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Heteroglucan-dendrimer glycoconjugate: a modulated construct with augmented immune responses and signaling phenomena

K. Sanjana P. Devi [a], Birendra Behera [a], Banalata Sahoo [b], Tapas K. Maiti [a,*]

[a] Department of Biotechnology, Indian Institute of Technology Kharagpur, Kharagpur 721302, West Bengal, India
[b] Department of Chemistry, Indian Institute of Technology Kharagpur, Kharagpur 721302, West Bengal, India

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ABSTRACT

Background: Newer strategies for augmenting immune responses of pharmacologically active glucans may serve to improve the medicinal potential of these biomolecules. With this aim, the present work was focused on generating targeted high molecular size glucan particles with magnified immune response activity.
Methods: Heteroglucans were conjugated with PAMAM dendrimers using a Schiff base reductive amination reaction to generate a polytethered molecule with multiple glucan motifs. The modulated construct was characterized by FTIR, TEM, ^{1}H NMR and dynamic light scattering (DLS) methods. Effects of conjugated glucans were examined in RAW 264.7 macrophage cells as well as in S-180 murine tumor models.
Results: Dendrimer-conjugated glucans were found to exhibit a two-fold increase in immune stimulation in comparison to unconjugated glucans. This may be corroborated by the predominant enhancement in immunological functions such as nitric oxide production, ROS generation and immune directed tumor inhibition in murine models. Immune cell surface markers (CD4, CD8, CD19, MHC-II) and cytokine levels were also found to be highly up-regulated in the splenocytes of mice subjected to particulate glucan administration. Our study also demonstrated that conjugated glucan treatment to RAW 264.7 cells strongly enhanced the phosphorylation of two downstream signalling molecules of the mitogen activated protein kinase (MAPKs) family: p38 and MEK1/2 relative to single glucans thereby relating molecular mechanisms with enhanced immune stimulation.
Conclusions and general significance: The results obtained thus support that particulate format of soluble heteroglucan will thereby improve its functionality and identify leads in therapeutic competence.

1. Introduction

Glucans (linear or multi-branched polymeric sugar entities), referred to as "biological response modifiers" (BRMs), can modulate the immune system to effectively encounter foreign pathogens, rectify self anomalies as well as maintain normal body homeostasis and may be regarded as 'immune regulatory switchers' in the arena of therapeutics. Unlike other customary antitumor drugs, most glucans isolated from various medicinal fungus have been shown to mediate antitumor efficacies by stimulating the immune response mechanism of the body without evoking harmful side-effects [1]. In contrast to lipopolysaccharides (present in gram-negative bacteria), these biomacromolecules have the additional advantage of mounting a controlled immunostimulatory response, thereby avoiding any chance of septic shock which may be detrimental to the host [2]. Indeed, the immune response induced by glucans retains a balanced limit which is beneficial and protective for the organism. These glucans behave as ligands and bind to their corresponding receptors protruded on the cell membranes of macrophages, monocytes, dendritic cells, NK cells as well as few T-cells. The major receptors of β-glucans in immune cells consist of Dectin-1 [3], TLRs [4], lactosylceramide [5], several scavenger receptors [6] and complement receptor 3 [7]. On recognition, various effector immune responses such as generation of ROS [8], production of NO (nitric oxide), activation of signalling molecules and release of proinflammatory cytokines/chemokines occur [9]. The downstream signalling processes enthused on glucan binding to cell surface receptors have been explored over the last few decades and published reports have revealed that immune cell maturation is an outcome of the up-regulation of NF-κβ, Akt kinase, p38 as well as other mitogen activated protein kinase (MAPK) pathways [10–12].

Recent studies have shown that different forms of glucan elicit varying degree of immune augmentation. It has been observed that particulate glucans such as those derived from *Saccharomyces cerevisiae* (yeast) produce amplified immune up-regulation such as maturation of dendritic cells, phagocytosis by macrophages and differentiation and priming of T_h and T_c cells while soluble glucans augment antibody mediated therapy [13]. The degree of immune response generated is

Abbreviations: BRM, biological response modifiers; PAMAM, poly amido amine; FTIR, Fourier transform infrared spectroscopy; TEM, transmission electron microscopy; NK cells, natural killer cells; ROS, reactive oxygen species; NO, nitric oxide; PBS, phosphate buffered saline; PBST, phosphate buffered saline with Tween 20; TNBS, 2,4,6-trinitrobenzene sulfonic acid; MAPK, mitogen activated protein kinase

* Corresponding author. Tel.: +91 3222 283766; fax: +91 03222-278707.
E-mail address: tkmaiti@hijli.iitkgp.ernet.in (T.K. Maiti).

