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Influence of Baicalin Addition for Cd4+Cd25- Population, Isolated From Wistar Rat Spleens

KEYWORDS

Edyta BANCYR	K PRIYADARSHINI
Wroclaw University of Environmental and Life Science, Department of Immunology, Pathophysiology and Preventive Medicine, Wroclaw, Poland	University of Mysore, Karnataka, India

Isaura FELCENLOBEN	Marcin ZYROMSKI
Wroclaw University of Environmental and Life Science, Department of Chemistry, Wroclaw, Poland	Wroclaw University of Environmental and Life Science, Department of Chemistry, Wroclaw, Poland

Edyta KOSTRZEWA-SUSŁOW	Mirosław ANIOŁ
Wroclaw University of Environmental and Life Science, Department of Chemistry, Wroclaw, Poland	Wroclaw University of Environmental and Life Science, Department of Chemistry, Wroclaw, Poland,

ABSTRACT *Baicalin and baicalein belong to group of compounds found in parts of Scutellaria baicalensis and Oroxylum indicum. From almost 2000 years, extracts from Scutellaria baicalensis are used in eastern medicine, as an important component pharmaceuticals. As many scientist show (Sahebakar 2012, Gaire et al. 2014, Shang et al. 2013), baicalin and baicalein play important role during treatment of many diseases, like liver cancer or skin problems. Many projects have undertaken the subject of baicalin and baicalein in order to better understand their chemical and biological potential.*

INTRODUCTION

Flavonoids are a group of plant secondary metabolites. The structure of most known flavonoids consists on 2-phenylchromane core. Flavonoids can be divided into 7 subgroups: flavones, isoflavones, flavonols, flavanols, flavanones, chalcones and anthocyanes. Since the first year of analysis of flavonoids, a large variety of their properties were discovered. The most described properties are antioxidant activity (Williams et al. 2004, Galleano et al. 2010, Stojadinovic et al. 2013), anticancer properties (Kang et al. 2011, Qin et al. 2012, Zhao et al. 2013, Pan et al. 2014, Tao et al 2014), antifungal activity (Buzzini et al. 2008, Galeotti et. 2008), antibacterial activity (Cushnie and Lamb 2011, Wu et al. 2013, Bahrin et al. 2014), anti-diabetic properties (Li-Weber 2009), hipocholesterolic activity anti-inflammatory activity (Shalini et al. 2012, Sithisarn P. et al. 2013) and ability to form complexes with protein and protein-like molecules (enzymes, DNA, RNA, peptides) (Janjua N.K. et al. 2009, Walle T. et al. 2003, Zhang X et al. 2013). Such wide range of discoveries had put flavonoids in front of pro-health compounds, promoting their usage in many branches of industry e.g. food industry pharmaceutical industry and chemical industry. Baicalin (7-glucoronic acid-5,6-dihydroxyflavone)and aglycone baicalein (5,6,7-trihydroxyflavone) belong to the flavone subgroup of flavonoids. Both can be found in leaves of *Oroxylum indicum* or in roots and leaves of *Scutellaria baicalensis*. Baicalin and baicalein, as well as other flavonoids, show a wide range of properties such as anti-inflammatory (Cui L. et al. 2010, Nakajima T. et al. 2001), anti-cancer (Zhao et al. 2010,), antibacterial (Sato Y. et al. 2000) and antioxidant activity while being a low toxic compound toward live cells.

The amount of flavonoid compounds leads to physicochemical and biological analysis resulting in recognition of their new, unknown properties.

MATERIALS AND METHODS

Animals:

Six female, 6-week-old, inbred Wistar rats weighing 150-200 g from a colony bred at Wroclaw Medical University, Department of Pathology, Poland were used. The experiment was conducted with the permission of Local Ethical Committee II in Wroclaw (Poland) no. 60/2011.The animals received water and maintenance food at libitum, and were kept in an animal room which was illuminated for 12 hours a day (7:00-19:00) at room temperature (24°C).

Flow cytometry:

Lymphocytes were isolated from the spleen and lymph nodes by sieving into PBS. After that 0.5x106 lymphocytes were suspended in a FACS buffer (40 mL PBS, 0.8 mL fetal calf serum, 40 mg NaN2). Cells were stained with rat anti-CD4 FITC (e-Bioscience, Cat# 11-0040-85) and anti-rat CD25-PE (E-Bioscience, Cat# 12-0390-82) for 30 minutes at 4°C, washed twice with FACS buffer, fixated and permeabilized with Fix/Perm buffer (eBIOSCIENCE, cat. 00-5123-43, cat. 00-5223-56, USA) and Permeabilization buffer (eBIOSCIENCE, cat. **1600044**, USA) according to the manufacturer's instructions. FOXP3 protein was stained with anti-Mouse/Rat Foxp3 Alexa Fluor 647 (E-Bioscience, cat # 51-5773-82, USA) for 30 minutes at 4°C. Cells were washed twice with FACS buffer and fixed in 2% paraformaldehyde. Cells were analyzed using a FACSCalibur flow cytometer and Weasel 2.0 software.

