

Original Article

Ursolic acid ameliorates autoimmune arthritis via suppression of Th17 and B cell differentiation

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Aim: Ursolic acid (UA) is a pentacyclic triterpenoid found in most plant species, which has been shown anti-inflammatory and anti-oxidative activities. In this study, we examined the effects of UA on collagen-induced arthritis (CIA) in mice, and to identify the mechanisms underlying the effects.

Methods: CIA was induced in mice. Two weeks later, the mice were treated with UA (150 mg/kg, ip, 3 times per week) for 4 weeks. The expression of cytokines and oxidative stress markers in joint tissues was measured with immunohistochemistry. The numbers of CD4⁺IL-17⁺, CD4⁺CD25⁺Foxp3⁺ and pSTAT3 cells in spleens were determined using confocal immunostaining or flowcytometric analyses. Serum antibody levels and B cell-associated marker mRNAs were analyzed with ELISAs and qRT-PCR, respectively. CD4⁺ T cells and CD19⁺ B cells were purified from mice spleens for *in vitro* studies.

Results: UA treatment significantly reduced the incidence and severity of CIA-induced arthritis, accompanied by decreased expression of proinflammatory cytokines (TNF- α , IL-1 β , IL-6, IL-21 and IL-17) and oxidative stress markers (nitrotyrosine and iNOS) in arthritic joints. In CIA mice, UA treatment significantly decreased the number of Th17 cells, while increased the number of Treg cells in the spleens, which was consistent with decreased expression of pSTAT3, along with IL-17 and ROR γ t in the splenocytes. In addition, UA treatment significantly reduced the serum CII-specific IgG levels in CIA mice. The inhibitory effects of UA on Th17 cells were confirmed in an *in vitro* model of Th17 differentiation. Furthermore, UA dose-dependently suppressed the expression of B cell-associated markers Bcl-6, Blimp1 and AID mRNAs in purified CD19⁺ B cells pretreated with IL-21 or LPS *in vitro*.

Conclusion: UA treatment significantly ameliorates CIA in mice via suppression of Th17 and differentiation. By targeting pathogenic Th17 cells and autoantibody production, UA may be useful for the treatment of autoimmune arthritis and other Th17-related diseases.

Keywords: ursolic acid; rheumatoid arthritis; collagen-induced arthritis; Th17 cell; regulatory T cell; B cell; spleen; proinflammatory cytokine; STAT3

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Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by chronic joint inflammation that can lead to destruction of the adjacent cartilage and bone. Although the exact molecular mechanisms underlying RA pathogenesis have yet to be elucidated, the current understanding defines RA as a T cell-mediated disease driven primarily by interleukin (IL)-17-producing helper T cells (Th17)^[1]. IL-17 is abundantly expressed in arthritic synovium, and it induces numerous proinflammatory cytokines, including tumour necrosis factor (TNF)- α and IL-1 β ^[2]. Th17 also contributes to antibody

production by B cells^[3] and enhances osteoclastogenesis by upregulating receptor activator nuclear kappa ligand expression^[4], resulting in bone erosion in arthritic joints. Accordingly, Th17 cells have emerged as a promising therapeutic target in RA, with inhibition of these cells sufficient to ameliorate many of the symptoms of RA.

The phosphorylation of signal transducer and activator of transcription (STAT)3 by Janus kinase (JAK) is a critical process in Th17 cell differentiation^[5]. Upon phosphorylation, STAT3 forms a dimer that is translocated to the nucleus, where it regulates the transcription of its corresponding gene, as well as that of IL-17 and retinoic orphan receptor (ROR) γ t, the primary transcription factor controlling Th17 differentiation. Modulation of the JAK-STAT signalling pathway is therefore an effective approach for regulating pathogenic

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Th17 development. Although a number of therapeutics have been approved for the treatment of RA, a substantial number of patients do not respond to these therapies, resulting in significant morbidity and joint destruction. Novel therapeutics, including those targeting Th17 development, may therefore hold promise for patients unresponsive to existing therapies. Recently, we reported the efficacy of several STAT3 inhibitors in the suppression of collagen-induced arthritis (CIA)^[6,7]. STAT3 inhibitors reduced STAT3 phosphorylation significantly in CIA mice, resulting in diminished Th17 populations, which corresponded to clinical improvements in both arthritis and joint damage.

High-throughput screening of candidate molecules that regulate Th17 activity identified ursolic acid (UA) as a compound with significant therapeutic potential. UA is pentacyclic triterpenoid that is ubiquitously expressed in most plant species^[8]. UA has been shown to exert anti-cancer^[9], anti-oxidative^[10], and anti-inflammatory^[11] effects, in addition to conferring benefits in cognitive function^[12], asthma^[13], and colon cancer^[14,15]. Of note, Pathak *et al* reported that UA inhibited activation of the STAT3 pathway, leading to the suppression of proliferation in human multiple myeloma cells^[16]. This study suggests that UA also acts as an inhibitor of STAT3 activation in T cells, resulting in the suppression of Th17 differentiation. We therefore sought to examine the effects of UA on pathogenic Th17 responses in a CIA model of arthritis.

Materials and methods

Induction of CIA and treatment with UA

Bovine Type II collagen (CII, Chondrex, WA, USA) was dissolved overnight in 0.1 mol/L acetic acid (4 mg/mL) with gentle rotation at 4°C. Eight-week-old male DBA/1J mice (Orientbio, Sungnam, Korea) were injected intradermally at the base of the tail with 100 µg of CII emulsified in complete Freund's adjuvant (Chondrex). To assess the influence of UA on symptom severity in the CIA model, mice were treated with UA (150 mg/kg) in 10% dimethyl sulfoxide or with vehicle alone by intraperitoneal injection three times a week for 4 weeks beginning 14 days after CII treatment.

Assessment of arthritis

The severity of arthritis was determined by three independent observers. The mice were examined two times a week for the onset and severity of joint inflammation for up to 8 weeks after primary immunization. The severity of arthritis was assessed on a scale of 0–4 using the following criteria, as described previously^[17]: 0=No evidence of erythema and swelling, 1=Erythema and mild swelling confined to the mid-foot (tarsals) or ankle joint, 2=Erythema and mild swelling extending from the ankle to the mid-foot, 3=Erythema and moderate swelling extending from the ankle to the metatarsal joint, and 4=Erythema and severe swelling encompass the ankle, foot, and digits. The arthritis score for each mouse was expressed as the sum of the scores for all four limbs. The highest possible arthritis score for a mouse was therefore 16. The mean arthritis index was used to compare the data among the control and

experimental groups.

