

RESEARCH ARTICLE

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Activation of Human T-Helper/Inducer Cell, T-Cytotoxic Cell, B-Cell, and Natural Killer (NK)-Cells and induction of Natural Killer Cell Activity against K562 Chronic Myeloid Leukemia Cells with Modified Citrus Pectin

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Abstract

Background: Modified citrus pectin (MCP) is known for its anti-cancer effects and its ability to be absorbed and circulated in the human body. In this report we tested the ability of MCP to induce the activation of human blood lymphocyte subsets like T, B and NK-cells.

Methods: MCP treated human blood samples were incubated with specific antibody combinations and analyzed in a flow cytometer using a 3-color protocol. To test functionality of the activated NK-cells, isolated normal lymphocytes were treated with increasing concentrations of MCP. Log-phase PKH26-labeled K562 leukemic cells were added to the lymphocytes and incubated for 4 h. The mixture was stained with FITC-labeled active form of caspase 3 antibody and analyzed by a 2-color flow cytometry protocol. The percentage of K562 cells positive for PKH26 and FITC were calculated as the dead cells induced by NK-cells. Monosaccharide analysis of the MCP was performed by high-performance anion-exchange chromatography with pulse amperometric detection (HPAEC-PAD).

Results: MCP activated T-cytotoxic cells and B-cell in a dose-dependent manner, and induced significant dose-dependent activation of NK-cells. MCP-activated NK-cells demonstrated functionality in inducing cancer cell death. MCP consisted of oligogalacturonic acids with some containing 4,5-unsaturated non-reducing ends.

Conclusions: MCP has immunostimulatory properties in human blood samples, including the activation of functional NK cells against K562 leukemic cells in culture. Unsaturated oligogalacturonic acids appear to be the immunostimulatory carbohydrates in MCP.

Background

Pectin is a complex carbohydrate soluble fiber. Dietary fibers, such as pectin, have been shown to have positive effects on a wide spectrum of pathological conditions. Their positive influence on human health is explained by their anti-oxidative, hypocholesterolemic, and anti-cancer effects [1-12]. The effect on the immune system has been previously attributed to the down regulation of the inflammatory response, moderating the production

of pro inflammatory cytokines and immunoglobulins in murine models for irritable bowel syndrome [13]. A diet rich in soluble fiber in an animal model showed protection against endotoxin-induced sickness behavior by cytokine modulation and promotion of alternative activation of macrophages [14]. Citrus pectin has the capacity to exert a favourable immunomodulatory response in human peripheral blood cells through its effect on cytokine production [15]. High methoxy citrus pectin inhibits the binding of fibroblast growth factor-1 (FGF-1) to its receptor in the presence of heparin [16]. The rhamnolacturonan I-arabinan fraction of pectin

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from a medicinal herb enhances secretion of granulocyte colony-stimulating factor (G-CSF) by murine colonic MCE 301 cells [17]. Rhamnogalacturonan I-arabinogalactan was also reported to activate macrophages and dendritic cells [18]. Methyl-esterified pectic oligosaccharides with 4,5-unsaturated non-reducing ends enhanced T-helper1 (Th1) dependent delayed-type hypersensitivity in a murine influenza vaccine model, reduced Th2 cytokine (IL-4, IL-5 and IL-10) production in splenocytes *in vitro* [19] and decreased allergic asthma in mice [20]. Therefore, the carbohydrate composition of pectin is very important in determining different immune responses.

The modified citrus pectin (MCP) used in this study, is composed of short, slightly-branched, carbohydrate chains derived from the soluble albedo fraction of citrus fruit peels altered by decreasing the molecular weight and degree of esterification using pH, temperature and a controlled enzymatic process, in order to increase its absorption into the circulatory system. MCP is relatively rich in galactose, and antagonizes a binding protein galectin-3 (Gal-3), which results in suppression of cancer cell aggregation, adhesion, and metastasis [5,6]. MCP acts as a ligand for Gal3, which plays a major role in tumor formation and progression [12,21-24]. It has been shown using a combination of fluorescence microscopy, flow cytometry, and atomic force microscopy, that specific binding of a pectin galactan to the recombinant form of human galectin-3 has been physically observed [25]. Moreover, MCP also showed anti-metastatic effects on cancer cells *in vitro* or *in vivo* [8,10,11,24,26-28]. Human clinical trials with MCP showed an increase in prostate specific antigen doubling time, a marker of slowing the progression of prostate cancer [9], and significant improvement of quality of life and stabilization of disease for patients with advanced solid tumors [29]. Besides the therapeutic roles against cancer, MCP has been shown to remove toxic metals from the body [30,31], and reduce experimentally induced kidney injury and fibrosis *in vivo* by reducing galectin-3 levels [32]. In the United States of America, MCP is registered as a food supplement and is generally regarded as safe (GRAS).

