

Silybin acts synergistically with mesenchymal stem cells to ameliorate R848-induced lupus-like disease in mice

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Abstract

Objective: To determine the therapeutic effects of silybin (SBI) and its synergistic effects with mesenchymal stem cells (MSCs) in a lupus mouse model and to explore the therapeutic mechanisms. **Methods:** TLR7/8 agonist resiquimod (R848) was applied for the induction of lupus mice. R848-induced B6 mice were randomly divided into a normal saline control group, SBI group, MSCs group, and SBI plus MSCs group, and treated with daily SBI by gavage or received MSCs injection once via the tail vein. Mice were sacrificed at week 12, with urine, serum, kidney, and spleen collected. The proportion of cell subsets was detected by flow cytometry using splenocytes. **Results:** SBI treatment significantly decreased total IgG, anti-ds-DNA antibody, and urinary protein levels, as well as renal IgG and C3 deposition in R848-induced mice. It also increased the ability of MSCs to suppress splenomegaly and serum antinuclear antibody levels. In vivo and in vitro studies showed a decrease in the percentage of Tfh cells after SBI treatment, which was most pronounced when combined with MSC therapy. When splenocytes of R848-induced mice were treated with SBI and MSCs in vitro, the expression of genes related to Tfh cell differentiation, including IL-6, Stat3, and Bcl-6, was reduced, and the phosphorylation of AKT, S6, and STAT3 proteins in Tfh cells was decreased. **Conclusion:** SBI acts synergistically with MSCs to ameliorate lupus-like features in R848-induced mice. It may enhance the ability of MSCs to inhibit Tfh cell production by counteracting the activation of IL-6 and its downstream pathways.

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Highlights:

- Silybin relieved lupus-like disease in TLR7/8 agonist (R848)-induced mice.
- Silybin increased the efficacy of mesenchymal stem cells (MSCs) in the treatment of lupus mice.
- Silybin synergized with MSCs to inhibit Tfh cell production, which was mostly achieved by suppressing the expression of Tfh differentiation genes such as IL-6 and STAT3.
- The effects of silybin on Tfh cells could be counteracted by the activation of IL-6 and its downstream pathways.

Abstract :

Objective:To determine the therapeutic effects of silybin (SBI) and its synergistic effects with mesenchymal stem cells (MSCs) in a lupus mouse model and to explore the therapeutic mechanisms.

Methods: TLR7/8 agonist resiquimod (R848) was applied for the induction of lupus mice. R848-induced B6 mice were randomly divided into normal saline control group, SBI group, MSCs group and SBI plus MSCs group, and treated with daily SBI by gavage or received MSCs injection once via the tail vein. Mice were sacrificed at week 12, with urine, serum, kidney and spleen collected. The proportion of cell subsets was detected by flow cytometry using splenocytes. Quantitative PCR and western blot were used to detect mRNA and protein levels. The regulatory mechanism of SBI on follicular helper T (Tfh) cells was explored through *in vitro* splenocyte experiments.

Results : SBI treatment significantly decreased total IgG, anti-dsDNA antibody and urinary protein levels, as well as renal IgG and C3 deposition in R848-induced mice. It also increased the ability of MSCs to suppress splenomegaly and serum antinuclear antibody levels. *In vivo* and *in vitro* studies showed a decrease in the percentage of Tfh cells and an increase in the percentage of regulatory T (Treg) cells after SBI treatment, which was most pronounced when combined with MSC therapy. SBI inhibited Tfh cell production in a dose-dependent manner. When splenocytes of R848-induced mice were treated with SBI and MSCs *in vitro*, the expression of genes related to Tfh cell differentiation, including *Icos*, *Stat3* and *Bcl-6*, was reduced, and the phosphorylation of AKT, S6, and STAT3 proteins in Tfh cells was decreased. Correspondingly, addition of IL-6 or STAT3/mTOR agonists to the culture system completely or partially blocked the inhibitory effect of SBI on Tfh cells.

Conclusion: SBI acts synergistically with MSCs to ameliorate lupus-like features in R848-induced mice. It may enhance the ability of MSCs to inhibit Tfh cell production by counteracting the activation of IL-6 and its downstream pathways.

