5′-GTCACTGTCTGCCATGTGGG-3′
Bcl-2	5′-TCACAGAGGGGCTACGAGTG-3′	5′-CCGTAGAGGCGACGTCCTG-3′
Foxp3	5′-GTCATGGTGGCACCCTCTG-3′	5′-CATCGCTGGGTTGTCCAGTG-3′
CD196	5′-CAAGGTGTTCGTGCCAATCG-3′	5′-CTGCCCAGAATGGTAGGGTG-3′
RORγt	5′-AGTGCCCCGTCTGTCGTTAT-3′	5′-TCCACTGGTGCCTCACACTA-3′
CD25	5′-ACAAGGCTCTACAGAGAGGTCC-3′	5′-AGCCTCACTCTCTGGGAAAG-3′
β-actin	5′-ACCACCATGTACCCAGGCATT-3′	5′-CCACACAGAGTACTTGCGCTCA-3′

The proliferation of AA-FLS cells was assessed using annexin V/PI staining as described by the manufacturer's protocol (eBiosciences, MA, USA). Briefly, isolated AA-FLS cells were stained with FITC-Annexin V labeling for 15 min in the dark at room temperature. Propidium iodide (PI) was added prior to the final wash, followed by flow cytometry analysis. Flow cytometry analysis of cells was performed in triplicates.

Isolation of T cells from spleen and Th17/Treg differentiation

T cells were isolated from adjuvant-induced arthritic rats' spleen using EasySep^{TM} kit previously described as per the manufacturer's protocol with certain modifications (STEMCELL Technologies, Vancouver, Canada). The purity of isolated T cells was analyzed using FITC tagged mAb raised against CD3. The cells were cultured in RPMI complete media until further analysis (BioLegend, CA, USA). The secretion of cytokines was inhibited by brefeldin A treatment (BioLegend, CA, USA).

For Th17 differentiation, splenocytes were cultured with RPMI medium containing PMA (10 ng/ml)/ionomycin (2.5 ng/ml) under minimal levels of TGF-β and IL-6 followed by IL-21 (20 ng/ml) stimulation. After stimulation, cells were treated with varying concentration of BBR (15-45 μM) or LY294002 (20 μM). Secretion of cytokines was inhibited by brefeldin A. Surface staining was performed with CD4 followed by fixation and permeabilization. Further, the $CD4^+$ Th17 cells were stained with specific fluorescent tagged mAbs targeted against CD196 and RORγt.

For Treg differentiation, freshly isolated T cells were grown in RPMI medium containing minimal levels of TGF-β and IL-10 followed by IL-21 (20 ng/ml) stimulation. Further, the cells were stained with fluorescent tagged anti-CD4 and anti-CD25 mAbs (BioLegend, CA, USA). For intracellular Foxp3 expression, lymphocytes were fixed and permeabilized with Foxp3 detection cocktail. Cell fluorescence was estimated by fluorescence-activated cell sorting (FACS Calibur, BD Biosciences, San Jose, CA, USA) and data were analyzed using the CellQuest 3.3 software (Becton-Dickinson, New Jersey, USA). All experiments were performed in triplicates.

AhR silencing

Small interfering RNA (siRNA) targeted against rat AhR gene was used to attenuate the expression of AhR in T cells. The siRNA for AhR (siAhR) and negative control (Ctrl) siRNA were designed and purchased from RiboBio (Guangzhou, China). Knockdown of AhR gene was performed as previously described with minor modifications [35]. In brief, T cells isolated from rat spleen tissues were cultured in six well tissue culture plates and introduced with the siRNA (Ctrl and siAhR) oligonucleotides prepared with Xfect RNA transfection reagent (Clontech, CA, USA) in serum-free medium for 4 h. Then the T cells were cultured in serum rich RPMI medium post 48 h transfection. At the end of the incubation period, T cells were stimulated under minimal levels of Treg differentiation conditions (TGF-β/IL-10) with or without BBR (45 μM) treatment for 24 h. The levels of $CD25^+$ $Foxp3^+$ Treg cells were detected using FACS sorting and expression levels of AhR and CYP1A1 were estimated using qPCR analysis as described previously [43].

Statistical analysis

The data were presented as mean ± standard error mean (SEM). The statistical analysis between the experimental groups was carried out using one-way analysis of variance (ANOVA) by Bonferroni's post-test using graph pad 5.0 for windows. $^{*\#\dagger¥}P < 0.05$ implies statistical significance.

Results

Purity analysis of AA-FLS and the effect of BBR on cellular proliferation

Purity and phenotype of the isolated AA-FLS were analyzed using CD90.2 (Thy-1 molecule—highly glycosylated membrane-bound protein) and CD55 mAbs. Cells obtained were trypsinized and stained with FITC-tagged CD90.2 and CD55 monoclonal antibodies at 4 °C for 30 min. Subsequently, the cells were subjected to flow cytometry analysis. The results of flow cytometry analysis showed that > 98% of the cells were stained positive for CD 90.2/Thy-1 fibroblast marker and > 95% of the cells were stained positive for CD55 (Fig. 1a, b).

The effect of varying doses of BBR (15–45 μM) on cellular proliferation of AA-FLS cells were detected in real time using Real-time cell migration assay (xCELLigence, OMNI LifeSciences, Germany). We witnessed a dose-dependent decrease in cellular proliferation from the 18th hour in a steady-state manner, thus providing evidence for the inhibiting nature of BBR towards the inflammatory expansion of AA-FLS cells (Fig. 1c). Thus, three individual doses of BBR (15–45 μM) were used for further analysis.

Autophagy in AA-FLS cells

Autophagy in AA-FLS cells mediated through IL-21/IL-21R was found to be essential for its proliferation and survival. We initially explored the gene and protein expression levels of Atg5 and Beclin-1 in FLS/AA-FLS cells stimulated with IL-21 (20 ng/ml) (Fig. 2a–d). We witnessed that IL-21

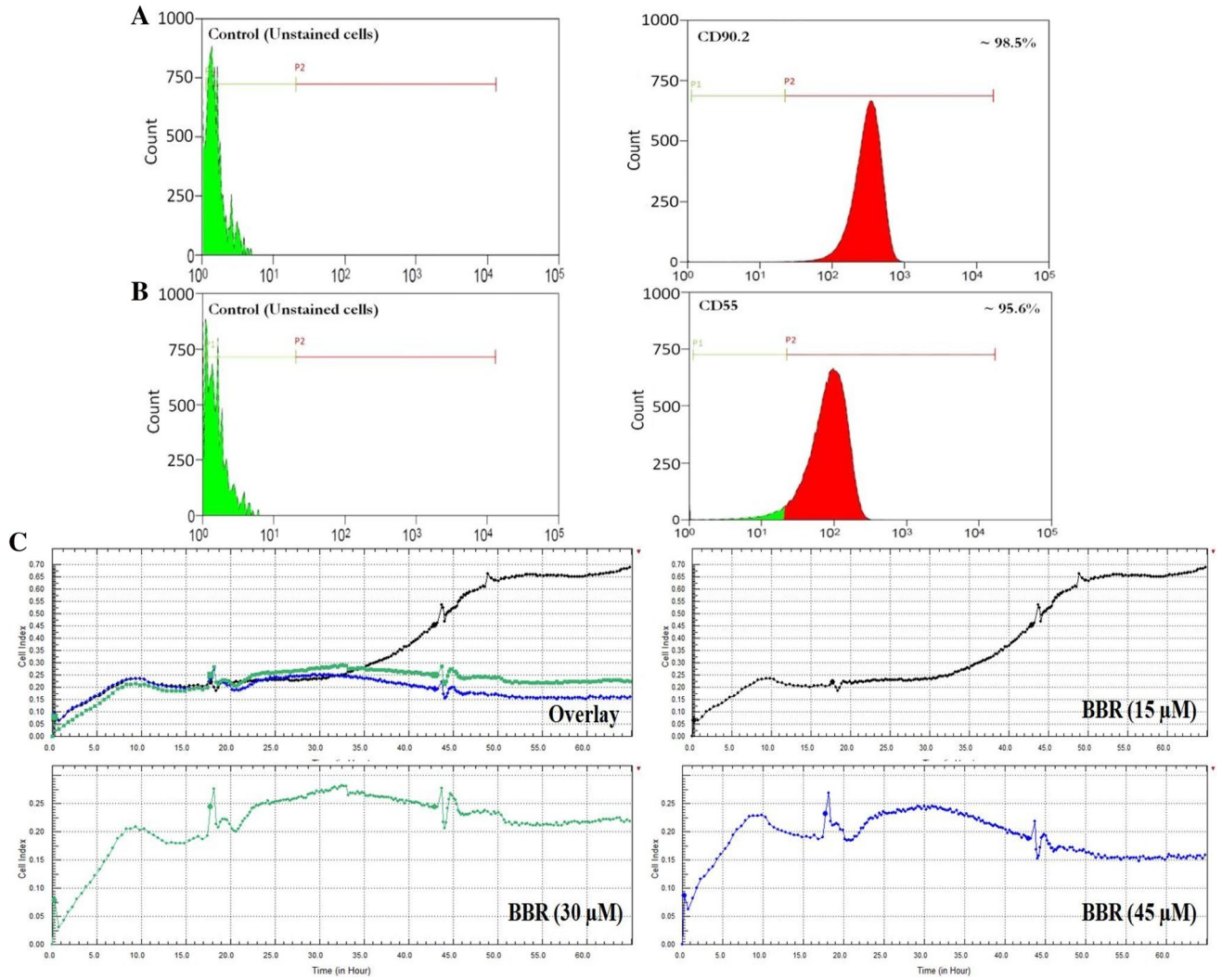

Fig. 1 Effect of BBR (15–45 µM) on cellular proliferation of AA-FLS stimulated with IL-21 (20 ng/ml). **a**, **b** Purity of isolated AA-FLS cells sorted using FITC tagged CD90.2 and CD55 markers at the third passage. **c** xCELLigence analysis of varying concentrations of BBR (15, 30 and 45 µM) on AA-FLS stimulated with IL-21 (20 ng/ml)

stimulated FLS/AA-FLS cells showed elevated levels of Atg5 and Beclin-1 compared to their respective controls. Treatment with varying concentrations of BBR (15–45 µM) attenuated the expression levels of Atg5 and Beclin-1 at the molecular levels. This elicits the autophagy inhibiting potential of BBR in AA-FLS cells.

