

## Original Article

# Betulinic acid regulates generation of neuroinflammatory mediators responsible for tissue destruction in multiple sclerosis *in vitro*

Jana BLAŽEVSKI<sup>1, #</sup>, Filip PETKOVIĆ<sup>1, #</sup>, Miljana MOMČILOVIĆ<sup>1</sup>, Reinhard PASCHKE<sup>2</sup>, Goran N KALUĐEROVIĆ<sup>2, 3</sup>, Marija MOSTARICA STOJKOVIĆ<sup>4</sup>, Djordje MILJKOVIĆ<sup>1, \*</sup>

<sup>1</sup>Department of Immunology, Institute for Biological Research “Siniša Stanković”, University of Belgrade, Serbia; <sup>2</sup>Biozentrum, Martin-Luther-Universität Halle-Wittenberg, Weinbergweg 22, 06120 Halle (Saale), Germany; <sup>3</sup>Institut für Chemie, Martin-Luther-Universität Halle-Wittenberg, Kurt-Mothes Str 2, 06120 Halle (Saale), Germany; <sup>4</sup>Institute for Microbiology and Immunology, School of Medicine, University of Belgrade, Serbia

**Aim:** To investigate the influences of betulinic acid (BA), a triterpenoid isolated from birch bark, on neuroinflammatory mediators involved in the pathogenesis of multiple sclerosis and experimental autoimmune encephalomyelitis *in vitro*.

**Methods:** Encephalitogenic T cells were prepared from draining lymph nodes and spinal cords of Dark Agouti rats 8 to 10 d after immunization with myelin basic protein (MBP) and complete Freund’s adjuvant. Macrophages were isolated from the peritoneal cavity of adult untreated rats. Astrocytes were isolated from neonatal rat brains. The cells were cultured and then treated with different agents. IFN- $\gamma$ , IL-17, iNOS and CXCL12 mRNA levels in the cells were analyzed with RT-PCR. iNOS and CXCL12 protein levels were detected using immunoblot. NO and ROS generation was measured using Griess reaction and flow cytometry, respectively.

**Results:** In encephalitogenic T cells stimulated with MBP (10  $\mu\text{g}/\text{mL}$ ), addition of BA inhibited IL-17 and IFN- $\gamma$  production in a dose-dependent manner. The estimated  $\text{IC}_{50}$  values for IL-17 and IFN  $\gamma$  were 11.2 and 63.8  $\mu\text{mol}/\text{L}$ , respectively. When the macrophages were stimulated with LPS (10 ng/mL), addition of BA (50  $\mu\text{mol}/\text{L}$ ) significantly increased ROS generation, and suppressed NO generation. The astrocytes were stimulated with ConASn containing numerous inflammatory mediators, which mimicked the inflammatory milieu within CNS; addition of BA (50  $\mu\text{mol}/\text{L}$ ) significantly increased ROS generation, and blocked ConASn-induced increases in iNOS and CXCL12 mRNA levels, but did not affect iNOS and CXCL12 protein levels. Importantly, in both the macrophages and astrocytes, addition of BA (50  $\mu\text{mol}/\text{L}$ ) inhibited lipid peroxidation.

**Conclusion:** Besides inhibiting encephalitogenic T cell cytokines and reducing NO generation, BA induces tissue-damaging ROS generation within CNS.

**Keywords:** betulinic acid; experimental autoimmune encephalomyelitis; multiple sclerosis; neuroinflammation; T cell; macrophage; astrocyte; cytokines; NO; ROS; CXCL12

Acta Pharmacologica Sinica (2013) 34: 424–431; doi: 10.1038/aps.2012.181; published online 4 Feb 2013

## Introduction

Multiple sclerosis (MS) is a chronic inflammatory, demyelinating and degenerative disease of the CNS which affects more than two million people worldwide and represents a leading cause of non-traumatic neurologic disability in young adults. The autoimmune nature of MS pathology is substantiated by evidence obtained in its laboratory analogue,

experimental autoimmune encephalomyelitis (EAE)<sup>[1]</sup>. The widely accepted mechanism of EAE and MS pathogenesis suggests that encephalitogenic proteins or cross-reactive antigens in the periphery prime autoreactive  $\text{CD4}^+$  T cells (T helper, Th), which differentiate into effector Th1 and Th17 cells specific for myelin antigens and then migrate to the CNS<sup>[2]</sup>. On reactivation by target antigen in the CNS, Th1 and Th17 cells induce inflammation through the production of various mediators, including their signature cytokines, IFN- $\gamma$  and IL-17, respectively<sup>[2]</sup>. Both resident (astrocytes, microglia) and hematogenous (macrophages) cells respond to Th cytokines, further generating inflammatory mediators, such

<sup>#</sup> The two authors contributed equally to this work.

<sup>\*</sup> To whom correspondence should be addressed.

