



Research Paper

An acidic polysaccharide (AGC3) isolated from North American ginseng (*Panax quinquefolius*) suspension culture as a potential immunomodulatory nutraceutical



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ABSTRACT

Polysaccharides isolated from *Panax quinquefolius* roots are widely used as nutraceuticals due to their immunomodulatory properties. Despite their popularity, several challenges exist in isolating ginseng root polysaccharides such as batch-to-batch structural inconsistencies and bacterial endotoxin contamination. A plant tissue culture-based platform offers a potential solution to isolate natural polysaccharide fractions with consistent chemical characteristics and reduced endotoxin content. In this study, an acidic polysaccharide fraction (AGC3) with immunomodulatory properties was isolated from *Panax quinquefolius* suspension cultures. The heterogeneous fraction (molecular weight: 4.81 and 32.14 kDa), purified by anion exchange chromatography, was predominantly composed of galactose (>60%) along with the presence of rhamnose, arabinose, glucose, glucuronic acid and galacturonic acid. The major glycosidic linkages were found to be t-Galp (47.7%), 4-Galp (15.6%), 2,4-Rhap (8.1%), 6-Galp (8.1%) and 4-GalAp (6.8%). Structural analyses indicated the presence of a pectic rhamnoglucuronan I polysaccharide in AGC3. AGC3 significantly ($p < 0.05$) stimulated RAW 264.7 murine macrophage cells and primary murine splenocytes by enhancing the production of several immunomodulatory mediators such as IL-6, TNF- α , GM-CSF and MCP-1. The results also indicated the putative roles of NF- κ B (p65/RelA) and MAPK (p38) signaling pathways in the immunostimulatory response. Additionally, AGC3 induced murine splenocyte proliferation, another major indicator of immunostimulation. Overall, AGC3 has the potential to be used as an immunostimulatory nutraceutical.

1. Introduction

Panax quinquefolius (North American ginseng; NAG), one of the two most prominent species of ginseng, is a well-known medicinal herb used widely in traditional medicine. Biological activities of NAG include antioxidant, immunomodulatory and anti-tumor properties (Luo and Fang, 2008; Biondo et al., 2008; Lemmon et al., 2012; Azike et al., 2015; Ghosh et al., 2019). These health-promoting properties have been largely attributed to two classes of compounds i.e., ginsenosides (triterpenoid saponins) and polysaccharides (Lemmon et al., 2012). Several studies indicate that complex polysaccharides are responsible for the immune stimulating properties of NAG extracts (Biondo et al., 2008; Lemmon et al., 2012; Azike et al., 2015; Ghosh et al., 2019; Yu et al., 2014, 2017).

The promising immunomodulatory effects of NAG polysaccharides have also led to the formulation of nutraceuticals such as CVT-E002 (commonly available as Cold-FX), a poly-furanosyl-pyranosyl polysaccharide-rich extract, that claims to have beneficial effects on influenza and common cold (Biondo et al., 2008; Predy et al., 2005). Novel immunostimulatory complexes from NAG continue to be formulated and research on their mechanism of immunomodulation is a topic of interest to the nutraceutical community.

Different classes of plant polysaccharides including glucans, mannans, pectins, arabinogalactans, galactans, fructans and xylans have shown promising immunomodulatory activity *in vitro* and *in vivo* (Ferreira et al., 2015). These compounds can bind to cell surface receptors such as Toll-like receptors (TLR) and C-type lectins on monocytes and

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macrophages triggering an innate immune response (Ferreira et al., 2015; Schepetkin and Quinn, 2006; Loh et al., 2017). The induction of innate immune response leads to several coordinated processes such as production of reactive oxygen species (ROS) and proinflammatory cytokines, enhanced phagocytosis, chemokinesis and chemotaxis (Schepetkin and Quinn, 2006). Activation of immune system either directly or indirectly might be responsible for the therapeutic effects of plant polysaccharides. The broad-spectrum therapeutic potential along with minimal cytotoxicity make polysaccharides attractive candidates for novel immunostimulatory complexes and functional food. Crude polysaccharide extracts as well as homogeneous polysaccharide fractions from NAG are examples of immunostimulatory complexes that continue to show promise as natural health products.

Despite the therapeutic potential of plant polysaccharides, certain challenges such as standardization of polysaccharide-based nutraceuticals and proper quality control persist. Polysaccharides isolated from natural sources often contain variable chemical characteristics (e.g. variability in size, monosaccharide composition, glycosyl linkages and degree of branching) (Giavasis, 2014), which can arise due to environmental growth conditions and stress. The inconsistent structural characteristics may lead to poor standardization of nutraceuticals leading to concerns regarding safety and efficacy. Another problem that has been identified in botanical extracts and polysaccharide preparations is the presence of bacterial lipopolysaccharides (LPS) (Pugh et al., 2008; Gertsch et al., 2011). The presence of LPS can lead to false positives in immunostimulatory assays. Thus, proper quality control is a necessity to ensure safe and effective use of these nutraceuticals.

One of the ways to overcome the issues of standardization and LPS contamination in botanical preparations is the use of plant tissue culture. Using this technique, a large biomass of tissue can be grown under a controlled and sterile environment. It is well known that tissue culture is a viable method for the production of large quantities of metabolites *in vitro* without regard for seasons and environmental conditions (Bourgaud et al., 2001; Murthy et al., 2014). Although, tissue culture has been widely used for the production of secondary metabolites, the technique has been underutilized for the production of primary metabolites such as polysaccharides. Successful production of standardized bioactive polysaccharides in cell culture has been reported by only a few studies (Wagner et al., 1988; Luettig et al., 1989; Puhlmann et al., 1991; Popov et al., 1999). Ghosh et al. (2019) utilized the tissue culture platform to produce bioactive polysaccharides in NAG. The study reported a neutral arabinogalactan type II polysaccharide with immunomodulatory properties and highlighted that NAG suspension cultures offer a potential source of bioactive polysaccharides with negligible LPS or endotoxin content that is suitable for use as a nutraceutical.

In the current study, we extracted, isolated and elucidated the structural features of an acidic polysaccharide fraction named AGC3 from NAG cell suspension cultures. AGC3 showed high immunostimulatory activity in RAW 264.7 murine macrophage cells and primary murine splenocytes. It stimulated a range of proinflammatory mediators including nitric oxide (NO), tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6) *in vitro* and *ex vivo*. The roles of nuclear factor- κ B (NF- κ B; p65/RelA) and mitogen activated protein kinase (MAPK; p38) signaling pathways in the immunostimulatory process were also demonstrated *in vitro*. Murine splenocyte proliferation in response to AGC3 was also evident in *ex vivo* studies. The acidic polysaccharide AGC3 has the potential to be formulated into a bioactive nutraceutical capable of boosting the immune system.