In vitro lymphocyte activation represents a standard approach for evaluating cell-mediated responses to a variety of stimuli including immunostimulatory botanical extracts. An appropriate assay system monitors the expression of the early activation marker CD69 in whole blood after stimulation with extracts. CD69 is expressed in all activated lymphocyte subsets and hence it represents a generic marker to monitor individual subset responses to specific stimuli [33]. CD4 antigen is expressed on the T- helper/inducer lymphocyte subset (CD3/CD4). CD8 antigen is expressed on the human

cytotoxic T-lymphocyte subset (CD3/CD8). Once activated both CD4 and CD8 positive T cells express CD69. T-lymphocyte subsets can be identified and quantified by using fluorochrome-labelled antibody combinations such as CD4/CD69/CD3 and CD8/CD69/CD3. CD19 antigen is present on human B-lymphocytes at all stages of maturation and is not present on resting or activated T-lymphocytes. CD19/CD69/CD45 labelled antibody combination can be used to identify an activated B cell population. CD56 antigen is present on Natural Killer (NK)-cells and antigen intensity increases with NK-cell activation. Hence, CD56/CD69/CD45 labelled antibody combination can be used to identify activated NK-cells. The ability of NK-cell subset in normal lymphocytes to induce death in leukemia cells is analyzed by co-incubating MCP treated lymphocytes with K562 T-cell leukemia cells. In this report we tested the ability of MCP to induce the activation of human blood lymphocyte subsets (T-helper/inducer, T-cytotoxic, B-cell, and NK-cell), and the induced NK-cell's activity against K562 chronic myeloid leukemia cells. Carbohydrate composition analysis was also performed to propose a structural mechanism of action of this immune enhancement.

Methods

MCP (PectaSol-C[®] MCP, Econugenics, Inc., Santa Rosa, CA, USA) was initially solubilized in phosphate buffered saline (PBS). The solubility of MCP in PBS was 76.4%. The volume was adjusted to get accurate amounts of the compound for treatment based on solubility factor.

T-, B- and NK-cell activation assay: Blood samples were collected from three unidentified healthy volunteers based on an exempt study protocol submitted and approved by the Miami Children's Hospital Institutional Review Board. Blood samples (250 µl) were incubated in 48-well plates with increasing concentrations of compound along with appropriate positive controls (recommended by Becton Dickinson Biosciences, CA, USA) for each subset. CD2/CD2R and Phorbol ester (PMA) were used as controls for T-cytotoxic cell activation studies. Pokeweed (PWM) mitogen was used as positive control for B-cell activation and IL-2 was used for activating NK-cells in blood cultures. The blood cultures were incubated at 37°C in a CO₂ incubator for 24 h. On the next day, 20 µl of specific antibody mix, [CD4-FITC/CD69-PE/CD45-PerCP (T-helper/inducer cell activation), CD8-FITC/CD69-PE/CD3-PerCP (T-cytotoxic cell activation), CD19-FITC/CD69-PE/CD45-PerCP (B-cell activation) and CD56-FITC/CD69-PE/CD45-PerCP (NK-cell activation)] was dispensed into separate flow tubes (in duplicate) and 50 µl blood sample was mixed with antibody and incubated for 30 min at room temperature in the dark. The blood-antibody mix was lysed in a Coulter Epics Q-prep work station using

Immunoprep kit and run on a Coulter Elite flow cytometer using a 3-color protocol. The percentage of activated T-helper cells, T-cytotoxic cells, B-cells and NK-cells and the percentage increase over untreated control were calculated and plotted against compound concentrations.

Functional NK Cell Activity Assay: Normal lymphocytes were isolated from three healthy volunteers using histopaque 1077 gradient centrifugation. The cells were washed twice with PBS and re-suspended in phenol-free RPMI 1640 medium supplemented with 10% fetal bovine serum and antibiotics (complete medium). Cells were plated in special 48-well deep plates (conical bottom) at 10^6 cells/ml/well and treated with increasing concentrations (0-800 ug/ml) of MCP in a 5% CO₂ incubator at 37°C for 24 h. On the next day, log-phase K562 leukemic cells were labelled with PKH26 membrane dye (Sigma, St. Louis, MO, USA) for 5 min according to manufacturer's protocol and 0.2×10^6 cells each were added to the normal lymphocytes in the 48 well plates. The plates were centrifuged for 1 min at 250 × g and returned to the CO₂ incubator for another 4 hours for inducing cell death.

