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IN VIVO ELUCIDATION OF ANTI-ARTHRITIC EFFECT OF PLANT ALKALOID BERERINE IN EXPERIMENTAL MOUSE JOINT INFLAMMATION

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ABSTRACT

Berberine is a protoberberine alkaloid possessing various pharmacological activities, known as a Janus kinase (JAK) inhibitor. The aim of the present study was to elucidate the mechanism of its action in relation to JAK2/3 and sclerostin expression in synovium and cartilage, the presence of Receptor activator of factor kB Ligand (RANKL) positive cells in synovial fluid, the presence of dendritic cells (DC) in cartilage and the expression of TNF-related apoptosis-inducing ligand (TRAIL) and its receptor DR5 on bone marrow (BM) cells. The experiments were conducted in a mouse model of erosive inflammatory joint disease, relevant to human rheumatoid arthritis, induced through intraarticular injection of zymosan. Berberine decreased the phosphorilation of both JAK2 and JAK3 in arthritic joints, the expression of RANKL, TRAIL and DR5. The alkaloid did not affect sclerostin expression and prevented the decrease of the number of 33D1

positive dendritic cells. Present results contribute to more clear understanding of the ameliorative effect of berberine on erosive processes in joint inflammation.

Keywords: berberine, JAK inhibitors, erosive arthritis, dendritic cells

Abbreviations: Janus kinase (JAK); Receptor activator of nuclear factor kappa-B ligand (RANKL); TNF-related apoptosis-inducing ligand (TRAIL); zymosan-induced arthritis (ZIA); rheumatoid arthritis (RA); TRAIL-R2 (DR5)

INTRODUCTION

The most common form of chronic inflammatory joint disease is rheumatoid arthritis (RA), characterized by synovial inflammation, pannus formation and irreversible destruction of cartilage and bone, leading to loss of function and disability [1, 2]. Systemic complications often appear due to an involvement of different organs such as cardiovascular system, lungs, eyes, and skin. Its therapy is expensive and in many cases is not enough efficient which necessitates the development of new therapeutic approaches. The mechanisms underlying RA pathology are not enough clear as they are result of complex interactions between genetic and environmental factors. RA is generally triggered by both, innate cells such as neutrophils, macrophages, mast cells, and dendritic cells, adaptive immune cells, and aberrant production of cytokines and inflammatory mediators [3, 4]. Proliferating synovial fibroblasts and infiltrated inflammatory cells form a pannus which invades the adjacent articular cartilage and subchondral bone, further leading to erosion of joint tissues [5]. Pannus cells secrete a variety of cytokines and chemokines, which are reported to be abundant in synovial tissues and fluid from RA patients [6, 7], thus being key therapeutic targets in RA [8]. Cytokines exert their action through transmembrane receptors whose cytoplasmic region is bound to janus kinases (JAKs) [9]. JAKs are activated through receptor-ligand interactions, resulting in tyrosine phosphorilation of receptors and subsequent activation of signal transducers and activators of transcription (STATs), which translocate to the nucleus and act as transcription factors of target genes [10]. JAK family comprises the four nonreceptor tyrosine kinases JAK1, JAK2, JAK3 and TyK2. IL-6, IL-10, IL-

11, IL-19, IL-20, IL-22 and IFN-γ use JAK1 and JAK2. Hormone-like cytokines, GM-CSF, IL-3 and IL-5 use JAK2, while IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 use JAK3 [9]. IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 are key players in T and NK cell development and B cell function and proliferation [11]. Increasing data shows that JAK inhibitors are promising for the treatment of RA [12-15]. Tofacitinib was the first pan-JAK inhibitor approved for treatment of RA in humans [16]. Baricitinib is a new generation JAK1/2 inhibitors showing in clinical trials to be more efficient than tofacitinib [17, 18]. It was reported that blocking of signal transduction of JAK1 and JAK2 may be potentially dangerous as their gene disruption appears lethal in mice [19, 20]. Based on the specificity of JAK3, it is suggested that a specific JAK3 inhibitor should have limited but precise effects on immune cells and should leave other cell types unaffected [9]. Many accumulating data suggests that temporary and reversible JAK inhibition contrary to permanent inhibition, may provide safe and efficacious treatment for many autoimmune diseases [15]. Selectivity against the four individual JAK family enzymes is now a key goal since each plays different roles in cytokine-induced cell signaling. Hence, the high degree of identity within the kinase domains of all members of JAK family has made it exceedingly difficult to design inhibitors specific for one or other member.

