Effects of polysaccharide fractions isolated from *Caltha palustris* L. on the activity of phagocytic cells & humoral immune response in mice with collagen-induced arthritis: A comparison with methotrexate

Agnieszka Suszko & Bożena Obmińska-Mrukowicz

*Department of Biochemistry, Pharmacology & Toxicology, Faculty of Veterinary Medicine, Wrocław University of Environmental & Life Sciences, 50-375 Wrocław, Poland*

Received May 17, 2014

**Background & objectives:** The extracts from *Caltha palustris* L. have been shown to be beneficial for treating arthritis and rheumatism. In this study, the immunomodulatory effects of polysaccharide fractions B and C of *C. palustris* extracts were studied, using the collagen-induced arthritis (CIA) mouse arthritis experimental model. The aim was to determine the activity of blood phagocytic cells and humoral immune response in CIA mice treated with polysaccharide fractions from *C. palustris*.

**Methods:** The effects of fractions B and C of *C. palustris* were explored by evaluating phagocytic activity of peripheral blood granulocytes and monocytes and humoral immune response in sheep red blood cell (SRBC)-immunized mice. The results were compared with methotrexate (MTX) treatment. Following the onset of CIA, DBA/1J mice were treated for 21 days with B or C fractions (10 mg/kg; i.p.) or MTX (every 48 h, 6.6 mg/kg; i.p.).

**Results:** The results showed that fraction B reduced the level of interleukin (IL)-1β, boosted nitric oxide synthesis in murine peritoneal macrophages stimulated in vitro with lipopolysaccharide and enhanced the monocyte phagocytic activity. Exposure of SRBC-immunized mice to fraction B and MTX during the course of CIA resulted in decreased total anti-SRBC haemagglutinin titres.

**Interpretation & conclusions:** Fraction B of *C. palustris* polysaccharides modulated macrophage function and exerted beneficial effects on the clinical course of CIA in mice. The results also suggested efficacy of fraction B was comparable to that of MTX treatment for certain parameters.

**Key words** Antibodies - *Caltha palustris* - collagen-induced arthritis - interleukin-1 - nitric oxide - phagocytic cells

Rheumatoid arthritis (RA) is an autoimmune systemic inflammatory disease¹. RA pathogenesis involves different pathways, in which pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6 and tumour necrosis factor-α (TNF-α) play a crucial role²,³. IL-1β has the strongest destructive effect on bone and cartilage tissues, and it impairs repair processes by inhibiting the synthesis of cartilage matrix proteins⁴,⁵. In RA, the joint synovial tissue is infiltrated by various inflammatory cells such as macrophages,
T-cells, B-cells, granulocytes and monocytes. Macrophages are profusely present in the inflamed synovial membrane and cartilage and their activity contributes to joint inflammation, pannus formation and cartilage destruction. Overexpression of TNF-α, IL-1β, and IL-6 is induced by infiltration or activation of macrophages. Furthermore, a large number of synovial lining macrophages produces nitric oxide (NO) that promotes high expression of TNF-α and additionally increases synovitis frequency. Another cellular population determining the severity and duration of RA, includes the B-cells. Their reactivity in RA patients is usually prominent, and they secrete a large amount of autoantibodies that can promote tissue destruction and release of autoantigens.

Collagen-induced arthritis (CIA) in mice is a well-known model of human RA, due to its numerous clinical and histological similarities to human RA. This model has been extensively used to estimate prospective anti-arthritic substances.

_Caltha palustris_, a plant species from the _Caltha_ genus (Ranunculaceae family), is a widespread plant in Europe, Asia and North America. Our previous experiments confirmed that polysaccharide fraction B from _C. palustris_ beneficially affected the course of CIA in mice. The presented results were comparable to those of methotrexate (MTX) treatment, especially for alleviating joint swelling severity, erythema and inhibition of CIA-induced peripheral blood leucocytosis. Moreover, fraction B exhibited immunomodulating activity toward thymic T lymphocyte subpopulations, lowered the percentage of splenic Tregs and reduced TNF-α concentration in peripheral blood, previously enhanced during the course of CIA. In this study, we explored the effects of polysaccharide fractions B and C from _C. palustris_ extract on the activity of blood phagocytic cells and humoral immune response in CIA, a mouse arthritis experimental model.