The cell mixture was permeabilized by incubating in 2% paraformaldehyde solution at 4°C followed by incubation in 0.2% Tween 20 solution (PBS) at 37°C. The cell mixture was washed with ice-cold PBS and stained with human specific FITC-labeled active form of caspase 3 antibody for 30 minutes at room temperature according to the procedure published earlier [34,35]. The stained cells were washed with 0.1% Tween 20 solution, resuspended in 0.5 ml of staining buffer and analyzed by a two-color flow cytometry protocol with FL1 and FL2 measuring PKH26 and FITC, respectively in a Beckman Coulter Elite Flow cytometer. The percentage of K562 cells positive for PKH26 and FITC are dead cells induced by NK-cells.

Statistical Analysis: Mean and standard deviation values were calculated and graphs prepared by Microsoft Excel. Kruskal-Wallis one-way Analysis of Variance (ANOVA) was used to analyze the data by the GraphPad Prism 5 software and *p* values were estimated. Multiple comparisons of treatments in all possible combinations were performed by Tukey-Kramer post test using the GraphPad Prism 5 software.

Carbohydrate Composition Analysis of MCP: Monosaccharide analysis was performed by high-performance anion-exchange chromatography with pulse amperometric detection (HPAEC-PAD) following methanolysis according previously published method [36]. HPAEC-PAD was also used for oligosaccharide analysis [32]. Weight average intrinsic viscosity analysis was performed by high-performance size-exclusion chromatography (HPSEC) with multiple detectors (multi-angle

laser light scattering, refractive index and differential pressure viscometer according to previously published methods [30].

Results

T-Lymphocyte Subset Activation

The percent increases in T-helper/inducer lymphocyte and T-cytotoxic cell activation are shown in Figure 1 & 2, respectively. All positive controls induced expected responses. Results show that MCP does not have a significant effect on T-helper/inducer cell activation as compared to positive controls like CD2/CD28 (*p* < 0.01) and PMA (*p* < 0.001). However, MCP activated T-cytotoxic cells at lower levels and in a dose-dependent manner between 50-800 ug/ml concentrations. MCP-induced T-cytotoxic cell activation was significant at 400 ug/ml (*p* < 0.05) and highly significant at 800 ug/ml (*p* < 0.01) concentrations.

B-Cell Activation

The percentage increase of MCP on B-cell activation over untreated control is given in Figure 3. The positive control PWM induced highly significant activation at 10 ug/ml (*p* < 0.01) and 25 ug/ml (*p* < 0.001) concentrations. MCP induced a dose-dependent activation of B-cells, although the level of activation was lower than that of PWM and not significant by Tukey-Kramer test.