Keywords:

Systemic lupus erythematosus; Silybin; Mesenchymal stem cells; Follicular helper T cells; Interleukin-6

Introduction :

Systemic lupus erythematosus (SLE) is a complex autoimmune disease manifested by the damage of multiple organs and systems^[11] Relle, Manfred et al. “Epigenetic Aspects of Systemic Lupus Erythematosus.” *Rheumatology and therapy* vol. 2,1 (2015): 33-46. doi:10.1007/s40744-015-0014-y¹. The pathogenesis of SLE is characterized by the abnormal activation of T and B lymphocytes, and the formation of autoantibodies^[22] Lou, Hantao et al. “Autoantibodies in systemic lupus erythematosus: From immunopathology to therapeutic target.” *Journal of autoimmunity* vol. 132 (2022): 102861.

doi:10.1016/j.jaut.2022.102861¹. Among which, the imbalance between regulatory T (Treg) cells and follicular helper T (Tfh) cells has attracted much attention^[33] He, Jing et al. “Low-dose interleukin-2 treatment selectively modulates CD4(+) T cell subsets in patients with systemic lupus erythematosus.” *Nature medicine* vol. 22,9 (2016): 991-3. doi:10.1038/nm.4148¹. Tfh cells represent a distinct subset of CD4+ T cells specialized in providing help to B cells and are required for the formation of germinal centers. In lupus, abnormal proliferation and function of Tfh cells will lead to pathological processes like autoantibody production and tissue damage, and thus become a promising therapeutic target ^[44] Feng, Xuebing et al. “Inhibition of aberrant circulating Tfh cell proportions by corticosteroids in patients with systemic lupus erythematosus.” *PloS one* vol. 7,12 (2012): e51982. doi:10.1371/journal.pone.0051982^[55] Wei, Xindi, and Xiaoyin Niu. “T follicular helper cells in autoimmune diseases.” *Journal of autoimmunity* vol. 134 (2023): 102976. doi:10.1016/j.jaut.2022.102976¹.

Our previous studies have shown that MSCs could suppress Tfh cell differentiation and proliferation, which was mediated by inducible nitric oxide synthase (iNOS) ^[6]. Recently, it has been proven that bone marrow-derived mesenchymal stem cells (MSCs) from SLE patients were inefficient to maintain Treg and Tfh balance, while transplantation of healthy MSCs effectively inhibited the expansion of Tfh cells and alleviate lupus symptoms^[66] Zhang, Zhuoya et al. “Human Umbilical Cord Mesenchymal Stem Cells Inhibit T Follicular Helper Cell Expansion Through the Activation of iNOS in Lupus-Prone B6.MRL-Fas Mice.” *Cell transplantation* vol. 26,6 (2017): 1031-1042. doi:10.3727/096368917X694660^[77] Jang, Eunkyeong et al. “Infusion of Human Bone Marrow-Derived Mesenchymal Stem Cells Alleviates Autoimmune Nephritis in a Lupus Model by Suppressing Follicular Helper T-Cell Development.” *Cell transplantation* vol. 25,1 (2016): 1-15. doi:10.3727/096368915X688173¹. However, this treatment is only effective for a subset of patients^[88] Wang, Dandan et al. “A Long-Term Follow-Up Study of Allogeneic Mesenchymal Stem/Stromal Cell Transplantation in Patients with Drug-Resistant Systemic Lupus Erythematosus.” *Stem cell reports* vol. 10,3 (2018): 933-941. doi:10.1016/j.stemcr.2018.01.029¹, for reasons that may be related to the insufficient inhibitory effect or short persistence time^[99] Nie M, Chen G, Zhao C, Gan J, Alip M, Zhao Y, Sun L. Bio-inspired adhesive porous particles with human MSCs encapsulation for systemic lupus erythematosus treatment. *Bioact Mater.* 2020 Aug 10;6(1):84-90. ¹. Therefore, it is necessary to find effective strategies to maximize the therapeutic effect of MSCs.

