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Cranberry (*Vaccinium macrocarpon*) Proanthocyanadin Complexes with Proteins Modulate the Macrophage Activation

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Abstract

In this work we characterize the interaction of cranberry (*Vaccinium macrocarpon*) proanthocyanidins (PAC) with bovine serum albumin (BSA) and hen egg-white lysozyme (HEL) and determine the effects of these complexes on macrophage activation and antigen presentation. We isolated PAC from cranberry and complexed the isolated PAC with BSA and HEL. The properties of the PAC-protein complexes were studied by matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS), gel electrophoresis and zeta-potential. The effects of PAC-BSA complexes on macrophage activation were studied in RAW 264.7 macrophage like cells after treatment with lipopolysaccharide (LPS). Fluorescent microscopy was used to study endocytosis of PAC-BSA complexes. The effects of PAC complexes on macrophage antigen presentation was studied in an in vitro model of HEL antigen presentation by mouse peritoneal mononuclear cells to a T-cell hybridoma. Mass spectra of PAC complexes with BSA and HEL differed from spectra of the proteins alone by the presence of broad shoulders on the singly and doubly charged protein peaks. Complexation with PAC altered the electrophoretic mobility shift assay in native agarose gel and the electrophoretic mobility (ζ-potential) values. These results indicate that the PAC-protein complexes are stable and alter protein structure without precipitating the protein. Fluorescent microscopy showed that RAW 264.7 macrophages endocytosed BSA and PAC-BSA complexes in discrete vesicles that surrounded the nucleus. Macrophages treated with increasing amounts of PAC-BSA complexes had significantly reduced COX-2 and iNOS expression in response to treatment with lipopolysaccharide (LPS) in comparison to controls. PAC-HEL complexes modulated antigen

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uptake, processing and presentation in murine peritoneal macrophages. After 4 h of pre-incubation, only trace amounts of IL-2 were detected in the co-cultures treated with HEL alone, whereas a PAC-HEL complex had already reached maximum IL-2 expression. Cranberry PAC may increase rate of endocytose of HEL and subsequent expression of IL-2 by the T-cell hybridomas. These results suggest that PAC-protein complexes modulate aspects of innate and acquired immune responses in macrophages.

Keywords

Cranberry, proanthocyanidins, protein complexes, macrophage activation.

1. Introduction

Research in our laboratory and others has demonstrated that cranberry proanthocyanidins (PAC), are bioactive in cell culture and in vitro models of disease processes related to microbial adhesion, oxidation and inflammation. Research with animal models, clinical and epidemiological studies indicate that consumption of cranberries is associated with decreased risk of cancer and cardiovascular disease [1]. However, PAC have low bioavailability and putative mechanisms of bioactivity are poorly understood. Ability of PAC to complex with proteins is the most important aspect of their nutritional and health effects and may provide insight regarding their bioactivity. Formation of soluble PAC-protein complexes has previously been demonstrated using size exclusion chromatography (SEC), or nuclear magnetic resonance (NMR) [2-7] but SEC cannot provide accurate information on the molecular weights and stoichiometry of the complexes and size of protein is a limiting factor for high-resolution NMR analysis. Mass spectrometry techniques that use "soft" ionization are an alternate approach for examining non-covalent interactions between proteins and ligands [8-11]. Matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) and electrospray ionization mass spectrometry (ESI-MS), were successfully used to characterize protein-tannin interactions.[12-15] Attenuation of macrophage activation by uptake of PAC protein complexes may be an alternative explanation for the anti-inflammatory effects of PAC. In this paper, cranberry PAC were complexed with bovine serum albumin (BSA) and hen egg-white lysozyme (HEL) in order to study the effects of PAC-protein complexes on macrophages in cell culture. The effect of

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PAC-BSA complexes on LPS-induced activation of murine macrophage cells was measured by expression of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS). The effects of complexes of PAC with HEL on antigen presentation by mouse peritoneal macrophages to a T-cell hybridoma line was measured by IL-2 expression. Our hypothesis was that macrophage endocytosis of PAC-protein complexes modulates subsequent macrophage activation in response to LPS, in the case of the PAC-BSA complex, or antigen presentation to T-cells, in the case of the PAC-HEL complex.

2. Materials and methods

2.1. Isolation and characterization of the PAC fraction

Oligomeric PAC (degree of polymerization 2 to 11 with a high content of A-type interflavan bonds)[14] were isolated from cranberry juice powder, (CJP) prepared for the National Institute of Health-National Center for Complementary and Alternative Medicine (NIH-NCCAM) program entitled "Cranberries: Urinary Tract Infections and other Conditions" (data not shown). CJP was reconstituted in H2O and applied to a preparative LH-20 column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) equilibrated in water. The column was eluted sequentially with water, ethanol, ethanol:methanol (1:1 v/v) and methanol to remove hydroxycinnamic acids, anthocyanins, and flavonols. The resin was then eluted with aqueous acetone (70% v/v), until the column was white, to recover PAC. Finally, the aqueous acetone fraction was concentrated by vacuum to remove the acetone, and its gallic acid equivalent (GAE) was calculated by the Folin-Ciocalteau assay (GAE = 33.4 mg GAE/mL).

2.2. Protein-PAC complexation and characterization

PAC were complexed to proteins (BSA or HEL) at different PAC to protein ratios (0.5:1.0, 1.0:1.0, 1.5:1.0, and 2.0:1.0, wt:wt), aqueous acetic acid (1% v/v) was used for dilutions. Samples were mixed under continuous stirring for 30 minutes and kept under refrigeration for further characterization.

Native agarose gel electrophoresis was carried out using a submerged horizontal electrophoresis tank and voltage unit (BioRad Protean II). The horizontal 0.7% agarose gel (8 cm x 5.5 cm x 3 mm) was prepared in Buffer A (25 mM Tris–HCl, pH 8.5, 19.2 mM glycine) and the comb

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placed in the center of the gel. The gel was submerged in a reservoir containing Buffer A and electrophoresis was performed at a constant voltage of 60 V for 2 h at room temperature. The samples (5 mg) were mixed 1:1 with Buffer B (20% glycerol, 0.2% Bromophenol blue, 0.12 M Tris base) prior to loading. Gels were stained in 0.12% Coomassie brilliant blue R, 45% methanol, 10% acetic acid for 30 min and destained in 45% methanol, 10% acetic acid and dried between two layers of cellophane membrane. Shift was calculated as percentage of the migration values of the main protein and the protein-tannin complexes, as follows:

$$Shift\ (\%) = \frac{Protein\ gel\ migration\ (cm) - [PAC\text{-}Protein]\ gel\ migration\ (cm)}{Protein\ gel\ migration\ (cm)} \times 100$$

Electrophoretic mobility measurements (ζ–potential) were carried out with a ZetaPlus instrument (Brookhaven Instruments Corporation, New York, USA). The samples were obtained as stated above and afterwards diluted to 1:10. All the dilutions were prepared using an aqueous solution with the same ionic strength (10^{-5} M NaCl). Five samples were prepared for each protein-tannin molar ratio. The error was the highest standard deviation for the five samples. All the ζ -potential values were approximated by the Smoluchowski's equation, using the following values: $\varepsilon_o = 8.9\times10^{-12}$ Fm^{-1} and $\varepsilon_r = 79$.