To further witness the potential of BBR towards autophagic response, we estimated the levels of various other elements essential for promoting autophagy in AA-FLS cells including utilization of p62, LC3-II subunit upregulation and CHOP inhibition (Fig. 3a–d). Upon IL-21 stimulation, there was decreased expression of p62 (active autophagy) and upregulation of LC3-II with inhibition of CHOP expression in FLS and AA-FLS cells compared to their respective controls (Figs. 4a, b; 5a, b). This depicts that IL-21 modulates the overall survival potential of FLS through induction of autophagy. To alter this phenomenon, we treated the cells with several concentrations of BBR that sequestered the expression levels of p62 which were utilized for autophagic complex formation in the cytosol through inhibition of LC3-II ubiquitination and via induction of CHOP expression. Furthermore, we witnessed that the autophagic influx was provoked through PI3K/Akt signaling pathway by treating the cells with PI3K specific inhibitor LY294002 (20 µM). Overall, it is evident that IL-21 promotes the inflammatory proliferation of FLS cells through induction of autophagic response mediated through the PI3K/Akt signaling pathway and treatment with BBR attenuates this process.

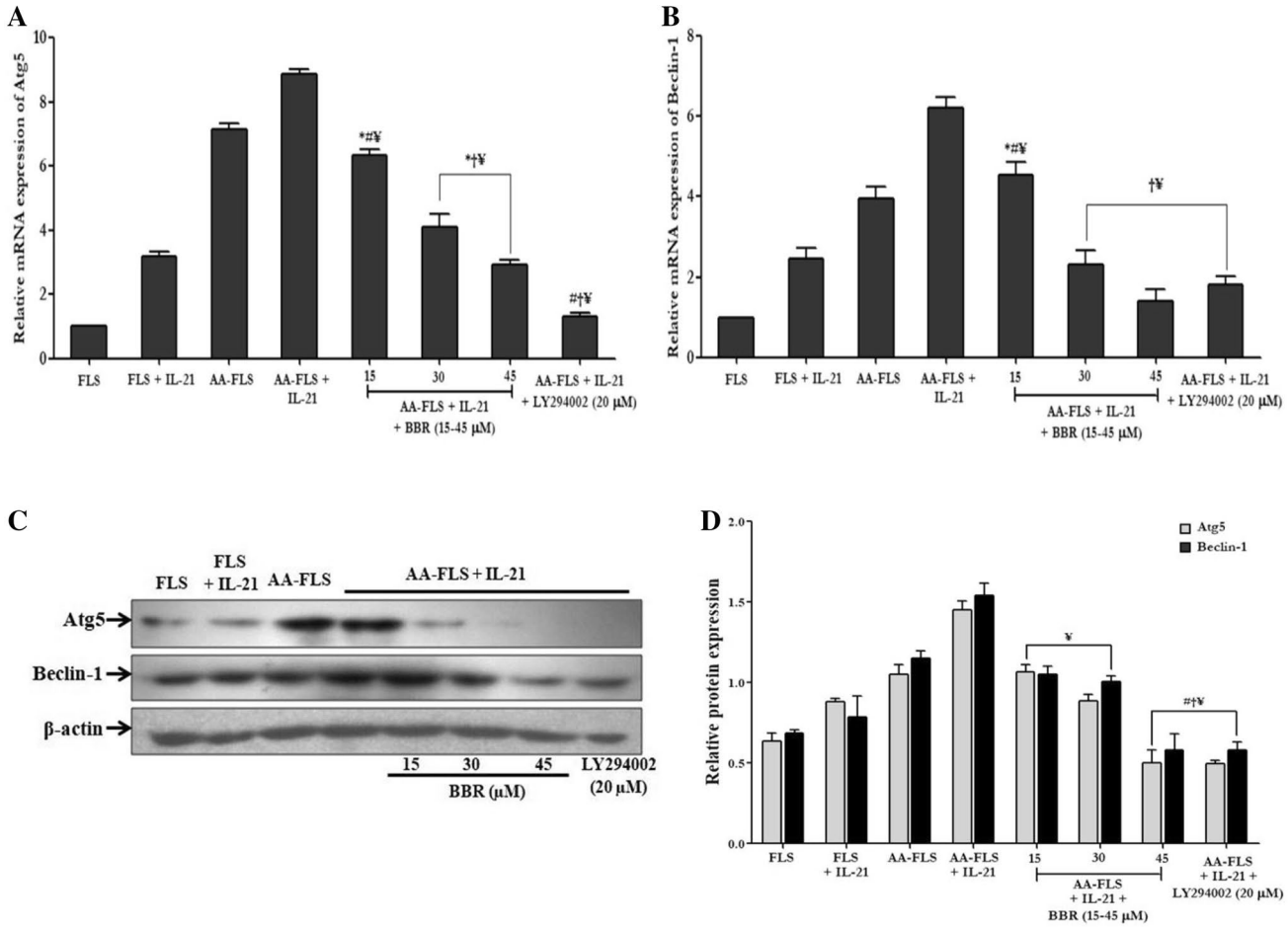

Fig. 2 Effect of BBR (15–45 μM) on mRNA and protein expression levels of Atg5 and Beclin-1 in IL-21 (20 ng/ml) stimulated AA-FLS cells. The mRNA expression of: **a** Atg5 and **b** Beclin-1 were measured using quantitative RT-PCR analysis. RQ values were calculated relative to the β-actin gene. **c**, **d** Western blot analysis of Atg5 and Beclin-1 were measured in whole cell lysates using densitometry analysis. β-actin was used as an internal loading control. Comparisons are made with: *FLS versus AA-FLS + IL-21 + BBR (15–45 μM)/LY294002 (20 μM); #FLS + IL-21 versus AA-FLS + IL-21 + BBR (15–45 μM)/LY294002 (20 μM); †AA-FLS versus AA-FLS + IL-21 + BBR (15–45 μM)/LY294002 (20 μM); ¥AA-FLS + IL-21 versus AA-FLS + IL-21 + BBR (15–45 μM)/LY294002 (20 μM). The results are expressed as mean ± SEM of the data from three individual experiments. $^{*\#\dagger¥}P < 0.05$ implies statistically significant

BBR inhibits AA-FLS proliferation

We further wanted to check whether IL-21 (20 ng/ml) stimulation with/without BBR (15–45 μM) or LY294002 (20 μM) induced proliferation of cells through apoptotic regulation. BAX (pro-apoptotic) and Bcl-2 (anti-apoptotic) being the major mediators of cell survival/apoptosis were detected after IL-21 stimulation (Fig. 6a–d). We witnessed that upon IL-21 stimulation, there was an increased expression level of Bcl-2 and downregulation of BAX in FLS/AA-FLS compared to their controls. Upon treatment with varying concentrations of BBR, the proliferation of FLS/AA-FLS was attenuated in a dose-dependent manner through induction of BAX and suppression of Bcl-2 transcription factor.