FACS sorting:

From each healthy rat used as a donor lymphocytes were isolated from the spleen and lymph nodes were mixed together, and in the other experiment lymphocytes after cell culture were suspended in PBS + 2% FCS and stained with rat anti-CD4 FITC (e-Bioscience, cat # 11-0040-85, USA), anti-rat CD25-PE (e-Bioscience, no. 12-0390-82, USA), for

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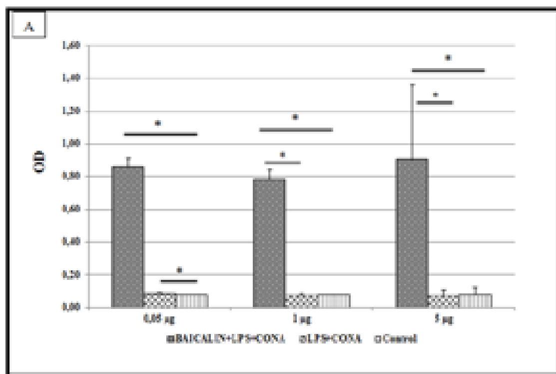
30 minutes at 4°C. After that cells were washed twice with PBS + 2% FCS, and disaggregated on sterile FACS tubes with a 35 µm nylon mesh cell strainer cap (BD Biosciences, cat.352235, USA). 50 x 10^6 cells/ml of such prepared cells were sorted on the BD FACSAria II at a speed of 15 000 cells/s. After sorting $CD4^+$ $CD25^-$ cells were immediately centrifuged in PBS+2% FCS. Then cell viability was determined by trypan blue (Sigma Aldrich, cat. T8154, USA) and transferred to the culture medium.

Culture of CD4+CD25- cells:
After sorting cells were cultured in 24-well plates coated with 10 µg/ml anti-CD3 and 1 µg/ml anti-CD28 in the presence of 100 U/ml interleukin-2 in RPMI 1640 medium supplemented with 100 unit/ml penicillin/streptomycin, 20 mM L-glutamine, 5 ng/mL TGF-β, 10% fetal calf serum (FCS), 10 µg/mL LPS and/or 10 µg/mL ConA and 0.5µM or 2 µM of baicalin (for FACS analysis) or 0.05 µg, 1 µg or 5 µg baicalin (for MTT analysis). Cells were cultured in conditions of 37°C and 5% CO_2 for four days. After cell culture, cells were phenotyped to identify purity of CD4+CD25+FOXP3+ by flow cytometry, as described in flow cytometry.

MTT test:
2 × 10^5 cells were suspended in 180 µL of complete RPMI-1640 with 0.05 µg, 1 µg or 5 µg baicalin, as described above for culture of CD4+CD25- cells, and cultured in 96-well tissue culture plates (FALCON, Becton Dickinson, USA) for 24, 48, 96 hours in conditions of 5% CO_2 at 37°C. Then20 µL of (5 mg/mL) 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was added (Sigma, cat. M2128, USA). Cells were cultured with MTT for the next 4 h in conditions of 37°C, 5% CO_2. Then 100 µL of lysing buffer (225 mL DMF, 67.5 g SDS, 275 mL H_2O per 1 litre) was added and cells were cultured with MTT for the next 4 h in conditions of 37°C, 5% CO_2. Optical density of stained and lysed cells were measured at 570 nm and 630 nm in the BioTek µQuantum.

Statistical analysis: Results were analyzed in Statistica software version 10.0, by analysis of variance (ANOVA) and Duncan's post-hoc test for statistical significance, with significance levels p <0.05 and p <0.01.