E-mail georgije\_zw@yahoo.com

Received 2012-07-03 Accepted 2012-12-05

as nitric oxide (NO) and reactive oxygen species (ROS)<sup>[3-5]</sup>. Reactive oxygen and nitrogen species largely contribute to tissue destruction in MS and EAE<sup>[5-7]</sup>. NO also restrains the encephalitogenic T cell response<sup>[8]</sup>. Astrocytes are intensively involved in limiting the encephalitogenic immune response<sup>[9]</sup>. For instance, they express CXCL12, which has been attributed an anti-inflammatory role in neuroinflammation<sup>[10]</sup>.

Although several drugs approved for therapy of MS patients have demonstrated respectable efficacy and safety, MS is still an incurable disease. These drugs alter the natural course of MS but their long-term impact on its progression is uncertain and different adverse effects of such therapies have been described<sup>[11]</sup>. Therefore, additional treatments that may curtail MS attacks or improve the neurological functions of the patients, including complementary therapy, have to be explored. Natural products are the organic molecules that are produced by living organisms. They have been used as traditional remedies or supplementary diet constituents from ancient times. Also, numerous natural products have become conventional therapeutics, *eg* penicillin and taxol. Betulinic acid [3b, hydroxy-lup-20(29)-en-28-oic acid], BA is a naturally occurring pentacyclic triterpene that exhibits a variety of biological activities, including antimicrobial, anti-inflammatory, anticancer and antioxidant properties<sup>[12, 13]</sup>. BA is widely distributed in the plant kingdom, *eg* substantial amounts of BA are present in the outer bark of birch trees. White birch bark has been used by Native Americans as a folk medicine, while the reduced BA betulin (3b-lup-20(29)-en-3, 28-diol), was one of the first natural products to be identified and isolated from plants in 1788.

The influence of BA on neuroinflammation has not been investigated so far. Here, we analyzed its influence on key mechanisms in neuroinflammation, including IFN- $\gamma$  and IL-17 release from T cells, NO and ROS generation in macrophages and astrocytes and CXCL12 expression in astrocytes. A profound effect of BA on the examined parameters was observed, thus suggesting that BA could have a potent effect on neuroinflammation.

## Materials and methods

### Reagents

RPMI-1640 medium and fetal calf serum (FCS) were from PAA Laboratories (Pasching, Austria). DMSO was from Sigma-Aldrich (St Louis, MO). BA was a kind gift from BioSolutions Halle GmbH.

### Cells and cell cultures

Dark Agouti rats were obtained from the Animal House Facility of the Institute for Biological Research "Siniša Stanković", Belgrade, Serbia. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the institute (App No 02.1/09-01-348). Cells were maintained in HEPES-buffered RPMI-1640 medium with 5% FCS, 2 mmol/L L-glutamine, antibiotics, and sodium pyruvate (culture medium). Spleen cells (SPC) and peritoneal cells (PC) were isolated from healthy, untreated rats. Draining lymph node cells (DLNC) were obtained from popliteal lymph nodes at 8-10 d and inflammatory cells were obtained from the spinal cord (SC) at 12-14 d after immunizing the rats with myelin basic protein (guinea pig MBP, kind gift from Professor Alexander Fluegel, University of Goettingen, Germany) and complete Freund's adjuvant (CFA, Difco, Detroit, MI). Lymph nodes and spleen were mechanically disrupted, passed through a 40- $\mu$ m nylon mesh filter and the resulting suspension was collected by centrifugation. Red blood cells from the single cell suspensions obtained from the spleens were lysed using RBC Lysis Buffer (eBioscience, San Diego, CA). PC were obtained by lavage of the peritoneal cavity with ice-cold PBS. Rats were perfused with sterile PBS before SC extraction. SC was homogenized, adjusted to 40% Percoll (Sigma-Aldrich) and overlaid on a 70% Percoll gradient. Following centrifugation at 870 $\times$ g for 50 min the SC cells (SCC) were recovered from the 40%/70% Percoll interface and washed in the culture medium. Astrocytes were isolated from mixed glial cell (MGC) cultures prepared from the brains of newborn rats as previously described<sup>[14]</sup>. Astrocytes were grown in the culture medium supplemented with 4 g/L glucose and they were