Furthermore, we confirmed the proliferation of FLS/AA-FLS mediated through IL-21 by detecting it using cellular migration assay and FITC annexin V/PI staining with/without BBR (15–45 μM) or LY294002 (20 μM) treatment (Fig. 7a–d). We witnessed that upon IL-21 stimulation, FLS/AA-FLS showed reduced apoptotic state with increased proliferation compared to control groups. Upon treating the cells with varying doses of BBR, there was a decrease in their proliferation through induction of apoptosis from 18.8 to 48.6% at the highest concentration compared to stimulated groups. We further treated the cells with LY294002 (20 μM), which promoted cellular apoptosis via inhibition of the PI3K/Akt signaling pathway. Overall, these evidences reveal that IL-21 promotes the proliferation of AA-FLS through increased expression of Bcl-2 and diminished expression

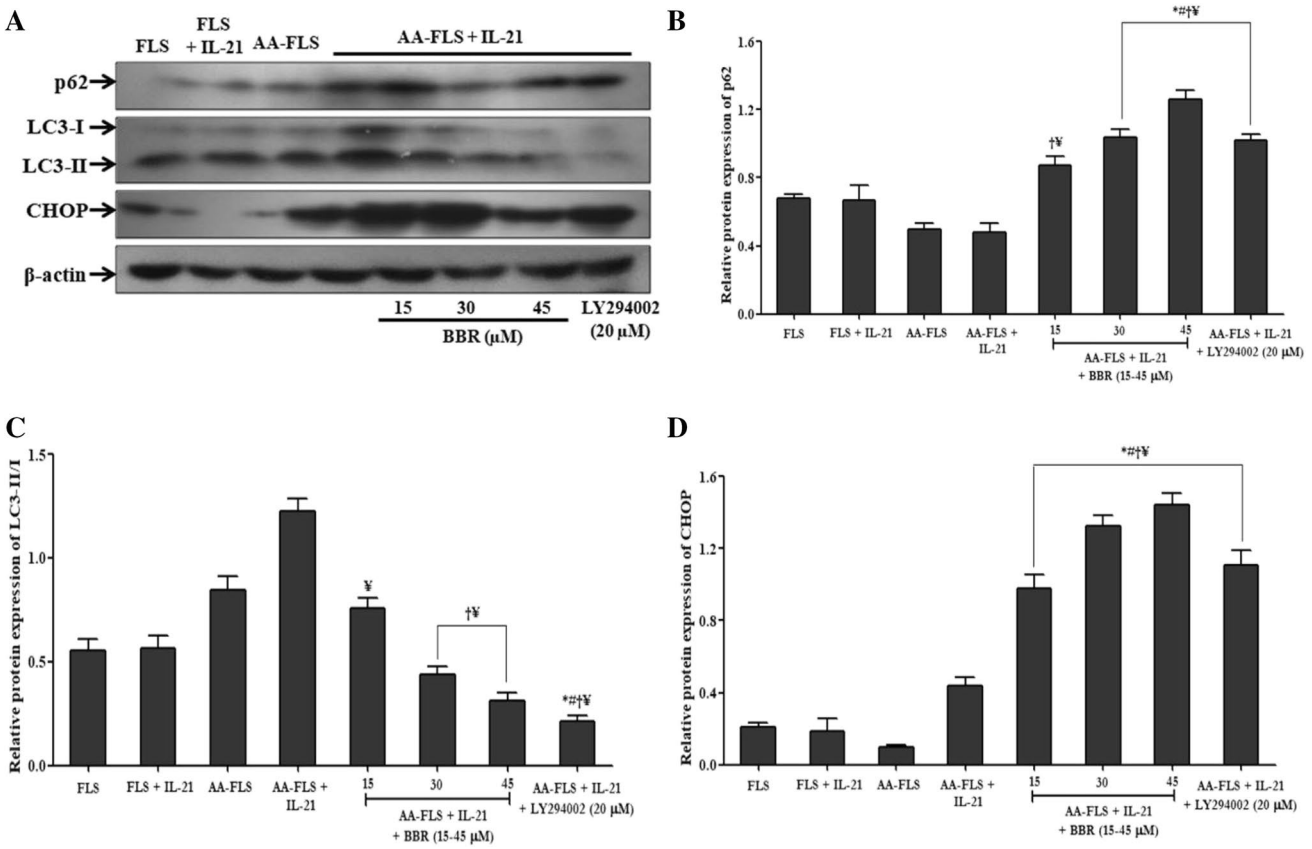

Fig. 3 Effect of BBR (15–45 μM) on protein expression levels of autophagic factors in IL-21 (20 ng/ml) stimulated AA-FLS cells. **a–d** Western blot analysis of p62, LC3-I/II and CHOP were measured in whole cell lysates using densitometry analysis. β-actin was used as an internal loading control. Comparisons are made with: *FLS versus AA-FLS + IL-21 + BBR (15–45 μM)/LY294002 (20 μM); #FLS + IL-21 versus AA-FLS + IL-21 + BBR (15–45 μM)/LY294002 (20 μM); †AA-FLS versus AA-FLS + IL-21 + BBR (15–45 μM)/LY294002 (20 μM); ¥AA-FLS + IL-21 versus AA-FLS + IL-21 + BBR (15–45 μM)/LY294002 (20 μM). The results are expressed as mean ± SEM of the data from three individual experiments. *#†¥$P < 0.05$ implies statistically significant

of BAX via PI3K/Akt pathway, which was attenuated after BBR treatment in a dose-dependent manner.

Purity of isolated T cells

Purity of isolated T cells from the spleen of rats was analyzed using FITC tagged mAb targeted against CD3. Isolated T cells were stained with FITC-tagged CD3 mAb at 4 °C for 1 h. Further, the cells were subjected to flow cytometry analysis. The results of flow cytometry analysis showed that > 94% of the cells were stained positive for T cells specific CD3 marker (Fig. 8a).

BBR inhibits Th17 differentiation

Isolated T cells (AA-T cells and Naïve T cells) from the spleen of rats were grown under Th17 differentiating conditions followed by IL-21 (20 ng/ml) stimulation in the presence/absence of BBR (15–45 μM) or LY294002 (20 μM) (Fig. 8b). We estimated the levels of $CD4^+$ T cells for the expression levels of Th17 specific cell surface markers such as CD196 and RORγt transcription factor using FACS analysis and gene expression studies (Fig. 8c–e). CCR6 or CD196 is a Th17 specific marker that is highly expressed during a disease condition such as RA. RORγt is a transcription factor that is overexpressed under Th17 skewing conditions which regulate a wide variety of genes essential for its differentiation.

Under IL-21 stimulation, there was upregulated expression of CD196 and RORγt elucidating its Th17 subtype differentiating potential. However, the proportion of Th17 cells decreased from 42.6 to 8.6% by treatment with varying concentrations of BBR. Furthermore, BBR inhibited the levels of CD196 and RORγt in a dose-dependent manner near to that of normal cells. LY294002 (20 μM) treatment diminished the proliferation of Th17 differentiation through inhibition of PI3K/Akt signaling, which essentially supported their survival. This depicts that IL-21/IL-21R interaction in Th17 cells helps in their survival/proliferation through activation of PI3K/Akt signaling pathway. Prominently,