2.3. MALDI–TOF MS analysis

Mass spectra were collected on a Bruker Reflex II-MALDI-TOF mass spectrometer (Billerica, MA) equipped with delayed extraction and N2 laser (337 nm). Positive linear mode was used to characterize proteins and PAC-protein complexes. Spectra were the sum of 100-300 shots using trans-3-indoleacrylic acid (t-IAA; 5 mg/100 μL 80% aqueous acetone; Aldrich Chemical Co., Milwaukee, WI) as the matrix. Spectra were calibrated with bradykinin (1060.6 MW, Sigma Chemical Co., St. Louis, MO) as an external standard.

2.4. Macrophage Activation Response

RAW 264.7 macrophage like cells (American Type Culture Collection, Manassas, VA) were maintained at 37 ˚C and 5% CO_2. Cells for experiments were transferred to 24-well plates and grown to confluence. Experiments were carried out in DMEM without phenol red, supplemented

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with 100 units/mL penicillin/100ug/mL streptomycin, 2mM L-alanyl-L-glutamine and 0.5% FBS. Experiments consist of a negative and positive LPS (100 ng/mL media, Sigma Chemical, St. Louis, MO) control, and four PAC-BSA ratios (0.5:1.0 to 2.0:1.0). PAC-BSA complexes were isolated using a spin filter (cut off 3,000). PAC-BSA complexes were retained, removed and added to the cell culture. The media was removed and replaced with media containing no LPS and no PAC-BSA complex, LPS alone, or LPS with a gradient of PAC-BSA complexes, and incubated for 4 hours. Media was removed and cell viability was assessed by trypan blue, visual observation, or colorimetrically with a Dojindo CCK8 assay (Dojindo Molecular Technologies, Kumamoto, Japan, measuring cell metabolism by NADH/NADPH reduction of a tetrazolium salt). The cells were prepared for Western blot by removing the media rinsing the cells with PBS rinse, followed by cell lyses with RIPA buffer plus Pierce HALT protease inhibitor (Pierce Biotechnology, Rockford, IL). Protein concentration was measured with a BioRad Bradford protein assay (BioRad Laboratories, Hercules, CA). An amount of 30-50 ug of protein equivalents was loaded onto a SDS-PAGE (sodium dodecylsulfate-polyacrylamide gel electrophoresis) gel and separated by electrophoresis. Proteins were transferred to a 0.45 μm membrane (PVDF, Osmonics, Westborough, MA). The percentages of COX-2 and iNOS were detected with polyclonal primary antibodies (Santa Cruz, Santa Cruz, CA), measured by chemiluminescence (Pierce SuperSignal West Pico reagent and x-ray film) and quantified (BioRad Quantity One analysis software).

Uptake studies were conducted on RAW 264.7 murine macrophages cultured in 35 mm glass bottom culture plates (P35G-1.0-14-C, MatTek Corp., Ashland, MA 01721) and treated with BSA alone and PAC-BSA complexes. Subsequent proteolysis of the PAC-BSA complexes in the endosomes was studied by fluorescent microscopy of proteins labeled by a quenched BODIPY dye conjugate (A-20181, Molecular Probes, Eugene, OR), which only fluoresce after proteolysis, and fluorescence was proportional to proteolysis. BSA was labeled according to kit instructions and subsequently mixed with appropriate ratios of PAC, as previously described. Macrophages were incubated with the labeled proteins and their PAC complexes for 0.25-8 hours and imaged with a Zeiss fluorescent microscope (Carl Zeiss Microimaging, Thornwood, NY 10594, with 450-490 nm excitation and a 510-565 nm emission filters). The microscope was fitted with a chamber to maintain the cells at 37°C and 5% CO_2.

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2.5. Macrophage Immune Response

HEL antigen uptake, processing and presentation studies were conducted in mouse macrophages according to previously describe methods.[15] Briefly, mouse peritoneal macrophages were isolated from 6-8 week B10.Br, I-Ak haplotype female mice (Jackson Laboratories, Bar Harbor, Maine), the mice were stimulated by an interperitoneal injection of LPS. Cranberry PAC were mixed with HEL at different PAC to protein ratios (0.5:1.0 to 2.0:1.0), aqueous acetic acid 1% v/v was used for dilutions. The PAC-HEL complexes were stored under refrigeration at 4 °C for further analysis. For experiments, media was removed from the macrophages and rinsed once with PBS. Media for the macrophages supplemented with HEL and PAC-HEL complexes was added and incubated for 0.25–4.00 h. The media was removed and cells washed once with PBS. The T-cell hybridomas (concentration equals 2x the number of macrophages) in 3A9 media (without phenol red) were added to the macrophages and incubated (24 h). Media was removed, placed in microfuge tubes, centrifuged for 3–4 min at 10,000 rpm and IL-2 was measured by ELISA kit (cat. no. 555148, BD Biosciences, San Diego, CA). Data was expressed as pg IL-2/mL media. Macrophage cell viability was assessed with a cell counting kit (kit-8, CCK-8, CK04-11, Dojindo Molecular Technologies, Gaithersburg, MD). Viability was based on the reduction of a tetrazolium salt by NADH/NADPH. Upon removal of the 3A9 cells, the macrophages were rinsed once with PBS and 0.4 mL media (25 uL CCK-8/mL) was added and incubated 0.5–2.0 h. The media was removed to a 96 well plate and read at 450nm on a plate reader. Data was expressed as absorbance/mL media.

Uptake studies were conducted on mouse peritoneal macrophages cultured in 35 mm glass bottom culture plates (P35G-1.0-14-C, MatTek Corp., Ashland, MA 01721) and treated with the PAC-HEL complexes. Endocytosis of the HEL and PAC-HEL complexes was studied by fluorescent microscopy with both labeled HEL and PAC. HEL was labeled with rhodamine dye (Rhod, NHS Rhodamine Labeling Kit, Invitrogen/Molecular Probes, Eugene, OR) according to kit instructions and cranberry PAC was labeled with 5-([4,6-dichlorotriazin-2-yl] amino) fluorescein (DTAF, Invitrogen, Carlsbad, CA), according to previously described methods.[16] After labeling, PAC/DTAF was mixed with HEL/Rhod at a 1.0:1.0 ratio, as previously described. Macrophages were incubated with the labeled PAC/DTAF-HEL/Rhod (1.0:1.0) complex and imaged with a Zeiss fluorescent microscope (Carl Zeiss Microimaging,

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Thornwood, NY 10594, with 450-490 nm excitation and a 510-565 nm emission filters). The microscope was fitted with a chamber to maintain the cells at 37 °C and 5% CO_2.

The study complied with all institutional and national guidelines, as per the Laboratory Animal Welfare Public Health Service Assurance (A3368-01), the protocol was approved by University of Wisconsin-Madison College of Agriculture and Life Sciences (CALS) Animal Care and Use Committee (IACUC #AO-1331).

2.6. Statistical analysis

Statistical analysis was performed using commercial software (AssistatVR) (Statistics, Arlington, TX). The iNOS, COX-2 and IL-2 results are presented as mean ± SD values. To compare the control group and experimental groups, the data were analyzed by generalized linear model followed by LSM (SAS; Cary, NC). The differences were considered statistically significant at $P < 0.05$.