Silybin (SBI), a flavonoid extracted from the seeds and fruits of silymarin in the compositae^[1010] Křen V, Valentová K. Silybin and its congeners: from traditional medicine to molecular effects. *Nat Prod Rep.* 2022 Jun 22;39(6):1264-1281. doi: 10.1039/d2np00013j.¹, is mainly used as a liver-protective drug in clinical practice^[1111] ederico, Alessandro et al. “Evaluation of the Effect Derived from Silybin with Vitamin D and Vitamin E Administration on Clinical, Metabolic, Endothelial Dysfunction, Oxidative Stress Parameters, and Serological Worsening Markers in Nonalcoholic Fatty Liver Disease Patients.” *Oxidative medicine and cellular longevity* vol. 2019 8742075. 15 Oct. 2019, doi:10.1155/2019/8742075¹, while studies have shown it has anti-tumor and anti-diabetic effects^[1212] Wei, Zibo et al. “Silybin suppresses ovarian cancer cell proliferation by inhibiting isocitrate dehydrogenase 1 activity.” *Cancer science* vol. 113,9 (2022): 3032-3043. doi:10.1111/cas.15470^[1313] Liu, Panwen et al. “Silibinin ameliorates STZ-induced impairment of memory and learning by up-regulating insulin signaling pathway and attenuating apoptosis.” *Physiology & behavior* vol. 213 (2020): 112689. doi:10.1016/j.physbeh.2019.112689¹. Recently, evidence has suggested that SBI has a therapeutic effect on collagen-induced arthritis models and patients with rheumatoid arthritis^[1414] Tong, W W et al. “Silibinin alleviates inflammation and induces apoptosis in human rheumatoid arthritis fibroblast-like synoviocytes and has a therapeutic effect on arthritis in rats.” *Scientific reports* vol. 8,1 3241. 19 Feb. 2018, doi:10.1038/s41598-018-21674-6^[1515] Xie, Ying et al. “Suppression of up-regulated LXR α by silybin ameliorates experimental rheumatoid arthritis and abnormal lipid metabolism.” *Phytomedicine : international journal of phytotherapy and phytopharmacology* vol. 80 (2021): 153339. doi:10.1016/j.phymed.2020.153339¹, which opens new perspectives for the treatment of systemic autoimmune diseases. Moreover, SBI could up-regulate the gene expression of MSC-related markers in a pulmonary arterial hypertension rat model^[1616] Zhang, Tingting et al. “Silibinin Upregulates CXCR4 Expression in Cultured Bone Marrow Cells (BMCs) Especially in Pulmonary Arterial Hypertension Rat

Model.” *Cells* vol. 9,5 1276. 21 May. 2020, doi:10.3390/cells9051276¹, suggesting that it may also have an impact on the function of MSCs. In this study, we systematically observed the efficacy of SBI alone and in combination with MSCs in the treatment of lupus mice for the first time and elaborated its regulatory mechanisms on Tfh cells.

Materials and methods

Mice models

Four-week-old female C57BL/6 mice (B6) were purchased from Beijing Spefford Biotechnology Co. Ltd (China) and maintained in the specific pathogen-free grade environmental animal facility of Nanjing Drum Tower Hospital. All mice were adaptive fed for one week, and then randomly divided into two groups: acetone control group and TLR7/8 agonist resiquimod (R848)-induced group. To induce lupus-like disease, 40ul R848 (2 mg/ml), dissolved in acetone and stored a -80°C, was applied to the auricle of one side of mice every 3 days for 7 weeks. All animal experiments were approved by the Medical Ethics Committee of Nanjing Drum Tower Hospital.

Study design

The experimental grouping of mice was shown in Table S1. Thirty-six R848-induced B6 mice were randomly divided into 4 groups (n= 9 in each group): normal saline treatment group, SBI treatment group, MSCs treatment group, and SBI plus MSCs treatment group. Umbilical cord derived MSCs were kindly provided by the Stem Cell Bank of Jiangsu Province (Beike Biotechnology) and cultured as previously described [11] Yuan, Xinran et al. “Mesenchymal stem cell therapy induces FLT3L and CD1c+ dendritic cells in systemic lupus erythematosus patients.” *Nature communications* vol. 10,1 2498. 7 Jun. 2019, doi:10.1038/s41467-019-10491-8]. Both SBI and MSCs were suspended in normal saline. SBI was administered by gastric gavage at 200mg/kg daily from week 5, and MSCs (1×10^6 cells) were injected via the caudal vein at week 9. Meanwhile, acetone-induced B6 mice (n=6) were applied as normal controls and received normal saline treatment. All mice were sacrificed by cervical dislocation at week 12. Prior to this urine was collected by massaging the bladder for urinary protein and creatinine, and later serum was collected for antibody levels and kidneys were collected to assess histologic changes. In addition, mouse spleens were ground to cell suspensions, resuspended in red blood cell lysis buffer for 5 min, passed through a 70µm cell filter, and centrifuged to obtain splenocytes.