### Material & Methods

Detailed information on animals, induction of CIA in mice, treatment and study design were as described in our previous study. Briefly, the studies were conducted on male and female DBA/1J mice (8-10 wk old). Experiments were performed on non-immunized and sheep red blood cells (SRBCs)-immunized mice. The immunization was conducted on day 43 of the experiment with 0.2 ml of 10 per cent SRBC suspension (4 × 10⁸ cells/mouse) administered intraperitoneally (i.p.). The sheep blood was collected (SRBC, Department of Physiology and Biostucture, Faculty of Veterinary Medicine, Wroclaw University of Environmental and Life Sciences, Poland) into the Alsever’s solution and kept at 4°C for at least three days. The SRBC suspension was prepared _ex tempore_ in phosphate-buffered saline (PBS, Institute of Immunology and Experimental Therapy Polish Academy of Sciences, Wroclaw, Poland).

Animals were divided into five groups: group 1 (negative control) - healthy animals, group 2 (positive control) - animals with induced CIA, group 3 - CIA mice treated with fraction C, group 4 - CIA mice treated with fraction B, and group 5 - CIA animals treated with MTX (methotrexate, Ebewe Pharma GmbH Nfg., Austria). Each control and experimental group comprised eight mice. The experimental protocol is presented in Fig. 1.

The study protocol was approved by the Local Ethics Committee in Wroclaw, Poland (No. 95/2009). The experiments were conducted from August 2009 to July 2011, at Wroclaw, Poland.

The plant material was collected from Trzebnica (Western Poland), identified and a voucher specimen (number WR-GN0058095) was deposited at the Herbarium of Museum of Natural History, Wroclaw University, Poland. Polysaccharide fractions B and C from _C. palustris_ extract were prepared and kindly provided by Professor Janina Kuduk-Jaworska as described previously. Stock solutions of the plant extract fractions were prepared _ex tempore_ by dissolving 2 mg of B or C fraction in PBS solution.

**Cytokine assay and NO production:** The mice were anaesthetized with isoflurane (Aerrane, Baxter, USA). Peritoneal exudate macrophages were acquired and cell culture procedure were conducted as described by Szczypka et al, except one modification that the medium was replaced after shorter incubation time, 18 h instead of 20 h.

Murine IL-1β level (pg/ml) in macrophage culture supernatants was established by using a commercial ELISA kit (Quantikine R&D Systems, Minneapolis, USA) in compliance with the manufacturer’s instruction. Each sample was tested in duplicate.

NO released from murine peritoneal macrophages, stimulated _in vitro_ with lipopolysaccharide (LPS) from _Escherichia coli_, was measured as nitrite using the procedure described by Szczypka et al. The level of IL-1β, NO, and the phagocytic and oxidative burst...
activity of the blood monocytes and granulocytes were determined after finishing treatment at two time points: day 43 of the experiment (24 h after the last administration of fraction C and B and 72 h after the last administration of MTX) and day 47 of the experiment (5 days after the last administration of fraction B or C and 7 days after the last administration of MTX).

**Phagocytic and the oxidative burst activity of blood granulocytes and monocytes:** The blood samples were collected into the heparinised tubes (Equimed, Kraków, Poland) from retro-ocular arteries of isoflurane-anaesthetized mice. The animals were then euthanized by cervical dislocation. Commercial Phagotest and Bursttest (Phagoburst) kits were used following the manufacturer’s instructions (ORPEGEN Pharma, Heidelberg, Germany). A flow cytometer (FACSCalibur, Becton Dickinson Biosciences, USA) with software - CellQuest 3.1f (Becton Dickinson, USA) was used to analyze the fluorescence. To determine the percentage of phagocytic blood granulocytes and monocytes (ingestion of one or more bacteria per cell), and their mean fluorescence intensity (MFI) (number of bacteria per cell) the Phagotest kit (fluorescein-labelled opsonized E. coli and necessary reagents) was used. The Bursttest was used to establish the leucocyte oxidative burst by measuring the percentage of blood granulocytes and monocytes producing reactive oxygen species (ROS) and their MFI (enzymatic activity). The measurements were made on days 43 and 47 of the experiment.