**Table 1.** Cell culture conditions.

Cells	Number/well	V/well (mL)	Plate	Stimulation	Read-out	Incubation time
SPC	5.0 $\times$ 10 <sup>6</sup>	1	24-well	ConA 2.5 $\mu$ g/mL	MTT assay, Griess reaction	24 h
DLNC	2.5 $\times$ 10 <sup>6</sup>	1	24-well	MBP 10 ng/mL	AP assay, ELISA, RT-PCR	24 h
PC	2.0 $\times$ 10 <sup>6</sup>	1	24-well	LPS 10 ng/mL	CV assay, Griess reaction	24 h
PC	2.0 $\times$ 10 <sup>6</sup>	1	24-well	LPS 10 ng/mL	Cytofluorimetry	30 min DHR+1 h
PC	4.0 $\times$ 10 <sup>6</sup>	1.5	24-well	LPS 10 ng/mL	TBARS assay	24 h
MGC	1.5 $\times$ 10 <sup>5</sup>	1	24-well	ConASn 20%	Cytofluorimetry	30 min DHR+1 h
Astrocytes	1.5 $\times$ 10 <sup>5</sup>	1	24-well	ConASn 20%	Cytofluorimetry	30 min DHR+1 h
Astrocytes	0.7 $\times$ 10 <sup>6</sup>	1.5	6-well	ConASn 20%	TBARS assay	24 h
Astrocytes	1.5 $\times$ 10 <sup>5</sup>	1	24-well	ConASn 20%	RT-PCR, immunoblot	24 h
C6 cells	1.5 $\times$ 10 <sup>5</sup>	1	24-well	ConASn 20%	Cytofluorimetry	30 min DHR+1 h
C6 cells	1.5 $\times$ 10 <sup>5</sup>	1	24-well	ConASn 20%	Immunoblot	24 h
C6 cells	1.0 $\times$ 10 <sup>6</sup>	1.5	6-well	ConASn 20%	TBARS assay	24 h
SCC	0.5 $\times$ 10 <sup>6</sup>	0.2	96-well	No	MTT assay, ELISA	24 h

purified by repetition of trypsinization (0.25% trypsin and 0.02% EDTA) and re-plating. The cells used in these experiments were obtained after a third or fourth passage. The rat C6 astrocytoma cell line (kindly donated by Dr P Tranque, Universidad de Castilla-La Mancha, Albacete, Spain) was maintained in the culture medium until it was used for the experiments. Details of the cell culturing conditions are given in Table 1. PC were left at 37°C for 2 h and then non-adherent cells were removed by intensive washing of the wells with PBS, thus leaving adherent cells (macrophages) in the cultures. ConA and LPS (E coli 055:B5) were from Sigma-Aldrich (St Louis, MO). ConASn was a supernatant obtained from cultures of SPC stimulated with ConA (2.5 µg/mL) for 48 h.

#### Cell viability assays

DLNC viability was determined using an acid phosphatase (AP) assay. The AP substrate contained 11 mg/mL *p*-nitrophenylphosphate, 4 µL/mL Triton-X and 0.3% sodium acetate. 100 µL of the substrate was added to 200 µL of the cell culture supernatants, directly into the cell cultures, and the cultures were incubated at 37°C for 2 h. The AP reaction was stopped with 100 µL of 1.3 mol/L NaOH. In order to assess the viability of the SPC and SCC we used the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) to formazan. At the end of the appropriate treatments, the cells were collected in tubes, centrifuged, supernatants removed and the cell pellets was dissolved in a 0.5 µg/mL MTT (Sigma-Aldrich) solution. Incubation with MTT lasted for 30 min at 37°C and cells were centrifuged once more. DMSO was added to the pellets to dissolve the formazan crystals. In order to determine the viability of the PC, crystal violet (CV) staining was performed. Cell culture supernatants were collected and the cells were fixed with methanol (10 min at room temperature). Subsequently, cells were stained with CV solution (2% in PBS *v/v*, 10 min, room temperature) and then thoroughly washed with tap water. Finally, the crystal violet dye remaining in the cells was dissolved in 33% acetic acid. Absorbance in the AP assay was measured at 405 nm and in the MTT assay and CV assay it was measured at 570 nm, with a correction at 690 nm, using an automated microplate reader (LKB 5060-006, LKB, Vienna, Austria).

#### ELISA

Cytokine concentration in the cell culture supernatants was determined by sandwich ELISA using MaxiSorp plates (Nunc, Roskilde, Denmark) and anti-cytokine paired antibodies according to the manufacturer's instructions. Samples were analyzed in duplicate for murine/rat IL-17 (eBioscience) and rat IFN-γ (R&D, Minneapolis, MN, USA). The results were calculated using standard curves made on the basis of known concentrations of the appropriate recombinant cytokines.

#### Immunoblot

Whole-cell lysates were prepared in a solution containing 62.5

mmol/L Tris-HCl (pH 6.8), 2% *w/v* sodium dodecyl sulfate (SDS), 10% glycerol, 50 mmol/L dithiothreitol (DTT), 0.01% *w/v* bromophenol blue, 1 mmol/L phenylmethanesulphonyl-fluoride or phenylmethylsulphonyl fluoride (PMSF), 1 µg/mL aprotinin, and 2 mmol/L EDTA. Samples containing 20 µg of proteins (measured by Lowry protein assay) were electrophoresed on a 12% SDS-polyacrylamide gel for iNOS detection or on a 15% gel for CXCL12 detection. The samples were electro-transferred to polyvinylidene difluoride membranes at 5 mA/cm<sup>2</sup>, using a semi-dry blotting system (Fastblot B43, Biorad, Muenchen, Germany). The blots were blocked with 5% *w/v* nonfat dry milk in PBS containing 0.1% Tween-20 and probed with specific antibodies to iNOS (Sigma-Aldrich, USA), to CXCL12 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and to β-actin (Abcam, Cambridge, England, UK), followed by incubation with secondary antibody at a 1:10000 dilution [ECL donkey anti-rabbit horseradish peroxidase (HRP)-linked, GE Healthcare, Buckinghamshire, England, UK]. Detection was performed using chemiluminescence (ECL, GE Healthcare) and photographs were made by X-ray films (Kodak, Rochester, NY). Densitometry was performed with Scion Image Alpha 4.0.3.2 (Scion Corporation, Frederick, MD). Results are presented relative to β-actin.