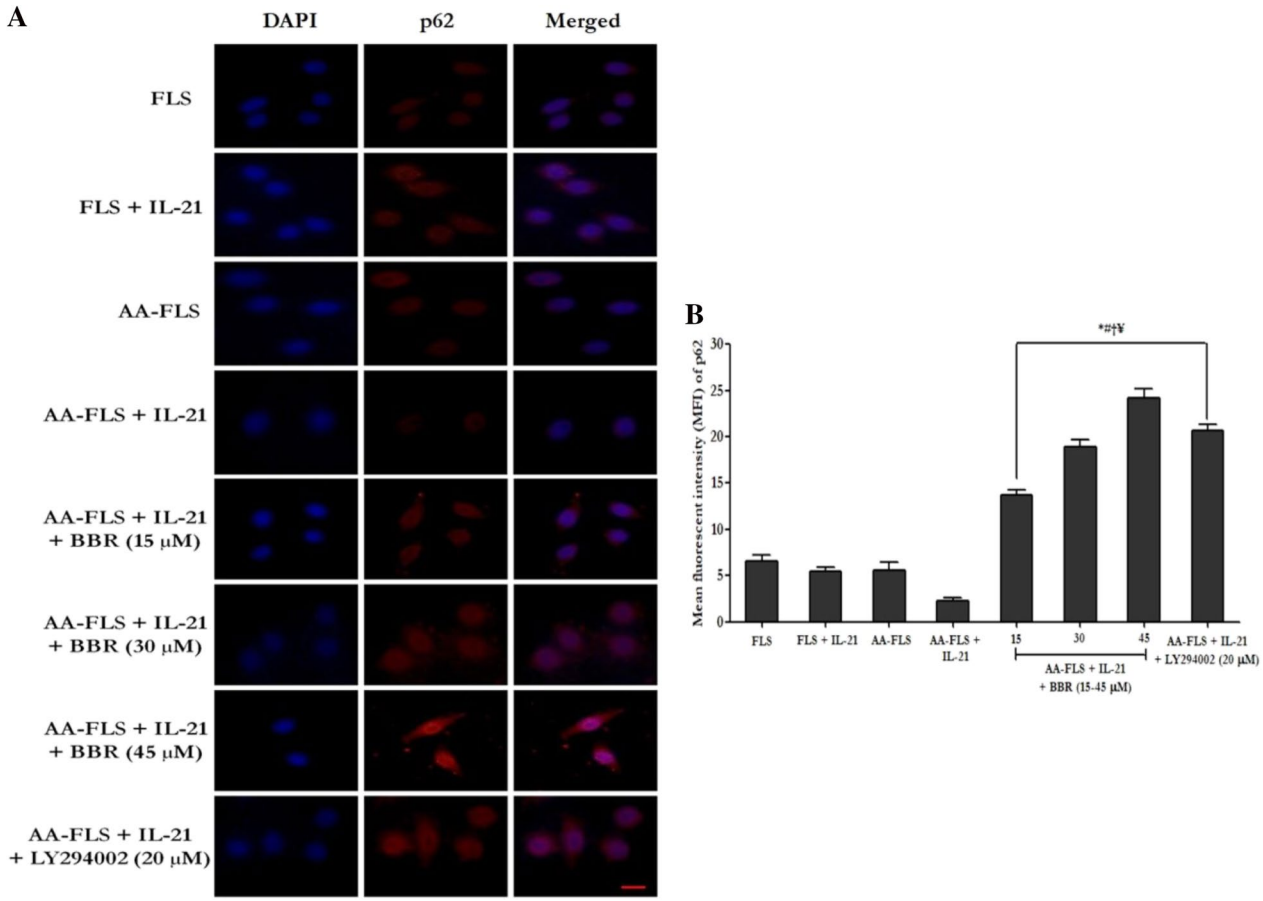

Fig. 4 Effect of BBR (15–45 μM) on cellular expression of **a**, **b** p62 in IL-21 (20 ng/ml) stimulated AA-FLS cells was determined by immunofluorescence analysis visualized using confocal microscopy. Comparisons are made with: *FLS versus AA-FLS + IL-21 + BBR (15–45 μM)/LY294002 (20 μM); #FLS + IL-21 versus AA-FLS + IL-21 + BBR (15–45 μM)/LY294002 (20 μM); †AA-FLS versus AA-FLS + IL-21 + BBR (15–45 μM)/LY294002 (20 μM); ¥AA-FLS + IL-21 versus AA-FLS + IL-21 + BBR (15–45 μM)/LY294002 (20 μM). The results are expressed as mean ± SEM of the data from three individual experiments. *#†¥$P < 0.05$ implies statistically significant. Scale bars, 10 μm

these outcomes elucidate that BBR inhibits differentiation/proliferation of Th17 cells through attenuation of CD196 and RORγt transcription factor.

BBR induces Treg differentiation through activation of aryl hydrocarbon receptor (AhR)

T cells isolated from the spleen of rats under Treg differentiation conditions along with stimulation from IL-21 (20 ng/ml) showed diminished levels of $CD4^+$ $CD25^+$ $Foxp3^+$ Treg cells compared to their respective controls as witnessed in FACS analysis and gene expression studies (Fig. 9a–d). Upon treatment with varying concentrations of BBR (15–45 μM), there was dose-dependent increase of Treg cells from 18.6 to 48.8%. BBR counteracted the effect of IL-21 through upregulation of Foxp3 expression inside the cells in a dose-dependent manner.

To further check as to how BBR promotes Treg differentiation, we estimated the levels of aryl hydrocarbon receptor (AhR) expression levels and CYP1A1 (cytochrome p450 family of enzymes) at the gene level (Fig. 10a–d). AhR is a transcription factor localized in the cytosol that has been indicated in recent years to promote differentiation and activity of Treg cells through the induction of Foxp3 expression [35]. Our data elucidates that BBR treatment resulted in induction of AhR transcription factor with increased levels $CD4^+$ $CD25^+$ $Foxp3^+$ Treg cells. Furthermore, BBR promoted the activation and sequestration of CYP1A1 family of enzymes (downstream element of AhR). We further confirmed this outcome through knockdown of siAhR inside the cells. Silencing of AhR resulted in downregulation of CYP1A1 compared to that of the siCtrl group and reduced population of $CD4^+$ $CD25^+$ $Foxp3^+$ Treg cells compared to its control. The group treated with siCtrl and BBR elicited increased expression levels of $CD4^+$ $CD25^+$ $Foxp3^+$ Treg

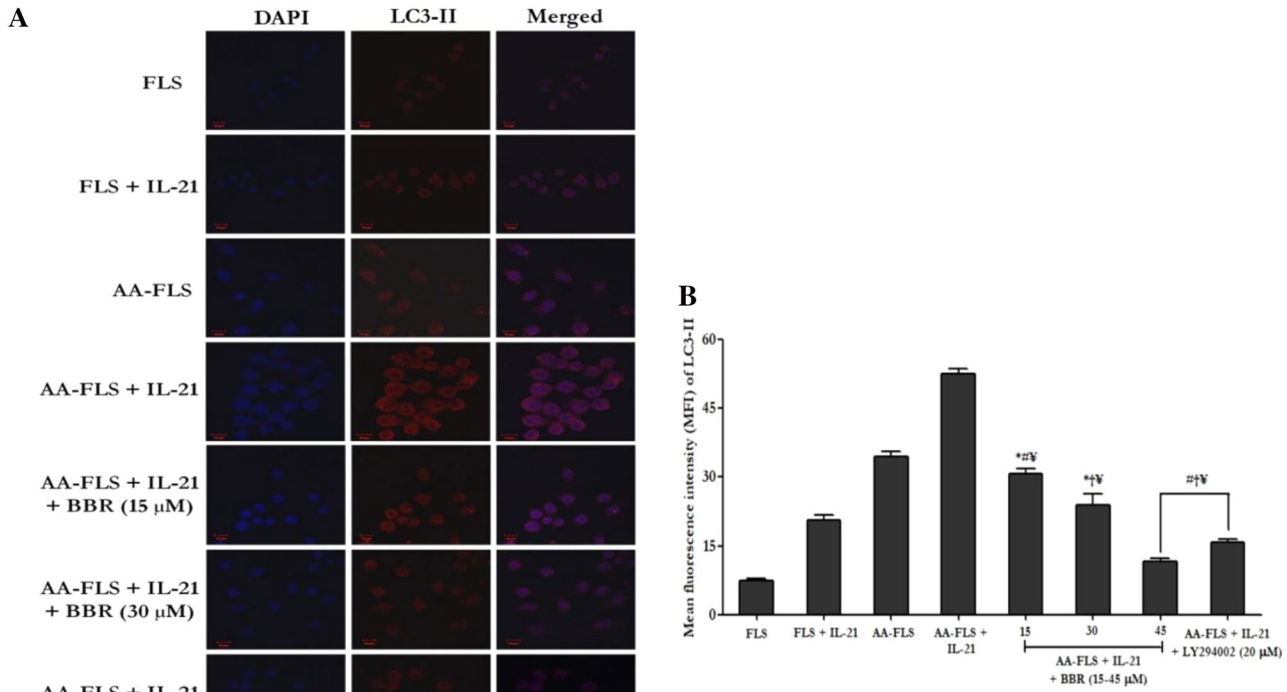

Fig. 5 Effect of BBR (15–45 μM) on cellular expression of **a**, **b** LC3-II in IL-21 (20 ng/ml) stimulated AA-FLS cells was determined by immunofluorescence analysis visualized using confocal microscopy. Comparisons are made with: *FLS versus AA-FLS + IL-21 + BBR (15–45 μM)/LY294002 (20 μM); #FLS + IL-21 versus AA-FLS + IL-21 + BBR (15–45 μM)/LY294002 (20 μM); †AA-FLS versus AA-FLS + IL-21 + BBR (15–45 μM)/LY294002 (20 μM); ¥AA-FLS + IL-21 versus AA-FLS + IL-21 + BBR (15–45 μM)/LY294002 (20 μM). The results are expressed as mean ± SEM of the data from three individual experiments. *#†¥$P < 0.05$ implies statistically significant. Scale bars, 10 μm

cells compared without BBR treatment. Thus, it is evident that AhR activation is essential for Treg differentiation and BBR has been elicited to be a potential candidate that promotes Treg differentiation through AhR transcription factor activation and induction of Foxp3 level inside the cells.

Discussion

RA, a systemic autoimmune disorder with an unknown etiology has been investigated in the recent years with respect to several inflammatory pathways [46]. Various factors circulating in the synovial joint space majorly contribute to the disease pathogenesis [2]. Today's treatment predominantly focuses on providing momentary relief through the prescription of drugs/mAbs targeted against major cytokines (TNFα, IL-6, and IL-17) and pain mediators. These strategies provoke reverting of the disease condition for which the cure becomes elusive [47]. Least studies have showcased on cytokines with a multifactorial role in the present years to promote the disease outcome of RA through various immunomodulatory effects. IL-21, which has been recently identified to be a key immunomodulator of various cell types in the RA synovium invokes survival/proliferation through various cellular survival mechanism [48].