RESULTS



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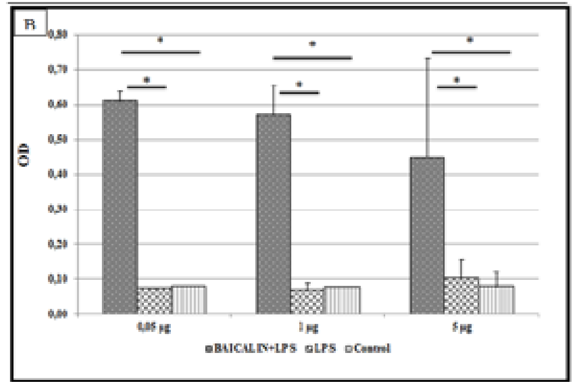


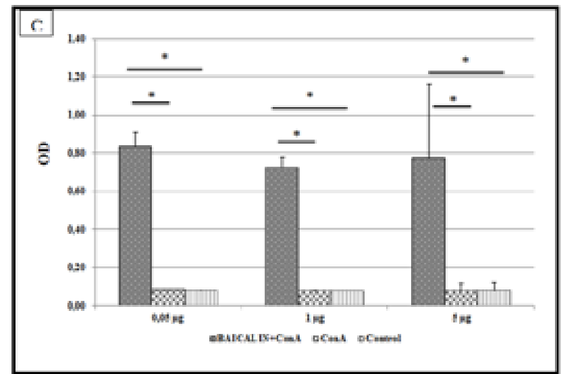
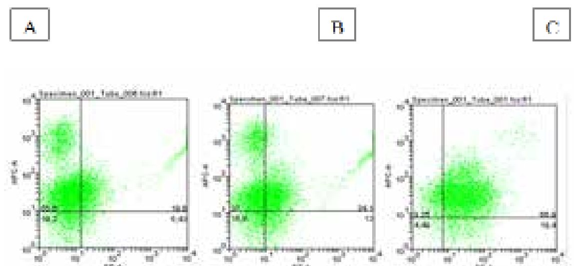


Fig. 1. Proliferation of CD4+CD25- cells cultured in the presence of diff baicalin and LPS+ConA (A); baicalin and LPS (B); and baicalin and ConA (C). The test was performed in 3 replicates. * – significant difference at p<0.01.

Sorted CD4+CD25- cells were cultured in medium with-three different concentrations of baicalin: 0.05µg/mL, 1 µg/mL or 5µg/mL for the proliferation analysis by MTT. The proliferation of cultured cells was estimated by the MTT test (Fig. 1). There were no significant differences in the proliferation between cultures with LPS+ConA or LPS or ConA; however, the proliferation of cultured cells was lower in cells culture with LPS. In every of examined option there were significant difference between culture with baicalin and mitogen (LPS and/or ConA) and control (without baicalin and mitogen) except culture without baicalin but with and 10 µg/mL LPS and 10 µg/mL ConA (where optical density was 0.08 ± 0,00) and control culture (where optical density was 0.8 ± 0,02) were significant difference at p<0.01



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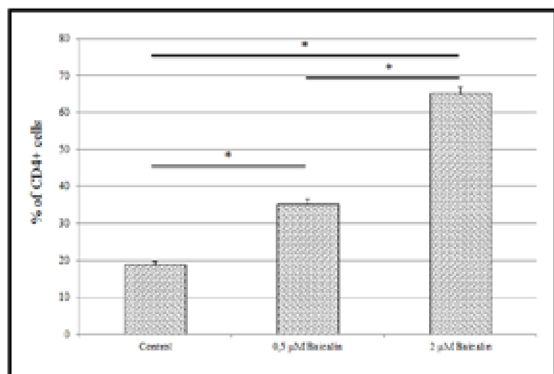

Fig. 2. Percent of CD4+ cells with phenotype CD4+CD25+FOXP3+; A - control; B -0.5 μM baicalin; C – 2 μM baicalin. N=2, * – significant difference at p<0.01.

On the other hand we had examined differentiation CD4+CD25- cells into regulatory T cells CD4+CD25+FOXP3+. Sorted cells CD4+CD25- were culture with 0.5 μM or 2 μM baicalin (control was without baicalin) for four days. Our results have shown significant differences at p<0.01 between 0.5 μM or 2 μM baicalin as a inductor of differentiation CD4+CD25- into Treg (35,3 ± 1.2 % in culture with 0.5 μM baicalin; versus 64.9 ± 2 % in culture with 2 μM baicalin). Also control culture was significant difference at p<0.01 with cultures with baicalin.

DISCUSSION AND SUMMARY

In 1995, Sakaguchi demonstrated that cryptic suppressor cells have the phenotype of CD4 + CD25 + and depletion of the cell population leads to schodze autoimmune disease (Sakaguchi et al., 1995). Following years of research made it possible to characterize the most important factor determining the function of regulatory T cells - the intracellular cell factor Foxp3 (Hori et al., 2003). Regulatory T cells to this day is an important research facility and provide answers to many questions related to the acquisition of resistance, the regulation of the immune response and the potential implementation of cellular therapy based on regulatory T cells.

Baicalin could be a one of the most important acitvation factors, which can be used in the future in medicine, as one of the potential compound for treatment.

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