Histology

Mouse joint tissues were fixed in 4% paraformaldehyde, decalcified in EDTA bone decalcifier, embedded in paraffin, and sectioned. The sections were stained with haematoxylin and eosin, safranin O, and toluidine blue to detect proteoglycans.

Immunohistochemistry

Mouse joint tissues were fixed in 10% formalin, decalcified in Calci-Clear Rapid bone decalcifier, embedded in paraffin, and sectioned^[18]. The sections were deparaffinised using xylene and dehydrated in a gradient of alcohol solutions. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide in methanol. Immunohistochemistry was performed using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA). The tissues were first incubated with primary antibodies against IL-21, IL-17A, IL-6 (Abcam, Cambridge, UK), IL-1β, TNF-α, nitrotyrosine, induced nitric oxide synthase (iNOS), and an isotype control (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. The tissues were then incubated with a biotinylated secondary antibody and streptavidin-peroxidase complex for 1 h. The final coloured product was developed using DAB chromogen (Thermo Scientific, Waltham, MA, USA). Finally, the sections were counterstained with haematoxylin and photographed using a photomicroscope (Olympus, Tokyo, Japan).

Measurement of CII-specific antibodies

Blood was drawn from the orbital sinuses of UA- and vehicle-treated mice; sera were stored at -20°C until use. Micro-titer plates were coated with CII (4 µg/mL in PBS) at 4°C overnight, followed by a blocking step for 30 min at room temperature. The serum samples were then diluted 1:10 000 in Tris-buffered saline (pH 8.0) containing 1% bovine serum albumin and 0.5% Tween-20, and incubated in the micro-titre plates for 1 h, after which the plates were washed five times. The concentrations of CII-specific IgG, IgG1, and IgG2a were measured using mouse IgG, IgG1, and IgG2a ELISA Quantitation Kits (Bethyl Laboratories, Montgomery, TX, USA), respectively. Absorbance values were determined with an ELISA microplate reader operating at 450 nm.

CD4⁺ T cell purification and stimulation

CD4⁺ T cells were purified from the spleens of DBA/1J mice using a CD4⁺ T cell MACS isolation kit with an AutoMACS separator, according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). To establish Th17 cell-polarising conditions, splenocytes were stimulated with plate-bound anti-CD3ε monoclonal antibody (0.5 µg/mL, BD Biosciences, San Jose, CA, USA), and anti-CD28 monoclonal antibody (1 µg/mL, BD), anti-IFN-γ antibody (2 µg/mL, R&Dsystems, Minneapolis, MN, USA), anti-IL-4 antibody (2 µg/mL, R&Dsystems), recombinant TGF-β (2 ng/mL, R&Dsystems), and recombinant IL-6 (20 ng/mL, R&Dsystems) for 72 h. Total RNA was extracted using TRI reagent (Molecu-

lar Research Center, Cincinnati, OH, USA) .

Cytotoxicity

Total splenocytes (2×10^5 cells/well) were seeded on 96-well flat-bottomed plate and stimulated with differential doses of UA and 1 $\mu\text{g}/\text{mL}$ of LPS for 24 h. Four hours before the termination of culture, Cell counting kit-8 (Dojundo Molecular Technologies, Rockville, MD, USA) solution was added in the culture for checking the absorbance at 450 nm using microplate reader.

Flow cytometry

For intracellular cytokine staining in mice, cells were stimulated with 25 ng/mL phorbol 12 - myristate 13 - acetate (PMA, Sigma, St Louis, MO, USA) and 250 ng/mL ionomycin (Sigma) in the presence of GolgiStop (BD) for 4 h. The following antibodies were used for intracellular staining: anti-CD4-PerCP, -CD25-APC, -IL-17A-FITC, and -FoxP3-PE antibodies (all eBioscience, San Diego, CA, USA). Events were recorded and analysed with FlowJo software (Tree Star, Ashland, OR, USA).

Confocal microscopy

Spleen tissues were snap-frozen in liquid nitrogen and stored at -70°C . Tissue sections (7 μm) were fixed in acetone and stained for the presence of Treg cells using anti-FoxP3-PE, -CD4-PerCP, and -CD25-APC antibodies (BD). To identify Th17 cells, tissue sections were stained with anti-IL-17A-FITC (eBioscience), -CD4-APC (eBioscience), and -pSTAT3-PE (Tyr705 or Ser727; BD) antibodies overnight at 4°C , and analysed using an LSM 510 Meta confocal microscopy system (Carl Zeiss, Germany). Positive cells were counted visually at a higher magnification by four individuals.

Real-time PCR

A LightCycler 480 II instrument (Roche Diagnostics, Basel, Switzerland) was used for PCR amplification and analysis. All reactions were performed with LightCycler480 SYBR Green I Master according to the manufacturer's instructions. The primer sequence was listed in the following Table 1. All mRNA expression levels were normalised to that of β -actin mRNA.

CD19⁺ B cell purification and stimulation

CD19⁺ B cells were purified from the spleens of DBA/1J mice using a B cell MACS isolation kit according to the manufacturer's instructions (Miltenyi Biotec). Cells were pre-treated with differential doses of UA for 1 h and then stimulated with lipopolysaccharide (1 $\mu\text{g}/\text{mL}$, Sigma) for an additional 96 h. Total RNA was extracted using TRI reagent (Molecular Research Center).

Statistical analysis

Statistical analyses were performed using GraphPad Prism (Version 4 for Windows; GraphPad Software, San Diego, CA, USA). When comparing pairs of groups, the Mann-Whitney

Table 1. Primer list used for this experiment.