Autophagy is one such cellular driven process essential for maintaining homeostasis of cells during stressful conditions, wherein its dysfunction leads to uncontrolled pleiomorphic changes to the cells [49]. In recent years, cytokine-driven cellular survival has been designated to be the major mediator of pathogenic response in various autoimmune disorders including RA. FLS cells being the major mediator of RA disease progression have been showcased to be modulated by inflammatory cytokines through induction of autophagy. Initial studies provided by Connor et al. elicited that TNFα stimulation to RA-FLS cells promoted their uncontrolled proliferation through aberrant autophagic responses [25]. Lin et al. provided a similar outcome in RA

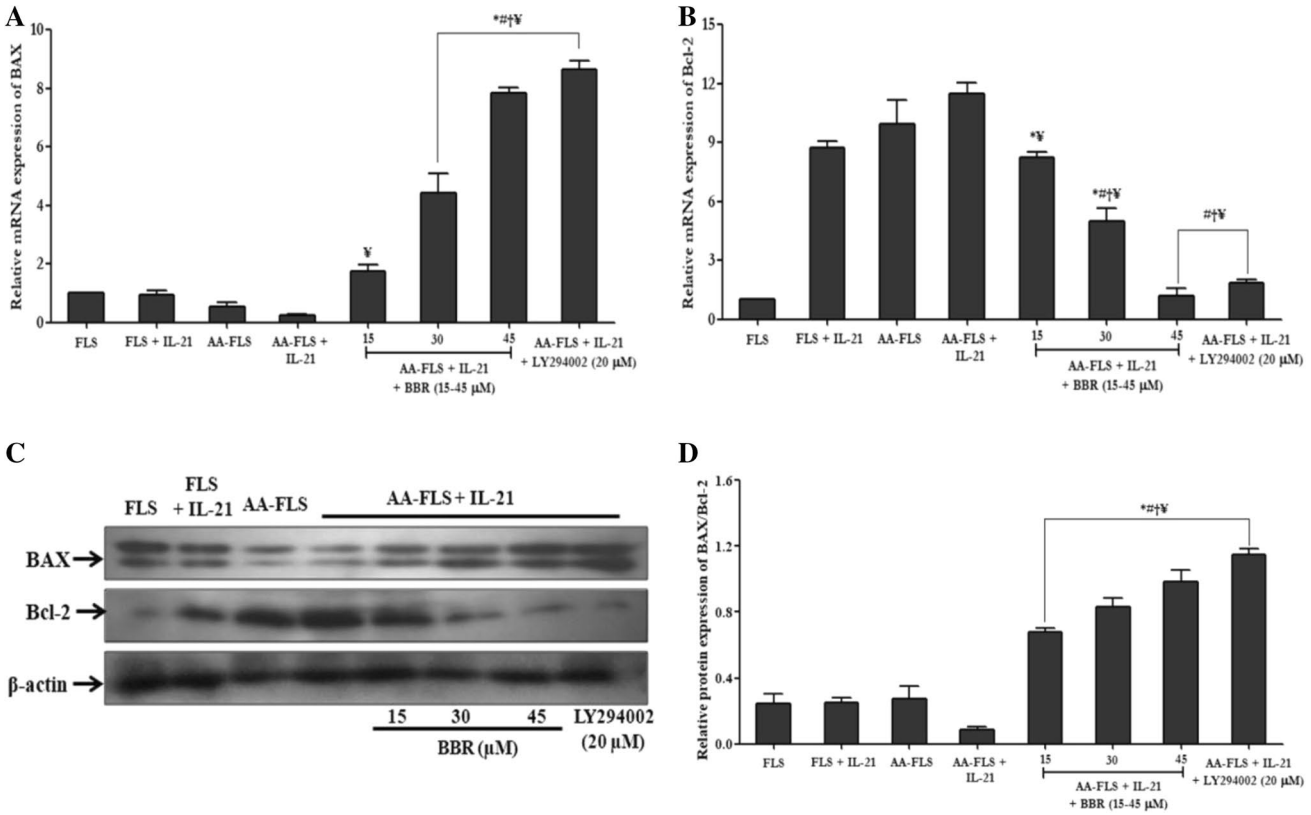

Fig. 6 Effect of BBR (15–45 μM) on mRNA and protein expression levels of apoptotic factors in IL-21 (20 ng/ml) stimulated AA-FLS cells. The mRNA expression of: **a** BAX and **b** Bcl-2 were measured using quantitative RT-PCR analysis. RQ values were calculated relative to the β-actin gene. **c**, **d** Western blot analysis of BAX and Bcl-2 were measured in whole cell lysates using densitometry analysis. β-actin was used as an internal loading control. Comparisons are made with: *FLS versus AA-FLS + IL-21 + BBR (15–45 μM)/LY294002 (20 μM); $^{\#}$FLS + IL-21 versus AA-FLS + IL-21 + BBR (15–45 μM)/LY294002 (20 μM); †AA-FLS versus AA-FLS + IL-21 + BBR (15–45 μM)/LY294002 (20 μM); $^{¥}$AA-FLS + IL-21 versus AA-FLS + IL-21 + BBR (15–45 μM)/LY294002 (20 μM). The results are expressed as mean ± SEM of the data from three individual experiments. $^{*\#\dagger¥}P < 0.05$ implies statistically significant

osteoclasts through induction of Beclin-1 and Atg7 [50]. Furthermore, Kim et al. depicted that IL-17 majorly produced by Th17 cells promoted RA-FLS survival through mitochondrial dysfunction and uncontrolled autophagy [26]. Moreover, Meng et al. depicted that IL-1β promoted RA-FLS survival through enhancement of autophagy in a PI3K/Akt/mTOR dependent manner [24]. Similarly, a recent study conducted by Chang et al. predicted that IL-17 promotes dysregulated RA-FLS proliferation through autophagic responses mediated through the activation of STAT3 signaling pathway [27]. However, to the best of our knowledge, there are no studies elucidating the role of IL-21/IL-21R interaction to mediate autophagic response in cell phenotypes of RA. Thus in this study, we unravel the mechanism of autophagic induction in adjuvant-induced arthritic fibroblast-like synoviocytes (AA-FLS) mediated through IL-21/IL-21R interaction via PI3K/Akt signaling pathway.

In the previous study, we have showcased that IL-21/IL-21R interaction promotes the inflammatory proliferation of AA-FLS through induction of PI3K/Akt signaling pathway [8]. In the present study, we elucidate the effect of IL-21 on the induction of autophagy response in AA-FLS cells through various checkpoints. Dysregulated autophagy has been implicated to play a major role in promoting the uncontrolled proliferation of FLS, aberrant osteoclastogenesis, T cell maturation and chondrocytes differentiation [28, 50, 51]. Several factors sequestered in the cytosolic fraction forms the autophagosome complex, which provides energy to support cellular survival [52]. FLS cells being the key player in RA pathogenesis has been recently elicited to express autophagy response and evade apoptosis [26]. Shin et al. provided initial evidence that rheumatoid arthritis synovial fibroblasts (RASFs) elicit resistance towards apoptosis through autophagy induction [20]. This was showcased through estimating the expression levels of Beclin-1 and LC3 conversion with reduced CHOP expression in ER stress model. Moreover, Xu et al. also predicted a similar outcome in RA-FLS cells through upregulation of Beclin-1