3. Results and discussion

We isolated PAC from cranberries and studied their interactions with hen egg-white lysozyme (HEL) and bovine serum albumin (BSA) (Fig. 1). We then determine the effects of these PAC-Protein complexes on macrophage endocytosis, activation and immune response and presentation of antigen. Our overall hypothesis was that cranberry PAC complex with proteins in the food matrix and gut and these PAC-Protein complexes modulate gut macrophage response to luminal antigens and pathogen associated molecular patterns.

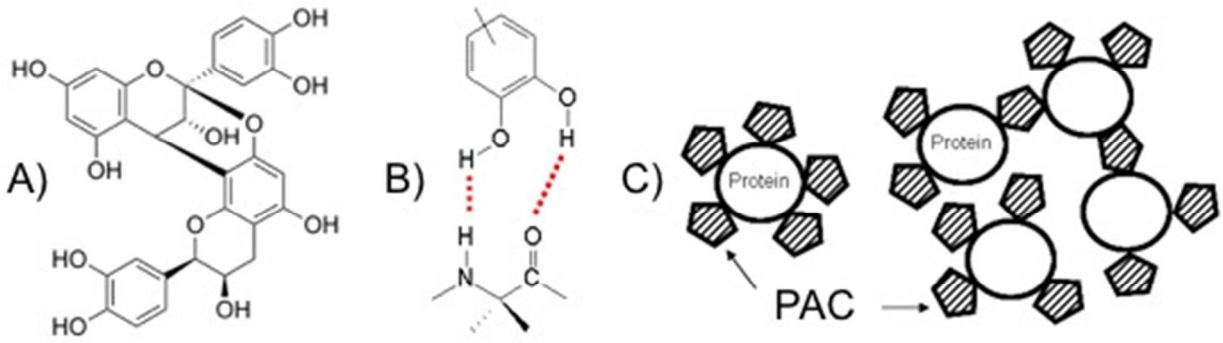

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Fig. 1 Schematic illustrative representation of A) the chemical structure of a cranberry proanthocyanidin (PAC) monomeric unit showing an A-Type interflavan bond; B) the proposed mechanism of interaction between PAC and proteins, based on hydrogen bonding, and C) the proposed structure of PAC-Protein complexes [8].

3.1. Characterization of PAC-Protein complexes

Structural characteristics of the cranberry PAC was determined by MALDI-TOF MS which showed that they had a degree of polymerization (DP) ranging from 4 to 7 with at least one A-type interflavan bond for each oligomer (data not shown).[14]

The formation of cranberry PAC-protein complexes may explain the effects of PAC on nutrition and health.[17] Unlike other antioxidants that are water-soluble (e.g., ascorbic acid) or lipid-soluble (e.g., tocopherols), PAC bind proteins in soluble or precipitated complexes.[3, 11] This phenomenon is responsible for the astringency of fruits, like cranberry, and fruit juices.[5, 10] Four mechanisms for interactions between proteins and PA have been postulated; covalent, ionic, hydrogen bonding and hydrophobic interactions.[11] The most frequent interaction involves hydrogen bond formation between protein amide carbonyl and phenolic hydroxyl groups. The aromatic portion of the polyphenol may interact hydrophobically with nonpolar amino acid side chains, such as phenylalanine.[6-8] When BSA was complexed with cranberry PAC (1.0:1.0), the complex was detected in the MALDI-TOF MS as a distinct shoulder at m/z 68.667 on the singly charged BSA peak at m/z 66.682 and at m/z 34.516 on the doubly charged BSA peak at m/z 33.442 (Fig. 2A and 2B). The appearance of this shoulder is difficult to interpret. According to previous publications the shoulder reflects the complex distribution of the PAC fraction.[12-14] The range of oligomers that are present in the cranberry PAC fraction and the high mass of BSA makes it difficult to detect specific PAC-Protein complexes in the spectrum, but the appearance of the shoulder is associated with the formation of a stable protein complex. The spectrum for the PAC-HEL complex showed greater resolution because HEL has a lower molecular weight than BSA and its tertiary structure is less globular (Fig. 2A and 2B). The spectrum shows masses that correspond to the singly charged HEL at *m/z* 14.182 and complexes with a more defined shoulder at higher masses (marked with black arrows). This pattern was repeated for the doubly charge HEL peak at *m/z* 7.104 with the appearance of the PAC shoulder as well.

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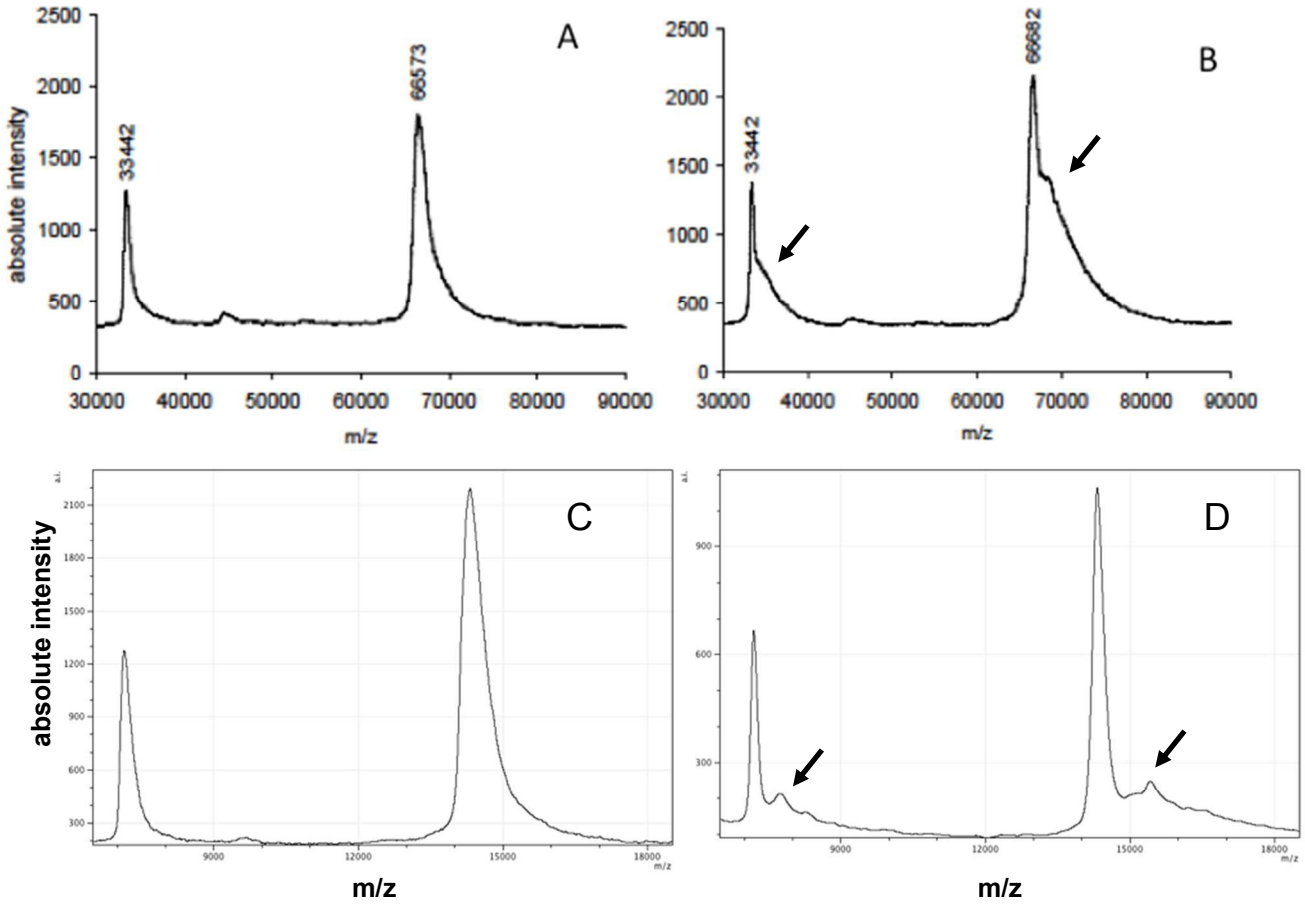