#### Measurement of NO release and ROS detection

Nitrite accumulation, as an indirect measure of NO release, was determined in cell culture supernatants using the Griess reaction. In brief, triplicate aliquots of cell-free supernatants were mixed with an equal volume of Griess reagent, of a 1:1 mixture of 0.1% naphthylethylenediamine dihydrochloride (Sigma-Aldrich) and of 1% sulphanilamide (Sigma-Aldrich) in 5% H<sub>3</sub>PO<sub>4</sub>. The absorbance at 570 nm was determined in a microplate reader (LKB 5060-006; LKB) and compared to a standard curve for NaNO<sub>2</sub>. ROS generation was measured by flow cytometry. Astrocytes, MGC, C6 cells and PC were incubated in the presence of 1 µmol/L dihydrorhodamine 123 (DHR, Sigma-Aldrich, USA) for 30 min prior to treatment and then treated for 1 h. Subsequently, the cells were washed twice in PBS and green fluorescence was analyzed with a FAC-SCalibur flow cytometer (BD, Heidelberg, Germany) using the Cell Quest Pro software (BD).

#### Determination of lipid peroxidation (TBARS assay)

Lipid peroxidation products were quantified by measuring thiobarbituric acid reactive substances (TBARS)<sup>[15]</sup>. One mL of 20% (*w/v*) trichloroacetic acid containing 0.8% (*w/v*) thiobarbituric acid was added to each culture dish. The cells were scratched off with a cell scraper, the suspensions were transferred to glass centrifuge tubes and incubated at 95°C for 45 min. After cooling to room temperature and centrifugation the absorbance of the supernatant at 535 nm was determined. In parallel, known concentrations of malondialdehyde (MDA) were used for standard curve construction. Also, parallel cultures of cells were used to determine cell viability by CV staining. Results are presented as relative values (µmol/L of MDA normalized to absorbance values determined in the CV test).

### Reverse transcription-real time polymerase chain reaction

In order to determine gene expression real time RT-PCR was performed. First, total RNA was isolated from DLNC ( $5 \times 10^6$  cells) or astrocytes ( $1.5 \times 10^5$ ) using an mi-Total RNA Isolation Kit (Metabion, Martinsried, Germany) and reverse transcribed using random hexamer primers and MMLV (Moloney Murine Leukemia Virus) reverse transcriptase, according to the manufacturer's instructions (Fermentas, Vilnius, Lithuania). Prepared cDNAs were amplified using a Maxima<sup>®</sup> SYBR Green qPCR Master Mix (Fermentas) according to the recommendations of the manufacturer in a total volume of 20  $\mu$ L in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Thermocycler conditions comprised an initial step at 50°C for 5 min, followed by a step at 95°C for 10 min and a subsequent 2-step PCR program at 95°C for 15 s and 60°C for 60 s for 40 cycles. The PCR primers (Metabion) were as follows:  $\beta$ -actin forward primer 5'-GCT TCT TTG CAG CTC CTT CGT-3';  $\beta$ -actin reverse primer 5'-CCA GCG CAG CGA TAT CG-3'; IFN- $\gamma$  forward primer 5'-TGG CAT AGA TGT GGA AGA AAA GAG-3'; IFN- $\gamma$ - reverse primer 5'-TGC AGG ATT TTC ATG TCA CCA T-3'; IL-17 forward primer 5'-ATC AGG ACG CGC AAA CAT G-3'; IL-17 reverse primer 5'-TGA TCG CTG CTG CCT TCA C-3'; CXCL12 forward primer 5'-GAT TCT TTG AGA GCC ATG TC-3'; CXCL12 reverse primer 5'-GTC TGT TGT TGC TTT TCA GC-3'; iNOS forward primer 5'-TTC CCA TCG CTC CGC TG-3'; iNOS reverse primer 5'-CCG GAG CTG TAG CAC TGC A-3'. The accumulation of PCR products was detected in real time and the results were analyzed using the 7500 System Software (Applied Biosystems) and presented as  $2^{-\Delta Ct}$ ,  $\Delta Ct$  being the difference between Ct values of specific genes and the endogenous control ( $\beta$ -actin).