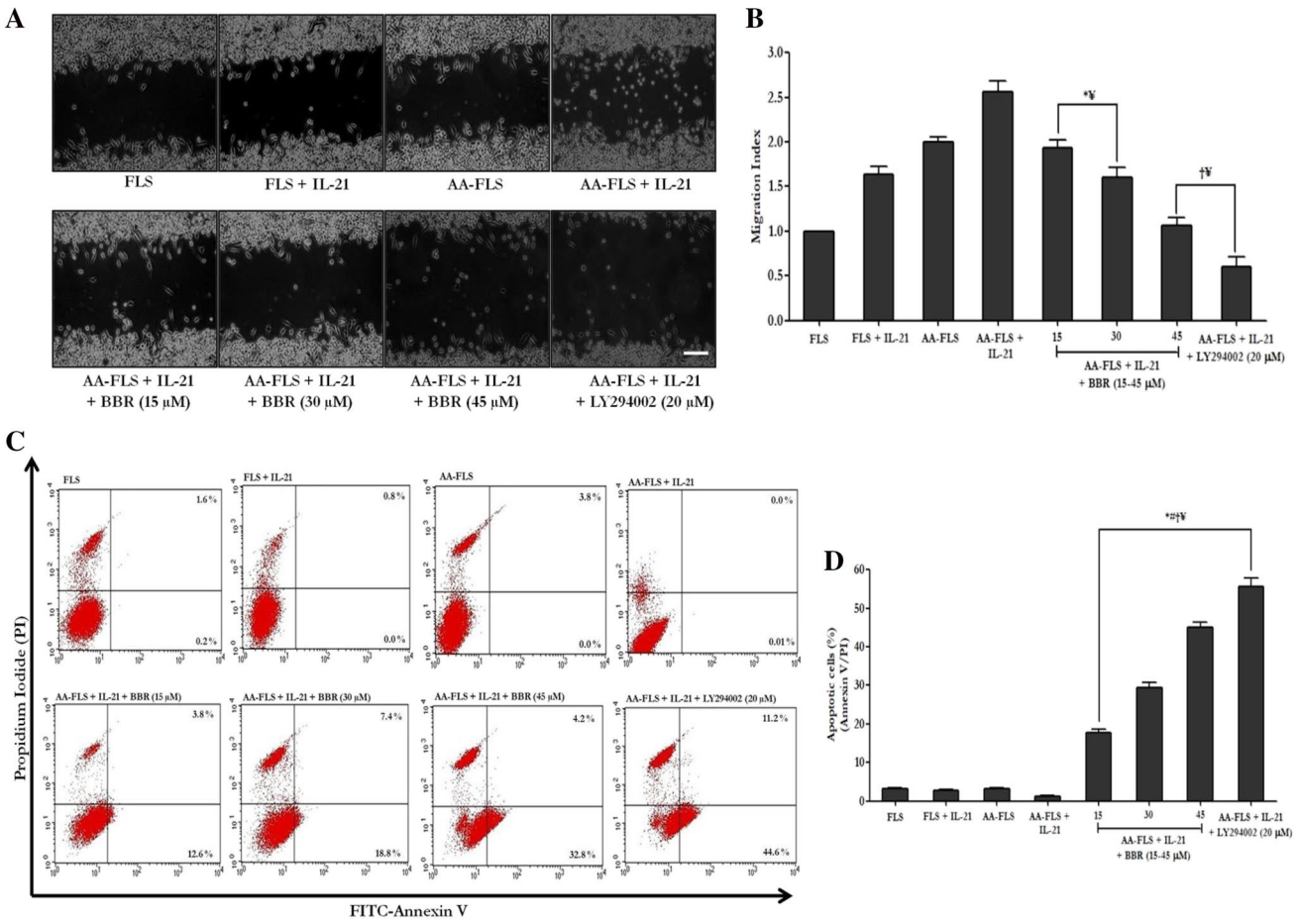

Fig. 7 BBR suppresses IL-21 mediated proliferation of AA-FLS cells. **a** Cell migration assay phenotypes of IL-21 stimulated FLS/AA-FLS cells treated with/without BBR (15–45 μM) or LY294002 (20 μM). **b** Cellular migration index. **c** Flow cytometry analysis of FITC Annexin V/PI staining. **d** Percentage of apoptotic cells in IL-21 stimulated FLS/AA-FLS treated with/without BBR (15–45 μM) or LY294002 (20 μM). Comparisons are made with: *FLS versus AA-FLS + IL-21 + BBR (15–45 μM)/LY294002 (20 μM); #FLS + IL-21 versus AA-FLS + IL-21 + BBR (15–45 μM)/LY294002 (20 μM); †AA-FLS versus AA-FLS + IL-21 + BBR (15–45 μM)/LY294002 (20 μM); ¥AA-FLS + IL-21 versus AA-FLS + IL-21 + BBR (15–45 μM)/LY294002 (20 μM). The results are expressed as mean ± SEM of the data from three individual experiments. *#†¥$P < 0.05$ implies statistically significant. Scale bars, 10 μm

and LC3-II, which were suppressed through miR-30a transfection [22]. Similar to these reports, our study elicited that upon stimulation with IL-21, autophagy was induced in FLS cells through upregulation of Beclin-1, conversion of LC3-I to LC3-II and inhibition of CHOP transcription factor inside the cells. Furthermore, the major autophagosome subunit composed of Atg5 is illustrated to promote its stability and functioning of the cells [21]. Deselm et al. initially demonstrated that ATG family of proteins including Atg5 promotes uncontrolled osteoclast differentiation resulting in aberrant bone loss [53]. Atg5 expression levels were witnessed in a similar fashion upon IL-21 stimulation in FLS cells with increased proliferation. Degradation of p62 inside the cells has been implicated in disease conditions such as RA to be a major indicator of dysregulated autophagy. Utilization of p62 by RA-FLS cells promotes autophagosome formation along with ALFY to evade caspase 3 dependent cell death response. Therefore, diminished p62 levels are associated with autophagy activation [23]. Moreover, all these factors have been predicted in the recent studies to be mediated via activation of PI3K/Akt signaling [54, 55]. In this regard, our report suggests that IL-21 promoted ubiquitination of p62 inside FLS cells and helped evade apoptosis. We also witnessed similar outcomes through the diminished expression levels of LC3-II, Beclin-1, and Atg5 with p62 sequestration/CHOP induction with LY294002 treatment. Overall, it is evident that BBR treatment attenuated IL-21 stimulated AA-FLS cells eliciting elevated expression levels of Atg5, Beclin-1 and LC3-II conversion with p62 sequestration through induction of CHOP, which was essential for autophagy-dependent cellular survival.

An influx between autophagy and apoptosis forms as a crux for many therapeutic treatment strategies. Aberrant autophagy has been reported in the recent years to diminish

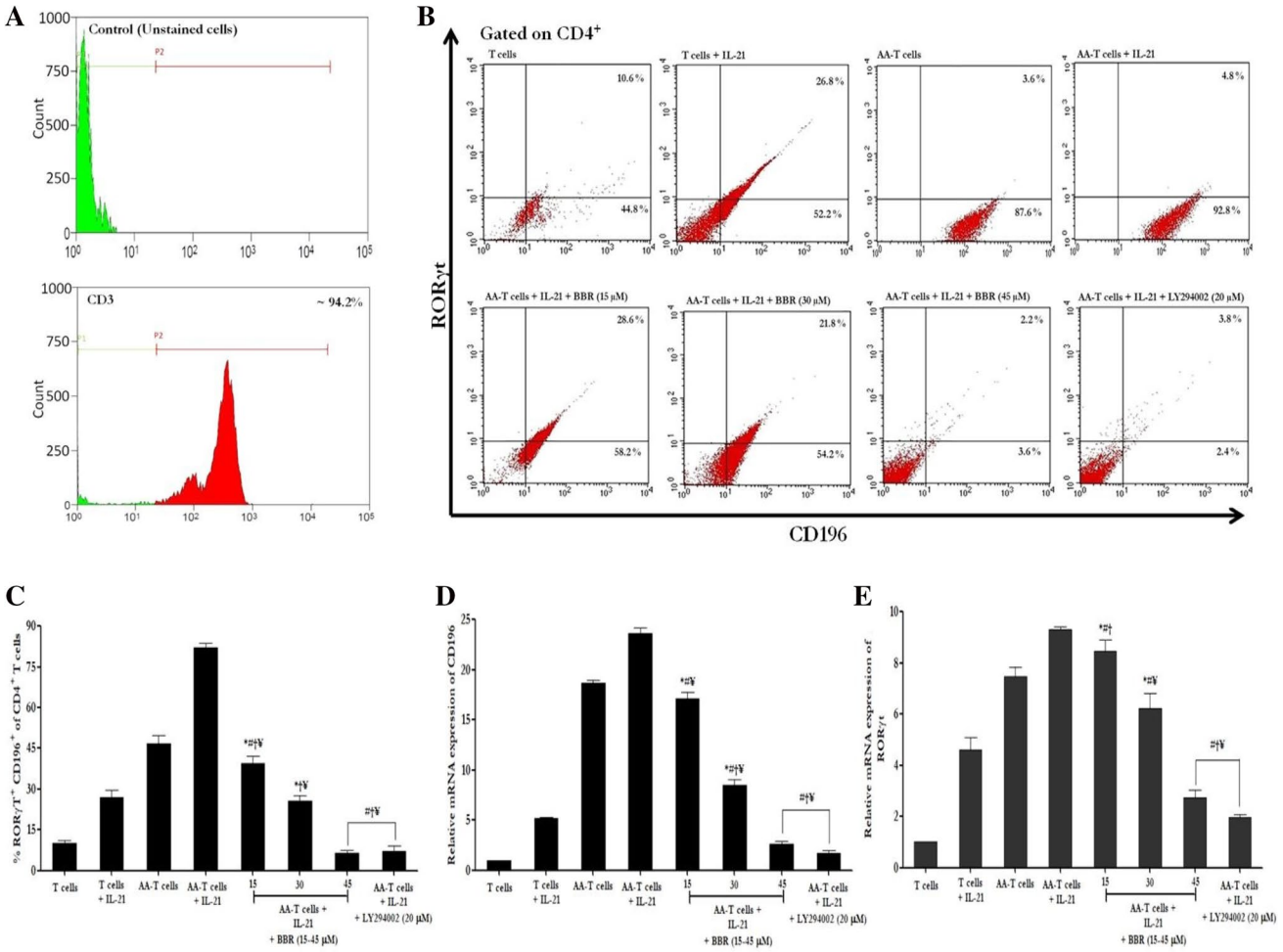

Fig. 8 Effect of BBR (15–45 μM) on proliferation of IL-21 (20 ng/ml) stimulated T cells. **a** Purity of isolated AA-T cells sorted using FITC tagged CD3 marker. **b** $CD4^+$ T cells were stained with fluorescent tagged CD196 and RORγt analyzed using FACS sorting. **c** Percentage of IL-21 stimulated $CD4^+$ Th17 cells with/without BBR (15–45 μM) or LY294002 (20 μM). Gene expression levels of **d** CD196 and **e** RORγt. Comparisons are made with: *T cells versus AA-T cells + IL-21 + BBR (15–45 μM)/LY294002 (20 μM); #T cells + IL-21 versus AA-T cells + IL-21 + BBR (15–45 μM)/LY294002 (20 μM); †AA-T cells versus AA-T cells + IL-21 + BBR (15–45 μM)/LY294002 (20 μM); ¥AA-T cells + IL-21 versus AA-FLS + IL-21 + BBR (15–45 μM)/LY294002 (20 μM). The results are expressed as mean ± SEM of the data from three individual experiments. $^{*\#\dagger¥}P < 0.05$ implies statistically significant

apoptosis and promote the uncontrolled proliferation of FLS cells in RA disease models [26, 27]. Increased autophagy response in FLS cells provide energy for its thriving in the synovial microenvironment for activation of other cell subtypes [56]. Similarly, IL-21 used in this study promoted survival of FLS cells through aberrant autophagy and promotion of anti-apoptotic element such as Bcl-2 transcription factor with downregulation of BAX. BBR, which has been recently shown to be an excellent candidate for inhibiting FLS proliferation in RA disease models elicited excellent inhibitory properties [57]. BBR intervened this uncontrolled proliferative nature of IL-21 stimulated FLS cells through induction of BAX and downregulation of Bcl-2 expression levels. This phenomenon has been well established to be controlled by the PI3K/Akt pathway, which triggers the survival mechanism of FLS cells in the synovium. We witnessed similar outcomes through inhibition of PI3K protein, which resulted in the apoptosis of FLS cells.