Flow cytometry

For surface staining, mouse-derived splenocytes were labeled for 30 min with directly labeled antibodies against cell surface antigens. For intracellular cytokine staining, cells were stimulated with 0.1 mg/ml foponol-1-myristate-37-acetate (PMA) plus 11 ng/ ml ionomycin for 4 h at 37°C (all from Enzo Life Sciences, Farmingdale, NY, USA), followed by fixation and permeabilization with BD Cytfix kits (BD Biosciences, the USA) to fix and permeabilize the cells, followed by staining with corresponding antibodies. To stain intranuclear factor Foxp3, membranes were broken for 30 min using a fixation/permeability kit (Invitrogen, USA) and then stained with APC-anti-Foxp3. To assess the cell viability, cells were directly stained with the reactive dye eFluor 506 for 20 min. For cell proliferation and apoptosis, intranuclear staining with the proliferation marker Ki67 was performed for 30 minutes, and cell apoptosis was assessed with Annexin V-PE/7-AAD Apoptosis Detection Kit (Vazyme Biotech, Nanjing). All antibodies used for flow cytometry detection were listed in Table S2. The assays were performed on a BD FACSAria III flow cytometer (BD Biosciences), and the data were analyzed using FlowJo X software.

Laboratory test

Proteinuria was quantitatively detected by a urine protein detection kit (Nanjing Jiancheng, China). Serum and urine creatinine levels were measured by a creatinine detection kit (Nanjing Jiancheng, China). Urea nitrogen levels were measured using a urea nitrogen kit (Nanjing Jiancheng, China). Immunoglobulin G (IgG) and antibody levels were measured using mouse IgG (Multi Sciences, China) and anti-nuclear antibody (ANA), anti-double strand DNA (dsDNA) (Aifang Bioscience, China) ELISA kits.

Renal histology and immunofluorescence

Renal tissue samples fixed in formaldehyde fixative were embedded in paraffin and cut into 10 μ m sections. Hematoxylin-eosin (H&E) staining was used to observe the changes in glomeruli under the microscope refer to the previous description^[11] Chen, Weiwei et al. “Lipocalin-2 Exacerbates Lupus Nephritis by Promoting Th1 Cell Differentiation.” *Journal of the American Society of Nephrology : JASN* vol. 31,10 (2020): 2263-2277. doi:10.1681/ASN.2019090937¹. Immunofluorescence staining of paraffin-fixed kidney sections with Rhodamine conjugated anti-IgG (Jackson Immuno Research, USA) and FITC conjugated anti-complement 3 (C3) (Abcam, UK) antibodies was performed to observe IgG and C3 deposition under a fluorescence microscope, as we described previously^[22] Zhang, Zhuoya et al. “Mesenchymal stem cells prevent podocyte injury in lupus-prone B6.MRL-Fas^{pr} mice via polarizing macrophage into an anti-inflammatory phenotype.” *Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association* vol. 34,4 (2019): 597-605. doi:10.1093/ndt/gfy195¹.

Polymerase chain reaction (PCR)

RNA was extracted from mouse splenocytes by Trizol reagent (Vazyme Biotech, Nanjing, China) and reverse transcribed by using Superscript qRT SuperMix kit (Vazyme Biotech) according to the manufacturer’s instructions. All the primers were synthesized by GenScript (Nanjing, China) and are listed in Table S3. Quantitative real-time RT-PCR (qRT-PCR) was performed by using the SYBR Green Premix kit (Vazyme) on a StepOne Plus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The relative expression of each gene was determined and normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and calculated by using the $2^{-[?][?]}Ct$ method.

Western blotting

Mouse splenocytes were lysed and proteins were extracted and blotted with signal transducer and activator of transcription-3 (STAT3) (1:1000), pSTAT3 (1:500), and β -actin (1:2000) antibodies (Affinity Biosciences, USA). Proteins were detected with Super Signal West Pico chemiluminescence Substrate solution (Thermo Fisher Scientific). Immuno-reactive bands were visualized by using a gel documentation system (Syngene, USA). The intensity blots were quantified by densitometry using Image J software.

Cell cultures

Splenocytes were placed in 48-well tissue culture plates at a density of 5×10^5 cells/well with or without SBI (0, 20, 50, 100, 200 μ M) (dissolved in DMSO), and the control was a DMSO solution without SBI. After incubation for 24h at 37 in 5% CO₂, the supernatant and the cells were collected for immediate assay or stored at -80. To verify the effect of MSCs on mice splenocytes, they were placed in 24-well tissue culture plates at a density of 6×10^4 cells/well with or without SBI (100 μ M) and incubated at 37 °C with 5% CO₂ for 4 hours, and then 5×10^5 splenocytes were added. 24 hours later, the supernatant and cells were collected for immediate assay or stored at -80. To confirm the impact of interleukin (IL) -6, STAT3 and mammalian target of rapamycin (mTOR) pathways on SBI efficacy, STAT3 agonist colivelin (MCE, USA) (50nM) and inhibitor Stattic (MCE, USA) (20 μ M), mTOR agonist MHY1485 (MCE, USA) (100nM) and inhibitor Rapamycin (MCE, USA) (100nM) as well as IL-6 (Genscript BioTECH, USA) (20 ng/ml) were added into mouse splenocyte cultures respectively with or without SBI and incubated for 24 hours. The above stimulant concentrations were provided as a reference by MCE Biotechnology and were used after *in vitro* validation.