Medicinal plants are widely used because they are readily available and cheaper than modern medicines. The place of plants was radically changed in the 19th century by the application of chemical analysis. The successful introduction of alkaloids as clinically perspective started with morphine from the poppy, and soon followed by quinine from the cinchona tree, and then many others. Several species of the genus *Berberis* such as *B. crataegina, B. aristata, B. vulgaris* and *B. calliobotrys* are witnessed as a source of anti-inflammatory compounds [21-23]. A protoberberine alkaloid berberine has been isolated from *Berberis* roots, rhizomes and stem barks. It is considered to be a predominantly JAK3 inhibitor by binding to the specific JAK3 kinase domain [24] but later it was shown that the substance activates also JAK2/STAT3 pathway [25]. This signaling pathway is constitutively activated in RA as some key proinflammatory cytokines like IL-1, IL-6, and interferons act through it [26]. Recent data point on berberine as a promising JAK inhibitor for RA treatment [21, 27-30].

In previous studies we have established that berberine reduces symptoms of zymosan-induced arthritis (ZIA) *via* amelioration of chronic synovitis and reduced cartilage and bone destruction [27, 28]. The substance decreases cell infiltration and prevents glucosaminoglycan loss [27] as

well as osteophyte formation related to the excessive accumulation of TGF-β3 positive cells and DC populations in the synovium [28]. Berberine also inhibits the generation of senescent cells in the synovium thus leading to ameliorated synovitis [27]. Concomitantly, berberine diminishes the appearance of senescent cells during osteoblast differentiation *in vitro* and enhances the processes of mineralization. The effect of the substance can be defined as a longtime since the assessment was done 6 weeks after the last treatment [27]. The aim of the present study was to evaluate whether berberine affects the activation of JAK2 and JAK3 and also to estimate the influence of berberine on dendritic cells, TRAIL, DR5, sclerostin and RANKL in different compartments.

MATERIALS AND METHODS

1. Substance and treatment

Berberine was isolated from the alkaloid fraction obtained from roots of *Berberis vulgaris* L. by procedures based on column chromatography. The structure of berberine was confirmed by comparison of the Rf value, and IR and ^{1}H NMR spectral data with these of authentic sample. The alkaloid was used as a sulphate [$(C20H18NO_4)2\text{-}SO_43H_2O$] fully dissolved in distilled H_2O and injected intraperitoneally (i.p.) in a dose of 10 mg/kg every other day from day 0 to day 12 of ZIA. The dose and time schedule of berberine's administration was determined in previous studies in a model of adjuvant arthritis [31].

2. Mice

BALB/c mice (Charles River Laboratories, Wilmington, MA, USA) were maintained at 12/12 light dark cycle in pathogen free conditions and were fed with pelleted food and tab water ad libitum. All experiments were conducted in accordance with the International and National Guidelines for the Care and Use of Laboratory Animals and were approved by the Animal Care Committee at the Institute of Microbiology, Sofia (Guidelines №352).

3. Zymosan-induced arthritis (ZIA)

Mice received an intra-articular (i.a.) injection of 180 μg/10 μl zymosan A from *Saccharomyces cerevisiae* (Sigma-Aldrich, Germany) under brief anaesthesia (sodium pentobarbital 50 mg/kg, i.p) (day 0). Control animals received an i.a. injection of an equal volume of sterile phosphate buffered saline (PBS). The development of inflammation might be divided into 3 phases: acute

phase (onset) from day 0 to day 7, active phase up to day 18, and established arthritis from day 18 to day 60.