After understanding this phenomenon, we wanted to explore as to how IL-21 promotes differentiation/proliferation of other cell subtypes. Major subsets of T lymphocytes (Th1, Th17, and Tfh) have been depicted in the recent years to promote disease pathogenesis of RA [58, 59]. Th17 cells thriving in the RA synovial microenvironment express high levels of CD196 and an essential transcription factor that is important for their survival designated as RAR-related orphan receptor gamma T (RORγt) [60]. With respect to this aspect, IL-21 has been recently showcased to provide a microenvironment for the proliferation of Th17 cells in RA [12]. Uncontrolled Th17 differentiation has been reported in

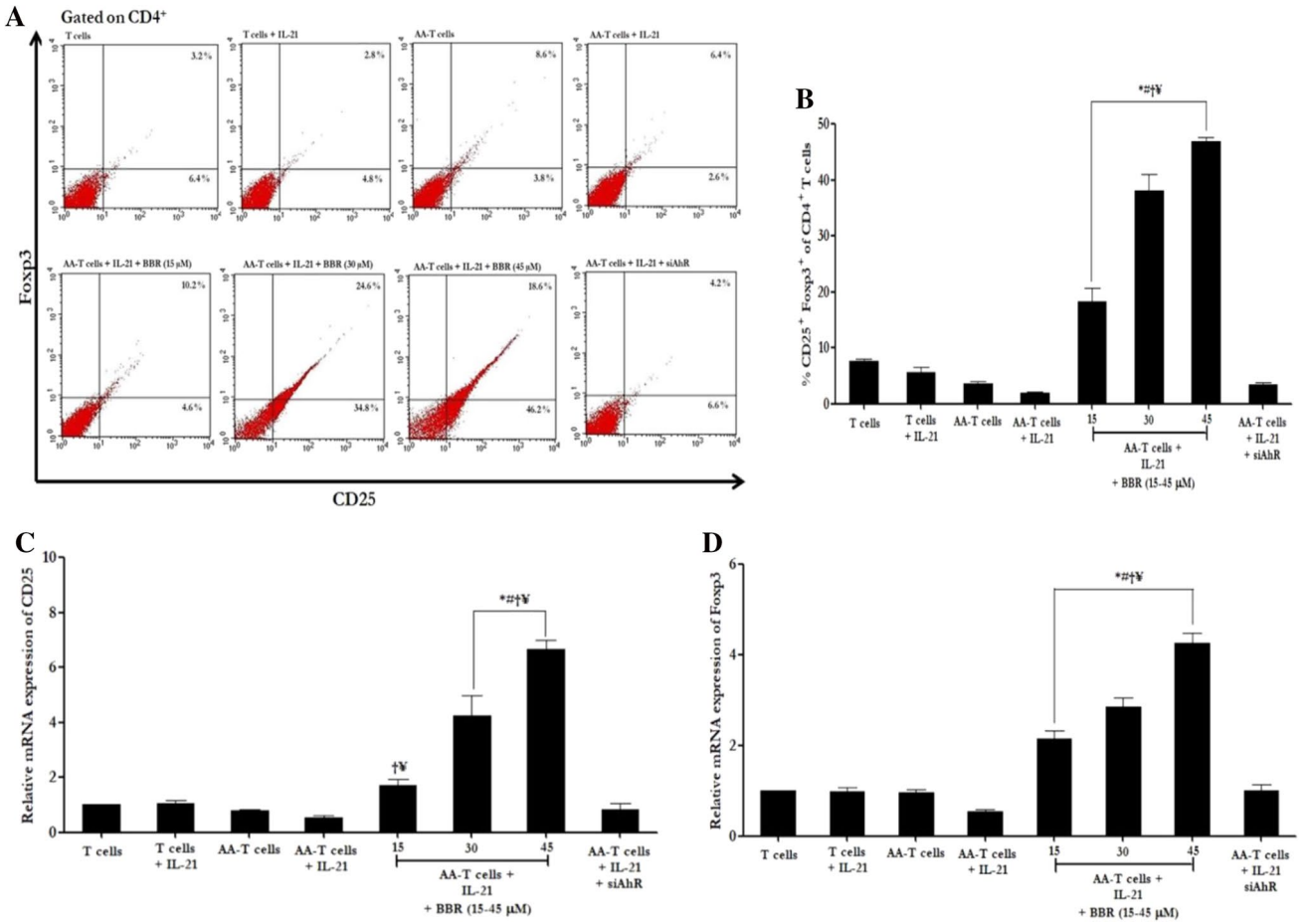

Fig. 9 BBR induces Treg differentiation. **a** FACS analysis of IL-21 stimulated $CD4^+$ T cells stained with fluorescent tagged CD25 and Foxp3 mAbs with/without BBR (15–45 μM) or transfected with siAhR. **b** Percentage of $CD4^+$ $CD25^+$ T cells treated with/without BBR (15–45 μM) or transfected with siAhR. Gene expression levels of **c** CD25 and **d** Foxp3. Comparisons are made with: *T cells versus AA-T cells + IL-21 + BBR (15–45 μM)/LY294002 (20 μM); #T cells + IL-21 versus AA-T cells + IL-21 + BBR (15–45 μM)/LY294002 (20 μM); †AA-T cells versus AA-T cells + IL-21 + BBR (15–45 μM)/siAhR; ¥AA-T cells + IL-21 versus AA-FLS + IL-21 + BBR (15–45 μM)/siAhR. The results are expressed as mean ± SEM of the data from three individual experiments. $^{*\#\dagger¥}P < 0.05$ implies statistically significant

the present years to contribute to progressive joint alteration [61]. IL-21, a cytokine majorly secreted by Tfh and Th17 cells has been elicited to promote co-stimulatory action on T cells through inducing $CD4^+$ Th17 differentiation [62]. Similarly, we witnessed that T cells upon stimulation with IL-21 promoted the differentiation of $CD4^+$ $CD196^+$ Th17 cells, which was mediated in a PI3K/Akt dependent manner. Moreover, the survival of IL-21 stimulated Th17 differentiation was sustained through RORγt transcription factor induction. However, upon BBR treatment, the differentiation of Th17 cells was diminished through inhibition of essential surface marker such as CD196 and downregulation of RORγt transcription factor.

We further checked whether BBR was able to promote differentiation of another class of $CD4^+$ T cells designated as Treg cells based on previous reports [43, 63]. The survival and proliferation of Treg cells depend on the activation of AhR and induction of Foxp3, which has been reported with respect to BBR treatment strategies in arthritic models [35, 42]. We witnessed that IL-21 stimulation reduced the proliferation of Treg population compared to control groups possibly through T cell class switching. However, BBR treated $CD4^+$ T cells with/without IL-21 stimulation differentiated into $CD4^+$ $CD25^+$ $Foxp3^+$ Treg cells with increasing cell volume in a concentration-dependent manner. Moreover, BBR sequestered the Foxp3 expression in the cytosol mediated through AhR activation, which resulted in $CD4^+$ $CD25^+$ $Foxp3^+$ Treg proliferation. Furthermore, BBR treatment induced Treg differentiation and upregulated p450 family of enzymes such as CYP1A1. This phenomenon was further witnessed after the AhR gene knockdown inside the cells. Overall, it is evident that BBR inhibits Th17 cell proliferation and helps to counteract the disease process through the induction