2. Material and methods

2.1. Material and reagents

RAW 264.7 murine macrophage cells (ATCC TIB 71) were purchased from American Type Culture Collection, USA. Male CD1 mice were obtained from Charles River Laboratories, USA. Hi Prep 16/10

DEAE-Sepharose anion exchange column was purchased from GE Healthcare Life Sciences, USA. TSK Gel PwXL G4000 analytical size exclusion column was purchased from Tosoh Bioscience LLC, USA. Tri-Sil HTP (HDMS:TMCS:Pyridine) reagent, Pierce™ bicinchoninic acid (BCA) assay kit, Pierce™ high capacity endotoxin removal resin and Invitrogen™ Click-iT™ Plus EdU Alexa Fluor™ 488 flow cytometry assay kit were purchased from ThermoFisher Scientific, USA. The Pyrostar™ ES-F/Plate kit for LPS quantitation was obtained from Fujifilm Wako Chemicals Corp., USA. Dextran standards, methanolic HCl, modified Griess reagent, LPS (*Salmonella enterica* serotype typhimurium), concanavalin A (ConA), RPMI 1640 medium, DMEM medium and reagents for maintenance of mammalian cells were obtained from Sigma Aldrich, USA. Reagents for Western blot analysis was either purchased from Sigma Aldrich, USA or Fisher Scientific, USA. Antibodies for Western blot analysis were purchased from Cell Signaling Technologies, USA and ThermoFisher Scientific, USA (see details in section 2.6). The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay kit was purchased from Cayman Chemical Company, USA. TNF- α and IL-6 ELISA kits were obtained from R&D Systems, USA.

2.2. Extraction and AGC3 sample preparation

NAG explant sourcing, generation of cell suspension culture and passaging methods have been reported previously by Ghosh et al. (2019). The cells were filter separated and completely dried in a 37 °C incubator prior to extraction of crude polysaccharides. Polysaccharides were extracted following the method reported by Hosain et al. (2019). Dried NAG suspension culture cells (10 g) were refluxed with 500 mL of 95% (v/v) ethanol at 80 °C for 4 h. The ethanol extract was discarded to exclude secondary metabolites and the leftover residue was further extracted with diH₂O at 80 °C for 16 h. The aqueous extract was filtered and concentrated to 100 mL. The concentrated aqueous extract was further deproteinated using Sevag reagent (1:4 n-butanol:chloroform) (Hosain et al., 2019; Sevag et al., 1938). Crude polysaccharides were precipitated from the deproteinated aqueous extract by the addition of four volumes of 95% (v/v) ethanol overnight at 4 °C. The precipitated crude polysaccharide was pelleted by centrifugation at 4000 rpm for 10 min and subsequently dissolved in diH₂O. The extraction was carried out multiple times from different batches of cultured cells and the extracted crude polysaccharides were pooled together for further processing.

The pooled crude polysaccharide extract was applied to a DEAE-Sepharose Fast Flow 16/10 anion exchange fast protein liquid chromatography (FPLC) column to separate the neutral and acidic polysaccharides. A stepwise gradient of NaCl (0 M, 0.1 M, 0.2 M and 0.3 M) at a flow rate of 3 mL/min was used to elute out the polysaccharides. Each gradient step consisted of four column volumes (column volume = 20.1 mL). The eluate was collected at 5 mL/tube. The presence of sugars in the tubes was assayed using a phenol sulfuric acid assay (DuBois et al., 1956) and a chromatogram was constructed. Based on the elution profile, four major fractions were identified, pooled and concentrated under reduced pressure. The neutral polysaccharide fraction, AGC1, was the focus of the study reported by Ghosh et al. (2019). Preliminary immunomodulatory activity of the acidic fractions indicated that the 0.2 M NaCl-eluted fraction (AGC3) was the most active acidic fraction (Ghosh et al., 2019). Hence, AGC3 was selected to be the topic of the present study. Protein concentration in AGC3 was determined using a Pierce™ BCA assay kit following manufacturer's protocol. AGC3 was subsequently eluted through a Pierce™ high capacity endotoxin removal resin to remove any bacterial LPS contamination. The presence of bacterial LPS in the sample was quantified using a Wako limulus amoebocyte lysate (LAL) Pyrostar™ kit according to manufacturer's protocol. The extraction and AGC3 sample preparation schematic is shown in Fig. 1.

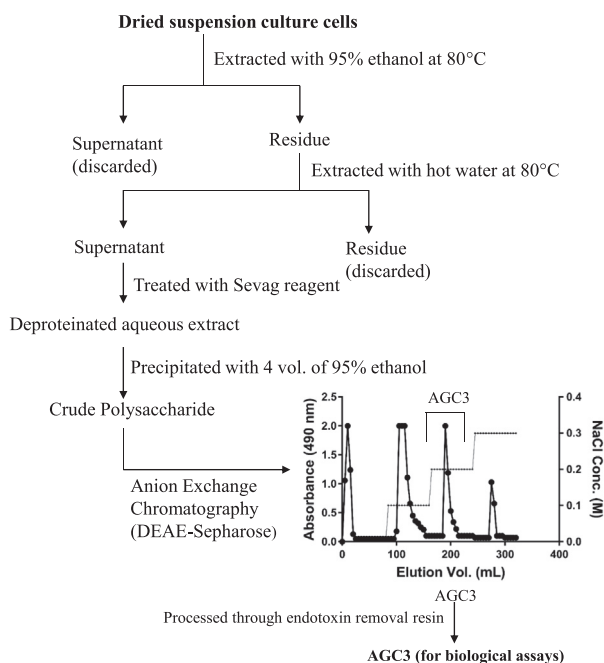


Fig. 1. Extraction and Fractionation Schematic of AGC3. Crude polysaccharides were extracted from dried NAG suspension culture cells followed by fractionation on a DEAE-Sepharose anion exchange column using a stepwise gradient of NaCl (0, 0.1, 0.2 and 0.3 M). The acidic fraction, AGC3, eluted by 0.2 M NaCl was further processed through an endotoxin removal column prior to biological assays.

2.3. Analysis of structural features of AGC3

The average molecular weight of polysaccharides present in fraction AGC3 was analyzed on a TOSOH-HLC-8320GPC EcoSEC instrument equipped with a refractive index detector. A TOSOH TSK Gel PwXL G4000 (7.8 mm X 30 cm) size exclusion column was used for the chromatographic analysis. An eluent of 0.5 M NaCl and 0.3 M NaPO₄ at a flow rate of 0.5 mL/min was used for the analysis. The average molecular weight of the polysaccharides present in the fraction was calculated based on a calibration curve constructed with dextran standards of known molecular weights (5 kDa–410 kDa).

AGC3 was sent to the Complex Carbohydrate Research Center (CCRC), University of Georgia, for further structural analysis. Linkage analysis was carried out following a method reported by Heiss et al. (2009). The sample was permethylated, hydrolyzed, reduced and acetylated. The resulting partially methylated alditol acetates (PMAA) were analyzed on an Agilent 7890A GC interfaced to a 5975C MSD (mass selective detector, electron impact ionization mode). Separation was performed on a Supelco SP-2331 bonded phase fused silica capillary column (30 m × 0.25 mm) (Heiss et al., 2009).

2.4. In vitro immunostimulatory assays

RAW 264.7 murine macrophage cells were grown and maintained in DMEM medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin in a humidified 5% CO₂ incubator. Cell concentrations were adjusted using a hemacytometer and seeded onto 96-well or 6-well plates followed by overnight incubation prior to treatments. Cells were then washed with 1X phosphate-buffered saline (PBS) and treated with different concentrations of AGC3 (1, 12.5, 25 and 50 µg/mL), positive control (LPS; 1 µg/mL) and culture medium. Culture supernatants were collected after 24 h treatments to assess the presence of proinflammatory mediators. Following 24 h treatments, the relative cell viability of the RAW 264.7 cells (5 × 10⁴ cells/well in a 96-well plate) was also measured using a MTT cell proliferation assay kit

(Cayman Chemical Company, USA) according to manufacturer's instructions.