Target		Sequence (5' to 3')
IL-17A	Forward	CCTCAAAGCTCAGCGTGTCC
	Reverse	GAGCTCACTTTTGCGCCAAG
FoxP3	Forward	GGCCCTTCTCCAGGACAGA
	Reverse	GCTGATCATGGCTGGGTTGT
ROR γ t	Forward	TGCTCTGGGCTACCCTACTG
	Reverse	GTGCAGGAGTAGCCACATT
Blimp1	Forward	CTGTCAGAACGGGATGAACA
	Reverse	TGGGGACTCTTTGGGTTAG
Bcl-6	Forward	CCCTGTGAAATCTGTGGCAC
	Reverse	ACACGCGGTATTGCACCTTG
AID	Forward	CGTGGTGAAGAGGAGAGATAGTG
	Reverse	CAGTCTGAGATGTAGCGTAGGAA
β -Actin	Forward	GTACGACCAGAGGCATACAGG
	Reverse	GATGACGATATCGCTGCGCTG

U test was used for continuous variables, while the chi-square test was used for categorical variables. $P < 0.05$ was considered statistically significant. Differences in the mean values of various groups were analysed using an ANOVA with a *post-hoc* test. $P < 0.05$ (two-tailed) was considered significant.

Results

UA suppresses CIA in mice

CIA mice were used to investigate the effects of UA on autoimmune arthritis. Mice were injected intraperitoneally with 150 mg/kg UA or vehicle control three times a week, beginning 14 d after CII treatment. UA treatment significantly reduced the incidence and severity of arthritis compared with the vehicle control (Figure 1A).

Histological examinations performed 49 d after the induction of CIA revealed a lower degree of inflammatory cell infiltration and cartilage loss in the ankles of UA-treated mice compared to those of vehicle-treated mice (Figure 1B). Consistent with reduced cartilage damage, the number of TRAP⁺ cells in the joints of the UA-treated mice was lower than that in the vehicle controls (Figure 1B). Next, we examined whether the anti-arthritic effects of UA were associated with changes in the humoral immune response. UA treatment efficiently inhibited the production of CII-specific total IgG, IgG1, and IgG2a in CIA mice (Figure 2), consistent with a role for B cells in the development of CIA.

The anti-inflammatory effects of UA were further demonstrated through immunohistochemical staining of arthritic joints, which revealed decreased expression of proinflammatory cytokines such as TNF- α , IL-1 β , IL-6, IL-21, and IL-17 (Figure 3A and 3B).

As oxidative stress is known to exacerbate arthritic inflammation, we also measured the expression of oxidative stress markers. Nitrotyrosine and iNOS were both suppressed by UA, consistent with the known anti-oxidative properties of UA (Figure 3B).