4. Immunohistochemistry

Knee joints were dissected at the active phase (day 18) of ZIA, fixed for 4 days in 10% formalin, decalcified by incubation in 10% EDTA/0.2% PFA in PBS at 4°C for 20 days and embedded in paraffin. Tissue samples 5 μm thick were dewaxed with xylene, dehydrated and immunohistochemical analysis was performed to estimate the expression of pJAK2, pJAK3, sclerostin and 33D1 markers (n = 7 per group in three separate experiments). After blocking of endogenous peroxidase activity with 0.3 % H2O2 in 60 % methanol (10 min), slices were incubated for 2 h with antibodies against pJAK2 ((10 μg/ml; GenScript, NJ, USA).), or pJAK3 (Y785, Cusabio Technology LLC), sclerostin (1:50 diluted, Abcam, Cambridge, UK), and against 33D1 (clone 33D1, 1:500 diluted, Biolegend). Isotype rabbit antibodies were used as specific controls. Slices were washed and stained with DAB solution (3,3′ diaminobenzidine kit, Sigma-Aldrich) for 10 min and counterstained with Gill's haematoxylin for 3 min. The number of cells stained positive for the examined proteins was determined by imaging system software (ImageJ 1.42; Research Services Branch, NIH, USA)

5. Flow cytometry

Synovial fluid cells were harvested by lavage of the synovial cavity with 25 μl of 1 mM ethylendiaminetetraacetic acid (EDTA)/PBS solution. The cell pellets from five mice were pooled and subjected to FACS analyses. Bone marrow cells were isolated from long bones of 8-10 weeks healthy mice or from mice at day 18 of ZIA. The suspension was gently aspirated to disrupt cell aggregates. Then, cells from both groups were resuspended at 2 × 105/ml in 2% fetal calf serum (FCS)/PBS solution and incubated for 15 min at 4°C with appropriately diluted antibodies against mouse RANKL (CD254, PE labeled, clone IK 22/5, Biolegend, San Diego, CA, USA), TRAIL (CD253, PE-labeled, Biolegend, USA), DR5 (CD2262, TRAILR-2, PE labeled, clone MD5-1, Biolegend, USA), and IgG isotype controls (Biolegend, USA). After washing four times with PBS, the samples were analysed by flow cytometry (BDTMLSR II), using FCS ExpressTM Diva Software (Beckton and Dickinson, San Jose, CA, USA).

6. Statistical analysis

Data represent mean ± SEM. Statistical significance was assessed using one-way ANOVA, considering a P value < 05 as significant. Statistical analyses were performed using InStat3.0 and GraphicPad Prism 5.0 (GraphPad Software Inc., La Jolla, CA, USA).

RESULTS AND DISCUSSION

We previously described that berberine ameliorates joint destruction in ZIA in a long term manner [27, 28]. In the present study immunohistochemical analyses showed that berberine inhibited the phosphorilation of JAK2 in the cartilage (Figure 1A, B, C). This effect was well expressed in the areas of osteophyte formation (Figure 1D, E, F and J). The number of pJAK2 positive cells was decreased in the synovium of berberine-treated mice also (Figure 1G, H, I and K).

Berberine inhibited phosphorilation of JAK3 in the cartilage of arthritic joints (Fig. 2 A, B, C). Several key cytokines such as IL-6, IL-10, GM-CSF, interferon (IFN)-γ use JAK2 receptors, whereas IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 use JAK3 to accomplish their proinflammatory activities in joint tissues damage [9]. Present results showed the decrease of JAK3 phosphorilation in the cartilage of berberine-treated arthritic mice compared to non-treated (Figure 2A, B, C and G).

