Baicalin attenuates collagen-induced arthritis via inhibition of JAK2-STAT3 signaling and regulation of Th17 cells in mice

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Abstract

This study focused on the potential therapeutic effect of Baicalin on collagen-induced arthritis (CIA) in mice and the underlying mechanisms. Baicalin (200 mg/kg) was administrated after the induction of CIA for 42 days. Therapeutic effects were evaluated by arthritic scores, X-rays and assessment of histopathological joint destruction. The production of TNF- α , IL-6, IL-17, IL-1 β were also measured. The percentage of Th17 cells in splenocytes were determined by flow cytometry analysis. Our results showed that Baicalin treatment attenuated the severity of arthritis of CIA mice and reduced the levels of several cytokines. Further mechanistic investigations revealed that Baicalin suppressed the expression and phosphorylation of JAK2 and STAT3 protein in splenocytes tissue. Moreover, the percentage of Th17 cells in splenocytes also downregulated by Baicalin. Our experiment indicate that CIA mice can be alleviated by Baicalin treatment via inhibition of JAK2-STAT3 signaling and regulation of Th17 cells in mice.

Keywords Collagen-induced arthritis · Baicalin · JAK2 · STAT3 · Th17 cells

Introduction

Rheumatoid arthritis (RA) is an autoimmune-mediated chronic inflammatory disease (Yang et al. 2017). It is characterized by chronic inflammation of synovial membranes and proliferation of the synovial lining, leading to cartilage destruction, bone erosion, and finally loss of joint function (Wu et al. 2017). RA affects approximately 1% of the population worldwide, decreasing the quality of patients' life as well as imposing a heavy financial burden on our society (Wang et al. 2014). How to effectively prevent the development of RA and let to the discovery of new treatments has now become a major issue that needs even more detail and thorough researches.

The etiology and pathogenesis of RA is not fully elaborated and may be the result of several factors, which exerts extreme difficulty in treating this disease. Previous studies demonstrated that several immune cells participated in the

development of RA (Keystone 2005; Kontinen et al. 1981; Stolina et al. 2009). Also, the presence of autoantibodies in the sera of RA patients has provided many clues to the underlying disease pathophysiology (Derksen et al. 2017). T cells, B cells and macrophages contribute to the pathogenesis of RA partially by secreting cytokines (Brennan and McInnes 2008). In recent years, with more detail and thorough research, researchers found that T cell-mediated immune abnormality is the main pathogenetic pathway of RA, such as Th1 and Th17. These cells can promote the genesis and development of immune response by secreting TNF- α , IL-17, IL-6, and IL-1 β (Edavalath et al. 2016; Niu et al. 2016; Wang et al. 2016). Many cytokines involved in RA, such as IL-4, IL-6, IL-7, IL-10, IL-12, IL-15 and IL-21, activate the Janus kinase/signal transducer and activator of transcription (JAK-STAT) pathways (O'Shea and Plenge 2012). JAKs represent a family of four non-receptor tyrosine kinases: Jak1, Jak2, Jak3, and Tyk2. STATs comprise a family of seven structurally and functionally related proteins: Stat1, Stat2, Stat3, Stat4, Stat5a, Stat5b, and Stat6 (Cai et al. 2015). JAK kinases can selectively phosphorylate STATs, leading to their activation. Once activated, STATs transfer to the nucleus and play a critical role in the transcription of target genes, regulating innate and acquired host immune responses. O'Shea JJ demonstrated that activated

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CD4⁺ T cells are differentiated into Th17 cells by activation of the IL-6-activated STAT3 pathway (Na et al. 2017). Son et al. showed that inhibiting the phosphorylation of STAT3 by SOCS3 suppressed the activation of Th17 (Son et al. 2017). Therefore, the inhibition of the activation of the JAK2/STAT3 signaling pathway and the regulation of Th17 differentiation will be important strategies for the treatment of RA.

There are various drugs for the treatment of RA, including non-steroidal anti-inflammatory drugs (NSAIDs), disease modifying anti-rheumatic drugs (DMARDs), biological agents, glucocorticoid, and plant medicine preparations. Baicalin (7-glucuronic acid, 5,6-dihydroxyflavone) is a flavonoid compound originally isolated from the root of the Chinese herb Huangqin (Li et al. 2000; Lin and Shieh 1996). Baicalin has been used as an anti-inflammatory and anti-allergic agent to treat a variety of inflammatory diseases such as bronchitis, nephritis, hepatitis, asthma, and atopic dermatitis (Kubo et al. 1984). Yang et al. (2013) found that Baicalin inhibited Th17 cell differentiation in vitro and in vivo and that Baicalin suppressed Th17 cell infiltration into the kidney. Baicalin also has an apparent antagonistic role in Th17 cell-mediated inflammation in lupus-prone MRL/lpr mice (Yang et al. 2013). However, the exact mechanisms of Baicalin on the development and treatment of RA remain unknown. In this study, we observed the role of Baicalin in treating CIA mice and examined the levels of several inflammatory cytokines, the expression and phosphorylation of JAK2 and STAT3 protein, as well as the percentage of Th17 cell, trying to clarify the therapeutic effects of Baicalin on CIA at molecular level.