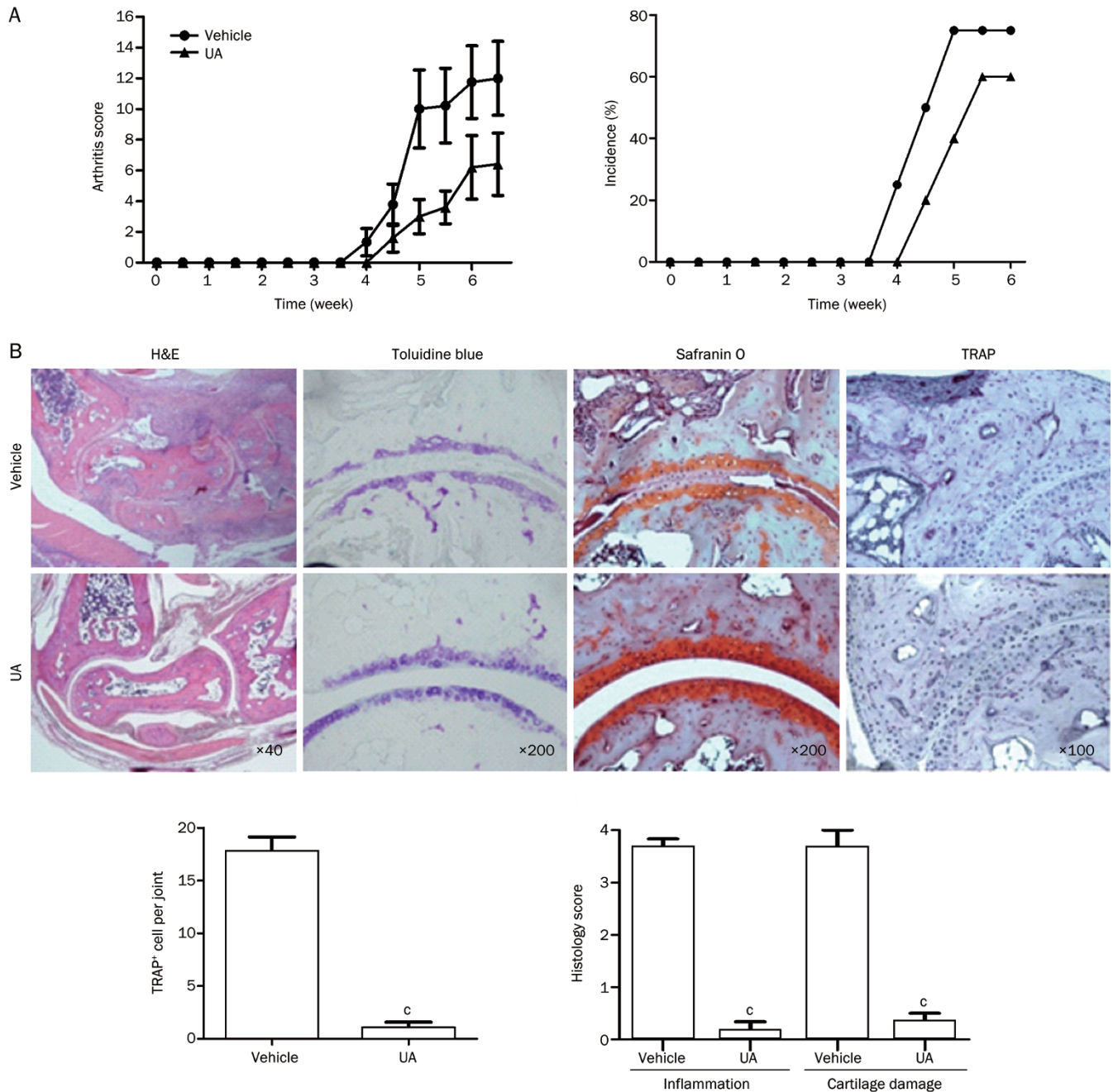


Figure 1. Ursolic acid (UA) suppresses collagen-induced arthritis (CIA). To assess the influence of UA on CIA, DBA/1J mice were treated with UA (150 mg/kg) or vehicle control three times a week for 4 weeks, beginning 14 d after type II collagen (CII) treatment ($n=5$ mice per group). (A) Arthritic scores and the incidence of arthritis in CIA-induced DBA/1J mice during the experimental period. (B) Representative histological features of the joints of CIA mice following treatment with either UA or vehicle control. Hematoxylin and eosin (H&E), safranin O, toluidine blue, and TRAP staining results are shown (top). The graph depicts the average histology score and number of TRAP⁺ cells per joint (bottom). The data are presented as the mean \pm SD of 6 joints per group. ^b $P < 0.05$, ^c $P < 0.01$ relative to the control mice.

UA influences the proportion of Th17 and Treg cells in mice with CIA

As Th17 cells play a central role in the pathogenesis of RA, we hypothesised that the reduced arthritic inflammation in UA-treated CIA mice resulted from Th17 inhibition. To address this issue, we used confocal microscopy to compare

the number of CD4⁺IL-17⁺ cells (Th17) in the spleens of either vehicle- or UA-treated CIA mice 49 d after the induction of CIA. We also examined the number of CD4⁺CD25⁺Foxp3⁺ (Treg) cells in the spleens of the mice, as these cells are known to be reciprocally regulated with Th17 cells and suppress autoimmune inflammation. UA-treated mice had significantly

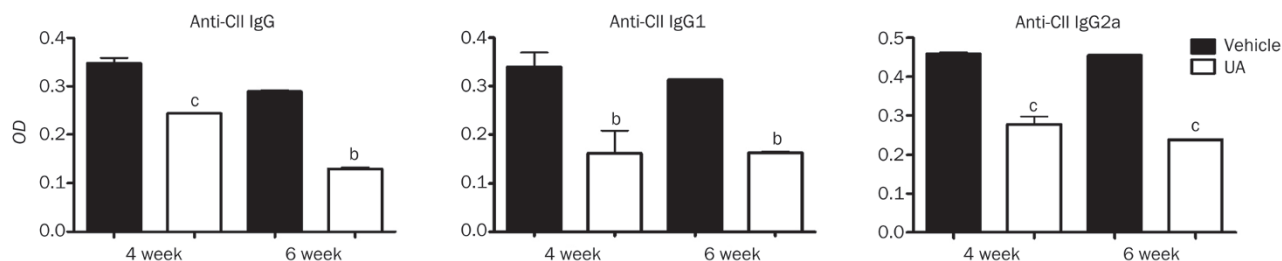


Figure 2. UA decreases the CII-specific antibody production in CIA mice. Blood was drawn from the orbital sinuses of UA- and vehicle-treated mice on d 28 and d 42 after first immunization. The concentrations of CII-specific serum IgG and IgG2a were determined by ELISA. The data are presented as the mean±SD. ^b $P<0.05$, ^c $P<0.01$.

lower numbers of Th17 cells, and a higher number of Treg cells than vehicle-treated controls (Figure 4A). The decrease in Th17 cells with UA treatment was consistently observed when the frequency of CD4⁺IL-17⁺ cell in *ex vivo* splenocytes was assessed by flowcytometry (Figure 4B). To confirm our confocal staining results, the mRNA expression of Th17-associated genes, including ROR γ t, IL-17, and IL-21, was measured in the splenocytes of CIA mice. The expression of these genes was significantly reduced in the splenocytes of UA-treated mice compared with vehicle-treated controls (Figure 4C).

A key step in Th17 differentiation is the phosphorylation of STAT3 via the JAK-STAT pathway. To investigate whether UA suppressed STAT3 phosphorylation, we counted the number of phosphorylated (p)STAT3⁺ (either at tyrosine [Y]705 or serine [S]727) CD4⁺ T cells in the spleens of UA-treated and control mice. The UA-treated mice exhibited a marked reduction in the number of pSTAT3⁺CD4⁺ T cells relative to the vehicle-treated controls (Figure 4D). Collectively, these data suggest that UA suppresses Th17 production and enhances Treg cell populations in CIA mice via the inhibition of STAT3 phosphorylation.

UA suppresses Th17 differentiation *in vitro*

Next, we assessed whether the inhibitory effects of UA on Th17 cells in the CIA model could be confirmed using an *in vitro* model of Th17 differentiation. CD4⁺ T cells were isolated from DBA/1J mice and incubated in Th17-skewing conditions (anti-IL-4 Ab, anti-IFN- γ Ab, IL-6, and TGF- β) in the presence or absence of various concentrations of UA. None of the doses used in these experiments was cytotoxic, as represented by cell viability assay using tetrazolium-based cell counting kit-8 (Dojindo) (Figure 5A). Flow cytometry revealed that UA inhibited Th17 cell differentiation in a dose-dependent manner (Figure 5B). Suppression of Th17 cell differentiation was also confirmed via the reduced mRNA expression of IL-17, IL-21, and ROR γ t (Figure 5C). Interestingly, in contrast to the enhanced Treg population observed in the CIA model, the frequency of Treg cells was not increased by UA treatment *in vitro* (data not shown). Similarly, Foxp3 expression was unaffected by UA (Figure 5C).

UA represses B cell activation and differentiation *in vitro*

UA-mediated reductions in the CII-specific antibody responses observed in the CIA model may be due to reduced numbers of Th17 cells, which function as B cell helpers. To clarify this issue, we examined the direct effects of UA on B cell activation *in vitro*. First, to confirm that UA reduces antibody production *in vitro*, total splenocytes from DBA/1J mice were stimulated with LPS and then treated with various concentrations of UA. After 4 d, antibody levels were measured in the culture supernatant. UA significantly reduced IgG, IgG1, and IgG2a production, consistent with what had been seen *in vivo* (Figure 6A). Next, to investigate the direct effects of UA on B cells, CD19⁺ B cells were isolated from DBA/1J splenocytes and pre-treated with various concentrations of UA. These cells were then cultured in the presence or absence of LPS or IL-21, and the expression of B cell-associated markers were measured by real time RT-PCR. No cytotoxicity was observed at any of the doses used in these experiments (data not shown). The mRNA expression of B cell lymphoma (Bcl)-6, Blimp1, and activation-induced cytidine deaminase (AID) was diminished following UA treatment (Figure 6B). These data suggest that UA suppresses B cell activation not only through its effects on Th17 cells, but also by directly inhibiting B cell activation and differentiation.