NK-Cell Activation

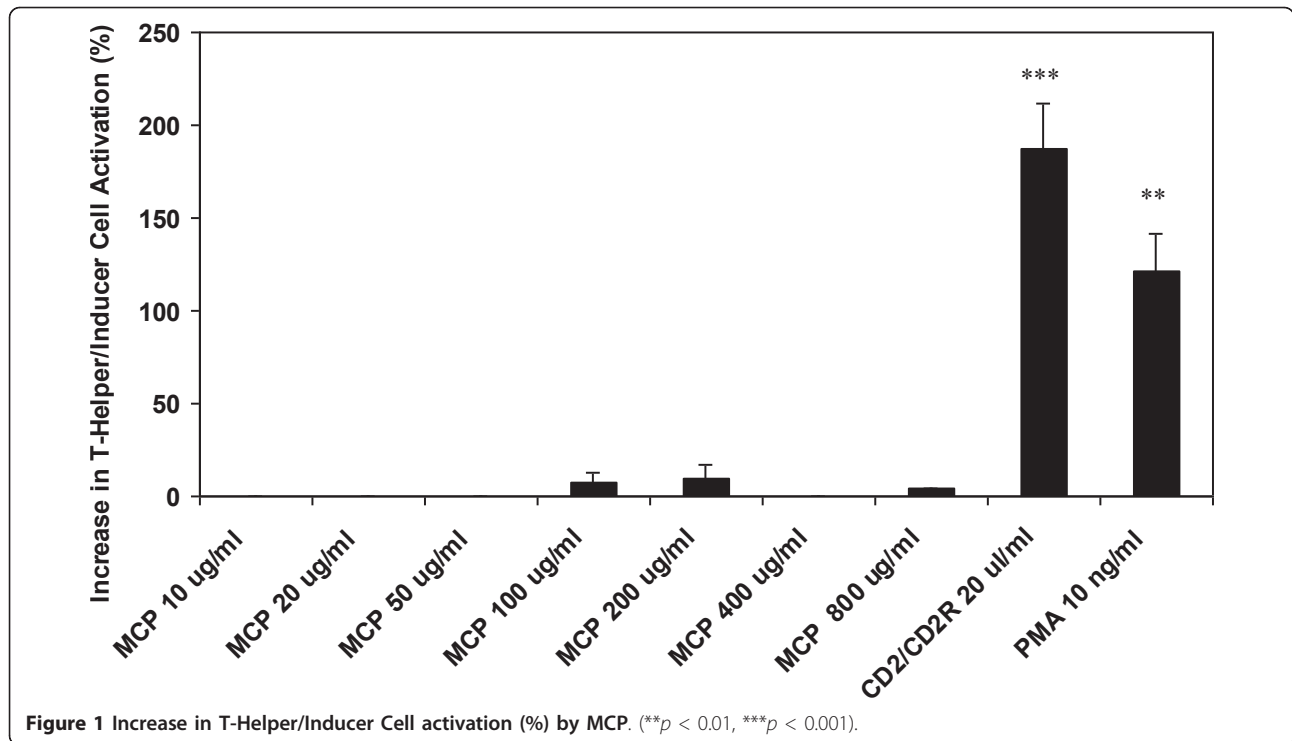
The effects of MCP on the percentage increase of NK-cell activation over untreated control is given in Figure 4. The positive control IL-2 at 6.6 ng/ml induced a significant level of NK-cell activation (*p* < 0.05). MCP demonstrated a dose-dependent activation of NK-cells with a lower significance level (*p* < 0.05) attained at 200 ug/ml and a highly significant level at 400 and 800 ug/ml concentrations (*p* < 0.01).

MCP activated NK-Cell activity on K562 Chronic Myeloid Leukemia Cells

The results of MCP-treated blood samples on increase in percent of functional NK-cell activity is shown in Figure 5. MCP induces NK-cell activity in a dose-dependent manner with 800 ug/ml dose causing about 53.60% increase in NK-cell activity (*p* < 0.05). The dose-dependent increasing trend in NK-cell activity apparently corresponds with the activation of NK-cells obtained by staining with NK-cell activation markers (CD56/CD69/CD45).