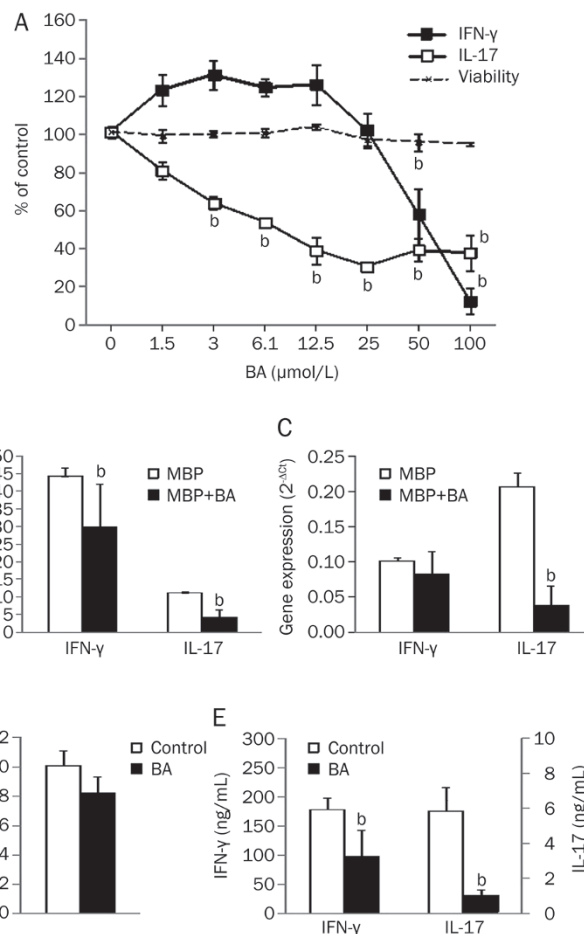
### Statistical analysis

A Student's *t* test was performed for statistical analysis. A *P* value less than 0.05 was considered statistically significant.

## Results

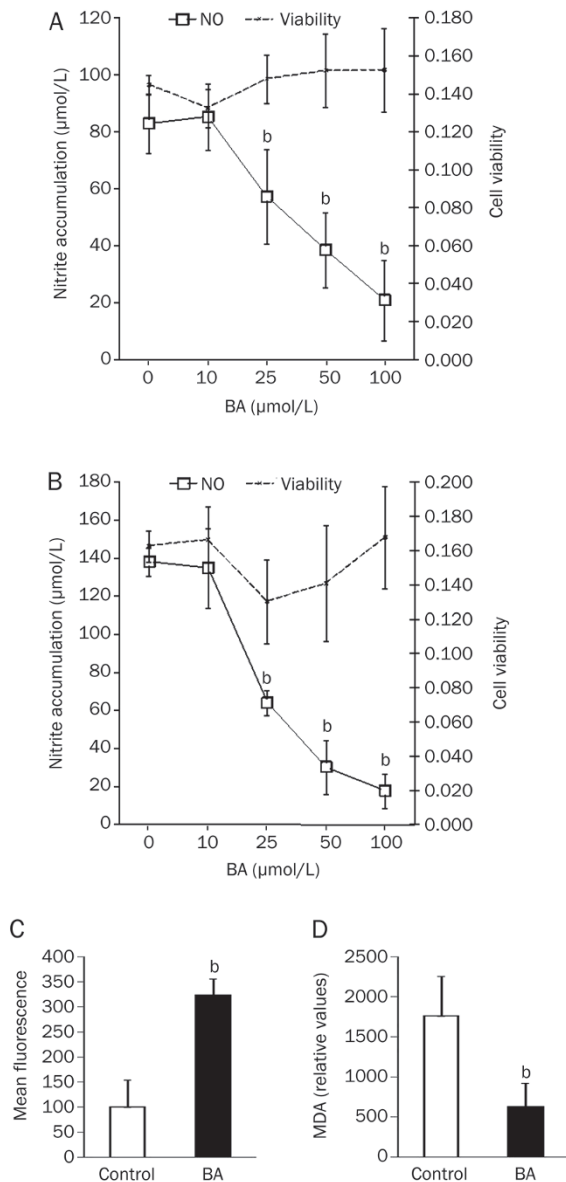
### The effect of BA on IFN- $\gamma$ and IL-17 generation in encephalitogenic T cells

DA rats were immunized with MBP and CFA. Eight to ten days after immunization DLNC were isolated and stimulated *in vitro* with MBP for 24 h. BA applied simultaneously with MBP did not significantly affect DLNC viability in concentrations up to 100  $\mu$ mol/L (Figure 1A). However, it did affect IFN- $\gamma$  and IL-17 production in DLNC in a dose dependent manner (Figure 1A). Inhibition of IL-17 was achieved with lower concentrations of BA than required for the reduction of IFN- $\gamma$  (estimated IC<sub>50</sub> values were 11.2  $\mu$ mol/L for IL-17 and 63.8  $\mu$ mol/L for IFN- $\gamma$ ). Indeed, when we applied 50  $\mu$ mol/L BA it markedly reduced IL-17 production, but less efficiently reduced IFN- $\gamma$  production in DLNC (Figure 1B). Moreover, 50  $\mu$ mol/L BA intensively down-regulated IL-17 gene expression, while the reduction of IFN- $\gamma$  gene expression was not statistically significant (Figure 1C). Still, applied in 100  $\mu$ mol/L, BA almost completely diminished IFN- $\gamma$  release from DLNC