Fig. 2 MALDI-TOF MS spectrum for A) BSA (1 mg/mL), B) PAC-BSA complex (1.0:1.0), C) HEL (1 mg/mL) and D) PAC-HEL complex (1.0:1.0). Arrows indicate the distinct shoulder on the single charged protein peak (BSA and HEL) peak and on the doubly charged protein peak.

Complexation to PAC changed the electrophoretic mobility shift assay in native agarose gel (EMSA-NAGE, Fig. 3A) of bovine serum albumin (BSA), and hen egg-white lysozyme (HEL). Proteins with a pI lower than the buffer pH (BSA, pI = 4.9) carry a net negative charge and migrate toward the anode, whereas proteins with a pI higher than the buffer pH (HEL, pI = 11.0) carry a positive charge and migrate toward the cathode. Higher ratios of PAC in the PAC-HEL complexes did not migrate as far as HEL. BSA and PAC-BSA migrated towards the anode and the PAC complexes migrated further than BSA.

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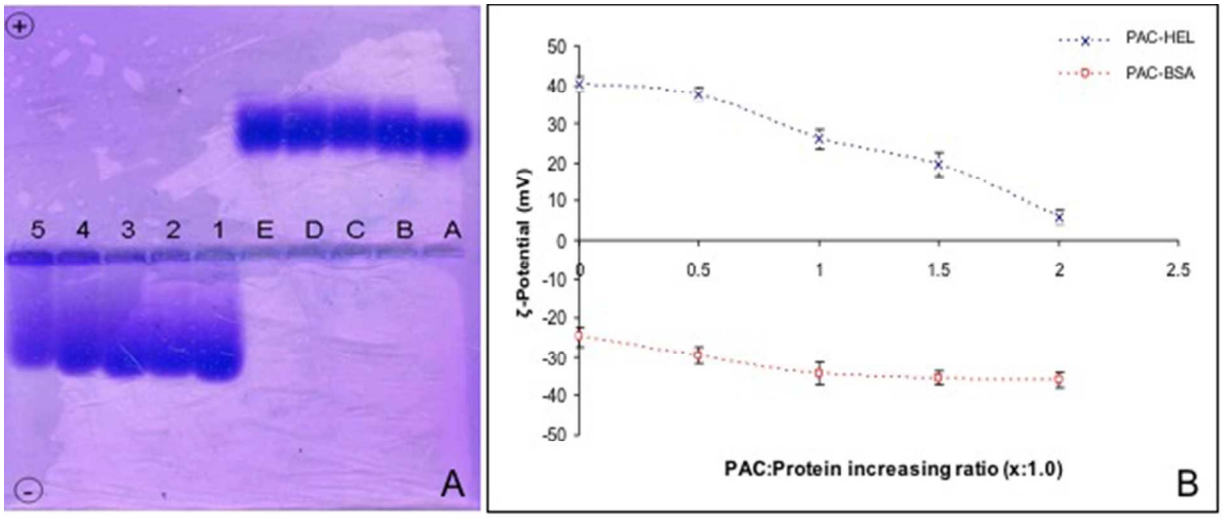

Fig. 3 Characterization of PAC-Protein complexes. A) Native agarose gel electrophoretic mobility shift (EMSA-NAGE) profile of PAC-protein complexes [1- HEL, 2- PAC-HEL (0.5:1.0), 3- PAC-HEL (1.0:1.0), 4- PAC-HEL (1.5:1.0), 5- PAC-HEL (2.0:1.0), A- BSA, B- PAC-BSA (0.5:1.0), C- PAC-BSA (1.0:1.0), D- PAC-BSA (1.5:1.0), E- PAC-BSA (2.0:1.0)]. B) Effect of the increasing PAC ratio on the ζ-potential of PAC-Protein complexes [mean ± SD, n=5; T=25°C, pH=8.5].

Analysis of the EMSA-NAGE of the PAC-Protein complexes (Table 1) showed positive shift values for the PAC-HEL complexes, suggesting that PAC complexation decreased the net positive charge of HEL. The PAC-HEL (2.0:1.0) complex showed a 15% shift, when compared to HEL alone. On the other hand, PAC-BSA complexes showed negative shift values, as indicated by increased mobility toward the anode and an increase in the negative net charge. The PAC-Protein migration profiles indicate that there are specific ion-dipole interactions between protonated amino groups of HEL and PAC hydroxyl groups, in addition to hydrogen bonding. In the case of BSA, these interactions seem to be lower than for HEL and this difference may be due to the globular nature of BSA and its higher molecular weight. Results also suggest that PAC-protein interactions were dependent on the molar ratio of PAC to protein in the complex.

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Table 1 Electrophoretic mobility shift assay values for cranberry proanthocyanidin complexes with bovine serum albumin (BSA) and hen egg-white lysozyme (HEL).

Sample	Migration on Agarose Gel (cm)	Shift (%)
HEL	2.65 ± 0.05	0.00
PAC-HEL (0.5:1.0)	2.51 ± 0.10	5.28
PAC-HEL (1.0:1.0)	2.44 ± 0.25	7.92
PAC-HEL (1.5:1.0)	2.32 ± 0.10	12.45
PAC-HEL (2.0:1.0)	2.25 ± 0.20	15.10
BSA	2.98 ± 0.25	0.00
PAC-BSA (0.5:1.0)	3.16 ± 0.30	-6.04
PAC-BSA (1.0:1.0)	3.27 ± 0.20	-9.73
PAC-BSA (1.5:1.0)	3.33 ± 0.25	-11.74
PAC-BSA (2.0:1.0)	3.36 ± 0.20	-12.75

The nature of the ion-dipole interactions between PAC and proteins was studied by ζ-potential measurements of the complexes. Electrophoretic mobility (ζ-potential) values agreed with EMSA-NAGE shift results (Fig. 3B). PAC complexation with HEL increased net negative charge, showing a linear decrease related to PAC:HEL ratio. On the other hand, PAC complexation with BSA also increased net negative charge of the protein complex, however the effect seems to reach a plateau at higher molar ratios, similar to the previous observation by EMSA-NAGE.