Discussion

In this study, we demonstrated that UA suppresses STAT3 phosphorylation and Th17 differentiation while enhancing Treg differentiation. UA also repressed B cell activation and differentiation, resulting in decreased antibody production. Together, these results provide a clear mechanistic basis by which UA decreases both the incidence and severity of CIA.

UA confers its anti-inflammatory activities through a number of different mechanisms, including inhibiting the production of proinflammatory cytokines such as IL-2, IFN- γ , and TNF- α ^[19], prostaglandin E2^[20], and cyclooxygenase 2. Earlier studies suggested that the anti-inflammatory properties of UA were mediated by the suppression of nuclear factor (NF)- κ B, a major transcription factor that regulates the expression of multiple proinflammatory cytokines^[12,21].

The effects of UA on immune cells have also been add-

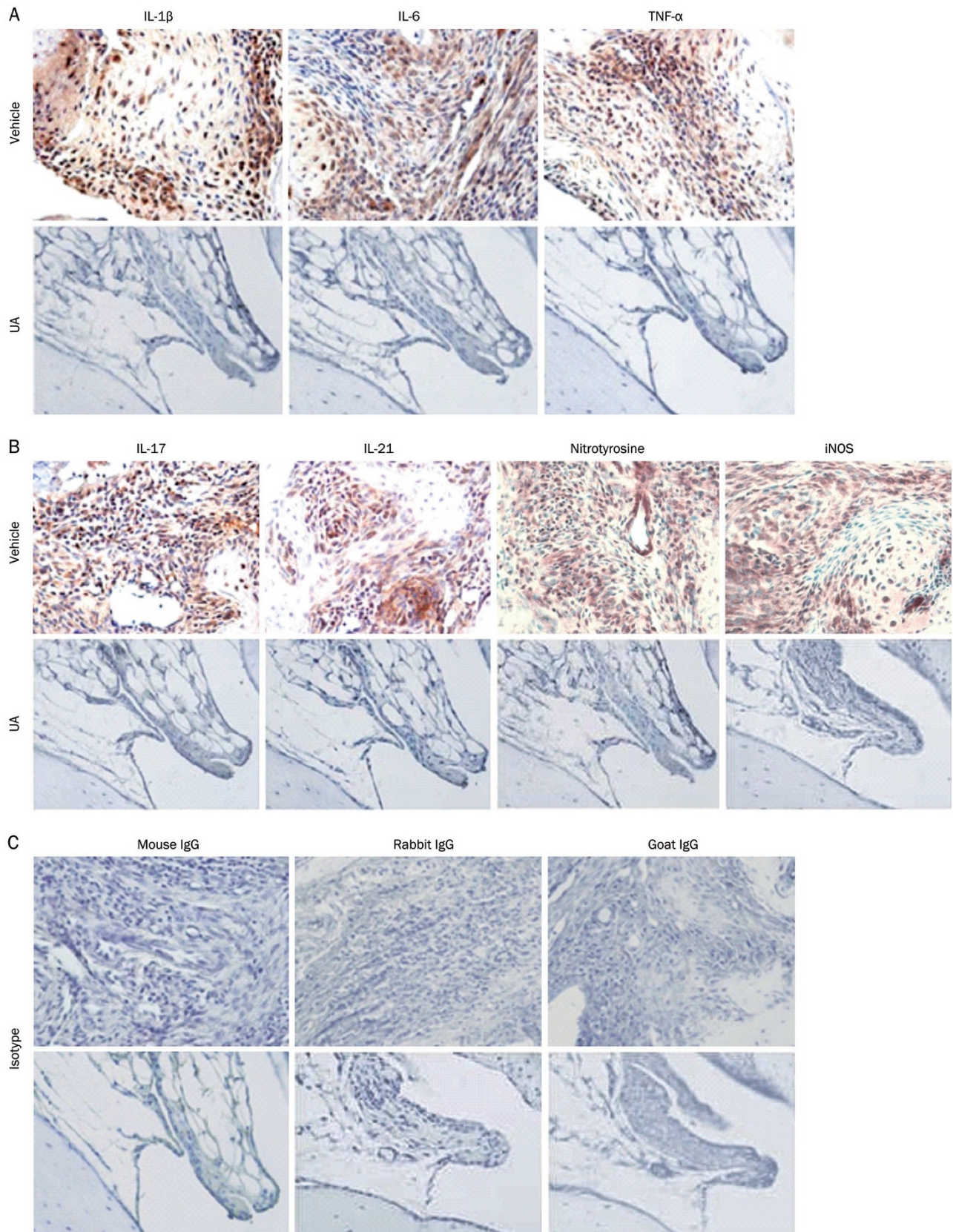


Figure 3. UA decreases the expression of proinflammatory cytokines and oxidative stress-related genes in the joints of CIA mice. Ankle joints (6 per group) of CIA mice treated with either UA or vehicle control were immunostained for (A) IL-1 β , IL-6, TNF- α , (B) IL-17, IL-21, nitrotyrosine, and iNOS, or (C) isotype control. Representative data are shown.