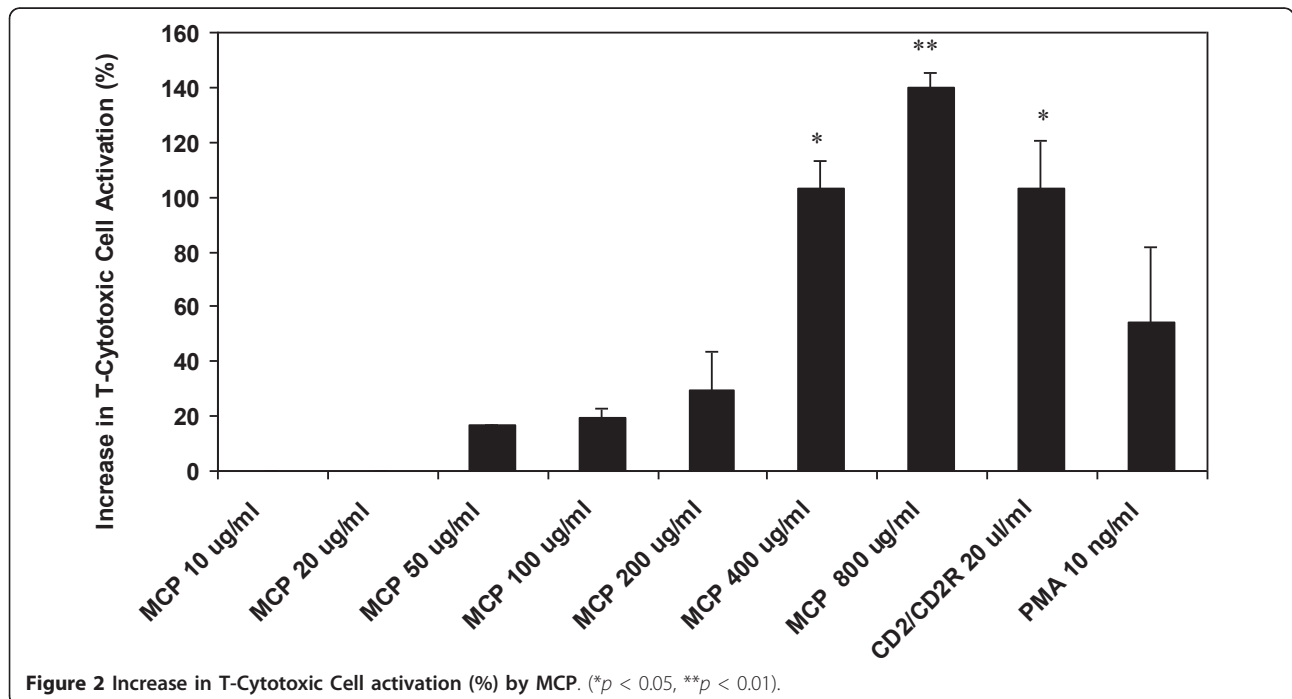
MCP Carbohydrate Composition Analysis

The monosaccharide composition of the MCP was identified and is reported in Table 1. The MCP consisted of mainly galacturonic acid with galactose and arabinose,



the most common neutral sugars. The MCP had a high amount of homogalacturonan compared to rhamnogalacturonan (high GalA/Rha ratio). On average the homogalacturonan backbone of the MCP was interrupted with a rhamnose residue once every 22 galacturonic acid residues (Table 1). Arabinan, galactan or

arabinogalactan neutral sugar side-chains in pectin are attached to the rhamnose residues in rhamnogalacturonan. The homogalacturonan consisted of both saturated and unsaturated oligogalacturonic acids (Figure 6). The unsaturated oligogalacturonic acids had longer retention times compared to saturated oligogalacturonic acids



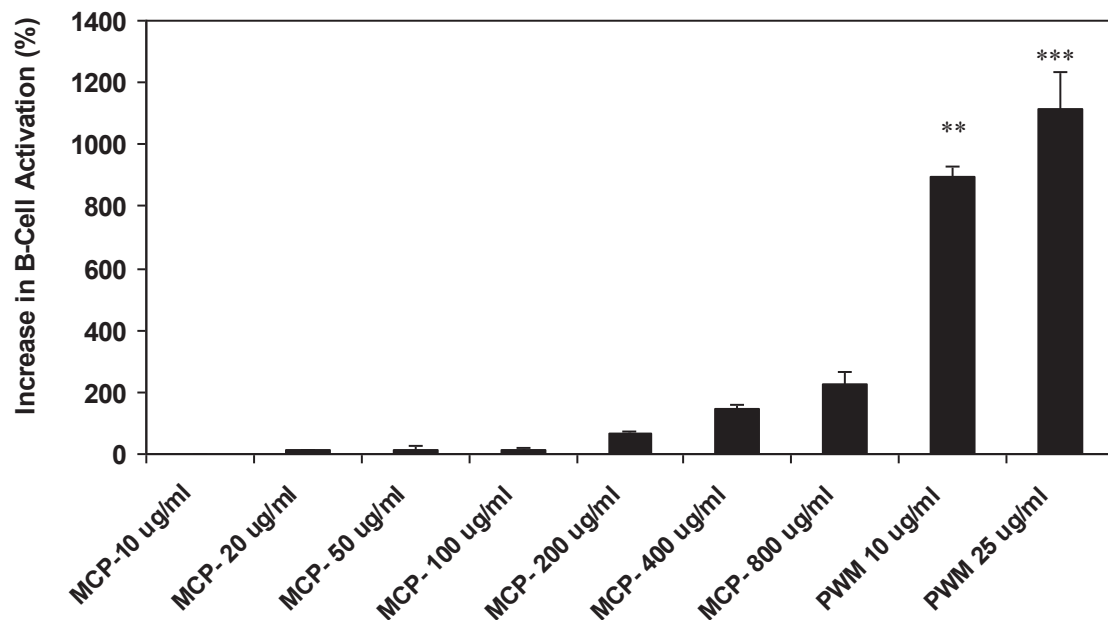


Figure 3 Increase in B-Cell activation (%) by MCP. (** $p < 0.01$, *** $p < 0.001$).

[36]. The MCP average molar mass (3,320 Da), average intrinsic viscosity (0.0648 dL/g), and Mark Houwink constant ($a = 0.824$) are reported (Table 2).