**Plaque forming cells (PFC) assay:** The mice were immunized i.p. with 0.2 ml of 10 per cent SRBC suspension (4 × 10⁸ cells/mouse) on day 43 of the experiment. The animals were anaesthetized with isoflurane (Aerrane, Baxter, USA) and then sacrificed by cervical dislocation on day 4 after priming (day 47 of the experiment). Briefly, following euthanasia, the murine spleens were removed, cell suspension acquired and the number of splenocytes producing haemolytic anti-SRBC antibodies (PFCs) was determined according to a method previously described

**Determination of anti-SRBC antibodies in serum:** The mice were treated for PFC assay as described above. The blood samples were taken from retro-ocular arteries of isoflurane anaesthetized mice. The number of anti-SRBC haemagglutinin titres in SRBC-immunized mice was determined on day 4 after priming (day 47 of the experiment)

**Statistical analysis:** One-way ANOVA and post hoc analysis using Duncan’s test were used to evaluate the differences between the groups using STATISTICA 10 software (StatSoft Inc., Oklahoma, USA).

**Results**

*Effects of polysaccharide fractions B and C of C. palustris and MTX on IL-1β production by peritoneal macrophages in CIA mice:* Synthesis and release of
IL-1β by murine peritoneal macrophages, stimulated in vitro with LPS from E. coli (2.5 μg/ml cell culture), was noticed on day 47. Administration of fraction B to the CIA mice inhibited (day 47) the stimulating effect of inflammation on the production and release of IL-1β by murine peritoneal macrophages stimulated in vitro with LPS from E. coli. MTX administration caused only a transient (day 43) induction of this stimulating effect (Fig. 2).

Effects of polysaccharide fractions B and C of C. palustris and MTX on NO production by peritoneal macrophages in CIA mice: Fraction B increased the NO output production by murine peritoneal macrophages stimulated in vitro with LPS from E. coli on both days (43 and 47). The strongest effect was observed on day 43 of the experiment. MTX suppressed NO production in peritoneal macrophages (Fig. 3).

Effects of polysaccharide fractions B and C of C. palustris and MTX on phagocytic activity of peripheral blood granulocytes and monocytes in CIA mice: Transient rise in the percentage of phagocytic granulocytes was perceived on the day 43 of the experiment in CIA mice. No effects on the phagocytic activity of monocytes were noticed in CIA mice (Table I).

No significant changes in the phagocytic activity of monocytes and granulocytes were observed on day 43, after administration of any fractions (Table I). On day 47, the exposure to fraction B increased the percentage of phagocytic monocytes, and it was accompanied by increased fluorescence intensity of these cells.

On day 43, a transient drop in the percentage of phagocytic granulocytes was noticed in MTX group. However, on day 47, a significant enhancement in the percentage of phagocytic monocytes and fluorescence intensity of these cells were observed.

Effects of polysaccharide fractions B and C and MTX on oxidative burst activity of peripheral blood granulocytes and monocytes in CIA mice: Reduced percentages of granulocytes producing ROS and lower enzymatic activity (fluorescence intensity) of monocytes were noted in CIA mice on day 43 of the experiment. However, reduced oxidative burst activity of granulocytes (and MFI of these cells) and monocytes was found on day 47. Administration of fraction B resulted in higher percentage of ROS producing monocytes, and this corresponded to increased fluorescence intensity of these cells. Fraction C did not significantly affect the burst activity of granulocytes and monocytes.

MTX administered to CIA mice strongly reduced the percentage of ROS producing granulocytes with augmented enzymatic activity, but only on day 43 (Table II).

Effects of polysaccharide fractions B and C and MTX on primary humoral immune response in SRBC-immunized mice during the course of CIA: The primary humoral immune response in SRBC-immunized mice was...
reduced by CIA, as evidenced by the lower number of splenocytes producing haemolytic anti-SRBC antibodies (PFC) (Fig. 4) and the total anti-SRBC antibody titres in serum (Fig. 5A). The suppressive effect of fraction B on the total anti-SRBC haemagglutinin titres in serum was equally strong in MTX samples.