Statistical analysis

All calculations were performed using GraphPad Prism 9.0 software. Data were presented as mean \pm standard error of the mean (SEM). T-tests were used to assess differences between the two groups. ANOVA followed by Bonferroni posthoc test was used for multiple comparisons. P value < 0.05 was considered significant.

Results

SBI alleviated lupus-like disease in R848-induced mice.

At 12 weeks, the survival rates of mice in the R848-induced group and the acetone- treated group were 66.7% and 100% respectively ($p = 0.065$) (Fig. 1A). In comparison to the control group, repeated treatment of B6 mice with R848 resulted in the development of lupus-like manifestations, such as splenomegaly, elevated levels of total IgG, ANA, anti-dsDNA, urine protein, urine creatinine, and serum creatinine (Fig. 1B-I), as well as increased infiltration of leukocytes and deposition of IgG/C3 in kidney sections (Fig. 1J).

In contrast, the survival rate of all mice induced with R848 and treated with SBI remained at 100% after 12 weeks (Fig. 1A). The administration of SBI exhibited a significant inhibitory effect on spleen enlargement and resulted in reduced levels of serum IgG, anti-dsDNA, urine protein, and urine creatinine in R848-induced mice (Fig. 1B-I). Additionally, SBI treatment demonstrated an improvement in renal pathology, a reduction in kidney inflammation, and a decrease in the sedimentation of IgG and C3 in the kidney (Fig. 1J), providing evidence that SBI effectively ameliorates lupus-like symptoms in mouse models.

SBI enhanced MSC efficacy in treating R848-induced mice.

Our data showed that the efficacy of SBI was comparable to that of MSCs, as evidenced by the inhibition of spleen enlargement and the reduction of serum IgG, anti-dsDNA, urine protein levels, and kidney IgG and C3 deposition (Fig. 1). There was one mouse in the SBI plus MSCs treatment group died of cellular embolism during tail vein infusion of MSCs. Furthermore, the addition of SBI to MSCs treatment resulted in an enhanced the ability of MSCs to inhibit splenomegaly and serum ANA levels (Fig. 1B,D). Additionally, SBI increased the extent to which MSCs improved urine creatinine and serum urea nitrogen levels (Fig. 1G,H), suggesting a synergistic effect of SBI with MSCs in the treatment of R848-induced mice.

SBI synergized with MSCs to restore immune balance.

In order to examine the impact of SBI and MSCs on lymphocytes, the proportions of major T and B cell subsets was analyzed by flow cytometry (Fig. 2A-C). In comparison to mice treated with acetone, the percentages of T helper-1 (Th1) cells ($CD4^+INF-\gamma^+$), T helper-2 (Th2) cells ($CD4^+IL-4^+$), T helper-17 (Th17) cells ($CD4^+IL-17^+$), Tfh cells ($CD4^+CXCR5^+PD-1^+$), germinal center (GC) B cells ($B220^+CD95^+GL7^+$) and plasmablast ($B220^+CD138^-$) in the splenocytes of R848-induced mice exhibited an increase, while the proportions of Treg cells ($CD4^+CD25^+Foxp3^+$) and memory B cells ($B220^+IgD^+CD38^+$) demonstrated a decrease. The administration of SBI resulted in a significant reduction in the proportion of Tfh cells and an increase in the proportion of Treg cells. Consequently, the percentage of plasmablast cells in splenocytes of mice induced with R848 was also decreased following SBI treatment (Fig. 2D). Compared to MSCs, combined treatment with SBI lowed the percentages of Th1 and Th2 cells, decreased the percentage of Tfh cells (Fig. 2), and further improved the imbalance of Tfh/Treg cells (Fig. 2). Meanwhile, the absolute number of Tfh cells was significantly decreased after SBI plus MSCs treatment (Fig. S1), supporting that SBI could act synergistically with MSCs to rebalance the immune system.

SBI regulated Tfh cell proportion in a dose dependent manner.