3.2. Macrophage activation

Consumption of cranberry proanthocyanidins (PAC) is associated with decreased risk of disease.[18-21] Cranberry PAC are oligomeric polyphenolic compounds that form multiple hydrogen bonds with proteins, resulting in decreased protein activity, solubility and digestibility. Therefore, PAC-protein interactions may modulate bioactivity of both molecules. Absorption of PAC from the gastrointestinal tract is low. Greater than 95% of PAC consumed are excreted in feces in complexes with proteins and polysaccharides from food or endogenous origins.[22-23] However, *in vitro* and cell culture experiments indicate that PAC are bioactive in disease processes such as inflammation, microbial adherence and oxidation. Therefore, the bioactivity of PAC may be a function of their interactions with proteins in the food matrix and gut, and not a function of post absorptive effects.

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The effects of PAC-BSA on molecular indicators of macrophage activation was explored in the next series of experiments. Cyclooxygenase 2 (COX-2) expression and inducible nitrogen oxide synthase (iNOS) expression, both induced by lipopolysaccharide (LPS), were used as indicators of macrophage activation. Activated macrophages increase expression of COX-2 and iNOS in response to bacterial infection and inflammation. [18-26] We therefore tested the effects of increasing levels of added cranberry PAC on the ability to attenuate COX-2 and iNOS expression in LPS stimulated macrophages (Fig. 4A). The PAC were added to media prior to LPS stimulation and PAC were not present in media when LPS was added. In a subsequent experiment, PAC-BSA complexes were formulated at the same ratios of PAC (0.5:1.0 to 2.0:1.0) in a fixed concentration of BSA (1 mg/mL) and added to the macrophage media prior to LPS stimulation (Fig. 4B).

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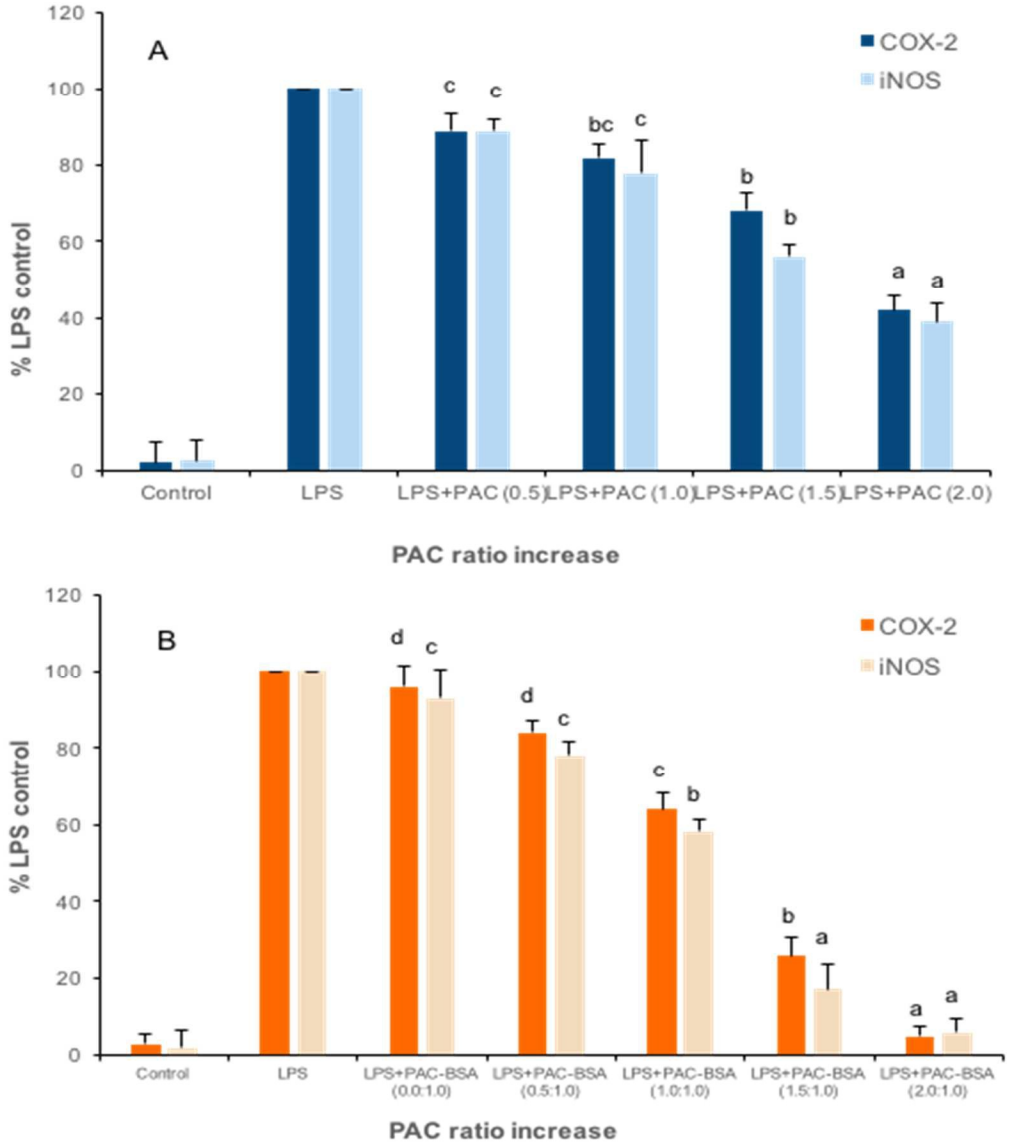

Fig. 4 Lipopolysaccharide (LPS) induced expression of COX-2 and iNOS in murine RAW 264.7 macrophages treated at A) increasing concentrations of cranberry PAC (based on PAC:Protein ratios) and B) at increasing concentrations of PAC:BSA complexes (BSA 1 mg/mL). Bars with different letters are significantly different ($p<0.05$, $n=3$).

Results indicate there was a dose dependent attenuation of expression of COX-2 and iNOS in response to addition of PAC-BSA complex. Macrophages actively endocytose BSA and PAC-BSA complexes (Fig. 5). Therefore, attenuation of COX-2 and iNOS expression was probably caused by endocytosed PAC-BSA complexes. COX-2 and iNOS expression was attenuated by

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cranberry PAC in absence of added BSA, but this effect was increased by approximately 25 to 30 % in the presence of BSA at a 2.0:1.0 PAC to protein ratio (Fig. 4). These results suggest that cranberry PAC-BSA complexes may be effective modulators of COX-2 and iNOS by down regulating expression of these proteins during inflammation. COX-2 is a regulatory enzyme in the conversion of arachidonic acid to prostaglandins and thromboxanes. The role of COX-2 in production of pro-inflammatory prostaglandins and their association with pain and fever suggest that COX-2 has a role in inflammatory diseases. On the other hand, iNOS is also associated with inflammation because iNOS produces nitric oxide (NO), an oxidant which attacks and kills the invading organisms. However, NO may also oxidize normal tissue if the inflammation proceeds unchecked. Activated macrophages increase expression of COX-2 and iNOS in response to bacterial infection and inflammation.[24-25] Our previously CI funded research demonstrated that cranberry PACS attenuated the expression of COX2 and iNOS in LPS stimulated macrophages. In the gut, lamina propria macrophages endocytose luminal protein and bacterial antigens. The subsequent responses of these cells affect mucosal immunity.[26-28]

The uptake of the PAC-BSA complexes by macrophages was determined by incubating murine RAW 264.7 macrophages with either BSA/DQ-B or PAC-BSA/DQ-B (1.0:1.0) for 1 hour. The macrophage cells endocytosed BSA/DQ-B as discrete vesicles in the cells that can be seen surrounding the nucleus in the cytoplasm of the macrophages (Fig. 5-1). Macrophages incubated with a PAC-BSA/DQ-B complex showed brighter fluorescence spots that still accumulates and disperse around the nucleus and the cytoplasm (Fig. 5-2). These experiments show that the BSA and PAC-BSA complexes are endocytosed *in vitro* by macrophages.