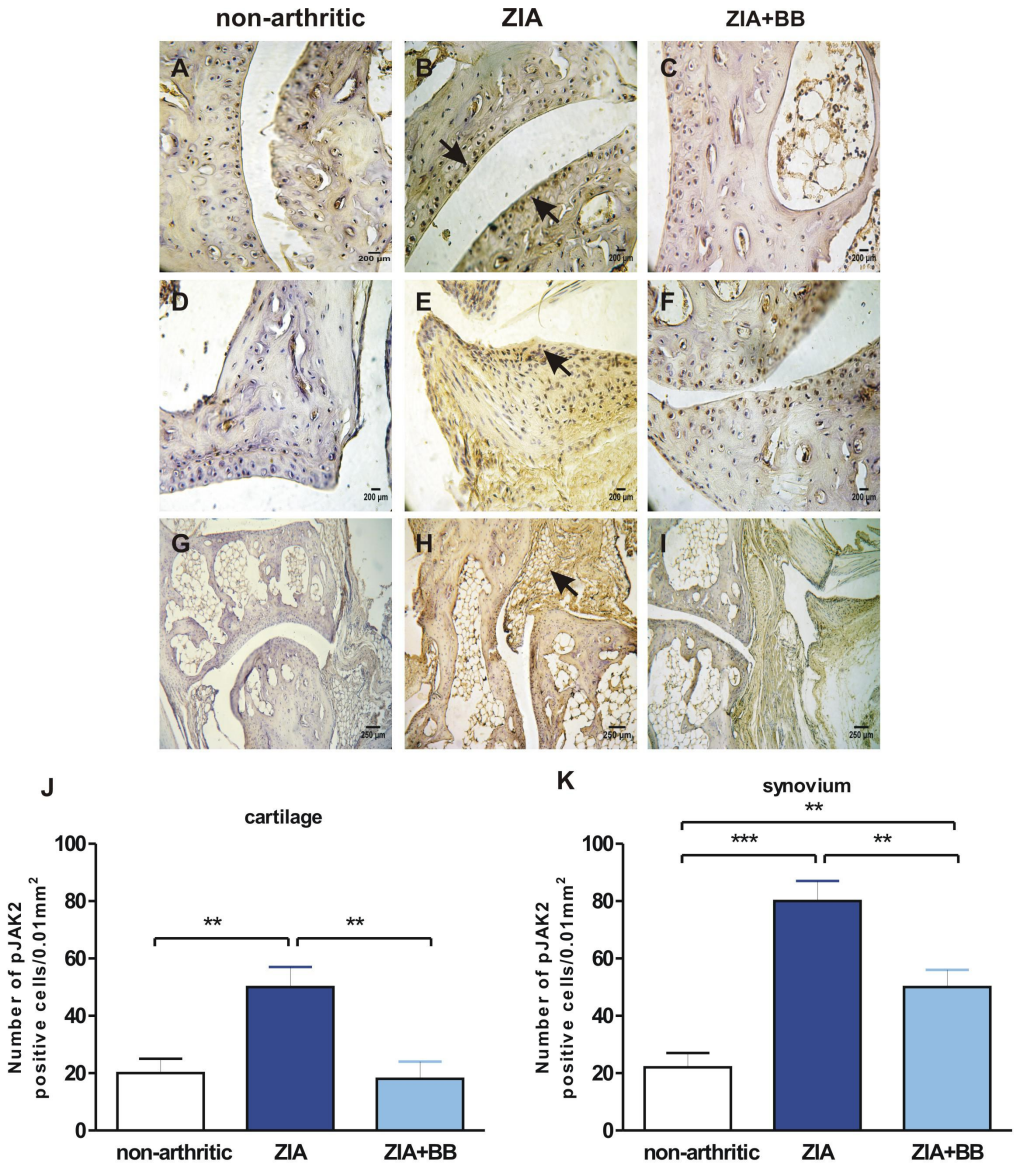

Figure 1. Immunohistochemical evaluation of the number of pJAK2 positive cells in non-arthritic, ZIA and BB-treated ZIA joints at active phase (day 18). **A, B, C –** cartilage**; D, E, F** – osteophytes; **G, H, I –** synovium and **J, K -** graphic presentation. **P < 0.01, ***P < 0.001 from the evaluation of 6 joints/group, one way ANOVA.

Common feature of the chronic, inflammatory rheumatic diseases, including RA, is impaired bone remodeling and homeostasis leading to loss of articular bone mass. Bone remodeling is performed through a sequence of cellular and mediator molecules interactions, engaging specialized bone cells such as bone-resorbing osteoclasts and bone-forming osteoblasts. Normally, a stable bone mass is maintained through coordinated functions of both cell types.