The production of mouse proinflammatory cytokines, chemokines and growth factors (interferon-γ, IFN-γ; IL-1β; IL-2; IL-4; IL-6; IL-10; IL-12 p70; granulocyte macrophage colony stimulating factor, GM-CSF; monocyte chemoattractant protein-1, MCP-1; and TNF-α) in response to AGC3 (25 µg/mL) or LPS (0.1 µg/mL) treatments in culture supernatants was analyzed at Eve Technologies, Canada. RAW 264.7 cells (5 × 10⁴ cells/well in a 96-well plate) were treated with AGC3 and controls for 24 h, supernatants collected, frozen at -80 °C and shipped in dry ice to Eve Technologies for multiplex bead-based analyses of cytokines. Dose-dependent production of IL-6 and TNF-α in culture supernatants was also measured by ELISA following manufacturer's protocol (R&D Systems, USA). Cell densities of 5 × 10⁴ cells/well and 1 × 10⁵ cells/well in a 96-well plate were used for the dose-dependent TNF-α and IL-6 ELISA experiments, respectively.

The production of nitrite, which serves as an indirect marker of NO, in culture supernatants of RAW 264.7 cells (1 × 10⁵ cells/well in a 96-well plate) treated with various concentrations of AGC3 (1, 12.5, 25 and 50 µg/mL), LPS (1 µg/mL) and culture medium for 24 h was measured using a modified Griess assay according to manufacturer's protocol (Sigma Aldrich, USA).

2.5. Role of NF-κB (p65) and MAPK (p38) in the immunostimulatory process

RAW 264.7 cells, seeded at a concentration of 5 × 10⁴ cells/well onto 96-well plates, were pre-treated with either BAY11-7082 (NF-κB/p65 signaling pathway inhibitor) (concentration: 5 µM) or SB202190 (MAPK/p38 signaling pathway inhibitor) (concentration: 30 µM) for 1 h. After pre-treatment, the cells were stimulated with LPS (1 µg/mL) or AGC3 (12.5 µg/mL) for 4 h. The cell culture supernatants were collected after 4 h and analyzed for the presence of TNF-α by ELISA (R&D systems, USA). TNF-α production in inhibitor pre-treated cells were compared to TNF-α production in control cells (no inhibitor pre-treatment; only LPS or AGC3 stimulation). A significant (*p* < 0.05) reduction in TNF-α production due to BAY11-7082 or SB202190 inhibitor pre-treatment served as an indicator of the putative roles of NF-κB (p65/RelA) or MAPK (p38) signaling pathways, respectively, in the immunostimulation triggered by AGC3.

RAW 264.7 macrophages, seeded at a concentration of 1.8 × 10⁶ cells/well onto 6-well plates and allowed to attach overnight, were treated with AGC3 (12.5 µg/mL), LPS (1 µg/mL) or culture medium for 15 min. After 15 min, the cells were lysed using a radio-immunoprecipitation assay (RIPA) buffer containing protease and phosphatase inhibitor cocktails and the resultant lysates were used to analyze the expression of p65, phospho-p65, p38 and phospho-p38 by western blotting. The protein concentration in the samples were quantified using the Pierce BCA assay kit and normalized by dilution with RIPA buffer. Total protein (10 µg) was denatured at 95 °C in laemmli buffer for 10 min, separated on a 4–12% Tris-glycine SDS PAGE gel and then transferred to a PVDF membrane. The Western blot analysis was carried out following the method reported by Park et al. (2017). Briefly, each membrane was blocked in either 5% milk in tris-buffered saline with 0.1% Tween-20 (TBS/T) or 5% bovine serum albumin (BSA) in TBS/T, washed, and probed overnight at 4 °C for each primary antibody. Proteins were detected using a horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (Cell Signaling Technologies, Cat#7074S) and an enhanced chemiluminescent substrate. The antibodies used were phospho-p65(Ser536) (93H1, Cat#3033S), p65 (D14E12, Cat#8242S), phospho-p38 (Thr180/Tyr182) (D3F9, Cat#4511S), and p38 (D13E1, Cat#8690S) from Cell Signaling Technologies, as well as β-Actin (Cat# PA1-16889) from ThermoFisher Scientific. All blots were imaged on the Bio-Rad Chemi-doc system (n = 3), and the relative chemiluminescent intensity of each protein was determined by densitometry using ImageJ (Schneider et al., 2012) standardized to β-Actin as a loading control.

Phosphorylation of transcription factors p65/RelA or p38 served as indicators of NF- κ B or MAPK (p38) pathway activation, respectively.

2.6. Ex vivo immunostimulatory assays

Male ICR mice (<200 g) were sacrificed by CO₂ asphyxiation followed by cervical dislocation (IACUC protocol ID: 17-3010). Murine splenocytes were harvested following the method reported by Wang et al. (2013) with modifications. The spleens were aseptically removed, homogenized, and placed in PBS. A lysis buffer solution (0.15 M NH₄Cl, 10 mM KHCO₃, 1 mM EDTA-Na₂, pH 7.2) was added to the cell suspension and vortexed for 2 min to lyse the red blood cells. Two mL of calf serum was added to the cell suspension followed by centrifugation at 500×g for 5 min. The bottom layer containing splenocytes were collected and washed with PBS buffer three times. The splenocytes were resuspended in RPMI 1640 medium with 10% heat-inactivated FBS and 1% penicillin/streptomycin. Three mice spleens were pooled together to generate sufficient splenocytes per biological replicate. Murine splenocyte proliferation in response to AGC3 and control treatments were then determined using i) MTT cell proliferation assay (Cayman Chemical Company, USA) and ii) Click-iT™ Plus 5-ethynyl-2'-deoxyuridine (EdU) flow cytometry assay (Invitrogen, USA).

Primary murine splenocytes were harvested, seeded onto 96-well plates (5 × 10⁵ cells/well), and treated with different concentrations of AGC3 (1, 12.5, 25 and 50 µg/mL), ConA (0.5 µg/mL), or RPMI 1640 complete medium for 48 h. After 48 h treatment, splenocyte proliferation was measured by the MTT cell proliferation assay kit (Cayman Chemical Company, USA) according to the manufacturer's protocol. Relative splenocyte proliferation was calculated using the following equation: $RSP = [(Abs_{sample}/Abs_{untreated\ control}) \times 100] - 100$.

For the flow cytometry analysis, murine splenocytes were seeded onto 96-well plates (5 × 10⁵ cells/well), and treated with different concentrations of AGC3 (1, 12.5, 25 and 50 µg/mL), ConA (0.5 µg/mL), or RPMI 1640 complete medium for 48 h. Following treatment, splenocytes were assessed for replicating DNA using the Click-iT™ Plus EdU Flow Cytometry Assay, which relies on the incorporation of 5-ethynyl-2'-deoxyuridine (EdU) in place of thymine during DNA synthesis and subsequent detection by conjugation of Alexa Fluor™ 488 picolyl azide by click reaction, as per manufacturer's instructions. (Invitrogen, USA) The number of EdU positive cells was then determined using 488 nm laser of the Guava® easyCyte™ HCT flow cytometer (Luminex Corp., Austin, TX USA). Cells were considered positive if the green fluorescence was greater than 10³ and the forward scatter exceeded 360.