**Figure 1.** The influence of BA on IFN- $\gamma$  and IL-17 generation and viability of MBP-specific T cells. Rats were immunized with MBP and CFA. DLNC were obtained from popliteal lymph nodes at 8–10 d after immunization. SCC were obtained from spinal cords at the peak of EAE. DLNC ( $2.5 \times 10^6$ /mL) were stimulated with MBP (10  $\mu$ g/mL) in the presence of various concentrations of BA (A) or 50  $\mu$ mol/L BA (B, C). SCC were cultivated without stimulation in the absence or presence of 50  $\mu$ mol/L BA (D, E). After 24 h of cultivation, cell-free culture supernatants were collected for cytokine determination (A, B, E), cell viability was measured by AP assay (A) or MTT assay (D), and DLNC were used for RT-PCR (C). Results are presented as means  $\pm$  SD of values obtained in samples from two (A), eight (B), or three (C, D, E) rats. <sup>b</sup>*P* < 0.05 represents a statistically significant difference between the cultures grown in the absence (Control) and presence of BA.

(to 11% of the value obtained in untreated cultures). On the contrary, the maximal effect on IL-17 release achieved with 25  $\mu$ mol/L BA was less potent (36% of the value obtained in untreated cultures). Higher BA concentrations did not decrease IL-17 production in DLNC additionally (Figure 1A). Further, SCC were isolated at the peak of EAE and cultivated for 24 h without stimulation, whereas the BA that was applied from the beginning of the cultivation in 50  $\mu$ mol/L concentration had limited effect on SCC viability (Figure 1D). Once again, BA was a potent inhibitor of IL-17 generation and was fairly effective against IFN- $\gamma$  production (Figure 1E). Thus,



**Figure 2.** The influence of BA on NO production and viability of SPC, PC, and ROS generation and lipid peroxidation in PC. SPC ( $5 \times 10^6$ /mL) stimulated with  $2.5 \mu\text{g/mL}$  ConA (A) and PC ( $1 \times 10^6$ /mL) stimulated with LPS  $10 \text{ ng/mL}$  (B, D) were cultured in the presence of various concentrations of BA. After 24 h of cultivation, cell-free culture supernatants were collected for nitrite determination and cell viability was measured by MTT assay (SPC) or CV (PC). PC ( $1 \times 10^6$ /mL) were cultivated in the presence of  $1 \mu\text{mol/L}$  DHR for 30 min and then stimulated with  $50 \mu\text{mol/L}$  BA for 1 h (C). PC ( $4 \times 10^6$ /1.5 mL) were cultivated in the presence or absence of  $50 \mu\text{mol/L}$  BA for 24 h and subsequently a TBARS assay was performed (D). Results are presented as mean  $\pm$  SD of values obtained in four (A, B) or three (C, D) independent experiments. <sup>b</sup> $P < 0.05$  represents a statistically significant difference between the cultures grown in the absence (Control) and presence of BA.

BA inhibited production of IFN- $\gamma$  and IL-17 in encephalito-genic cells that were re-stimulated *in vitro* with MBP or *in vivo* within the CNS.

### The effect of BA on NO generation in splenocytes and on NO generation, ROS production and lipid peroxidation in peritoneal macrophages

SPC and PC were isolated from untreated rats and stimulated with ConA and LPS, respectively. Various concentrations of BA, up to  $100 \mu\text{mol/L}$ , were added simultaneously and cultivation lasted for 24 h. Subsequently, nitrites were determined in culture supernatants and cell viability was analyzed. In both SPC (Figure 2A) and PC (Figure 2B), BA did not significantly influence viability in the applied concentrations, whereas it had a clear dose-dependent down-regulatory effect on NO production. Estimated  $\text{IC}_{50}$  values were  $58 \mu\text{mol/L}$  for SPC and  $42 \mu\text{mol/L}$  for PC. In contrast, ROS generation in PC was induced by 1 h treatment with BA ( $50 \mu\text{mol/L}$ ) (Figure 2C), but lipid peroxidation was reduced after 24 h of incubation (Figure 2D). Thus, BA had the opposite effect on the production of the main reactive species in macrophages, *ie* it inhibited synthesis of nitrogen and induced the generation of oxygen reactive species, yet its net effect on lipid peroxidation in these cells was negative.

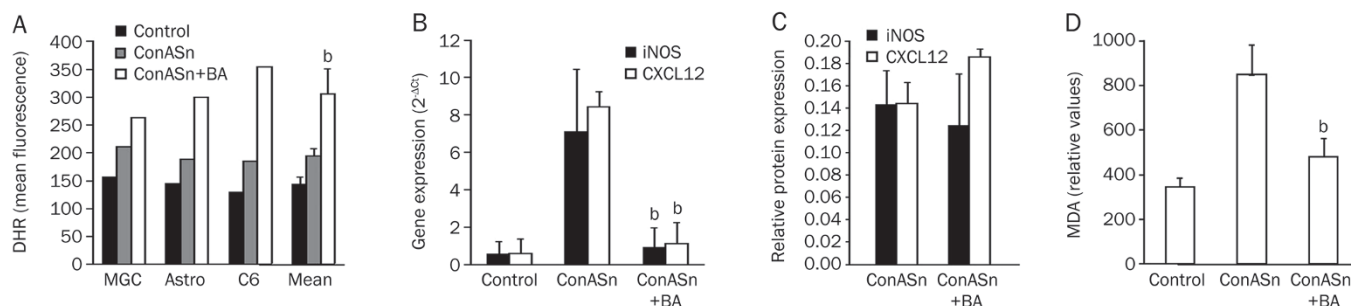
### The effect of BA on ROS generation, iNOS expression and CXCL12 expression in astrocytes