Critical for osteoblast differentiation and activation is the canonical Wnt signaling pathway. Its antagonists are few molecules, including frizzled-related protein (Dicckopf) and sclerostin. It was suggested that sclerostin restricts osteoblasts in bone remodeling, while it stimulates osteoclastogenesis signaling through RANKL/OPG pathway [32].

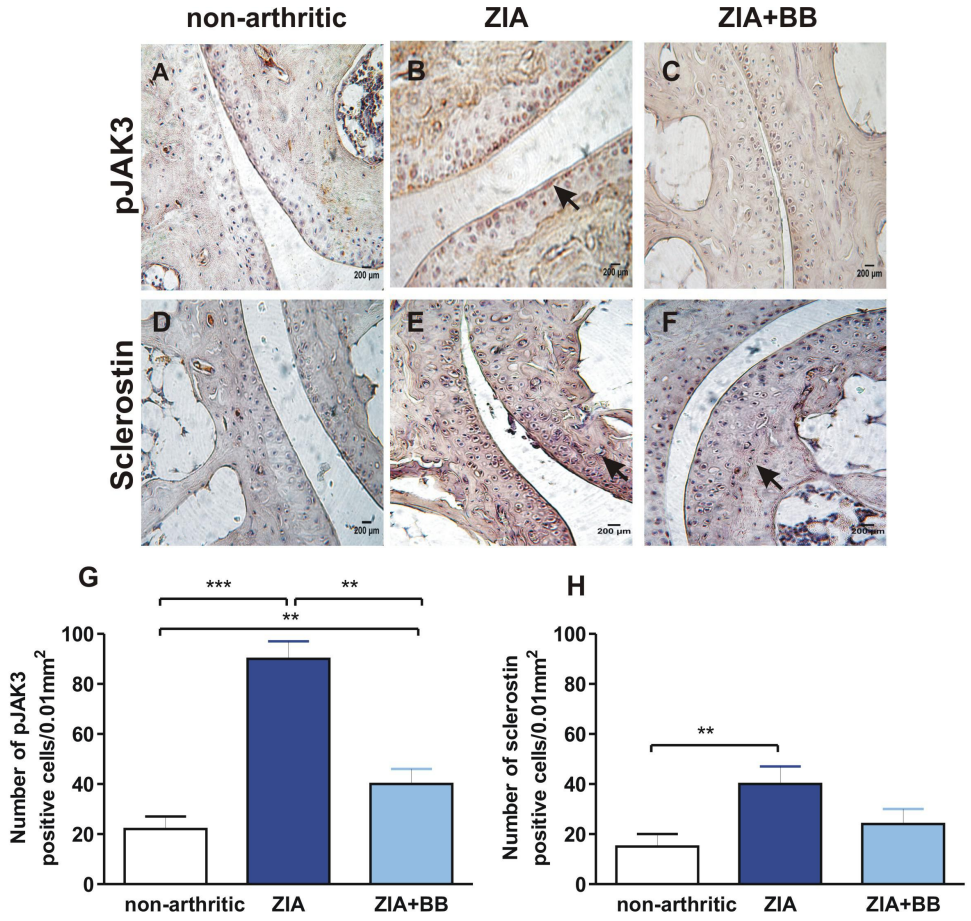

Figure 2. Immunohistochemical analyses of pJAK3 positive cells (**A, B, C and G,** arrows) and sclerostin positive cells (**D, E, F and H,** arrows) in cartilage of non-arthritic, ZIA and BB-treated ZIA mice at active phase (day 18). **P < 0.01 from the evaluation of 6 joints/group, one way ANOVA.

Our results showed that in both, berberine treated and nontreated ZIA groups the number of sclerostin expressing cells was elevated compared to non-arthritic animals, and berberine treatment did not significantly change this effect (Fig. 2D, E, F and H). It could be concluded that previously observed influence of berberine on osteoblast formation was not realized through sclerostin.