Discussion

The immune system protects people from disease causing microorganisms and other harmful materials. Circulating blood transports immune components between

organs of the immune system and sites of inflammation. The enumerative (activation marker assays) and functional assays (NK cell activity assay) used in the present study to evaluate the immunostimulative potential of MCP clearly demonstrated activation of T cytotoxic and NK cells in blood cultures *in vitro*. Furthermore, functional assay indicated that MCP enhanced cytotoxicity of leukemic cells by NK cells. It is well known that NK cell

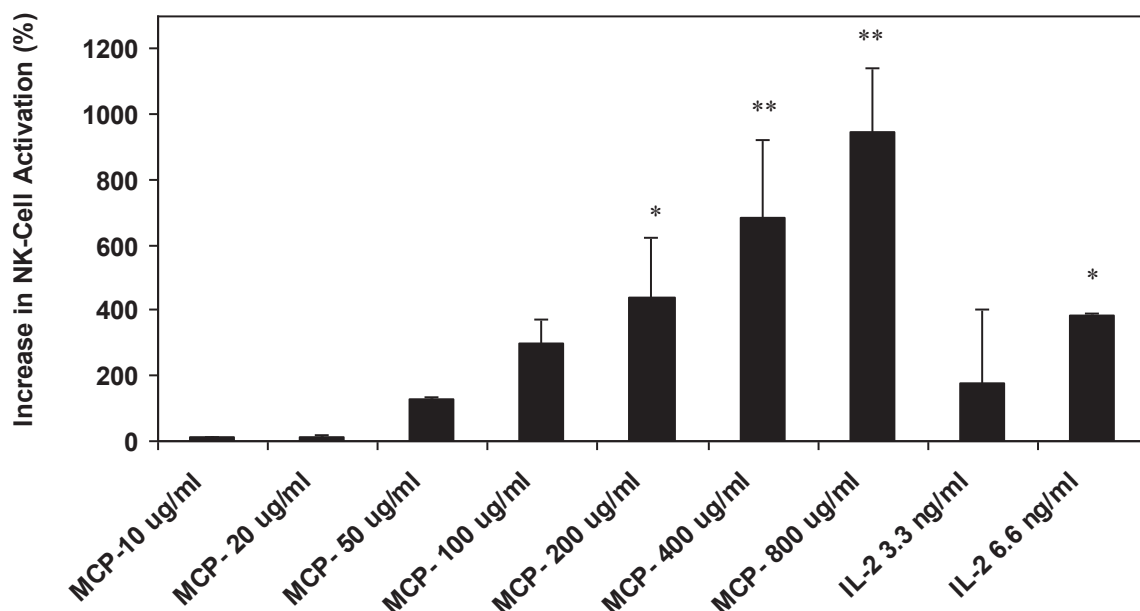


Figure 4 Increase in NK-Cell activation (%) by MCP. (* $p < 0.05$, ** $p < 0.01$).

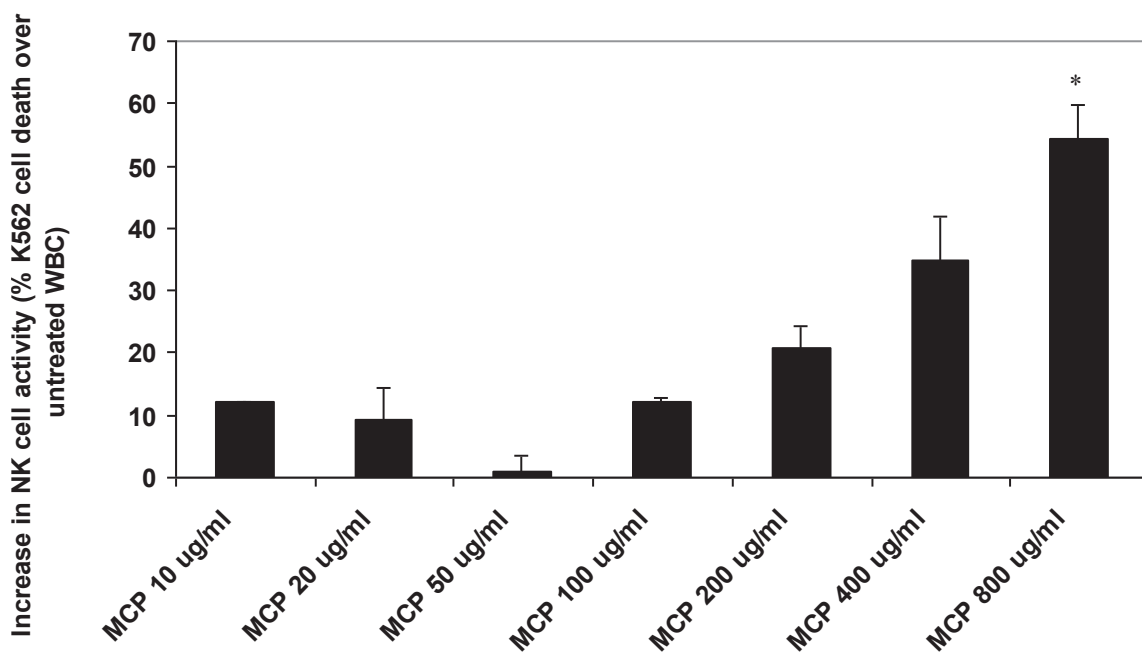


Figure 5 Increase in Percent NK-Cell activity by MCP. (* $p < 0.05$).