Astrocytes were stimulated with ConASn, which contains numerous inflammatory mediators and thus mimics the inflammatory milieu present in the CNS during neuroinflammation. To detect ROS generation, MGC, primary astrocytes and C6 cells were stimulated with ConASn in the presence of  $50 \mu\text{mol/L}$  BA for 1 h. BA potently stimulated ROS generation in all of the cells examined (Figure 3A). Astrocytes were also treated with ConASn for 24 h in the absence or presence of BA ( $50 \mu\text{mol/L}$ ) and iNOS and CXCL12 gene expression was determined. BA significantly reduced iNOS mRNA expression in the astrocytes, but the downregulation did not reach a significant level at the protein level (Figure 3B, 3C). Also, expression of the CXCL12 gene, but not of the CXCL12 protein, was inhibited by BA (Figure 3B, 3C). Finally, BA inhibited ConASn-induced lipid peroxidation in C6 cells (Figure 3D) and astrocytes (data not shown). Thus, similar to macrophages, astrocytes responded to the BA treatment with up-regulation of ROS, but the net effect of the BA on lipid peroxidation was negative.

### Discussion

Here, we present BA as a potent modulator of the synthesis of molecules that are relevant for neuroinflammation. BA inhibits generation of the pro-inflammatory cytokines IL-17 and IFN- $\gamma$  in T cells and NO in macrophages, but stimulates generation of CNS-tissue deleterious ROS in macrophages and astrocytes. Importantly, BA downregulates lipid peroxidation in macrophages and astrocytes.

BA potently inhibited IL-17 and IFN- $\gamma$  production in MBP-specific T cells isolated from lymph nodes or the CNS, which suggests that BA intensively inhibits Th17 and Th1 effector functions. IL-17-producing Th17 cells and IFN- $\gamma$ -producing Th1 cells are considered to be among the major pathogenic



**Figure 3.** The influence of BA on ROS production, lipid peroxidation, and CXCL12 and iNOS expression in astrocytes and C6 cells. Astrocytes, MGC, or C6 cells ( $1.5 \times 10^5$ /mL) were pre-incubated with  $1 \mu\text{mol/L}$  DHR for 30 min and then stimulated with 20% ConASn in the presence or absence of  $50 \mu\text{mol/L}$  BA. After 1 h of cultivation, ROS generation was determined by DHR staining and flow cytometry (A) and after 24 h the cells were used for detection of CXCL12 and iNOS gene expression by RT-PCR (B) or protein expression by immunoblot (C). C6 cells ( $1.5 \times 10^6$ /1.5 mL) were stimulated with 20% ConASn in the presence or absence of  $50 \mu\text{mol/L}$  BA for 24 h and subsequently a TBARS assay was performed (D). Results are presented as mean  $\pm$  SD of values obtained in at least three independent experiments. Also, results from individual experiments are presented in A. <sup>b</sup> $P < 0.05$  represents a statistically significant difference between the cultures stimulated with ConASn in the absence and presence of BA.

populations in the CNS autoimmunity<sup>[2]</sup>. Both IL-17 and IFN- $\gamma$  are continuously produced by DLNC and SCC in Dark Agouti rats immunized to develop EAE<sup>[16]</sup>. Furthermore, low production of IL-17 and IFN- $\gamma$  in DLNC, as well as low production of the cytokines that induce IL-17 and IFN- $\gamma$  generation (including IL-6, IL-12, and IL-23) in DLNC, have been associated with the resistance of Albino Oxford rats to EAE induction<sup>[17, 18]</sup>. Regarding MS, it has been shown that IL-17 is elevated in immune cells within the CNS and at the periphery and that it contributes to the pathogenesis of the disease, *eg* through distortion of the blood-brain-barrier<sup>[19-21]</sup>. It has also been shown that IFN- $\gamma$ -producing cells are present within the CNS and at the periphery of MS patients<sup>[22]</sup>, and that treatment of MS patients with IFN- $\gamma$  exacerbates the disease<sup>[23]</sup>. Therefore, inhibiting IL-17 and IFN- $\gamma$  generation is relevant when modulating the EAE course and also has relevance when evaluating therapeutic efficiency in MS. Regarding the higher efficacy of the BA influence on IL-17, relative to IFN- $\gamma$ , it is known that this agent potently inhibits STAT-3 signaling<sup>[24, 25]</sup>, which is essential for IL-17, but not IFN- $\gamma$  production<sup>[26]</sup>. Although it was previously reported that BA moderately inhibits IFN- $\gamma$  production in human peripheral blood cells<sup>[27]</sup>, this is the first report, to the best of our knowledge, that BA is effective against IL-17 generation.