Materials and methods

Animals

Male DBA/1 mice were purchased from the Shanghai SLAC Laboratory Animal Corporation (Production License No. 2015000509273, Shanghai, China). In our experiment mice weighing approximately 18 g to 20 g were used. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Shangdong Medical University.

Reagents

Bovine type II collagen (CII) was obtained from Chondrex, Inc. (20,021, WA, USA). IFA (Chondrex, Inc. 7002, WA, USA) and CFA (Chondrex, Inc. 7009, WA, USA) were used as adjuvants. Baicalin was purchased from Sigma.

Induction and assessment of CIA

CIA was induced as described previously (Brand et al. 2007). Briefly, 24 mg CII was dissolved in 6 ml glacial acetic acid to a concentration of 2 mg/ml and stored at 4 °C overnight. Then added the CII to 2 mg/ml CFA or IFA dropwise to get emulsion containing 2 mg/ml CII. Each DBA/1 mouse was immunized subcutaneously at the base of the tail with 100 µl emulsion, followed by a booster immunization on day 21. The severity of arthritis was determined according to an established scoring system (Kim et al. 2002). Each paw was graded as follows: 0, normal; 1, one finger inflamed and swollen; 2, more than one finger moderately inflamed and swollen; 3, severe inflammation and swell involving entire paw; and 4, severe arthritis resulting in ankylosis and loss of joint movement. The maximum score for each mouse was 16. The assessment is double blind.

Drug administration

Three weeks after the primary immunization, the mice were randomly divided into four groups: 1) normal control group (Con group): mice without CIA, received Phosphate Buffer solution (PBS); 2) CIA model control group (CIA group): mice with CIA, received PBS; 3) Baicalin group: mice with CIA were daily treated with Baicalin at the dose of 200 mg/kg; and 4) CIA + Vehicle group: mice with CIA, received Vehicle. Drugs were administrated intraperitoneally for a total of seven weeks.

Histopathological analysis

After being treated for seven weeks, the hind limbs were harvested and fixed in 10% formalin for 24–48 h, followed by decalcifying in EDTA. The hind limbs were stained with hematoxylin and eosin (H&E). The degrees of synovial hyperplasia, cell infiltration, and bone erosion were scored on a scale of 0–4: Grade 0, normal; Grade1, mild changes; Grade2, moderate changes; Grade3, severe changes; Grade4, disrupted joint architecture.

Detection of cytokines levels

Tissue samples from each mouse were collected after seven weeks' treatment. TNF- α , IL-6, IL-17 and IL-1 β were measured by ELISA kit according to the manufacturer's instructions.

Western blot analysis

Synovial tissues were lysed using radioimmunoprecipitation assay buffer for 30 min on ice, followed by centrifuging at 12,000 $\times$ g for 20 min at 4 °C. The concentration of the protein

Fig. 1 Assessment of CIA and the therapeutic effect of Baicalin on CIA. After the booster immunization, arthritic scores were assessed every 6 days for a total of 42 days. **a** Body weight changes of the mice. **b** The severity of arthritis was evaluated by mean arthritic scores. The data were analyzed using a one-way ANOVA. The error bars represent the standard error of the mean (S.E.M.). *: $P < 0.05$, versus normal control group (Con); #: $P < 0.05$, versus Vehicle-treated CIA group

was determined by bicinchoninic acid assay. Twenty microgram sample protein was mixed with 5X SDS loading buffer and loaded onto 10% SDS-PAGE. The separated protein bands in the gel were transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was then blocked with 5% non-fat milk in PBS with 0.1% Tween 20 (PBST) at room temperature for 2 h. PVDF membranes were then incubated with primary antibodies at 4 °C overnight and washed with PBST. Secondary antibodies were added to each blot and incubated at room temperature for 2 h; After washing the membrane three times with TBST, the membrane was incubated in a dilution of secondary monoclonal antibody

for 1 h at room temperature and developed with ECL western blotting Kit. Primary antibodies used were anti-JAK2 (1:2000; Abcam company), anti-p-JAK2 (1:2000; Abcam company), anti-STAT3 (1:3000; Abcam company), anti-p-STAT3 (1:1000; Abcam company), and anti- β -Actin (1:3000; Abcam company). The experiment was repeated three times.