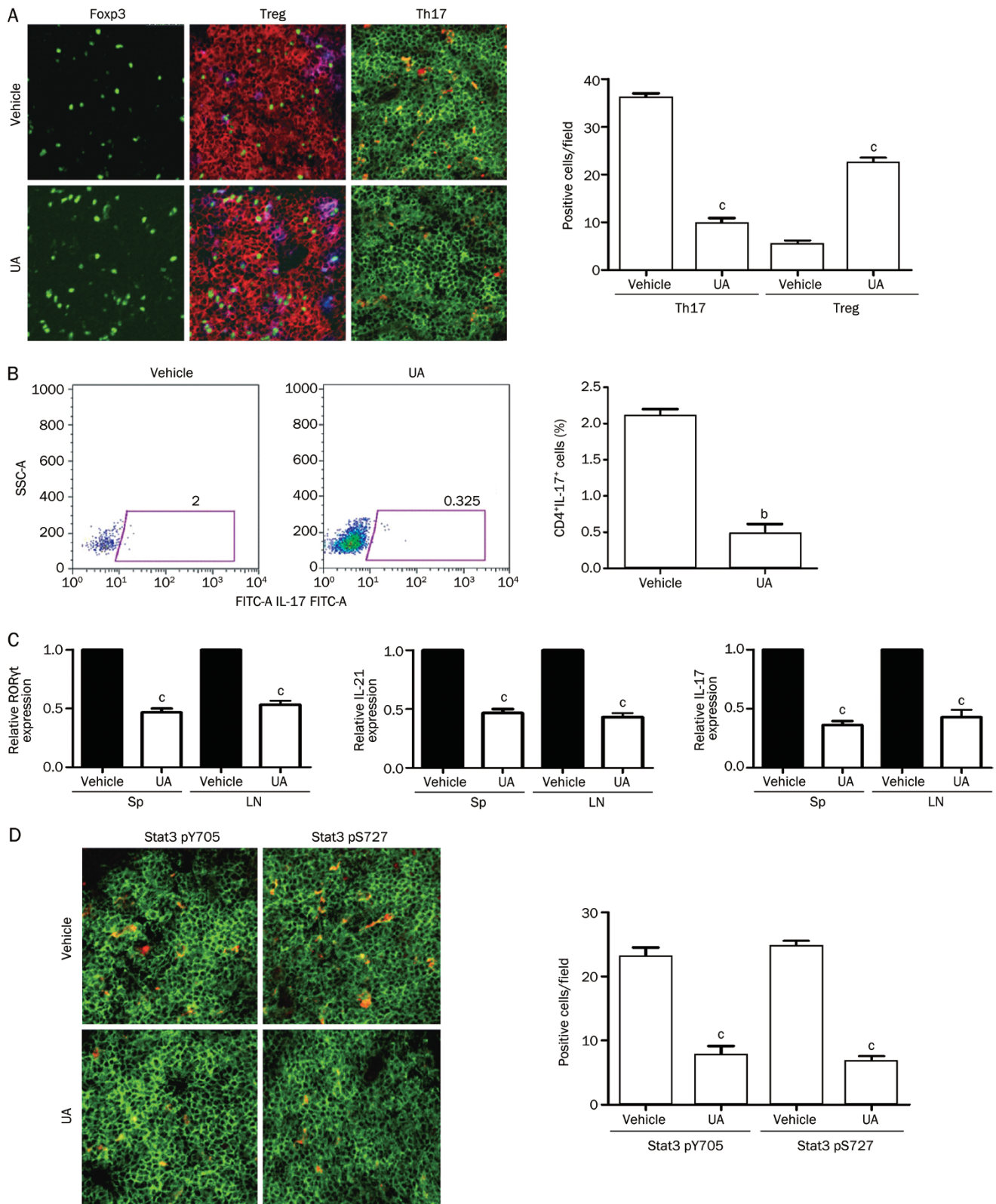


Figure 4. UA reduces STAT3 phosphorylation and decreases the frequency of Th17 cells within the population of CD4⁺ T cells in CIA mice. (A) Spleens of UA- or vehicle-treated CIA mice were subjected to immunostaining for CD4⁺IL-17⁺, CD4⁺CD25⁺, and CD4⁺Foxp3⁺. (B) The frequency CD4⁺IL-17⁺ cells among ex vivo total splenocytes was assessed using flow cytometry. (C) The mRNA expression of IL-17, IL-21, and RORyt was determined by real-time RT-PCR in cells obtained from the spleen (Sp) or draining lymph nodes (LN). (D) Spleens of UA- or vehicle-treated CIA mice were subjected to immunostaining of CD4⁺pSTAT3 Y705⁺ or CD4⁺pSTAT3 S727⁺ cells. The data are presented as the mean±SD of 3 animals per group. ^bP<0.05, ^cP<0.01.