The production of proinflammatory mediators (NO and TNF- α) in response to AGC3 treatments in murine splenocytes was also assessed. Splenocytes were seeded onto 96-well plates (5 × 10⁵ cells/well) and treated with different concentrations of AGC3 (1, 12.5, 25 and 50 µg/mL), LPS (1 µg/mL) or RPMI 1640 complete medium for 48 h. Following 48 h treatment, concentration of nitrite in the culture supernatants was measured using the modified Griess assay (Sigma Aldrich, USA). TNF- α concentration in the culture supernatants was determined using ELISA following manufacturer's protocol (R&D Systems, USA).

2.7. Statistical analysis

All biological assays were conducted independently at least three times. Results were reported as mean ± SEM. Statistical significance ($p < 0.05$) was determined using one-way analysis of variance (ANOVA), Student's t-test or non-parametric Mann Whitney's U test (GraphPad Prism 7, USA).

3. Results and discussion

3.1. Extraction and purification of polysaccharides

Crude polysaccharides were extracted from dried suspension culture cells using the classical method of hot ethanol extraction followed by hot

water extraction. The ethanol extraction removes low molecular weight secondary metabolites (Cui et al., 2014). Additional treatment of the aqueous extract with Sevag reagent deproteinates the sample (Sevag et al., 1938). The yield of the crude polysaccharide precipitated with ethanol from the deproteinated aqueous extract was 3% (w/w) of the total dry weight. Optimization of tissue culture medium to enhance polysaccharide yields for future commercial applications is currently underway. The crude polysaccharide was applied to an anion exchange DEAE-Sepharose column and fractionated using a stepwise gradient of NaCl (0, 0.1 M, 0.2 M and 0.3 M) (Fig. 1). The fraction eluted by 0.2 M NaCl (yield: 15% w/w of the crude polysaccharide dry weight), named AGC3, demonstrated the highest immunomodulatory activity among the acidic fractions in our initial studies (Ghosh et al., 2019). Based on our preliminary results, it was decided to further analyze the chemical composition and the dose-dependent immunomodulatory effects of AGC3 *in vitro* and *ex vivo*. All biological assays were conducted after AGC3 was passed through an endotoxin removal column.

3.2. Chemical composition of AGC3

The protein content in AGC3 was found to be below the limit of detection in a BCA assay. Additionally, a wavelength scan (200–800 nm) of AGC3 did not produce any peaks at 280 nm indicating lack of proteins in the sample (Fig. S1). A phenol sulfuric acid assay confirmed that AGC3 was composed of sugars. The polysaccharide profile as well as the size of AGC3 was then analyzed using a high performance size exclusion chromatography (HPSEC) analysis. AGC3 was found to be a heterogeneous fraction containing two peaks with average molecular weights of 32.14 kDa (minor peak) and 4.81 kDa (major peak) (Fig. S2). The heterogeneous fraction was deemed suitable for potential use as a nutraceutical and it was decided not to further purify the fraction.

The sugar composition as well as the glycosidic linkages present in AGC3 were analyzed by GC-MS. Analysis of the PMAA derivatives of AGC3 indicated the presence of arabinose (7.8%), rhamnose (8.1%), glucose (2%), galactose (74.3%), galacturonic acid (6.8%), glucuronic acid (1%) and trace amounts of xylose and mannose. AGC3 was primarily

Table 1
Glycosyl linkages present in AGC3.

Monosaccharides	Glycosyl Linkage	Mol %
Ara	Terminal Arabinofuranosyl residue (t-Araf)	3.4
	Terminal Arabinopyranosyl residue (t-Arap)	3.5
	3 linked Arabinofuranosyl residue (3-Araf)	0.2
	4 linked Arabinopyranosyl residue (4-Arap)	0.7
	or 5 linked Arabinofuranosyl residue (5-Araf)	
Xyl	Terminal Xylopyranosyl residue (t-Xylp)	0.1
	Rha	2, 4 linked Rhamnopyranosyl residue (2,4-Rhap)
Glc		Terminal linked Glucopyranosyl residue (t-Glcp)
	Gal	4 linked Glucopyranosyl residue (4-Glcp)
Terminal Galactofuranosyl residue (t-Galf)		0.1
Terminal Galactopyranosyl residue (t-Galp)		47.7
3 linked Galactopyranosyl residue (3-Galp)		1.1
4 linked Galactopyranosyl residue (4-Galp)		15.6
6 linked Galactopyranosyl residue (6-Galp)		8.1
3,4 linked Galactopyranosyl residue (3,4-Galp)		1.5
GalA	3,6 linked Galactopyranosyl residue (3,6-Galp)	0.2
	4 linked Galacturonopyranosyl residue (4-GalpA)	6.8
GlcA	Terminal linked Glucuronopyranosyl residue (t-GlcpA)	0.8
	4 linked Glucuronopyranosyl residue (4-GlcpA)	0.2

composed of terminal galactopyranosyl residues (47.7%) followed by 4-linked galactopyranosyl (15.6%), 2,4-linked rhamnopyranosyl (8.1%), 6-linked galactopyranosyl (8.1%), 4-linked galacturonopyranosyl (6.8%), terminal arabinopyranosyl (3.5%) and terminal arabinofuranosyl residues (3.4%) (Table 1 and Fig. S3). Other linkages were present in minor amounts (<1.5%). The 2,4-substituted rhamnopyranosyl and 4-substituted galacturonopyranosyl residues are commonly found in the pectic polysaccharide rhamnogalacturonan I (RGI). The backbone of RGI pectin is comprised of alternating units of 2-linked rhamnose and 4-linked galacturonic acid residues (Yamada et al., 2007). The 4-position of the rhamnose can be substituted with a range of side chains including arabinogalactans, arabinans and galactans. Interestingly, AGC3 contains 4-linked galactans as well as the core residues of type II arabinogalactans (i.e. 3-linked, 6-linked, 3,6-distributed galactopyranosyl and terminal arabinofuranosyl units) (Yamada et al., 2007). The results of the linkage analysis suggest the presence of a RGI type of pectin in AGC3. Additionally, the presence of a high percentage of terminal residues in AGC3 indicate the presence of low molecular weight polysaccharides. There have been only a few studies that have previously characterized

NAG polysaccharides (Ghosh et al., 2019; Yu et al., 2017; Zhu et al., 2012; Guo et al., 2015; Wang et al., 2015a, 2015b). Among them, Guo et al. (2015) characterized a pectic polysaccharide fraction containing RGI from mature NAG roots. RGI type of pectic polysaccharides have also been isolated from tissue culture system of higher plants such as sycamore (McNeil et al., 1980). The current study is the first to isolate a polysaccharide fraction containing RGI pectins in NAG suspension cultures. The composition of AGC3 reported in this study is essential for future structure-activity relationship studies as well as quality control of AGC3 for potential use as a nutraceutical. Absolute characterization of AGC3 by 2D NMR will require further purification of the fraction and is beyond the scope of this study. Further purification of AGC3 into homogeneous polysaccharides will likely result in low yields unsuitable for commercial application.