In order to validate the *in vivo* findings of SBI on lymphocytes, we measured the changes of T and B cell subsets within R848-induced mice splenocytes cultures subsequent to SBI treatment. Likewise, SBI treatment successfully rectified the anomalous proportions of Treg cells, Tfh cells and plasmablast of R848-induced mice *in vitro*, yet had no effect on the percentages of Th1 and Th2 cells (Fig. 3A). These results indicate that SBI primarily participates in the regulation of Tfh/Treg cell equilibrium. Moreover, our data showed that SBI inhibited the frequency of Tfh cells in a dose dependent manner. With the increase of SBI concentration (0- 400 μ M), the inhibitory ability of SBI on Tfh cells gradually increased (Fig. 3B).

SBI coordinated with MSCs to modulate IL-6/pSTAT3 signaling.

In the current and previous studies^[6], we revealed that Tfh cells were also an important cell type under MSC regulation. To explore the mechanism of SBI in collaboration with MSCs to inhibit Tfh cells, we examined the proliferation and apoptotic status of Tfh cells in splenocytes of R848-induced mice after different treatments

in vitro , and found that both treatments had no effect on the Ki67⁺ Tfh cell proportion, while the effect on the Annexin V⁺Tfh cell proportion was quite opposite (Fig. 4A).

Next the expression levels of genes closely related to Tfh cell differentiation were examined by qRT-PCR. Compared with acetone-treated mice, mRNA expression of inducible T cell costimulator (*Icos*), *Stat3* and *IL-6* was significantly increased in the splenocytes of R848-induced mice, while SBI treatment reduced mRNA expression of *Stat3* and *IL-6* (Fig. 4B). SBI enhanced the ability of MSCs to suppress mRNA expression of *Icos*, *Stat3*, and *IL-6* in the splenocytes of R848-induced mice. (Fig. 4C). Consistently, SBI inhibited the phosphorylation of STAT3 protein in the splenocytes of R848-induced lupus mice (Fig. 4D), implying that SBI may coordinate with MSCs to modulate IL-6/pSTAT3 signaling, thereby inhibiting Tfh cell differentiation.

IL-6 and its downstream pathways counteracted the effect of SBI on Tfh cell production.

To confirm that SBI regulated Tfh cell production through the inhibition of IL-6, we added IL-6 to R848-induced mice splenocyte cultures with the presence of SBI. As expected, IL-6 blocked the effect of SBI on Tfh cell expansion (Fig. 5A). Since IL-6 may initiate downstream signaling through the mTOR and STAT3 pathways, we subsequently measured the mean fluorescence intensity of the mTOR downstream targets phosphorylated serine/threonine kinase Akt (pAKT) and phosphorylated ribosomal protein S6 (pS6), as well as pSTAT3, by flow cytometry and found that their expression in Tfh cells was all inhibited by SBI and rebounded upon addition of IL-6 (Fig. 5B-C). Correspondingly, both the mTOR inhibitor rapamycin and the STAT3 inhibitor Stattic partially dampened IL-6-mediated Tfh cell expansion (Fig. 5D-E), whereas both the STAT3 agonist colivelin and the mTOR agonist MHY1485 partially attenuated the inhibitory effect of SBI on Tfh cells, with colivelin showing a more pronounced effect compared to MHY1485 (Fig. 5F). These findings suggest that SBI inhibits Tfh cell production by regulating IL-6 and its downstream pathways.

Discussion

In this study, we demonstrated for the first time that SBI could alleviate lupus-like manifestations and improve the efficacy of MSCs therapy in R848-induced mice. SBI synergized with MSCs to restore immune balance, particularly by suppressing aberrant Tfh cell production. On the molecular level, SBI increased the ability of MSCs to down-regulate the expression of Tfh differentiation-related genes, mainly through the inhibition of IL-6 and its downstream pathways.

So far, there is no study reported of SBI regulating Tfh cells. As the main active ingredient of silymarin, SBI has been previously reported to have immunomodulatory effects on Th1, Th17 cells^[11] Ribeiro, Vanessa Rocha et al. “Silibinin downregulates the expression of the Th1 and Th17 profiles by modulation of STATs and transcription factors in pregnant women with preeclampsia.” *International immunopharmacology* vol. 109 (2022): 108807. doi:10.1016/j.intimp.2022.108807¹. It also increases the function of regulatory T cells through the immunomodulatory effect of up-regulation of FOXP3 expression, and can be used as an adjuvant therapy to alleviate the adverse effects associated with interferon-beta treatment in patients with multiple sclerosis (MS)^[22] Abbasirad, Faezeh et al. “Significant immunomodulatory and hepatoprotective impacts of Silymarin in MS patients: A double-blind placebo-controlled clinical trial.” *International immunopharmacology* vol. 97 (2021): 107715. doi:10.1016/j.intimp.2021.107715¹. Our *in vivo* and *in vitro* study suggests that SBI mainly regulates Tfh and Treg cells in lupus mice. Among them, the inhibition of Tfh cell production was largely achieved by suppressing its differentiation-related genes, while the effect on cell proliferation and apoptosis was not so evident. Overall, SBI is a promising immunomodulatory agent with a wide range of immunomodulatory functions under different conditions.