RANKL is an essential factor for osteoclast differentiation in the presence of macrophage colony-stimulating factor (M-CSF) [33]. The osteoblasts and osteogenic stromal stem cells produce osteoprotegerin (OPG) thus limiting bone resorption by binding to RANKL and preventing an interaction with RANK. The RANKL/OPG ratio in bone marrow is an important determinant of bone mass in normal and disease states. Proinflammatory cytokines released within the inflamed synovial tissues induce osteoclast formation and inhibit osteoblast differentiation, resulting in focal articular bone erosions [34]. RANKL is expressed on osteoblasts and synovial fibroblasts and is upregulated by proinflammatory cytokines, including IL-1, IL-6, IL-17 and TNF.

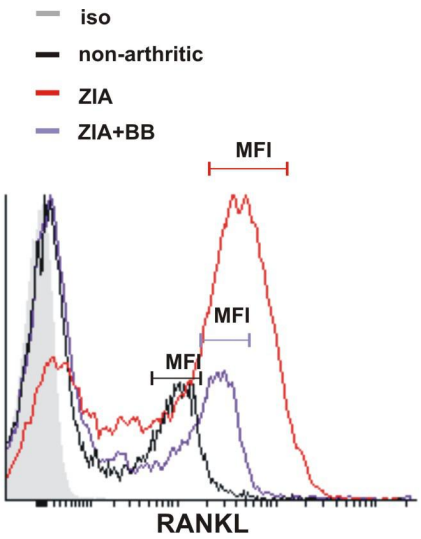

Figure 3. Flowcytometric analyses of cells expressing RANKL in the synovial fluid of non-arthritic, ZIA and ZIA mice treated with BB, mean fluorescence intensity (MFI) is shown, n=10 in each group in 2 experiments.

We assessed the number of RANKL positive cells in the synovial fluid of non-arthritic and ZIA mice at day 18. The results showed that the elevation observed in arthritic group was strongly reduced in BB-treated group (Figure 3). Mean fluorescent intensity (MFI) was 560±60, 1240±120*** vs non-artritic, and 620±60$^{###}$ vs ZIA, respectively.

Synovitis is a prominent feature of RA and it is suggested that dendritic cells (DC) may initiate and/or perpetuate synovitis by presenting arthritogenic antigens to CD4+ T cells thus inducing their differentiation and subsequent contribution to joint inflammation [35, 36]. Here, we examined immunohistochemically the presence of 33D1 positive DCs in the bone and cartilage tissues of arthritic joints at day 7 and day 18 of ZIA of mice nontreated or treated with berberine.

33D1 positive DCs were present in the cartilage and subchondral zones of healthy joints, but in ZIA animals their number was decreased. In berberine treated ZIA mice we observed high number of 33D1 positive DCs both during acute (day 7) and active phases of arthritis (day 18), similar to that in healthy animals (Fig. 4).