**Discussion**

So far, treatment modalities for RA have been limited by their high cost and side effects, especially...
immunosuppression and toxicity. Therefore, natural products have become a new therapeutic target for researchers. In the current study, the immunomodulatory effects of polysaccharide fraction B and C of *C. palustris* extracts were studied on the activity of phagocytic cells and humoral immune response in SRBC-immunized mice with CIA. MTX was used as a control treatment, due to the fact that it still remains as a standard in RA therapy\(^9\).

Administration of fraction B inhibited the stimulating effect of inflammation on the synthesis and release of IL-1β by murine peritoneal macrophages stimulated \textit{in vitro} with LPS from *E. coli*. The beneficial suppressing action of plant extracts on the synthesis of IL-1β in CIA mice was also demonstrated by Kim \textit{et al} \(^9\). Red ginseng saponin extract (RGSE) from the root of *Panax ginseng* C., administered orally at a dose of 10 mg/kg, reduced the level of serum IL-1β and TNF-α in CIA mice. Moreover, in the \textit{in vitro} studies on spleen cells isolated from RGSE-treated CIA mice, LPS-stimulated increase in cytokine levels was significantly inhibited. However, this suppressing effect on IL-1β production was quite different from most investigated plant polysaccharides as enhanced production of this cytokine was reported in several studies\(^{20,21}\).

Our results for MTX were consistent with the results, which showed a lack of MTX influence on IL-1β synthesis and release by mononuclear spleen cells obtained from MTX-treated CIA mice\(^{22}\). Another study also suggested that MTX might inhibit some IL-1β activities without affecting IL-1β production or secretion\(^{23}\).

Our results showed that fraction B enhanced NO production by peritoneal macrophages stimulated \textit{in vitro} with LPS from *E. coli*. Fraction B increased the percentage of phagocytic monocytes in the peripheral blood, which corresponded to increased fluorescence intensity of these cells, thus indicating a higher number of ingested bacteria per cell. Exposure to this fraction led to increased percentage of monocytes producing ROS, and their enhanced enzymatic activity. The polysaccharide fraction B has been shown to trigger monocyte phagocytic activity, burst activity and NO. Our results were in accordance with the studies which showed that most polysaccharides derived from higher...
plants activated macrophages\textsuperscript{24,25}. Unfortunately, this activation of macrophage function might pose a problem in the case of inflammation. Activation of T-cells and macrophages is a major pathogenic determinant of CIA\textsuperscript{26}. However, our findings regarding NO production were obtained only \textit{in vitro} and were limited to M1 phenotype of macrophage activation. To draw correct inference about the effects of polysaccharide fractions from \textit{C. palustris} extract on the macrophage activity in CIA mice, especially the populations that infiltrate the synovium, further and more detailed experiments are required.

Our data also showed that MTX caused a transient drop in the percentage of phagocytic granulocytes and a decrease in the percentage of ROS producing granulocytes with augmented enzymatic activity (day 43 of the experiment). These findings confirmed the inhibitory effect of MTX on granulocyte phagocytic function\textsuperscript{27}. Our data were also consistent with the results by Omata et al\textsuperscript{28}, who claimed that MTX reduced NO production by peritoneal macrophages from rats with adjuvant-induced arthritis. The polysaccharide fraction B and MTX showed inhibitory action of B-cell function. Park et al\textsuperscript{29} showed that low molecular weight fucoidans from brown algae reduced the intensity of arthritis and the levels of collagen-specific IgG 2a\textsuperscript{29}.

In conclusion, our study showed that fraction B enhanced macrophage activity (boosted NO synthesis in murine peritoneal macrophages stimulated \textit{in vitro} with LPS and enhanced the monocyte phagocytic activity), but reduced the level of pro-inflammatory cytokine IL-1β. In turn, humoral immune response was suppressed as fraction B showed inhibitory action on antibody formation. Taking into account the results of our present and previous experiments, it seems that at least part of the favourable effects of \textit{C. palustris} polysaccharides on the clinical course of CIA in mice may be due to the modulation of macrophage function.