activity is regulated by the expression levels of cytotoxic molecules, activating receptors and/or inhibitory receptors. Even though several CAM agents have been shown to activate NK-cells and improve NK cell activity, the precise mechanism is not clearly defined. The two main possibilities are: (i) augmentation of cytotoxic molecules in NK cells and/or (ii) up-regulation of activating NK receptors and/or down-regulation of inhibitory NK receptors [37,38].

The mechanism for selective immunostimulation of T cytotoxic and NK cells by MCP can only be speculated. It is suggested that, except for a lower degree of esterification, the MCP pectic oligosaccharide composition and molecular weight is quite similar to other pectic oligosaccharides reported to polarize the T-helper response toward Th1 in a murine influenza vaccination model [19] and those used in subsequent studies of murine

allergic asthma [20] and human infant formula feeding [39]. Therefore, unsaturated pectic oligosaccharides appear to be required for immunostimulation and a relatively high degree of esterification may be necessary for stimulation of T-helper cells.

The average intrinsic viscosity of pectin is directly proportional to the molar mass, thus demonstrating a lower molecular weight range of the MCP compared to 14,800 Da and 0.398 dL/g reported previously [30]. The lower Mark Houwink constant for MCP compared to the 1.45 value that we observed previously [30] means that the global structure has more random coil shape typical of citrus pectin, while a higher Mark Houwink constant (a) was interpreted as being more rigid and rod like. Therefore, MCP is a flexible low molecular weight pectin polymer enriched in saturated and unsaturated oligogalacturonic acids.

MCP doses used previously in pre-clinical studies *in vivo* (0.1% up to 4.0% orally administered) and human clinical studies (15 grams/day orally administered) have demonstrated an effect on both tumor growth, angiogenesis, cancer progression markers, and spontaneous metastasis [8-10,24,28,29] and the lowering of body burden of heavy metals as seen in their blood and urinary excretion levels [30,31]. These doses had an effect on galectin-3 levels in experimentally induced kidney injury which lead to reduced renal injury and fibrosis [32]. MCP concentrations used in the present investigation (up to 0.08%) are below the previously used doses in pre-clinical studies, which may be achievable and

Table 1 MCP Monosaccharide Composition (Mole %)

Monosaccharide	Mole %
Glucose	2.17
Arabinose	3.28
Galactose	10.31
Xylose	1.45
Rhamnose	3.53
Fructose	0.13
Galacturonic Acid	79.03
Glucuronic Acid	0.11
Galacturonic Acid/Rhamnose	22.39