3.3. *In vitro* immunostimulatory assays

Prior to immunostimulatory assays, the toxicity of NAG suspension culture polysaccharides was determined by the MTT assay in RAW 264.7

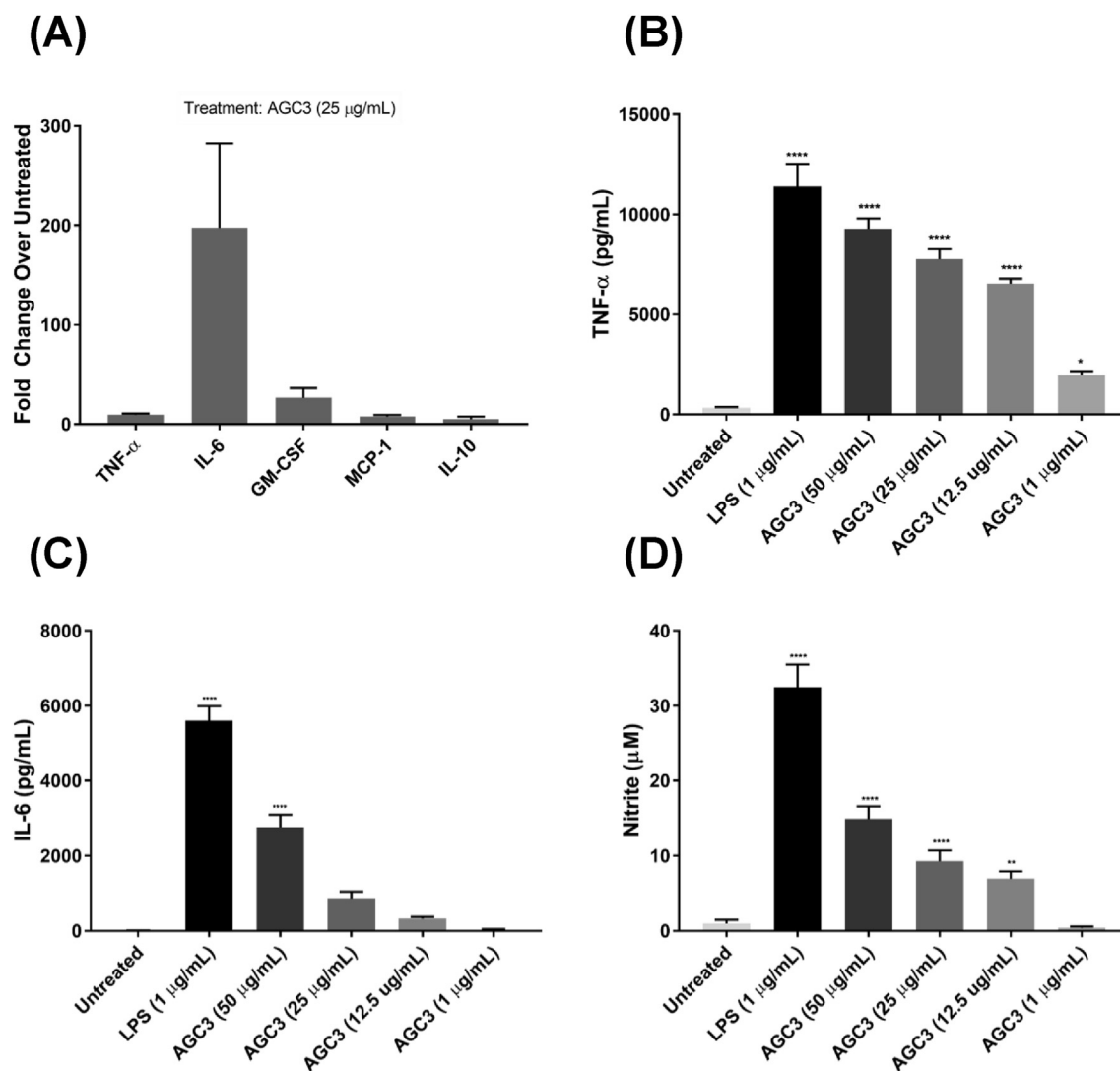


Fig. 2. AGC3 Stimulates Several Inflammatory Mediators in RAW 264.7 Murine Macrophages. Cells were treated with AGC3, LPS, or culture medium for 24 h and supernatants were collected for analysis. (A) represents a cytokine panel showing stimulation (fold change over untreated) of TNF-α, IL-6, MCP-1, GM-CSF, and IL-10 by AGC3 (25 µg/mL). (B), (C) and (D) represent production of TNF-α, IL-6 and NO respectively in response to different doses (1, 12.5, 25 and 50 µg/mL) of AGC3. TNF-α and IL-6 were measured by ELISA and NO production was measured by the modified Griess assay. Results are expressed as mean ± SEM for three independent replicates. Statistical significance was reported based on one-way ANOVA followed by Dunnett's multiple comparison test (**** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ compared to untreated control group).

murine macrophage cells. The cell viability of RAW 264.7 cells was found to be >95% in response to different doses (1, 12.5, 25, and 50 $\mu\text{g}/\text{mL}$) of AGC3 and other NAG suspension culture polysaccharide fractions (Fig. S4). Although, the cell viability in response to the two highest doses (25 and 50 $\mu\text{g}/\text{mL}$) of AGC3 decreased slightly, the values were statistically indistinguishable compared to the lower doses. The apparent non-toxic nature of NAG suspension culture polysaccharides is similar to other reports of plant polysaccharides being minimally toxic. The low toxicity and minimal side-effects of plant polysaccharides is considered

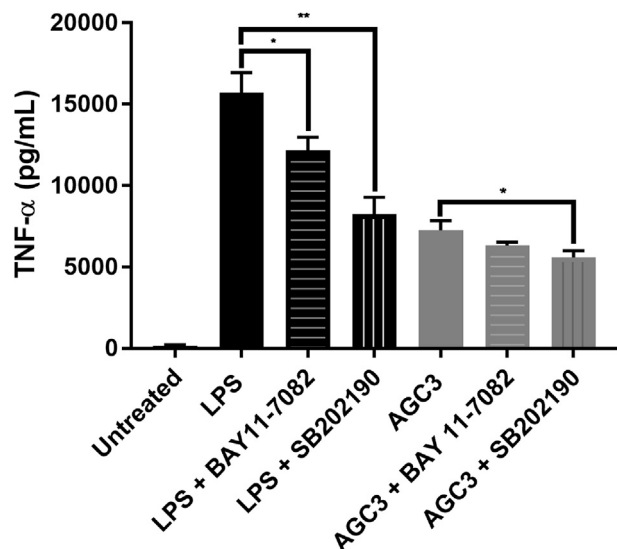


Fig. 3. Role of NF- κ B (p65/RelA) and MAPK (p38) Signaling Pathways in the Immunostimulation Triggered by AGC3. RAW 264.7 murine macrophage cells were pre-treated with BAY11-7082 (5 μM) or SB202190 (30 μM) for 1 h prior to stimulation with AGC3 (12.5 $\mu\text{g}/\text{mL}$) for 4 h. Following treatments, culture supernatants were collected and analyzed for TNF- α by ELISA. Results are expressed as mean \pm SEM for three independent replicates. Statistical significance was (* $p < 0.05$, ** $p < 0.01$) (LPS versus BAY11-7082+LPS; LPS versus SB202190+LPS; AGC3 versus BAY11-7082+AGC3; AGC3 versus SB202190+AGC3) was determined using a non-parametric Mann-Whitney's U test.

to be a major advantage compared to harmful bacterial polysaccharides and several synthetic immunomodulators (Schepetkin and Quinn, 2006).