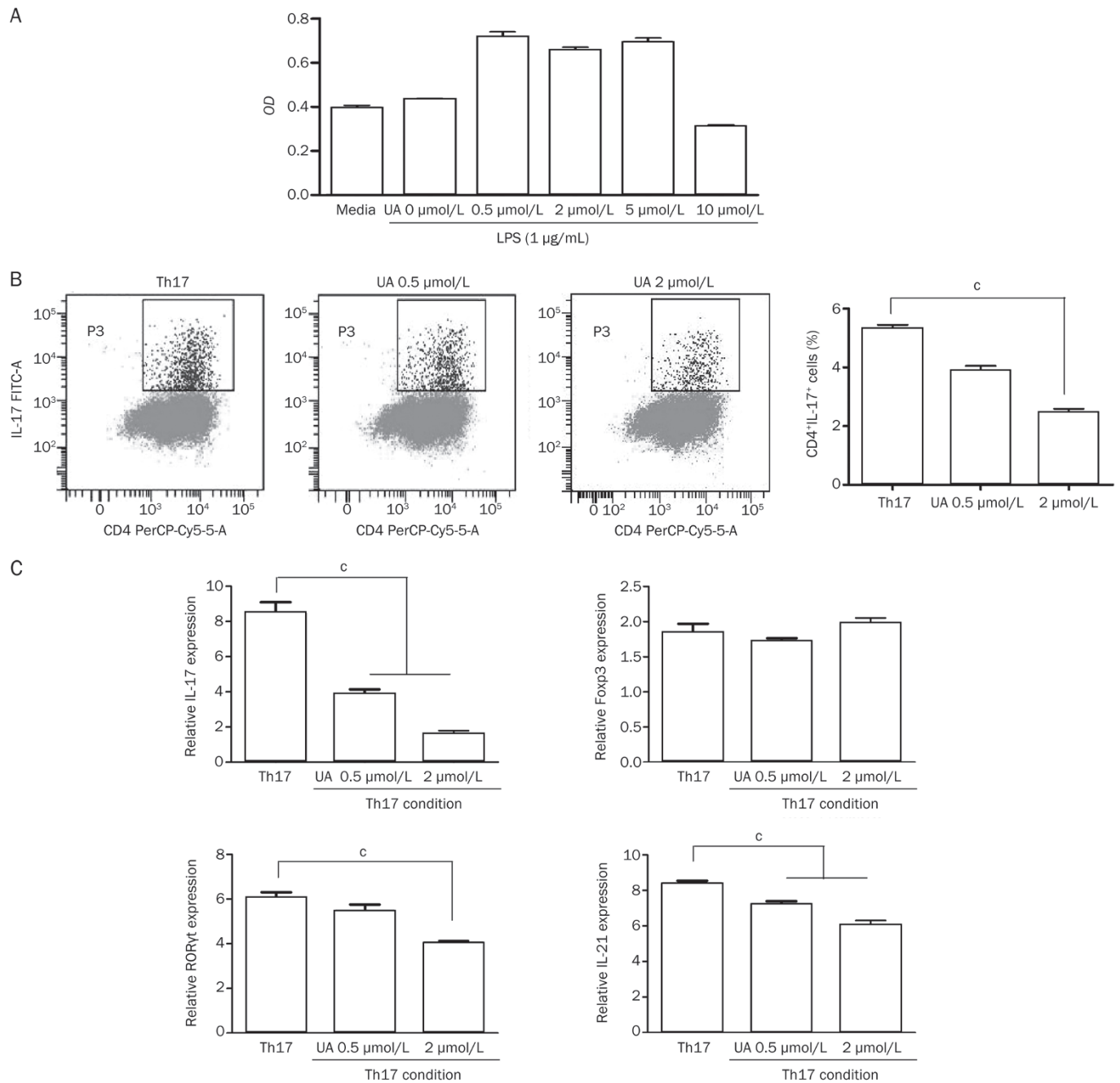


Figure 5. UA inhibits Th17 differentiation *in vitro*. CD4⁺ T cells were isolated from the spleens of DBA/1J mice and incubated for 3 d under Th17-polarizing conditions (0.5 μg/mL anti-CD3 mAb, 1 μg/mL anti-CD28 mAb, 2 μg/mL anti-IL-4 Ab, 2 μg/mL anti-IFN-γ Ab, 20 ng/mL IL-6, and 2 ng/mL TGF-β) in the presence or absence of UA. (A) UA doses ≤10 μmol/L did not affect cell viability. (B) The frequency of CD4⁺IL-17⁺ T cells was measured by flow cytometry. (C) The expression of IL-17, IL-21, RORγt, and Foxp3 was analysed by real-time PCR. The data are presented as the mean±SEM of three independent experiments. ^b*P*<0.05, ^c*P*<0.01 relative to the control mice.

ressed. The clear association between UA and NF-κB signaling strongly suggested a role for T cell activation. Indeed, Zeng *et al* demonstrated a dose-dependent decrease in T cell proliferation following UA treatment, as well as reduced IL-2 production^[22]. Similarly, UA was suggested to be involved in the suppression of Th1 cytokines (*eg*, IL-2 and IFN-γ) in combination with increased Th2 cytokines (*eg*, IL-4, IL-5, and

IL-10)^[23]; however, the mechanism underlying these effects was not clearly shown.

Recently, Xu *et al* reported that UA suppressed IL-17 production by selectively antagonizing the function of RORγt^[24]. They demonstrated that UA inhibited the binding of RORγt to its coactivator protein, thereby suppressing its transcriptional activity, even though the expression of RORγt was unaffected.