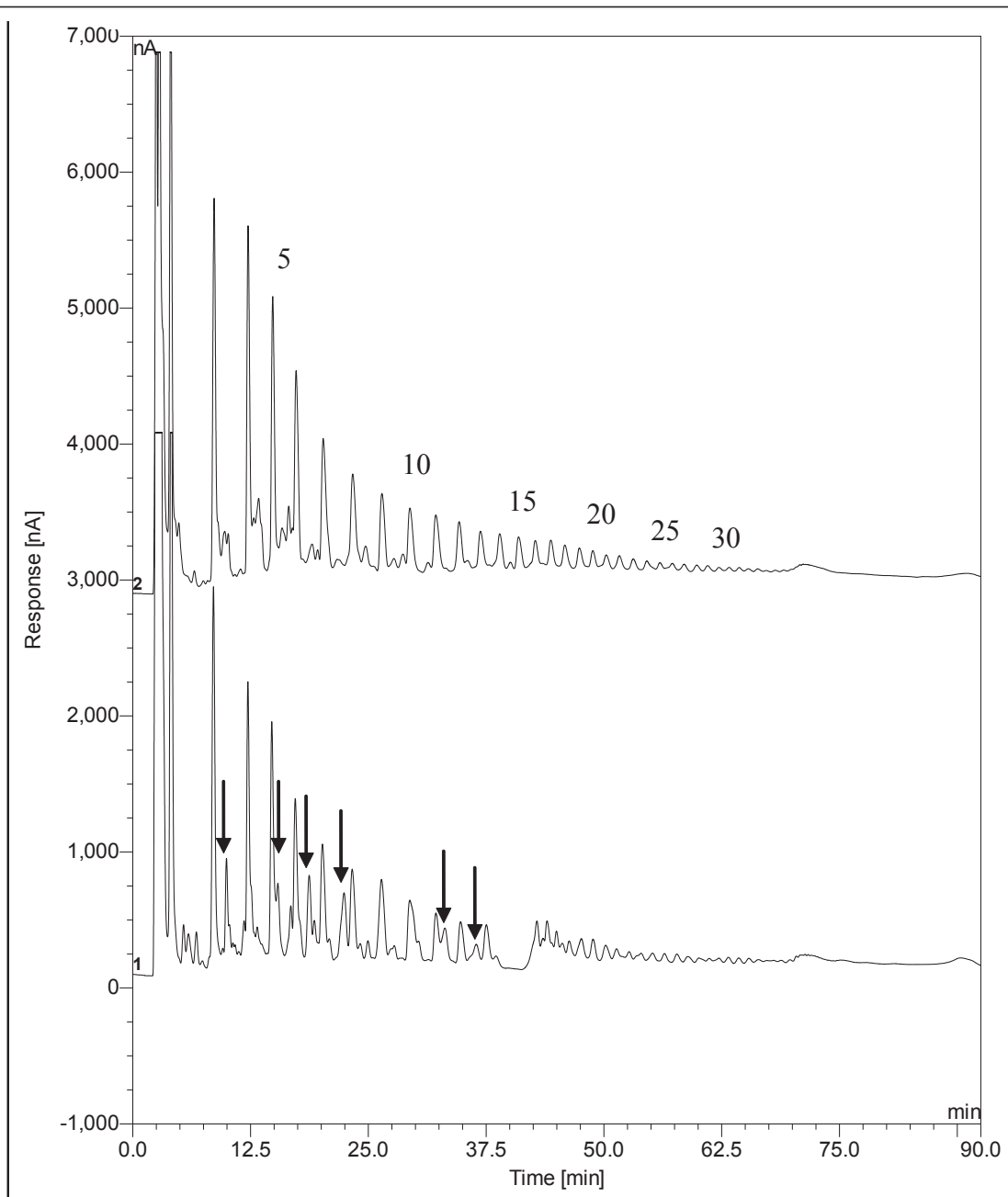


Figure 6 HPAEC-PAD analysis of MCP (1) compared to a polygalacturonic acid hydrolysate (2). The degree of polymerization is listed over the peaks. The arrows point to unsaturated oligogalacturonic acids.

Table 2 MCP Average Molecular Weight, Intrinsic Viscosity and Shape Analysis (Mark Houwink Constant)

Average Molar Mass (Da)	Average Intrinsic Viscosity (dL/g)	Mark Houwink Constant (<i>a</i>)
3,320 ± 300	0.0648 ± 0.001	0.824 ± 0.03

Values are mean ± SD

effective for modulation of immune system. However, detailed pharmacokinetic investigations of MCP by analysing the *in vivo* absorption and distribution are required to determine its effective oral dosage.

Conclusions

The data shows MCP as a substance with immunostimulatory properties in human blood samples, including the activation of functional NK cells against K562 leukemic cells in culture. The selective immunostimulatory properties are proposed to be attributed to the presence of low degree of methyl esterification and unsaturated oligogalacturonic acids. Additional research is necessary to determine if changing the degree of esterification of oligogalactouronic acid in MCP can alter the immune response. *In vivo* studies are necessary to better understand the applications of MCP as an immune enhancer.

List of abbreviations

DMSO: Dimethyl sulfoxide; MCP: Modified citrus pectin; NK: Natural killer; PBS: Phosphate buffered saline; SD: Standard deviation.

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Authors' contributions

CR designed and contributed to the interpretation of the immunological data. SJM designed, acquired data, performed analysis and interpretation of immunological data. AH designed, acquired data, performed analysis and interpretation of the molecular analysis data. HC acquired data, performed analysis and interpretation of the molecular analysis data. BJW and IE helped in the study design, data review, and interpretation of all data. All authors contributed in the drafting of the manuscript, contributing critically in its intellectual content, and have read and given their approval of the final manuscript.

Competing interests

Authors CR, AH, HC, and SJM declare that they have no competing interests. The authors BJW and IE are employed by EcoNugenics, Inc., the makers of the MCP used in this study. IE holds patents on the method and use of MCP.

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