Flow cytometry analysis

After being treated for seven weeks, cells in splenocytes were isolated and counted. The cells were prepared to single

Fig. 2 General photos of mice hind paws in different groups. The photos were taken on day 42 after the booster immunization. **a** Normal control group: mice without CIA, received PBS; **b** CIA group: mice with CIA, received PBS; **c** Baicalin-treated group, received Baicalin 200 mg/kg; **d** Vehicle-treated group: mice with CIA, received Vehicle

Fig. 3 X-ray pictures of mice hind paws in different groups. The X-ray was performed on day 42 after the booster immunization. **a** Normal control group showed neither paw swelling nor joint erosion; **b** CIA group and **(d)** Vehicle-treated group showed severe paw swelling and bone erosion; **c** 200 mg/kg Baicalin-treated group showed less damage.

e The radiologic scores of bones erosion in treated and untreated CIA mice. The data were analyzed using a one-way ANOVA. The error bars represent the standard error of the mean (S.E.M.). #: $P < 0.05$, versus Vehicle-treated CIA group

cell suspension with a final concentration of 1×10^6 /ml. For flow cytometry analysis of Th17, 1×10^6 cells were used. The cells were blocked with 2 ml 2% fetal serum, followed by centrifuging at 1500 rpm for 5 min. Then washed with 2 ml PBS and centrifuged at 1500 rpm for 5 min. CD4 (65–0193, Tonbo biosciences, San Diego, CA, USA) was added and incubated in dark place. 30 min after the incubation with antibodies, the cells were centrifuged at 1500 rpm for 5 min, followed by washing with PBS. The pellet was resuspended with 200 μ l PBS and protected from light. The flow cytometry analysis was performed with BD FACSCalibur (Becton, Dickinson and Company, Lake Franklin, New Jersey, USA).

Statistical analysis

Statistical analysis was performed using a one-way ANOVA. $P < 0.05$ was considered statistically significant.

Error bars represent mean $\pm$ SEM. Statistical analysis was performed using SPSS Statistics 17.0 (SPSS Inc., Chicago, IL) and GraphPad Prism 6.07 (GraphPad Software, Inc., La Jolla, CA).

Results

Baicalin improved clinical arthritic conditions in CIA mice

After the booster immunization, we evaluated the arthritic scores every 6 days for a total of 42 days. Experimental observation shows that the normal control group of mice is in good condition with steadily increased weight (Fig. 1a). Compared with the normal control group, on the 12th day of drug administration, the mice in the CIA group and other treatment groups were not in good condition and their weight

Fig. 4 Treatment with Baicalin significantly decreased histological damage in joints of CIA mice. The mice were sacrificed on day 42, and their joints were harvested and stained with H&E. The histopathological damage score was assigned using values between 0 and 4. **a** Normal control group showed normal cartilage, bone, and synovium without inflammation; **b** CIA group and **(d)** Vehicle-treated group showed severe synovitis with pannus formation, and extensive erosions of bone and cartilage; **c** Baicalin-treated group showed a conserved synovial

space similar to a normal mouse, with minimal pannus formation compared with CIA group and vehicle-treated group. **e** Histologic score was evaluated from the joints of treated and untreated CIA mice, according to the infiltration of inflammatory cells, synovial hyperplasia, pannus formation, cartilage destruction, and bone erosion. The data were analyzed using a one-way ANOVA. The error bars represent the standard error of the mean (S.E.M.). *: $P < 0.05$, versus normal control group (Con); #: $P < 0.05$, versus Vehicle-treated CIA group

initially decreased obviously and then increased slowly (Fig. 1a). The CIA group and CIA + Vehicle group exhibited the slowest body weight gain when compared with normal control group ($P < 0.05$). While the Baicalin treated group started steadily increased weight gain from the 21th day (Fig. 1a). The symptoms associated with arthritis appeared gradually in all CIA mice, comparing with normal control group (Fig. 1b). CIA group and CIA + Vehicle group continued to develop severe arthritis and reached a plateau between days 24 and 30. However, the administration of Baicalin significantly lowered the arthritic scores when compared with the Vehicle-treated CIA group ($P < 0.05$). The general photos and X-ray pictures in Figs. 2 and 3 showed similar results. There was no redness and inflammation in mice from normal control group. The Vehicle-treated CIA mice showed severe swelling, erythema and joint rigidity in the paw when compared with Baicalin treated mice (Fig. 2). The bone density and bone cortex were normal, and the joint surface was

smooth in normal control group, while the cartilage and bone erosion were evident in Vehicle-treated CIA mice ($P < 0.05$) (Fig. 3).