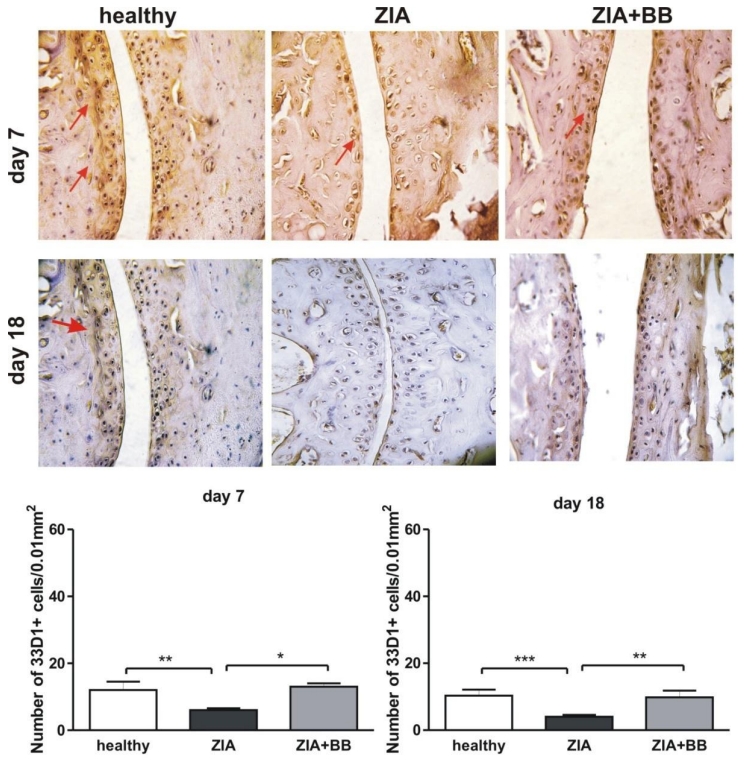

Figure 4. Immunohystochemical analyses of 33D1 positive dendritic cells in the cartilage of non-arthritic, ZIA and ZIA mice treated with BB at onset of arthritis (day 7) and in active phase (day 18) of ZIA. Red arrows point on the areas of 33D1+ cells. **P < 0.01 from the evaluation of 6 joints/group, one way ANOVA.

These findings may indicate that 33D1 positive DCs might have a protective, rather than deleterious function in joint tissues.

TRAIL is a member of TNF cytokine family expressed on activated T cells, NK cells, macrophages, and dendritic cells and is known to induce their apoptosis [37]. TRAIL acts as proapoptotic factor for fibroblast-like synoviocytes [38], decreases their production of OPG, thus facilitating RANKL-dependant osteoclastogenesis [39] and participates in hondrocytes apoptosis causing cartilage destruction [40]. In the present experiments we found that in bone marrow of berberine-treated ZIA mice the number of cells positive for TRAIL and its receptor DR5 was

significantly decreased compared to untreated ZIA animals (Figure 5). The appearance of pro-apoptotic cells during the course of arthritis might trigger local inflammatory foci prevented by berberine.

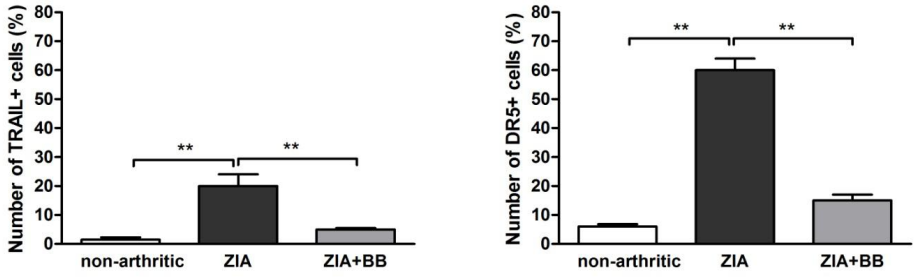

Figure 5. Flowcytometric analyses of TRAIL and DR5 expression on BM cells isolated at day 7. **P < 0.01 from the evaluation of 6 joints/group, one way ANOVA.

A number of JAK inhibitors have been developed over the past decade, with some compounds having greater specificity for certain JAKs than others. Although treatment with biologic agents is efficient for many patients with RA, only approximately 30% achieve complete remission, while most of the patients treated with biologics experience disease exacerbation after discontinuation of treatment. Our results prove that berberine represent an effective treatment alternative in patients who do not respond adequately to conventional drugs.

CONCLUSIONS

The present study extends our investigations on the mode of anti-arthritic action of berberine. The results demonstrated its suppressive effect on JAK2 and JAK3 phosphorilation, thus confirming its properties as a JAK2/3 kinase inhibitor. By inhibiting JAKs BB disrupts an important signaling pathway that is responsible for triggering inflammatory responses. Sclerostin appeared not to be a target of berberine's action. The alkaloid prevented the decrease of the number of 33D1+ cells in the cartilage of ZIA mice so it might be supposed, that the presence of dendritic cells was needed for amelioration of inflammation. The decrease of RANKL+ cells in the synovial fluid is a good prognostic sign for limitation of cartilage and bone erosion. In ZIA mice the number of TRAIL and DR5 positive cells was dramatically increased that was prevented by berberine treatment.

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