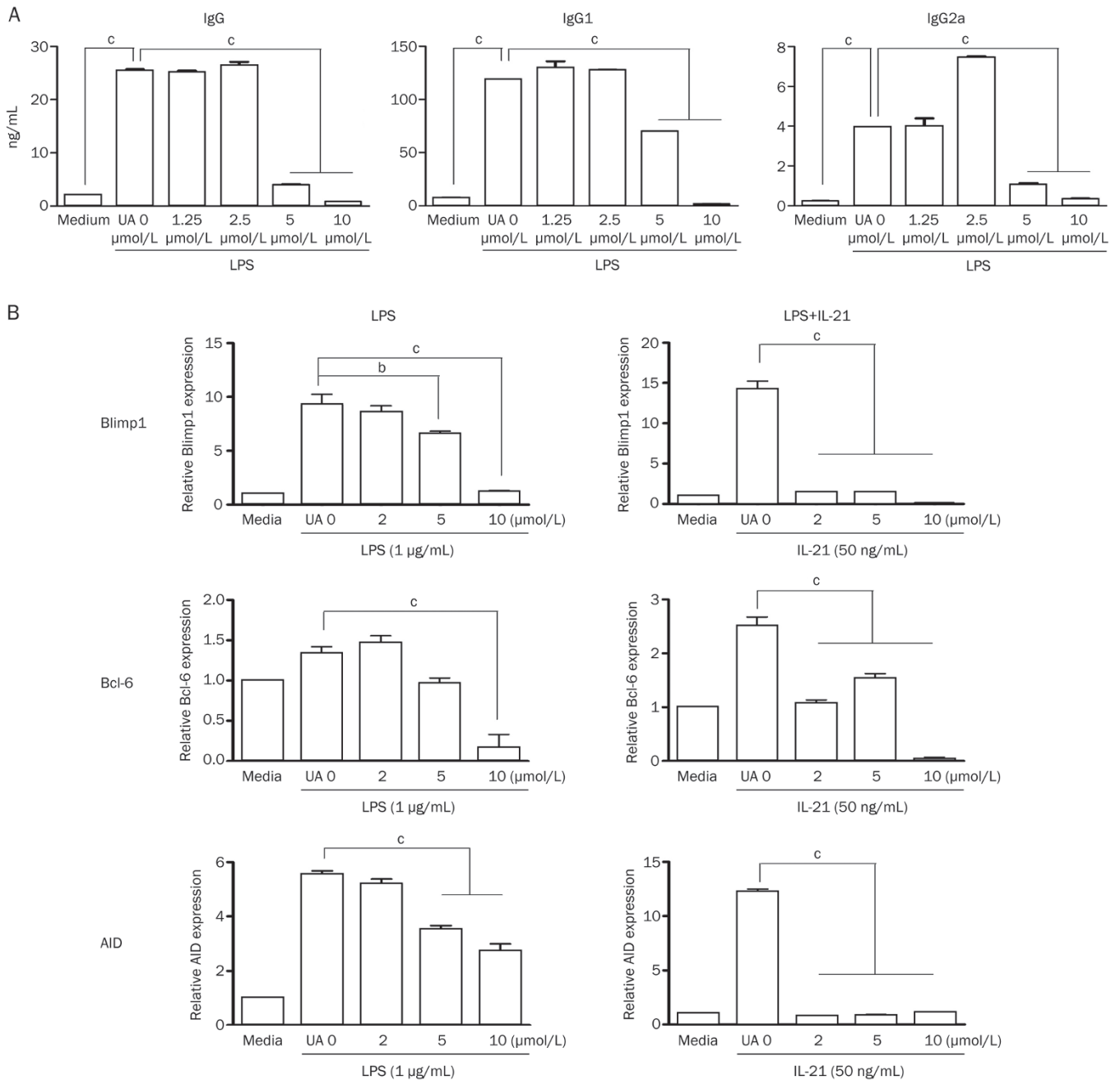


Figure 6. UA negatively affects B cell function *in vitro*. (A) Total splenocytes were obtained from DBA/1J mice and stimulated with 1 µg/mL LPS and incubated with various concentrations of UA. After 4 d, the levels of total IgG, IgG1, and IgG2a in the supernatant were determined by ELISAs. (B) CD19⁺ B cells were isolated from the spleens of DBA/1J mice and pre-treated with either 1 µg/mL LPS or 50 ng/mL IL-21, and then cultured with various concentrations of UA. After 4 d, the mRNA expression of Blimp1, Bcl-6, and AID was analysed by real-time RT-PCR. ^b*P*<0.05, ^c*P*<0.01 relative to the controls.

They also argued that UA did not affect STAT3 phosphorylation in Th17 cells. In contrast, our data clearly show a reduction in RORγt mRNA expression and the number of pSTAT3-positive cells following UA treatment. This discrepancy may have resulted from the slightly different cytokine cocktails used to induce Th17 differentiation or from differences in the cell types used in these experiments. Indeed, UA has been shown to inhibit STAT3 activation in human multi-

ple myeloma^[16] and colon cancer cells^[25]. Even though these results were obtained using cancer cell lines, these findings are consistent with our results showing that UA inhibits STAT3 activation.

We observed relative increases in the Treg populations in UA-treated CIA mice *in vivo*; however, this was not the case in our *in vitro* experiments. Considering that Foxp3 expression was not affected by UA treatment, it is unlikely that UA

increased Treg differentiation directly. When differentiation into Th17 cells is suppressed under inflammatory conditions, T cells may instead be converted into Treg cells, especially considering the plasticity of Th17 and Treg cells^[26].

Although RA is largely considered to be a T cell-mediated disease, the humoral immune response, as represented by autoantibody production, plays another key role in the pathogenesis. This prompted us to investigate the direct effects of UA on B cells. LPS- or IL-21-stimulated antibody production was markedly suppressed following UA treatment. We hypothesised that this was due to reduced plasma cell differentiation and isotype switching. Indeed, the expression of Blimp1, a master transcription factor that drives plasma cell differentiation, and AID, an essential enzyme in isotype switching, was markedly decreased following UA treatment. Interestingly, UA also suppressed Bcl-6 expression in these cells, a somewhat contradictory result given that Bcl-6 is known to be repressed during plasma cell differentiation. However, B cells express both Bcl-6 and Blimp1 in response to IL-21 stimulation and differentiate either into germinal centre B cells or plasma cells, respectively, in a context-dependent manner^[27]. Therefore, it can be concluded that UA generally inhibits B cell activation and differentiation mediated by IL-21 or LPS.

The inhibitory effects of UA on B cells appear to be mediated by the suppression of pSTAT3, similar to the effects seen in T cells. STAT3 activation was recently shown to play an important role in the differentiation of B and Th17 cells^[28], particularly IL-21-mediated plasma cell differentiation, which is dependent upon pSTAT3^[29]. These findings, together with our data showing pSTAT3 suppression by UA, suggest that the reduced expression of B cell differentiation markers resulted from the inhibition of STAT3 phosphorylation. Further investigation will be necessary to clarify whether UA is able to directly inhibit STAT3 phosphorylation in B cells.

From the results presented here, it appears that reduced CII-specific IgG production *in vivo* occurs as a result of both the direct inhibition of B cells and Th17-mediated B cell activation. Indeed, Th17 cells, which are decreased by UA treatment, are known to play a role in antibody production^[3]. These results are consistent with other known functions of STAT3, including the generation of follicular helper T cells^[30] and development of Th2-mediated B cells^[31]. Taken together, it is clear that UA ameliorates autoimmune arthritis by targeting multiple processes associated with STAT3 activation.

Conclusion

UA inhibits Th17 cell proliferation and promotes Treg cell differentiation by inhibiting the expression of ROR γ t and pSTAT3. UA also inhibits B cell activation and plasma cell differentiation, leading to a reduction in serum antibodies. By targeting pathogenic Th17 cell proliferation and autoantibody production, UA has potential as a novel therapeutic for the treatment of autoimmune arthritis. The consumption of UA-rich foods may help prevent autoimmune arthritis and other Th17-related diseases.

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Author contribution

Seung-ye BAEK, Jaeseon LEE, Dong-gun LEE, Mi-kyung PARK, Jennifer LEE, Seung-ki KWOK, and Sung-hwan PARK contributed to conception and design, or acquisition of data, or analysis and interpretation of data; Seung-ye BAEK, Jaeseon LEE, Jennifer LEE, Seung-ki KWOK, Mi-la CHO, and Sung-hwan PARK were involved in drafting the article or revising it critically for important intellectual content; Seung-ye BAEK, Jaeseon LEE, Jennifer LEE, Seung-ki KWOK, and Sung-hwan PARK approved the final version to be published.

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