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reported to be dependent on the mode of presentation of β-glucan (particulate versus soluble formats) for robust receptor mediated downstream signalling [14]. In this regard, generation of higher biofunctional molecules by using dendrimers as frameworks has gained wide recognition nowadays [15]. Dendrimers, which are synthetic low molecular weight molecules with an inner core and a series of branches, have become attractive tools as carriers of drugs or for conjugating pharmacologically bioactive molecules. The concept of receptor-ligand interactions are related to cell surface based signalling and new effective drugs possessing cooperative and multiple receptor binding capacities modulate this interactive mechanism. The functional groups of dendrimers have been exploited for conjugating with biological molecules possessing novel therapeutic traits to enhance their functionality by regulating the receptor-ligand based downstream machinery [16]. There are numerous reports which define that molecules with multiple side chain functional units enhance their functional potency by increasing molecular interactions at the receptor-ligand level. For example, Papp et al., 2008 have reported that dendritic-polygalactose conjugate exhibited many fold increase in their binding to selectins in comparison to a single galactose unit [17]. Another report has also revealed that polymers such as polyacrylamide-sialioside conjugates increased their binding affinity for Hemagglutinin (HA) present in the Influenza virus to 10^8 fold through multivalent interactions. Such multiple bindings were able to mask the virus particles and prevent their initiation of infection[18].

Pleurotus ostreatus is a well known edible mushroom which is easily cultivable and possesses high medicinal benefits. Bioactive compounds isolated from this organism exhibit properties such as immune stimulation as well as anti-tumor, anti-inflammatory and cholesterol lowering properties, among various others [19,20]. The mycelia of *Pleurotus ostreatus* may be considered as a better source for the isolation of therapeutic compounds as they can be grown in a defined temperature and pH conditions without any environmental influences. Our group has previously reported the isolation and characterization of heteroglucans derived from *Pleurotus ostreatus* mycelia. The isolated glucan was found to be of high molecular weight (~2.7×10^6 Da), water soluble in nature and structurally composed of heterosaccharide repeating units of glucose, mannose and fucose (3:2:1) with (1 → 2), (1 → 3), (1 → 4) and (1 → 6) linkages [21,22]. In this report, we describe a strategy to generate particulate heteroglucan molecules from the soluble form (isolated from the above mentioned source) by applying conjugation chemistry to enhance its immune stimulating property without crossing the immunological balance of causing septic shock. Hence, with an aim to incorporate higher immune stimulative effects, the amino groups of the dendrimer (PAMAM dendrimer) were conjugated to a number of aldehyde groups of the soluble glucan by creating a covalent secondary amide linkage between the two molecules. The conjugated glucan behaved as a particulate form with high molecular size and up-regulated immune reactions. Furthermore, probing into the augmented effects of particulate glucan in animal models and in signalling mechanisms, it was revealed that elevated immune competency was due to high cellular and molecular processes. To the best of our knowledge, this is the first description where a soluble glucan has been modified into particulate form using biocompatible polymeric dendrimers to enhance immune responses.

2. Materials and methods

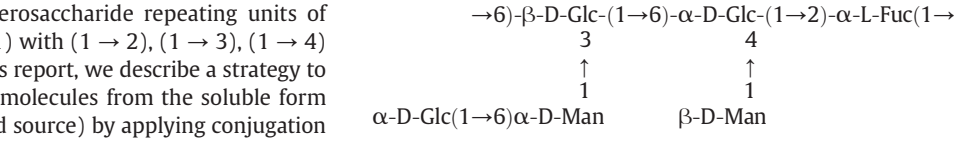
2.1. Materials

Dulbecco's modified Eagle's medium (DMEM) was purchased from Himedia while Roswell Park Memorial Institute (RPMI) and Foetal Bovine Serum (FBS) were from Gibco company. Essentials such as trypsin EDTA and antibiotics were obtained from Himedia. Other requirements included MTT ((3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium) which was procured from Loba chemicals, India while DEAE-Sephadex beads, PAMAM dendrimers, sodium cyanoborohydride, RNase, propidium iodide dye and lipopolysaccharides were obtained from Sigma, USA. Cytokines and fluorescent tagged antibodies against cell surface markers such as CD4, CD8, CD19 and MHC-II were purchased from BD Bioscience.

2.2. Glucan isolation

The mycelia of *Pleurotus ostreatus* was obtained as a kind gift from Agriculture Department, IIT Kharagpur and have its geographic origin in West Bengal, India. The fungal mycelium was nurtured in potato dextrose (PD) broth at pH 6.5 with regulated temperature conditions of 25 ± 2 °C in an incubator. The mycelial biomass was collected after every 21 days of culture and new inoculums were maintained regularly.