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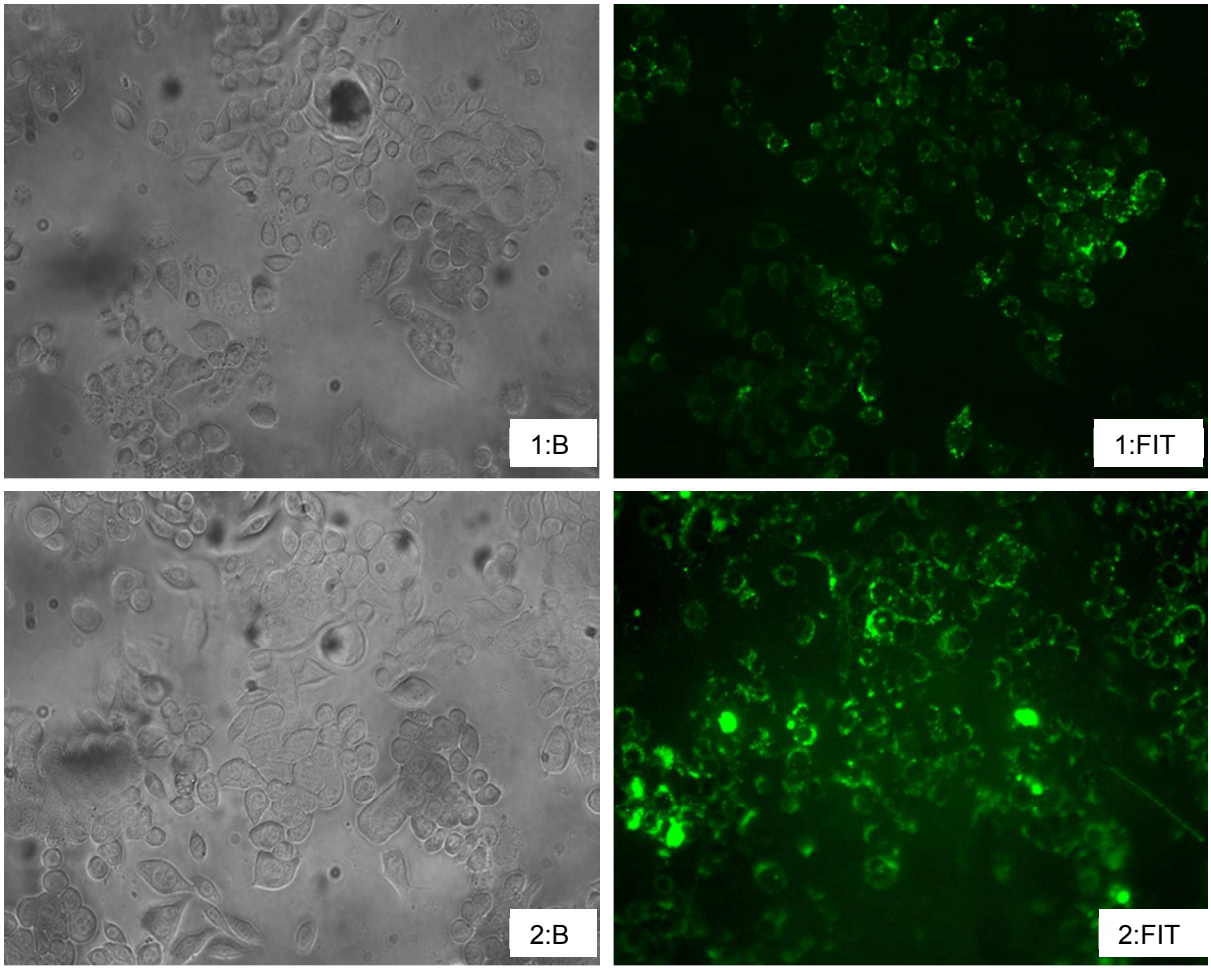

Fig. 5 Murine RAW 264.7 macrophage uptake of DQ BODIPY dye conjugated BSA (BSA/DQ-B) after 24 h incubation with 1) BSA/DQ-B and 2) PAC:BSA/DQ-B (1.0:1.0). The BSA/DQ-B conjugate does not fluoresce until it is partially digested in the macrophage endosome as shown in the bright spots inside the cells. (BF: bright field filter image; FITC: fluorescent filter image: 40x magnification).

3.3. Macrophage immune response

Macrophages are effector cells of innate immunity and link innate immune responses to acquired immunity through antigen presentation to memory lymphocytes. [28-30] The effect of cranberry PAC on macrophage antigen presentation of hen egg-white lysozyme (HEL) was measured by production of interleukin-2 (IL-2) by a T-cell hybridoma line co-cultured with murine peritoneal macrophages in the presence of HEL. When PAC-HEL complexes were added to the co-culture

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at increasing ratios of PAC to HEL (0.5:1.0 to 2.0:1.0), IL-2 production increased up to a ratio of 1.0:1.0 and then decreased (Fig. 6A). There was no effect of the PAC-HEL complexes on macrophage cell viability (data not shown).