IL-6/STAT3 axis is critical in the development of Tfh cells^[33] Wan, Siyuan et al. “Costimulation molecules differentially regulate the ERK-Zfp831 axis to shape T follicular helper cell differentiation.” *Immunity* vol. 54,12 (2021): 2740-2755.e6. doi:10.1016/j.immuni.2021.09.018¹. Consistent with literature reports^[44] Zheng, Rongjuan et al. “Chemopreventive Effects of Silibinin on Colitis-Associated Tumorigenesis by Inhibiting IL-6/STAT3 Signaling Pathway.” *Mediators of inflammation* vol. 2018 1562010. 25 Oct. 2018, doi:10.1155/2018/1562010¹, we experimentally verified the inhibitory effect of SBI on the IL-6/STAT3 path-

way. Besides STAT3, IL-6 may also activate the downstream PI3K/mTOR pathway through the action of signal transducer glycoprotein (gp130)^[55] Heinrich PC, Behrmann I, Haan S, Hermanns HM, Müller-Newen G, Schaper F. Principles of interleukin (IL)-6-type cytokine signalling and its regulation. *Biochem J.* 2003 Aug 15;374(Pt 1):1-20. doi: 10.1042/BJ20030407[]]. The role of SBI on the mTOR pathway remains controversial, as a study examining the protective effects of SBI against cerebral ischemia showed that SBI treatment activated Akt/mTOR signaling^[66] Wang, Chaohui et al. “Protection by silibinin against experimental ischemic stroke: up-regulated pAkt, pmTOR, HIF-1 α and Bcl-2, down-regulated Bax, NF- κ B expression.” *Neuroscience letters* vol. 529,1 (2012): 45-50. doi:10.1016/j.neulet.2012.08.078[]], and another research on LPS stimulated porcine mammary epithelial cells showed SBI increased the expression of mTOR and S6^[77] Xu, Shengyu et al. “Silibinin Alleviates Lipopolysaccharide Induced Inflammation in Porcine Mammary Epithelial Cells via mTOR/NF- κ B Signaling Pathway.” *Molecular nutrition & food research* vol. 67,14 (2023): e2200715. doi:10.1002/mnfr.202200715[]]. Our data indicate that SBI inhibits both STAT3 and mTOR pathways downstream of IL-6, thus further clarifying the molecular mechanism of SBI treatment.

A series of studies have shown a reduction in the dose of immunosuppressive drugs and a significant decrease in mortality in patients with SLE after MSC treatment^{[8][88]} Wang, Dandan et al. “Long-term safety of umbilical cord mesenchymal stem cells transplantation for systemic lupus erythematosus: a 6-year follow-up study.” *Clinical and experimental medicine* vol. 17,3 (2017): 333-340. doi:10.1007/s10238-016-0427-0[]], yet there are still many challenges to overcome before clinical application. MSCs have strong immunomodulatory plasticity and are susceptible to microenvironmental influences. Besides, MSCs can secrete cytokines with strong pro-inflammatory effects, such as IL-6, which may reduce the efficacy of MSC therapy in SLE patients^[99] Li, Aifen et al. “Mesenchymal Stem Cell Therapy: Hope for Patients With Systemic Lupus Erythematosus.” *Frontiers in immunology* vol. 12 728190. 30 Sep. 2021, doi:10.3389/fimmu.2021.728190[]]. In this study, we revealed that SBI synergized with MSCs to inhibit Tfh cell production. SBI could counteract IL-6 and its downstream pathways, thus may enhance the therapeutic effect of MSCs in lupus mice.

In summary, our data suggest that SBI is an effective and promising agent for the treatment of lupus autoimmunity. Moreover, SBI could improve the efficacy of MSCs in R848-induced lupus mice, which may provide a theoretical basis for better clinical application of MSCs. However, the prospective use of SBI in SLE patients still needs to be validated by further experimental and clinical evidence.

Ethics statement

This study was carried out by the recommendations of the Ethical Research Committee for Laboratory Animal Welfare of Drum Tower Hospital of Nanjing University Medical School. The protocol was approved by the ethical review of Drum Tower Hospital, Nanjing University Medical School.