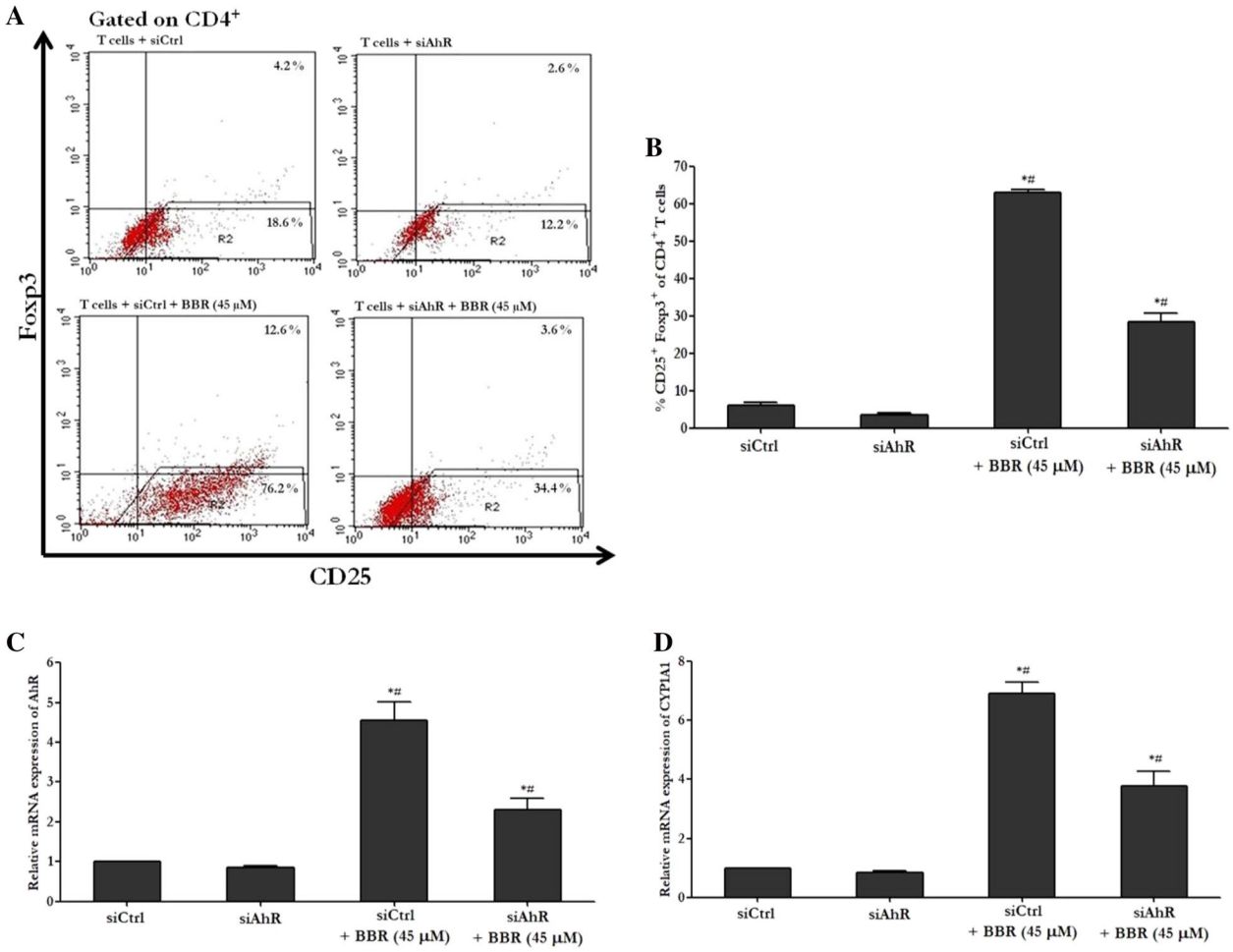

Fig. 10 BBR induces AhR and promotes Treg proliferation. **a**, **b** FACS analysis of $CD4^+$ T cells stained with fluorescent tagged CD25 and Foxp3 mAbs with/without BBR (45 μM) or transfected with siAhR/siCtrl. **c**, **d** Gene expression levels of AhR and CYP1A1. Comparisons are made with: *T cells + siCtrl versus T cells + siCtrl + BBR (45 μM)/T cells + siAhR + BBR (45 μM); #T cells + siAhR versus T cells + siCtrl + BBR (45 μM)/T cells + siAhR + BBR (45 μM). The results are expressed as mean ± SEM of the data from three individual experiments. *#$P < 0.05$ implies statistically significant

of Treg cells via activation of AhR transcription factor (Illustrated in Fig. 11).

Conclusion

The findings of the current study provide strong evidence that IL-21 promotes proliferation/survival of AA-FLS cells through induction of autophagy in a PI3K/Akt dependent manner. Various autophagic elements such as Beclin-1, Atg5 and LC3-II were upregulated with the utilization of p62 and diminished expression CHOP transcription factor upon IL-21 stimulation. BBR treatment was able to counteract this mechanism through induction of apoptosis via upregulation of various apoptotic factors including BAX and CHOP with sequestration of p62 in the cytosol and attenuation of Bcl-2. Furthermore, IL-21 plays a pivotal role in T cell differentiation through increased $CD4^+$ $CD196^+$ $ROR\gamma t^+$ Th17 proliferation mediated through PI3K/Akt signaling. BBR treatment intervened this process via suppressing its proliferation and promotes $CD4^+$ $CD25^+$ $Foxp3^+$ Treg differentiation through AhR transcription factor activation, thus providing a balance between Th17/Treg cells. Overall, this report elicits that IL-21 promotes uncontrolled proliferation/survival of AA-FLS cells through induction of autophagy and differentiation of Th17 cells. However, treatment with BBR suppresses autophagic response in AA-FLS cells, proliferation of Th17 cells and induces Treg cells differentiation, thus making it a potential candidate for IL-21/IL-21R targeted therapy in RA (Illustrated in Fig. 11).

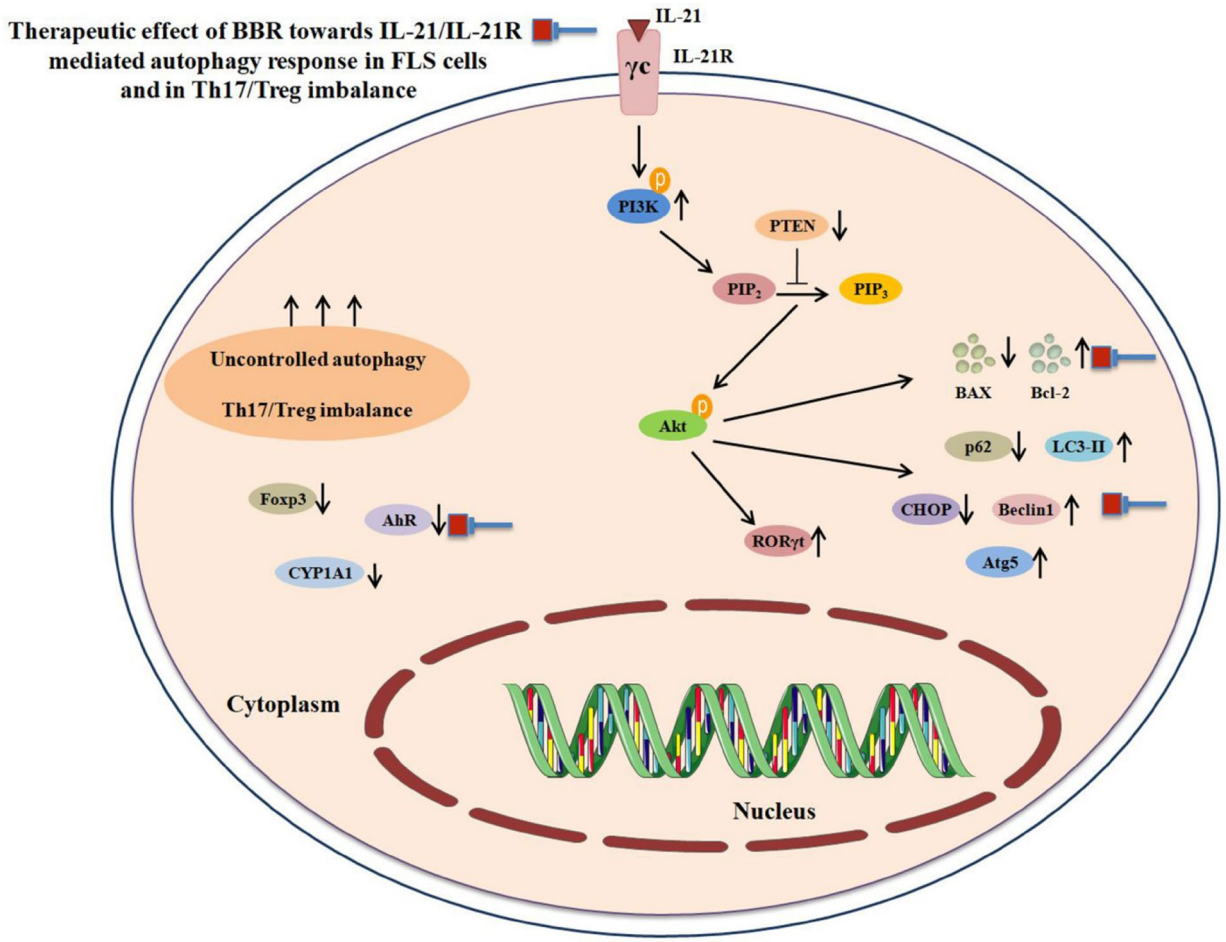

Fig. 11 Therapeutic role of BBR against IL-21 mediated autophagic response in FLS and in Th17/Treg imbalance. BBR diminishes the elements which promotes autophagy in AA-FLS and promotes Treg differentiation through induction of AhR levels. *AhR* aryl hydrocarbon receptor, *Atg5* autophagy related 5, *BAX* Bcl-2 associated X protein, *Bcl*-2 B-cell lymphoma 2, *BECN1* beclin-1, *CHOP* C/EBP homologous protein, *CYP1A1* cytochrome P450, family 1, subfamily A, polypeptide 1, *Foxp3* forkhead box P3, *IL-21* interleukin 21, *IL-21R* interleukin 21 receptor, LC3-II LC3 phophatidylethanolamine conjugate 3-II, *p62/SQSTM1* sequestosome 1, *PI3K* phosphoinositide 3 kinase, *PKB/Akt* protein kinase B, *PTEN* phosphatase and tensin homolog, *RORγt* RAR-related orphan receptor gamma

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

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