\textbf{Acknowledgment}

This study was financially supported by the State Committee for Scientific Research; Ministry of Science and Higher Education (Grant No. 5694/B/P01/2010/39) in Poland. The authors thank Dr R. Miedzybrodzki (Ludwik Hirszfeld Institute of Immunology and Experimental Therapy Polish Academy of Sciences) and Drs P. Pawłowski, M. Szczypka, M. Lis, A. Pawlak and Mgr. I. Zbyryt (Faculty of Veterinary Medicine, Wrocław University of Environmental & Life Sciences) for support.

\textbf{Conflicts of Interest:} None.

\textbf{References}

1. Smolen JS, Aletaha D. Developments in the clinical understanding of rheumatoid arthritis. \textit{Arthritis Res Ther} 2009; 11 : 204.
2. Feldmann M, Brennan FM, Maini RN. Role of cytokines in rheumatoid arthritis. \textit{Annu Rev Immunol} 1996; 14 : 397-440.
3. Lipsky PE. Interleukin-6 and rheumatic diseases. \textit{Arthritis Res Ther} 2006; 8 (Suppl 2) : S4.
4. Henderson B, Thompson RC, Hardingham T, Lewthwaite J. Inhibition of interleukin-1-induced synovitis and articular cartilage proteoglycan loss in the rabbit knee by recombinant human interleukin-1 receptor antagonist. \textit{Cytokine} 1991; 3 : 246-9.
5. Van Lent PL, Van De Loo FA, Holthuysen AE, Van Den Bersselera LA, Vermeer H, Van Den Berg WB. Major role for interleukin 1 but not for tumor necrosis factor in early cartilage damage in immune complex arthritis in mice. \textit{J Rheumatol} 1995; 22 : 2250-8.
6. Choy EH, Panayi GS. Cytokine pathways and joint inflammation in rheumatoid arthritis. \textit{N Engl J Med} 2001; 344 : 907-16.
7. Thurlings RM, Wijbrands CA, Bennink RJ, Dohmen SE, Voermans C, Wouters D, et al. Monocyte scintigraphy in rheumatoid arthritis: The dynamics of monocyte migration in immune-mediated inflammatory disease. \textit{PLoS One} 2009; 4 : e7865.
8. Kinne RW, Bräuer R, Stuhlmüller B, Palombo-Kinne E, Burmester GR. Macrophages in rheumatoid arthritis. \textit{Arthritis Res} 2000; 2 : 189-202.
9. Feldmann M, Maini SR. Role of cytokines in rheumatoid arthritis: An education in pathophysiology and therapeutics. \textit{Immunol Rev} 2008; 223 : 7-19.
10. Kinne RW, Stuhlmüller B, Burmester GR. Cells of the synovium in rheumatoid arthritis. Macrophages. \textit{Arthritis Res Ther} 2007; 9 : 224.
11. Holmdahl R, Jansson L, Larsson A, Jonsson R. Arthritis in DBA/1 mice induced with passively transferred type II collagen immune serum. Immunohistopathology and serum levels of anti-type II collagen auto-antibodies. \textit{Scand J Immunol} 1990; 31 : 147-57.
12. Courtenay JS, Dallman MJ, Dayan AD, Martin A, Mosedale B. Immunisation against heterologous type II collagen induces arthritis in mice. \textit{Nature} 1980; 283 : 666-8.
13. Asano K, Matsuishi J, Yu Y, Kasahara T, Hisamitsu T. Suppressive effects of \textit{Tripterygium wilfordii} Hook f. a traditional Chinese medicine, on collagen arthritis in mice. \textit{Immunopharmacology} 1998; 39 : 117-26.
14. Suszko A, Obmińska-Mrukowicz B. Influence of polysaccharide fractions isolated from \textit{Caltha palustris} L. on the cellular immune response in collagen-induced arthritis (CIA) in mice. A comparison with methotrexate. \textit{J Ethnopharmacol} 2013; 145 : 109-17.
15. Kuduk-Jaworska J, Rulk F, Gąsiorowski K. Searching for immunologically active polysaccharides. I preliminary investigations of polysaccharide fractions isolated from \textit{Caltha palustris} L. \textit{Herba Polonica} 1993; 39 : 103-12.
16. Szczypka M, Obmińska-Mrukowicz B. The effects of selevctive and nonselective phosphodiesterase cells inhibitors
on phagocytic cells in mice. *Immunopharmacol Immunotoxicol* 2010; 32 : 507-13.