Author contributions

NZ, MA contributed to the majority of experiments and data analysis. NZ drafted the manuscript. YZ, WL, and MH were involved in the analysis of cellular experiments. TA and YC were involved in the feeding of experimental animals. XT was involved in experimental teaching and manuscript editing. YX contributed to the conceptualization of this study. XF revised the manuscript and was responsible for conceptualizing and finalizing this study. All authors read and approved the final manuscript.

Conflict of Interest

The authors declare no conflict of interest.

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References

Figure legends

Fig. 1 Silybin ameliorated lupus-like features and improved the efficacy of MSCs in R848-induced mice. **(A)** The survival rate for 12 weeks old acetone-induced B6 mice, R848-induced B6 mice, and R848-induced mice treated with silybin, MSCs, and silybin plus MSCs (n =6 in B6 group, n =9 in the rest four groups). **(B)** Spleen size of mice among five groups. **(C-E)** Serum IgG, antinuclear antibody and anti-ds-DNA antibody levels among five groups. **(F-I)** Urine protein, urine creatinine, serum nitrogen and serum creatinine levels among five groups. **(J)** Representative renal histology and immunofluorescence among five groups (Magnification: ×400). *P<0.05, **P<0.01, ***P<0.001, ****P<0.00001, ns: not significant.

Fig. 2 Changes in T/B lymphocyte subsets *in vivo* after silybin and MSCs treatment. **(A)** Representative flow cytometry plots of Th1 cell (CD4⁺INF- γ ⁺), Th2 cell (CD4⁺IL-4⁺) and Th17 cell (CD4⁺IL-17⁺) percentages in splenocytes among five groups. **(B)** Representative flow cytometry plots of Tfh cell (CD4⁺CXCR5⁺PD-1⁺) and Treg cell (CD4⁺CD25⁺Foxp3⁺) percentages in splenocytes among five groups. **(C)** Representative flow cytometry plots of memory B cell (B220⁺IgD⁺CD38⁺), GC B cell (B220⁺CD95⁺GL7⁺) and plasmablast (B220⁺CD138⁺) percentages in splenocytes among five groups. **(D)** Statistical results of flow cytometry data for each group. n = 6 in B6 and R848 groups, n = 9 in silybin or MSCs treated groups, and n = 8 in silybin plus MSCs group after removal of dead mice. *P<0.05, **P<0.01, ***P<0.001, ****P<0.00001, ns: not significant.

Fig. 3 Effects of silybin on lymphocyte subsets *in vitro*. **(A)** The cell subset proportions were determined by flow cytometry using splenocytes from two groups (n = 6 for Th1, Th2, Th17, Treg and Tfh cells, n = 5 for GC B cells and plasmablast). **(B)** The percentages of Tfh cells of R848-induced mice treated with different concentrations of silybin *in vitro* (n=5). *P<0.05, **P<0.01, ***P<0.001, ****P<0.00001, ns: not significant.

Fig. 4 Regulatory effects of silybin and MSCs on Tfh cell proliferation, apoptosis and differentiation-related genes. **(A)** The percentages of apoptosis (n=5) and proliferation (n=6) of Tfh cells in R848-induced mice treated with silybin and MSCs *in vitro*. **(B)** Expression of Tfh-related genes in acetone-induced B6 mice and R848-induced B6 mice (n=6). **(C)** Expression of Tfh-related genes in R848-induced B6 mice and R848-induced mice treated with silybin and/or MSCs (n=6). **(D)** Western blot analysis of p-STAT3, STAT3 and β -actin in splenocytes of B6, R848-induced and silybin treated R848-induced mice. *P<0.05, **P<0.01, ***P<0.001, ****P<0.00001, ns: not significant.

Fig. 5 Impact of IL-6 and its downstream pathways on silybin regulated Tfh cells. **(A)** IL-6 counteracted the effect of silybin on Tfh cell expansion in splenocytes of R848-induced mice (n=6). **(B)** The mean fluorescence intensity of pAKT and pS6 in Tfh cells of R848-induced mice increased after IL-6 treatment (n=6). **(C)** The mean fluorescence intensity of pSTAT3 in Tfh cells of R848-induced mice increased after IL-6 treatment (n=6). **(D-E)** Both rapamycin and stattic partially inhibited the effect of IL-6 on Tfh cell expansion in normal B6 mice splenocytes (n=6). **(F)** Both colivelin and MHY1485 counteracted the effect of silybin on Tfh cell expansion in splenocytes of R848-induced mice (n=6). *P<0.05, **P<0.01, ***P<0.001, ****P<0.00001.

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