Although, the NAG suspension cultures were grown in a sterile environment, thus limiting microbial exposure, the polysaccharide fractions were processed through a Pierce™ high capacity endotoxin removal resin out of an abundance of caution. The endotoxin content in AGC3 was then determined using the LAL assay. Although, the LAL assay was used for endotoxin detection in this study, it must be noted that it is not ideal for analysis of carbohydrate samples as multiple studies have reported cross-reactivity and false-positives (Mikami et al., 1982; Pearson et al., 1984). However, due to lack of a better alternative, the LAL assay was used to quantify endotoxin in AGC3. The LAL-reactive material in AGC3 was found to be 0.2 ng/ μg . Due to limitations of the assay, it is difficult to ascertain the precise endotoxin levels, if any, in the AGC3 sample.

It has been reported that endotoxin or LPS contamination in samples can lead to false positives in immunostimulatory assays (Pugh et al., 2008; Gertsch et al., 2011). In order to rule out such false positives, it is important to determine the potential contribution of the contaminant LPS in the sample towards immunostimulation. An acceptable method to rule out false positives is to compare the immunostimulatory response elicited by the sample versus the immunostimulatory response triggered by an equivalent amount of contaminant LPS present in the sample. The approximate LPS content in 12.5 $\mu\text{g}/\text{mL}$ of AGC3 was calculated to be 2.5 ng/mL. In a comparative study, production of proinflammatory cytokine TNF- α was measured in RAW 264.7 cells in response to AGC3 (12.5 $\mu\text{g}/\text{mL}$) and LPS (2.5 ng/mL). AGC3 (12.5 $\mu\text{g}/\text{mL}$) produced significantly ($p < 0.05$) higher amounts of TNF- α compared to LPS (2.5 ng/mL) treatment (Fig. S5). The significantly higher production of TNF- α by AGC3 compared to equivalent amount of contaminant LPS indicates that the polysaccharide is a true immunostimulant. It should be noted that polysaccharide-based botanicals often contain minute amounts of endotoxin and the immunostimulatory effects of such products are likely to be triggered by a combination of LPS and polysaccharides (Pugh et al., 2008; Gertsch et al., 2011).

The immunostimulatory activity of AGC3 was determined by measuring the production of a range of cytokines, chemokines and growth factors (IFN- γ , IL-1 β , GM-CSF, IL-2, IL-4, IL-6, IL-1 β ; IL-12 p70, MCP-1, and TNF- α) in RAW 264.7 macrophages in response to AGC3 treatments. AGC3 at a dose of 25 $\mu\text{g}/\text{mL}$ significantly stimulated five (TNF- α , IL-6, MCP-1, GM-CSF, and IL-10) out of the ten cytokine mediators. IL-6 was stimulated 197-fold over untreated followed by GM-CSF

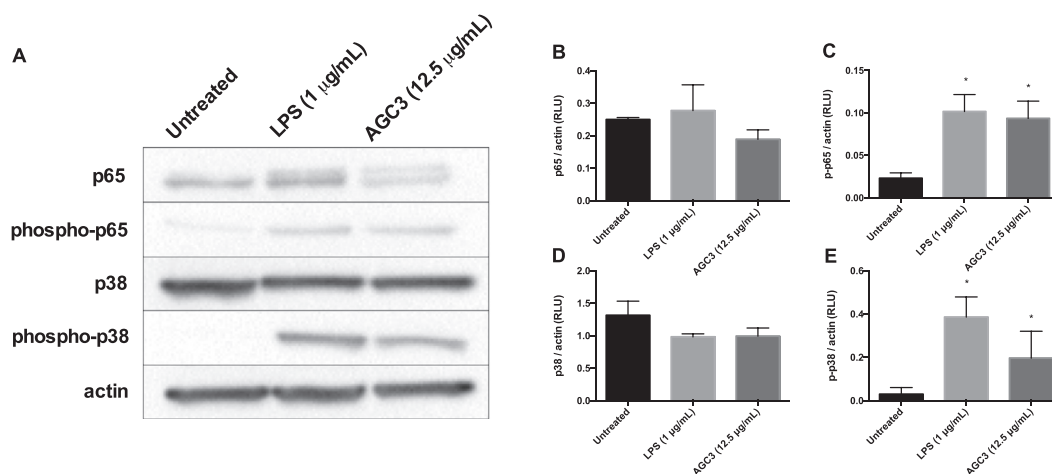


Fig. 4. AGC3 activates NF- κ B (p65) and MAPK (p38) in RAW 264.7 Macrophages. RAW 264.7 macrophages were treated with AGC3 (12.5 $\mu\text{g}/\text{mL}$), LPS (1 $\mu\text{g}/\text{mL}$), or culture medium for 15 min. Total protein was extracted using RIPA buffer and changes in phosphorylated p65 (phospho-p65) and phosphorylated p38 (phospho-p38) were analyzed using Western blots and densitometry. β -actin was used as a loading control. (A) Western blot of p65 and p38 activation; (B) Ratio of p65: β -actin; (C) Ratio of phospho-p65: β -actin; (D) Ratio of p38: β -actin; (E) Ratio of phospho-p38: β -actin. Results are shown as the mean of the ratio for each protein to β -actin \pm SEM for three independent treatments. Densitometry values were determined using ImageJ and compared (untreated versus LPS; untreated versus AGC3) by non-parametric Mann-Whitney's U test (* $p \leq 0.05$).

(26-fold), TNF- α (9-fold), and MCP-1 (7-fold) (Fig. 2A). IL-10, which is an anti-inflammatory cytokine was also stimulated 5-fold over untreated (Fig. 2A). Production of IL-10 is important to prevent excessive inflammatory response. The other cytokines analyzed in this study were either unaffected, not significantly upregulated, or not produced by the RAW 264.7 cells. Ghosh et al. (2019) reported a difference in the trends of cytokine production in response to NAG suspension culture polysaccharide AGC1 and positive control LPS. Similar to the results of that study, stimulation of IL-6 (197-fold) and GM-CSF (26-fold) by AGC3 were considerably lower compared to LPS (0.1 $\mu\text{g}/\text{mL}$) treatments. The stimulation of IL-6 and GM-CSF by 0.1 $\mu\text{g}/\text{mL}$ of LPS was 2964-fold and 350-fold over untreated, respectively (Fig. S6). The difference in the production of cytokines further indicates that the immunostimulation caused by AGC3 is not due to endotoxin contamination.

Production of proinflammatory cytokines TNF- α and IL-6 in response to different doses of AGC3 (1, 12.5, 25, and 50 $\mu\text{g}/\text{mL}$) was further evaluated in RAW 264.7 cells *in vitro*. At doses of 12.5–50 $\mu\text{g}/\text{mL}$ of AGC3, TNF- α production was significantly higher compared to untreated cells (Fig. 2B). TNF- α production is responsible for a wide range of functions including recruitment of lymphocytes, neutrophils and monocytes to the site of inflammation (Arango Duque and Descoteaux, 2014). Stimulation of TNF- α production is a major indicator of the immunostimulatory potential of AGC3. Similar to TNF- α , AGC3 significantly enhanced IL-6 production but only at a higher dose of 50 $\mu\text{g}/\text{mL}$ (Fig. 2C). IL-6 serves a broad range of roles that affect immunity, tissue repair and metabolism (Arango Duque and Descoteaux, 2014). Enhanced IL-6 production is another indicator of the immunostimulatory properties of AGC3.