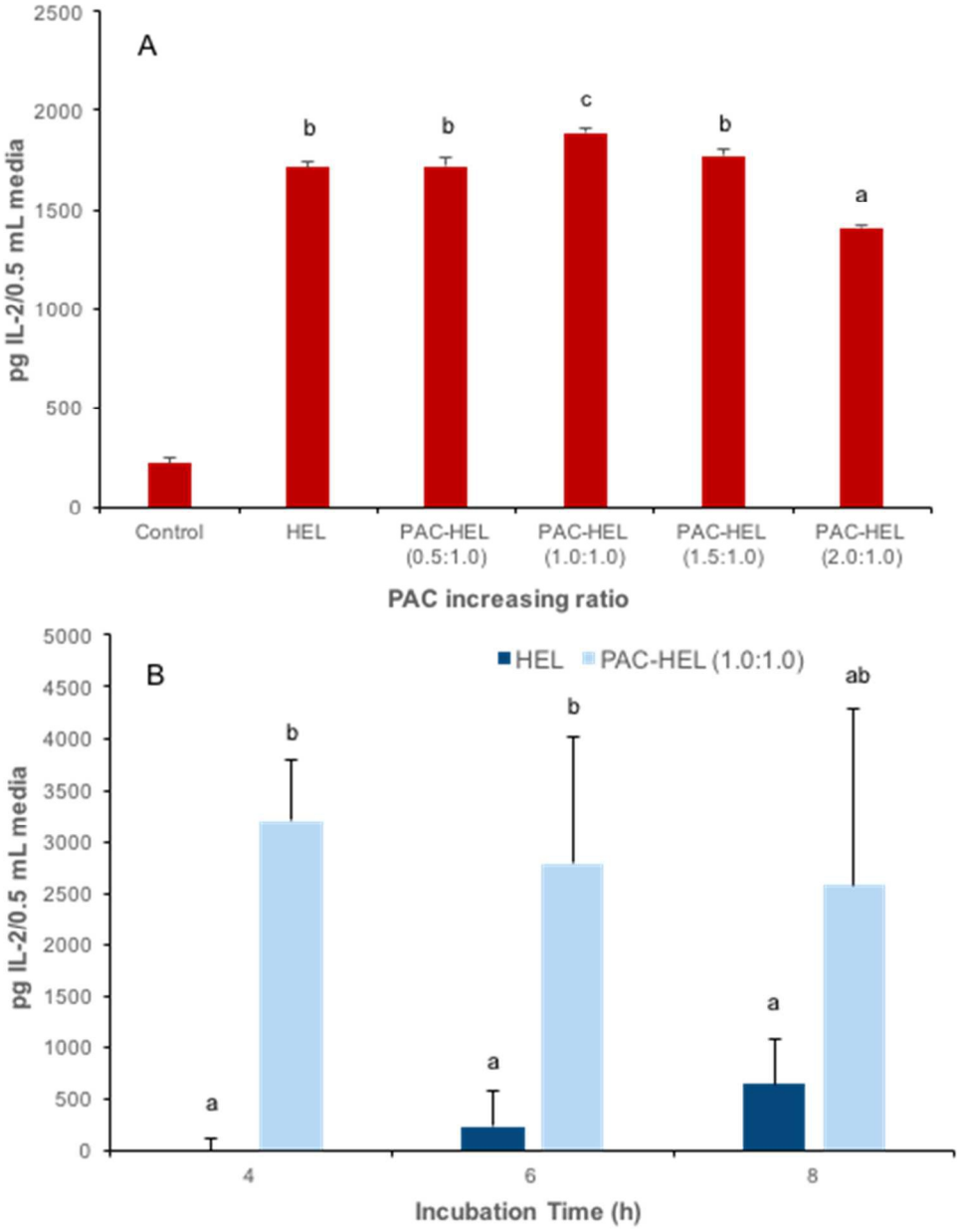

Fig. 6 PAC-HEL complexes modulate macrophage immune response. A) Effect of culturing PAC-HEL complexes at increasing PAC ratios (0.5:1.0 to 2.0:1.0) on interlukin-2 (IL-2) expression from 3A9 T-cell hybridoma cells co-cultured with murine peritoneal macrophages.

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Bars with different letters are significantly different ($p<0.05$, $n=4$). B) Effects of PAC:HEL complexes on IL-2 expression in cocultures of 3A9 T cell hybridomas cocultured with mouse peritoneal macrophages. HEL alone or PAC:HEL (1.0:1.0) complex were added to the macrophage culture for 4, 6, and 8 hours prior to addition of the T-cell hybridoma. Media containing the HEL and HEL PAC complex was removed and the T-cell hybridoma culture was added to the macrophages for 24 hours.

These results suggest that PAC modulates macrophage antigen presentation through altering rate and extent of proteolysis in the endosome. As the ratio of PAC to HEL increases, the extent of proteolysis in the endosome should decrease because PAC inhibit proteolysis. At lower ratios, more antigenic peptide should be produced in the endosome because of incomplete proteolysis and therefore IL-2 production increased. However, at higher ratios, the proteolysis of HEL may be inhibited to such an extent that less antigenic peptide is produced and IL-2 production decreased, suggesting PAC modulates HEL antigen processing and presentation by mouse peritoneal macrophages.

In a subsequent experiment (Fig. 6B), HEL alone and PAC:HEL (1.0:1.0) were added to the macrophage culture for different time periods (4, 6, and 8 hours) prior to addition of the T-cell hybridoma. Media containing the HEL and PAC-HEL complex was removed and the T-cell hybridoma culture was added to the macrophages for 24 hours. Results indicate that cranberry PAC may increase the uptake of HEL and subsequent expression of IL-2 by the T-cell hybridomas. After 4 hours of pre-incubation, only trace amounts of IL-2 were detected in the co-cultures treated with HEL alone, whereas co-cultures treated with PAC-HEL (1.0:1.0) complex had already reached maximum IL-2 expression. In the absence of PAC, IL-2 expression increased with increasing time of incubation, reaching similar values than those obtained in the presence of PAC, after 8 hours incubation. Thus, PAC may increase rate of endocytosis of HEL and subsequent expression of IL-2 by the T-cell hybridomas. Alternatively, PAC may decrease rate of HEL proteolysis in the macrophage and allowed more antigenic peptide to be presented to the T-cell hybridoma.

Fluorescent rhodamine labeled HEL (HEL/Rhod) was complexed to cranberry PAC, previously labeled with 5-([4,6-dichlorotriazin-2-yl]amino)fluorescein (PAC/DTAF)[16] and incubated with

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mouse peritoneal macrophages to study uptake of the PAC/DTAF-HEL/Rhod complexes by fluorescent microscopy (Fig. 7).