The mice were sacrificed on day 42, and their joints were stained with H&E. A considerable correspondence between the results of clinical signs and histopathological was exhibited (Fig. 4). Histopathological analysis showed that PBS and Vehicle-treated CIA mice presented dramatic synovial proliferation, cell infiltration, and cartilage erosion compared with normal mice ($P < 0.05$), whereas significantly improvement of the above pathological changes were observed in Baicalin treated groups ($P < 0.05$). Collectively, our results suggested that Baicalin exhibited a certain effect on CIA.

Baicalin suppressed cytokine production in CIA mice

To investigate the mechanism underlying the development of CIA. The levels of cytokines in paw, such as TNF- α , IL-6, IL-

Fig. 5 Baicalin treatment suppressed the production of TNF- α , IL-6, IL-17, and IL-1 β in CIA mice. 42 days after the booster immunization, the paw tissues of the mice were harvested and detected the levels of TNF- α , IL-6, IL-17, and IL-1 β . The concentration of these cytokines was extremely high in CIA mice and vehicle-treated CIA mice, while

Baicalin reduced their levels significantly. The data were analyzed using a one-way ANOVA. The error bars represent the standard error of the mean (S.E.M.). *: $P < 0.05$, versus normal control group (Con); #: $P < 0.05$, versus Vehicle-treated CIA group

IL-1 β were detected. The contents of TNF- α , IL-6, IL-17, IL-1 β in PBS and Vehicle-treated CIA mice increased significantly when compared with normal control group ($P < 0.05$) (Fig. 5), while the treatment with Baicalin significantly reduced the production of these cytokines compared with Vehicle-treated CIA mice ($P < 0.05$). This findings indicated that TNF- α , IL-6, IL-17, IL-1 β played a role in the pathogenesis of CIA.

Baicalin downregulated the relative expressions of p-JAK2/JAK2 and p-STAT3/STAT3

To further know the mechanism of Baicalin on CIA, we detected the expression of JAK2, p-JAK2, STAT3, and p-STAT3 in synovial tissues of CIA mice by western blot. Our result showed that the relative expressions of p-JAK2/JAK2 and p-STAT3/STAT3 in synovial tissues of PBS and Vehicle-treated CIA groups were higher than that of the normal control group ($P < 0.05$), while the expressions of p-JAK2/JAK2 and p-STAT3/STAT3 were significantly lowered by Baicalin ($P < 0.01$) (Figs. 6 and 7).

Baicalin suppressed the proportion of Th17 cells in the splenocytes of CIA mice

It has been demonstrated that Th17 cells are the major components of the adaptive immune response system and play a vital role in the regulation of immune responses. Thus, in our study we further measured the percentage of Th17 cells by flow cytometry. Our result showed that the proportion of Th17 cells in PBS and Vehicle-treated CIA groups was significantly higher than that in the normal control group ($P < 0.05$), while the proportion of Th17 cells in the Baicalin group was markedly lower than that in the Vehicle-treated CIA group ($P < 0.05$).

Discussion

In this report, we explored the role of Baicalin in treating CIA. Our results showed that Baicalin could inhibit the genesis and development of CIA in mice with the evidence that the arthritic scores and pathological damage recovered significantly.

Fig. 6 Effects of Baicalin on JAK2-STAT3 signaling pathways. On day 42 after booster immunization, the mice were sacrificed and total proteins extracted from the synovial were separated by SDS-PAGE as described in Materials and Methods. **a** showed the Western blot analysis of JAK2, p-JAK2, STAT3 and p-STAT3 using specific antibodies. **b** and **c** were the quantitation of Western blots of JAK2, p-JAK2, STAT3 and p-STAT3 in synovial of mice from different groups. The data were analyzed using a one-way ANOVA. The error bars represent the standard error of the mean (S.E.M.). *: $P < 0.05$, versus normal control group (Con); #: $P < 0.05$, versus Vehicle-treated CIA group

When administered Baicalin, the levels of several cytokines in paw tissues, such as TNF- α , IL-6, IL-17, IL-1 β were reduced compared with vehicle-treated CIA mice. Baicalin downregulated the relative expressions of p-JAK2/JAK2 and p-STAT3/STAT3, as well as suppressed the proportion of Th17 cells. Thus, we hypothesized that the therapeutic benefits of

Baicalin in CIA may lie in the inhibition of JAK2-STAT3 signaling and regulation of Th17 cells.