NO, an important mediator of classical M1 macrophage activation, was also measured *in vitro*. AGC3 significantly stimulated the production of nitrite, an indirect marker of NO production, at doses 12.5–50 $\mu\text{g}/\text{mL}$ in RAW 264.7 cells (Fig. 2D). NO is a dynamic marker of the immune system that is partly responsible for the tumoricidal and microbicidal properties of M1 macrophages (Bogdan, 2001). The enhanced NO production indicates murine macrophage activation in response to AGC3.

3.4. Role of NF- κB (p65) and MAPK (p38) pathways in AGC3 immunostimulation

Plant polysaccharides have been widely speculated to bind to TLR and C-type lectin receptors that can potentially lead to the activation of intracellular signaling cascades such as NF- κB and various MAP kinases (Schepetkin and Quinn, 2006). Two major signaling cascades that regulate the production of proinflammatory cytokines are the NF- κB (RelA/p65) and the MAPK (p38) pathways (Schepetkin and Quinn, 2006). Since AGC3 significantly stimulated TNF- α , IL-6 and NO, we decided to evaluate the putative roles of NF- κB (RelA/p65) and MAPK (p38) transcription factors in the immunostimulatory process. RAW 264.7 cells were pre-treated with BAY11-7082 (a known inhibitor of NF- κB pathway) and SB202190 (a known inhibitor of p38 MAPK) prior to stimulation with AGC3. As a result of the pre-treatments, TNF- α production was reduced 11% by BAY11-7082 and 22% ($p < 0.05$) by SB202190 (Fig. 3). A significant reduction in TNF- α production by SB202190 indicates that p38 MAPK is responsible for the murine macrophage activation and subsequent expression of proinflammatory mediators. Although, TNF- α production was not statistically significantly reduced by BAY11-7082, there is a possibility that p65 NF- κB transcription factor is partially responsible for the macrophage activation.

In order to further investigate the roles of the two pathways, a Western blot analysis was carried out in RAW 264.7 cells. Activation of the canonical NF- κB pathway is marked by an increase of phosphorylated p65 (phospho-p65/pp65) resulting in its nuclear translocation, while MAPK is marked by an increase of phosphorylated p38 (phospho-38/pp38). As expected, the total p65 and p38 pools are statistically indistinguishable among treatment groups (Fig. 4B, D,

respectively). However, we found that RAW 264.7 cells treated with AGC3 for 15 min increased levels of pp65 to 4.06-fold higher than basal levels ($p \leq 0.05$), in a similar manner to LPS (4.41-fold over basal levels, $p \leq 0.05$) (Fig. 4C). AGC3 also increased phosphorylation of p38 from basal levels (6.63-fold, $p \leq 0.05$). However, the increase of pp38 by AGC3 treatment was less than LPS (13-fold, $p \leq 0.05$) when compared to untreated cells (Fig. 4E). It has been shown previously that RGI-type pectins activated the canonical NF- κB pathway through various TLRs (mainly TLR2 and 4) and MyD88 (Prado et al., 2020). Prado et al. (2020) also showed that the lower molecular weight pectins were able to activate NF- κB through nucleotide-binding oligomerization domain-containing protein 2 (NOD2), and to a lesser extent, NOD1 in a dose dependent manner. In addition to NF- κB activation, NOD2 signaling has also been linked to activation of MAPK (Strober and Watanabe, 2011). The results of the current study indicate that

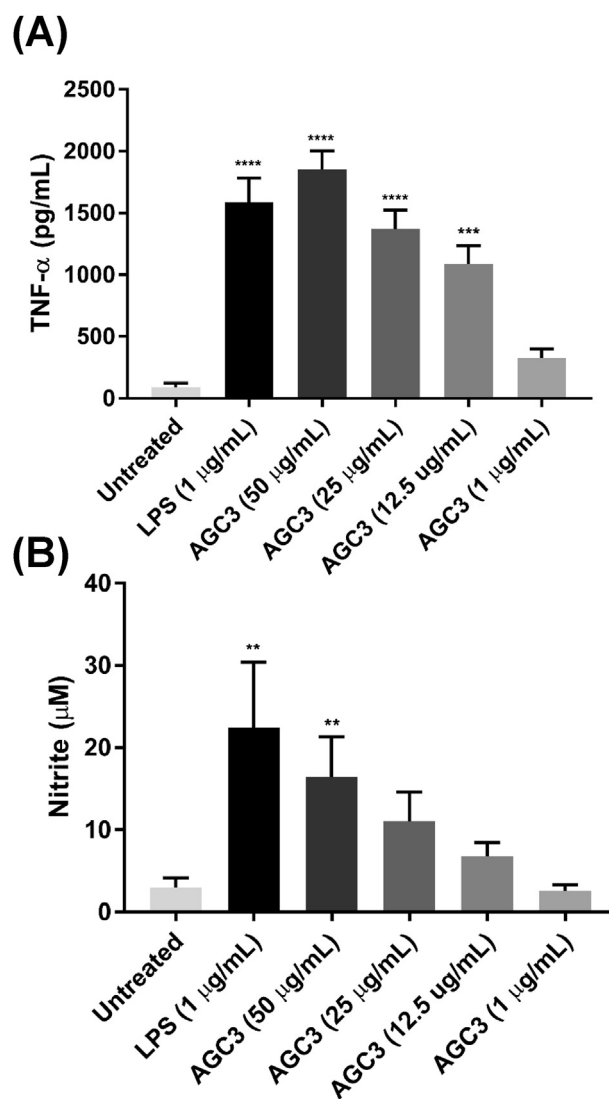


Fig. 5. AGC3 Stimulates Production of Proinflammatory Mediators in Primary Murine Splenocytes. Splenocytes were treated with different doses of AGC3 (1, 12.5, 25 and 50 $\mu\text{g}/\text{mL}$) or LPS (1 $\mu\text{g}/\text{mL}$) for 48 h. Following treatments, filtered culture supernatants were used to analyze the production of TNF- α (ELISA) and NO (modified Griess assay). (A) represents the production of TNF- α and (B) represents the production of nitrite in response to different doses of AGC3. Results are expressed as mean \pm SEM for three independent replicates. Statistical significance was reported based on one-way ANOVA followed by Dunnett's multiple comparison test (**** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ compared to untreated control group).