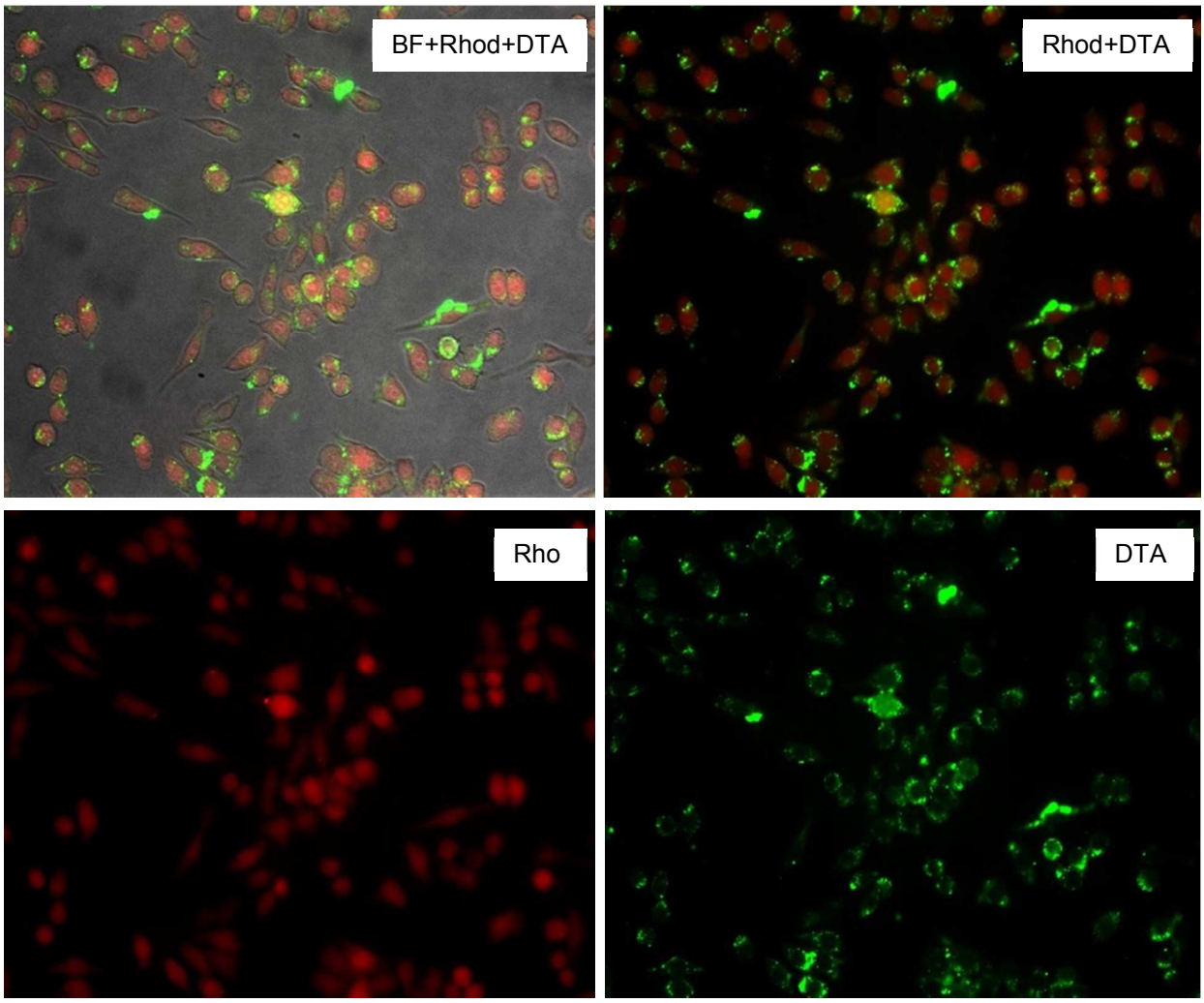

Fig. 7 Murine peritoneal macrophages after 4 h incubation with PAC-HEL (1.0:1.0) complex. HEL was labeled with rhodamine dye conjugated (HEL/Rhod) and cranberry PAC were labeled with DTAF dye conjugate (PAC/DTAF) prior to complexation reaction. (BF: bright-field filter image; Rhod: red fluorescent rhodamine dye filter image; DTAF: green fluorescent DTAF dye filter image; 40x magnification).

After 4 hours incubation, macrophages treated with PAC/DTAF-HEL/Rhod fluorescent complex contained fluorescent green endosomes, associated to PAC/DTAF and most of their cytoplasm

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showed red fluorescence, associated to HEL/Rhod. These experiments show that the PAC-HEL complexes are endocytosed *in vitro* by peritoneal macrophage cells and both green and red fluorescent signals are located inside the macrophages. Our results indicate that HEL complexation with cranberry PAC modulate uptake, processing and presentation of antigenic protein by mouse peritoneal macrophages.

4. Conclusions

In this work, we described methods to characterize cranberry PAC-protein complexes based on MALDI-TOF MS, EMSA-NAGE and ζ -potential. The experimental data obtained suggest that cranberry PAC effectively complex to proteins (BSA and HEL) that modulate macrophage activation. This will be the first step in the prevention of inflammatory responses associated to macrophage activation. Our results indicate that PAC-Protein complexes modulate uptake, processing and presentation of an antigenic model protein (HEL) by mouse peritoneal macrophages. These results suggest that there could be a relationship between PAC-Protein interactions in the food matrix and gut, to putative health benefits of cranberry PAC consumption. Therefore, our results suggest that PAC-protein complexes in the food matrix and gut may modulate gut immune response to luminal antigens and pathogen associated molecular patterns.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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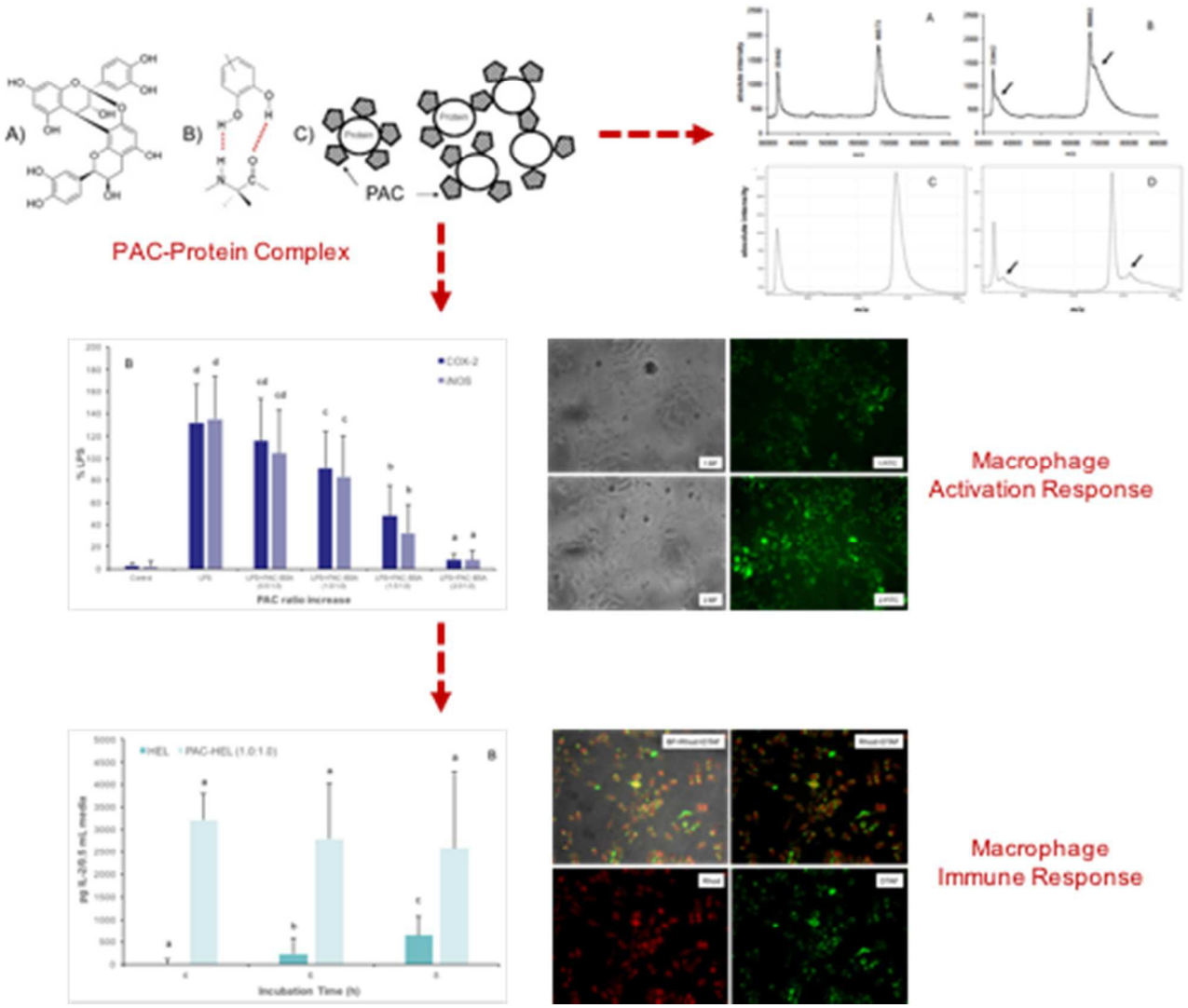
Graphical Abstract



Running title:

Cranberry *(Vaccinium macrocarpon*) Proanthocyanadin Complexes with Proteins Modulate the Macrophage Activation