17. Lis M, Szczypta M, Suszko A, Obmińska-Mrukowicz B. The effects of bestatin on humoral response to sheep erythrocytes in non-treated and cyclophosphamide-immunocompromised mice. *Immunopharmacol Immunotoxicol* 2013; 35 : 133-38.

18. Smolen JS, Landewé R, Breedveld FC, Buch M, Burmester G, Dougados M, *et al.* EULAR recommendations for the management of rheumatoid arthritis with synthetic and biological disease-modifying antirheumatic drugs: 2013 update. *Ann Rheum Dis* 2014; 73 : 492-509.

19. Kim KR, Chung TY, Shin H, Son SH, Park KK, Choi JH, *et al.* Red ginseng saponin extract attenuates murine collagen-induced arthritis by reducing pro-inflammatory responses and matrix metalloproteinase-3 expression. *Biol Pharm Bull* 2010; 33 : 604-10.

20. Luettig B, Steinmüller C, Gifford GE, Wagner H, Lohmann-Matthes ML. Macrophage activation by the polysaccharide arabinogalactan isolated from plant cell cultures of *Echinacea purpurea*. *J Natl Cancer Inst* 1989; 81 : 669-75.

21. Kouakou K, Schepetkin IA, Yapi A, Kirpotina LM, Jutila MA, Quinn MT. Immunomodulatory activity of polysaccharides isolated from *Alchornea cordifolia*. *J Ethnopharmacol* 2013; 146 : 232-42.

22. Neurath MF, Hildner K, Becker C, Schlaak JF, Barbulescu K, Germann T, *et al.* Methotrexate specifically modulates cytokine production by T cells and macrophages in murine collagen induced arthritis (CIA): A mechanism for methotrexate-mediated immunosuppression. *Clin Exp Immunol* 1999; 115 : 42-5.

23. Segal R, Mozes E, Yaron M, Tartakovsky B. The effects of methotrexate on the production and activity of interleukin-1. *Arthritis Rheum* 1989; 32 : 370-7.

24. Lee KY, Lee MH, Chang IY, Yoon SP, Lim DY, Jeon YJ. Macrophage activation by polysaccharide fraction isolated from *Salicornia herbacea*. *J Ethnopharmacol* 2006; 103 : 372-8.

25. Moretao MP, Buchi DF, Gorin PAJ, Iacomini M, Oliveira MBM. Effect of an acidic heteropolysaccharide (ARAGAL) from the gum of *Anadenanthera colubrine* (Angico branco) on peritoneal macrophage functions. *Immunol Lett* 2003; 89 : 175-85.

26. Myers LK, Rosloniec EF, Cremer MA, Kang AH. Collagen-induced arthritis, an animal model of autoimmunity. *Life Sci* 1997; 61 : 1861-78.

27. Hyams JS, Donaldson MH, Metcalf JA, Root RK. Inhibition of human granulocyte function by methotrexate. *Cancer Res* 1978; 38 : 650-5.

28. Omata T, Segawa Y, Inoue N, Tsuzuki N, Itokazu Y, Tamaki H. Methotrexate suppresses nitric oxide production *ex vivo* in macrophages from rats with adjuvant-induced arthritis. Research in experimental medicine. *Res Exp Med (Berl)* 1997; 197 : 81-9.

29. Park SB, Chun KR, Kim JK, Suk K, Jung YM, Lee WH. The differential effect of high and low molecular weight fucoidans on the severity of collagen-induced arthritis in mice. *Phytother Res* 2010; 24 : 1384-91.

Reprint requests: Dr Agnieszka Suszko, Department of Biochemistry, Pharmacology and Toxicology, Faculty of Veterinary Medicine, Wroclaw University of Environmental and Life Sciences, C. K. Norwida 31, 50-375 Wroclaw, Poland e-mail: asushi@o2.pl