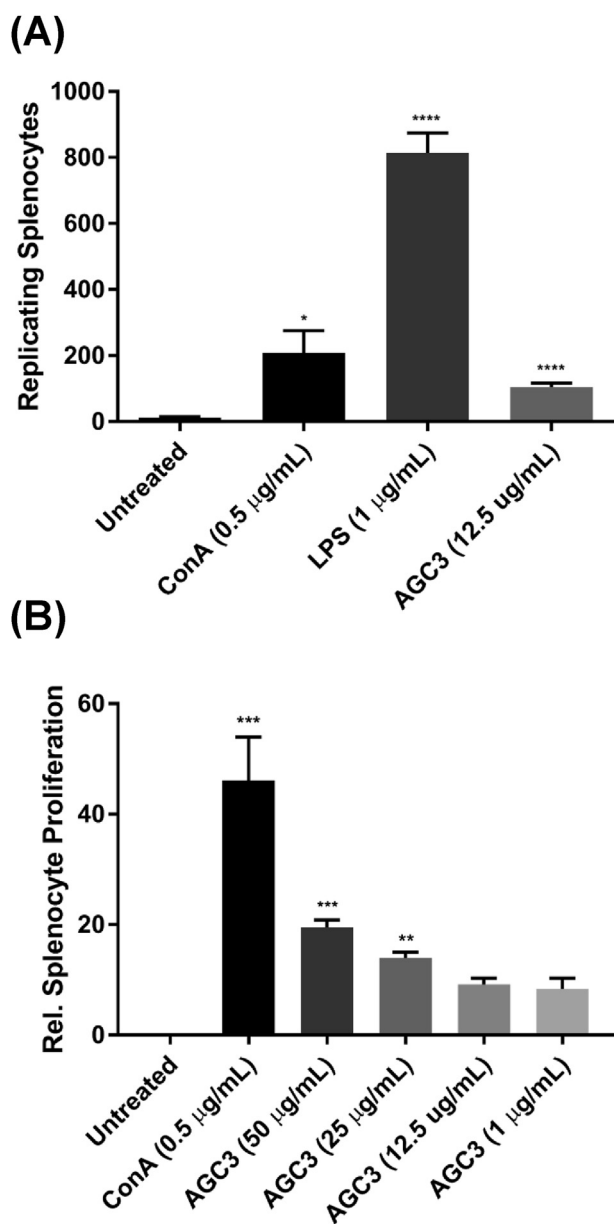


Fig. 6. AGC3 Induces Primary Murine Splenocyte Proliferation. Splenocytes were treated with different doses of AGC3 (1, 12.5, 25 and 50 µg/mL), ConA (0.5 µg/mL), LPS (1 µg/mL) or culture medium for 48 h. Following treatments, splenocyte proliferation was measured by the MTT assay and the Click-iT™ Plus EdU Flow Cytometry Assay. (A) represents the number of replicating splenocytes as determined by flow cytometry. The number of EdU positive cells was determined using the 488 nm laser of the Guava® easyCyte™ HCT flow cytometer. Results are expressed as mean ± SEM for three, independent replicates. Statistical significance was reported based on Student's t-test (**** $p < 0.0001$, * $p < 0.05$; Untreated versus ConA, Untreated versus LPS and Untreated versus AGC3). (B) represents relative splenocyte proliferation (RSP) as determined by the MTT assay. RSP was calculated using the following equation: $RSP = [(Abs_{sample}/Abs_{untreated\ control}) \times 100] - 100$. The results are expressed as mean ± SEM for three independent replicates. Statistical significance was reported based on one-way ANOVA followed by Dunnett's multiple comparison test (**** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$ compared with untreated control group).

AGC3 potentially activates both p65 and p38 signaling pathways (Fig. 4). A possible explanation is that AGC3 binds to NOD2 and possibly TLR4 causing crosstalk between the various pathways. Future research on the interaction of AGC3 with various macrophage cell surface receptors will further elucidate the mechanism of action.

3.5. Ex vivo immunostimulatory assays

The immunostimulatory properties of AGC3 was also tested in primary murine splenocytes *ex vivo*. Two proinflammatory mediators, TNF- α and nitrite, were measured in response to different doses (1, 12.5, 25, and 50 µg/mL) of AGC3. The production of TNF- α was significantly enhanced at doses 12.5–50 µg/mL of AGC3 following 48 h treatment of splenocytes (Fig. 5A). The production of nitrite was also significantly stimulated but only at a higher dose of 50 µg/mL (Fig. 5B). These results further support that AGC3 is a potent immunostimulator.

Several plant polysaccharides such as phytohemagglutinin have historically shown mitogenic properties (Nowell, 1960). Proliferation and differentiation of immune cells is considered to be a characteristic of a mitogen. The process of cell proliferation and differentiation is often triggered and regulated by cytokines (Schepetkin and Quinn, 2006). Since, AGC3 stimulated the production of several cytokines that potentially regulate cell proliferation, it was decided to test the effects of AGC3 on primary murine splenocyte proliferation *ex vivo*. The Click-iT™ Plus EdU Flow Cytometry Assay was used to determine the number of replicating splenocytes in response to AGC3 treatment (12.5 µg/mL). The number of replicating splenocytes in AGC3-treated cells was significantly higher compared to untreated control (Fig. 6A). The number of replicating splenocytes in AGC3-treated cells and ConA-treated cells (0.5 µg/mL) were not significantly different. Interestingly, both AGC3 and ConA treatments were significantly lower compared to LPS treatment (1 µg/mL). Additionally, the relative splenocyte proliferation was measured using the MTT assay that measures the net metabolic activity of the cells. AGC3 significantly stimulated relative murine splenocyte proliferation at doses of 25–50 µg/mL (Fig. 6B). Overall, the results of both the MTT assay and the flow cytometry analysis demonstrated that AGC3 induced murine splenocyte proliferation. These results further support the immunostimulatory potential of AGC3.

3.6. Pectins as immunomodulators

Immunomodulatory pectic RGI-type of polysaccharides have been previously isolated from plants such as *Carica papaya* (Prado et al., 2020), *Avicennia marina* (Fang and Chen, 2013) and *Centella asiatica* (Wang et al., 2005). Some of these pectins have reported complement fixing, mitogenic and macrophage activating properties. The arabino-3,6-galactans side chains of RGI type of pectins have been widely reported to be immunomodulatory. The arabinosyl side-chains as well as the galactan core of arabino-3,6-galactans are known to stimulate macrophages (Yamada et al., 2007; Peng et al., 2016). The rhamified regions of AGC3 have also been reported to possess immunomodulatory properties (Yamada et al., 2007). However, these studies are still preliminary and further research focusing on enzymatic or mild acid hydrolysis of RGI-type of pectins such as AGC3 will lead to the identification of specific bioactive glycan moieties. In our future studies, we intend to hydrolyze AGC3 into oligosaccharides. These oligosaccharides will be used to evaluate immunostimulatory properties and identify bioactive glycan residues.

4. Conclusions

This study highlights the potential of NAG plant tissue culture as a viable platform for the production of natural polysaccharides that is suitable for use as nutraceuticals. The acidic polysaccharide, AGC3, reported in this study predominantly contained immunomodulatory RGI-type pectins. AGC3 stimulated the production of several proinflammatory mediators such as TNF- α , IL-6 and NO in murine macrophages and splenocytes. Mechanism of action studies indicated the putative roles of NF- κ B (p65/RelA) and MAPK (p38) signaling pathways in the immunostimulatory process. Additionally, AGC3 demonstrated mitogenic properties by stimulating murine splenocyte proliferation. The *in vitro* and *ex vivo* immunomodulatory properties of AGC3 reported here

will serve as the basis for future *in vivo* as well as structure-activity relationship studies. AGC3 has the potential to be formulated into a bioactive nutraceutical that can be used as an exogenous immunomodulator either by itself or in combination with other therapeutics.

Author contributions

Rajarshi Ghosh: Conceptualization, Methodology, Validation, Formal Analysis, Investigation, Writing original draft, Visualization; **Daniel L. Bryant:** Formal Analysis, Investigation, Visualization; **Brock A. Arivett:** Formal Analysis, Investigation, Visualization; **Shannon A. Smith:** Investigation; **Elliot Altman:** Conceptualization, Writing-Review and Editing, Supervision, Funding acquisition; **Paul C. Kline:** Conceptualization, Writing-Review and Editing, Supervision, Funding acquisition; **Anthony L. Farone:** Conceptualization, Writing-Review and Editing, Supervision, Funding acquisition; Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.crfs.2020.07.002>.

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