NO and ROS are among the main culprits for tissue damage in neuroinflammation, especially when they are produced simultaneously and when they interact to generate peroxynitrite<sup>[6, 7]</sup>. BA applied *in vivo* has been shown to decrease both oxidative and nitrative stress in mice and rats<sup>[28, 29]</sup>. However, in our experimental setting BA consistently induced ROS generation and inhibited iNOS-mediated NO production. Consistent with our results, it was previously shown that BA inhibits NO generation in primary mouse macrophages and the RAW264.7 cell line<sup>[30]</sup>. We also show that BA inhibits synthesis of NO in mixed populations of spleen cells, as well as in astrocytes. Thus, the influence of BA on NO generation might be of pharmacological relevance when treat-

ing (neuro)inflammatory disorders. However, ROS induction in macrophages and astrocytes by BA is a greatly unwanted effect of this agent, as ROS present a threat to the CNS tissue in neuroinflammation<sup>[31]</sup>. Still, simultaneous inhibition of NO production should prevent the generation of peroxynitrite, which is the major mediator of oxidative stress and excitotoxicity in the pathogenesis of MS and EAE<sup>[6, 32]</sup>. Indeed, in our system, BA downregulated the oxidative degradation of lipids, *ie* lipid peroxidation, both in LPS-stimulated macrophages and cytokine-stimulated astrocytes. This clearly shows that although BA stimulates ROS generation in these cells its overall effect is cytoprotective. Also, as ROS generation by macrophages is one of the essential effector mechanisms for destroying pathogens, potentiating ROS production in macrophages by BA might be a favorable pharmacological property of the agent in the treatment of infectious diseases.

It has recently been determined that CXCL12 has a major regulatory role in the control of encephalitogenic cell entry into the CNS in neuroinflammation<sup>[10]</sup>. Here, we observed that BA inhibits CXCL12 gene expression, but not its generation, in astrocytes, thus suggesting a neutral effect of the agent on the CXCL12-mediated anti-inflammatory effect in the CNS. Besides astrocytes, endothelial cells are an important source of CXCL12 in regulating immune cell entry into the CNS, and therefore exploration of the influence of BA on endothelial cells is needed. Such studies would allow further insight into the influence of BA on neuroinflammation through the modulation of CXCL12 expression.

In conclusion, BA potently modulates the generation of various molecules that are important for the pathogenesis of neuroinflammatory disorders such as MS and EAE. While inhibiting the production of the proinflammatory cytokines IL-17 and IFN- $\gamma$  and NO, BA also stimulates the production of potentially toxic ROS. Thus, further investigation of the influence of BA is warranted, especially regarding the *in vivo* application of the agent in EAE and other animal models of neuroinflammation. Special attention should be given to the

way that BA is administered to experimental animals. It is clear that the application of BA systemically would affect the immune cells in lymphoid tissues and circulation. However, it is debatable whether BA would be able to affect cells within the CNS, either immune or resident, if applied in this way. Although data on the ability of BA to cross an intact blood-brain barrier (BBB) are, to the best of our knowledge, unavailable, it is conceivable that a complex triterpenoid compound such as BA does not passively cross this barrier. However, during neuroinflammation, *eg* in EAE, the BBB is affected as well, and it becomes more permeable for transport of complex molecules<sup>[33]</sup>. The possibility of barrier circumvention, through BBB disruption, prodrugs, molecular Trojan horses or nanocarriers, should also be considered<sup>[34]</sup>. Similarly, the possibility of chemically modifying the BA to make it able to cross the BBB seems very attractive, as it has been shown to be effective for other triterpenoids, *eg* oleanolic acid in EAE<sup>[35]</sup>. As a matter of fact, the strategy of chemical modification has already been applied to betulinic acid, as its derivatives have been investigated for their anti-cancer properties<sup>[36,37]</sup>.

Finally, besides demonstrating the potential therapeutic effects in neuroinflammation, our results also contribute to the understanding of BA anti-cancer properties. It is well appreciated that chronic inflammation paves the road for cancerogenesis, while its prevention or restriction might aid tumorogenesis impediment<sup>[38]</sup>. Thus, the anti-inflammatory properties of BA presented here might contribute to its overall anti-cancer therapeutic potential.

### Acknowledgements

This work was supported by the Ministry of Education and Science of the Republic of Serbia (173035, 175038, and 173013). The authors would like to thank BioSolutions Halle GmbH for betulinic acid.

### Author contribution

Jana BLAŽEVSKI, Filip PETKOVIĆ, Miljana MOMČILOVIĆ, and Djordje MILJKOVIĆ performed research; Marija MOSTARICA STOJKOVIĆ and Djordje MILJKOVIĆ designed research, analyzed data and wrote the paper; Reinhard PASCHKE and Goran N KALUĐEROVIĆ contributed new reagents.

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