The JAK/STAT signaling pathway is an important cytokine signal transduction pathway, playing an important role in the regulation of several physiological and pathophysiological processes. The JAK/STAT pathway is initiated when a peptide ligand binds to its receptors. The receptor-associated JAKs are then activated via the receptor oligomerization and conformational changes. The activated JAKs subsequently phosphorylate cytokine receptors and STATs. The activated STATs then translocate to the nucleus and subsequently activate or inhibit transcription of target genes (Rawlings et al. 2004). Up to now, many cytokine-specific JAKs and STATs have been determined. Parganas E demonstrated that JAK2-deficient cells failed to respond to IL-3, granulocyte/macrophage colony-stimulating factor (GM-CSF) and IFN- γ , leading to embryonic lethal (Parganas et al. 1998). Zhang's study showed that inhibition of JAK2/STAT3 signaling pathway could reduce the production of proinflammatory cytokines of TNF- α and IL-6 (Zhang et al. 2013). Modern studies show that synovial hyperplasia of RA is the pathological basis to cause joint damage. The JAK2/STAT3 pathway has been inherently linked with cell differentiation and proliferation and has become the important path for the study of the transfer of information and regulation of synovial cells. Our study showed that Baicalin can inhibit the expressions of JAK2, p-JAK2, STAT3, and p-STAT3 and the relative expressions of p-JAK2/JAK2 and p-STAT3/STAT3 in synovial tissues and can regulate the JAK2/STAT3 pathway, which is consistent with the articles (Xu et al. 2017).

In this study, DBA/1 mice were used to establish a CIA model with similar clinical manifestations and pathological changes of RA. Baicalin, a traditional Chinese medicine, was used to treat the CIA mice. Our results showed that Baicalin can markedly alleviate the development of CIA, reduce ankle swelling, arthritis score, bone destruction, as well as decrease the production of TNF- α , IL-6, IL-17, IL-1 β , which is consistent with previous articles. Western blot analysis revealed that the relative expressions of p-JAK2/JAK2 and p-STAT3/STAT3 also downregulated.

Type-17 helper T (Th17) cells are one of the major helper T cells involved in the pathogenesis and progression of RA (Park et al. 2005). Th17 serves as a main source of the proinflammatory cytokine IL-17. Previous study showed that IL-17 overexpression in the murine knee joint induces joint inflammation, bone erosion and cartilage proteoglycan loss (Lubberts et al. 2001). Proinflammatory cytokines, such as IL-6 and TNF- α induce naive CD4⁺ T cell differentiation into Th17 cells (Noack and Miossec 2014), as well as play direct roles in RA pathogenesis (McInnes and Schett 2007). Recently, drugs suppressing Th17 activities have drawn more and more researchers' attention as candidates

Fig. 7 Baicalin decreased the percentage of Th17 cells in splenocytes of CIA mice. The mice were sacrificed 42 days after the booster immunization, and the number of Th17 cells were evaluated by flow cytometry analysis (a–d). Treatment with Baicalin caused a remarkable decrease in the percentage of Th17 cells in CIA mice. e showed the

quantification of the flow cytometry analysis. The data were analyzed using a one-way ANOVA. The error bars represent the standard error of the mean (S.E.M.). *: $P < 0.05$, versus normal control group (Con); #: $P < 0.05$, versus Vehicle-treated CIA group

for RA treatment (van den Berg and Miossec 2009). Baicalin treatment induced a significant decrease in the levels of inflammatory mediators, including the myeloperoxidase activity, the levels of tumor necrosis factor α , IL-1 β , and Th1-related cytokines IL-12 and IFN- γ . Xue Yang et al. showed that Baicalin reduced joint inflammation by inhibiting splenic Th17 cell population expansion in vivo (Yang et al. 2013). Ji Yang et al. showed that Baicalin can act as an immunoregulatory compound by controlling Th17 cell differentiation (Yang et al. 2011). Here in our study, we observed that Baicalin suppressed the proportion of Th17 cells in CIA mice.

All the data in our paper demonstrated that Baicalin-inhibited inflammation reaction is a key therapeutic mechanism in treating CIA. Baicalin was able to alleviate the symptoms of CIA by regulating the inflammation reaction. The underlying mechanism may involve in the regulation of JAK2-STAT3 signaling and Th17 cells.

In this paper we demonstrated the therapeutic effect of Baicalin, through measuring several cytokines, the relative expressions of p-JAK2/JAK2 and p-STAT3/STAT3,

and the proportion of Th17 cells in CIA mice. Our study may help to elucidate the mechanism of the genesis and development of RA.

Compliance with ethical standards

Conflict of interest The author (s) declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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