Heteroglucans were isolated from the mycelium of *Pleurotus ostreatus* by alkali extraction method as described in the earlier reports [21]. Briefly, mycelial biomass (~1 Kg) was crushed in a grinder with distilled water and the residual fractions were separated from the aqueous portion by centrifugation at 8000 rpm for 30–45 min. The residual mass obtained was further subjected to 2% KOH treatment overnight followed by centrifugation and neutralization of the supernatant. Alkali soluble polysaccharides were obtained by ethanol precipitation of the neutralized supernatant solution. The dry pellet/extract obtained after ethanol treatment was then dissolved in 20 mM Tris Buffer with pH 8.0 and finally passed through an ion-exchange DEAE-Sephadex column for further purification. The unbound flow through of the column (0.5 ml/min flow rate) was collected and lyophilized to obtain the purified glucan. Physiochemical characterization of the extracted glucan revealed its nature to be water soluble with a heterogenous composition of glucose, mannose and fucose in a ratio of 3:2:1 and having terminal D-glucopyranosyl, terminal D-mannopyranosyl, (1 → 6) linked D-mannopyranosyl, (1 → 2) linked L-fucopyranosyl, (1 → 4,6) linked D-glucopyranosyl and (1 → 3,6) linked D-glucopyranosyl moieties (1:1:1:1:1:1) [21,22]. The repeating unit of the glucan molecule may be represented as follows:



2.3. Conjugation methodology

Heteroglucans were conjugated to branched dendrimers by generating a secondary amide bond between the aldehyde group of glucan and the amine functional group of the dendrimer. About 50 mg of heteroglucan was initially dissolved in 10 ml of an aqueous solution containing 0.4% sodium acetate trihydrate (w/v) and 0.2% boric acid (w/v). This was followed by the addition of a strong reducing agent such as sodium cyanoborohydride (1 M) in the above solution. Amine functionalized dendrimers (PAMAM, 4th generation) were then added to the above conjugating solution at varying glucan:dendrimer (w/w) ratios of 25:1; 50:1; 75:1; 100:1 and the derivatization reaction was carried out at 70 °C for one hour in hot water bath. The mixture was then cooled to room temperature and about 5 volumes of acetonitrile: water (97:3 v/v) solution added to precipitate the dendrimer conjugated glucans. Precipitates were collected by centrifugation at 5000 rpm for 10 min. Washing of the precipitates with acetonitrile/water was repeated two to three times to remove unconjugated dendrimers and other solvent mixtures. The final precipitate obtained was further dissolved in water and dialysed to purify the conjugated glucans from other undesired reagents and ultimately lyophilized.

The endotoxin levels in the soluble glucan as well as the dendrimer conjugated forms was found to be less than 0.01 ng/ml as ascertained by limulus amoebocyte lysate (LAL) test.

2.4. Physiochemical characterization

Degree of conjugation in the dendrimer conjugated glucan was determined by monitoring the reduction in amine density on dendrimer surface by TNBS assay, following the protocol as reported in the book of Bioconjugate Techniques [23]. Concisely, molecules before and after conjugation at different glucan: dendrimer ratios (25:1; 50:1; 75:1; 100:1) were initially dispersed into 0.1 M sodium bicarbonate solution at pH 8.5 to make a final concentration of 200 μg/ml. About 0.5 ml of TNBS (2,4,6-trinitrobenzene sulfonic acid) solution (0.01%) was then added and properly mixed with 1 ml of the above sample solution and incubated at 37 °C for 2 hours. 10% SDS (0.5 ml) and 1 N HCl (0.25 ml) were then carefully added to cease the reaction and absorbance of the final solution was taken at 335 nm. Amine density was estimated by plotting a curve using glycine as standard.

The size and microstructure of the dendrimer conjugated glucan was examined using high resolution transmission electron microscopic (HRTEM, JEOL 3010, Japan) technique. Consecutively, the hydrodynamic size of the conjugated and unconjugated molecules was also estimated by dynamic laser light scattering (DLS) at an angle of $\theta = 90°$ (at 27 °C) using a Brookhaven 90 Plus particle size analyzer.

Surface chemistry of the dendrimer conjugated glucan molecules was characterized by FTIR method using Perkin-Elmer Instruments, Spectrum One FT-IR Spectrometer to assess the occurrence of a covalent conjugation between the amine group of the dendrimer and the aldehyde group of glucan. Briefly, dried samples were pulverized with KBr powder (1:50 ratio) and pressed to obtain pellets of halide disc to be spectrally analyzed for FTIR measurement (4000–400 cm^{-1} frequency range). FTIR analysis of PAMAM dendrimers was performed by dissolving in an organic solvent such as chloroform at a concentration of 1 mg/ml. In addition, ^1H NMR was performed to further confirm the conjugation of dendrimer and glucan. About 10 mg/ml of conjugated and unconjugated samples were dissolved in DMSO-d_6/D_2O and its magnetic resonance spectra were recorded in an NMR spectrometer (Avance 400).

2.5. Cell lines and mice model

RAW 264.7 which is a murine macrophage cell line was obtained from National Center for Cell Sciences (NCCS), Pune and grown in DMEM medium supplemented with 10% FBS in a 5% CO_2 incubator at 37 °C.

Swiss albino mice which were used for experimental purpose was purchased from an authorized dealer in Kolkata, West Bengal and acclimatized in a suitable sterile laboratory conditions. The mice were given feed and sterile water *ad libitum* and maintained at proper room temperatures. All animal experiments were performed on female mice and protocols followed were in agreement with "Committee for the purpose of control and supervision of experiments on animals (CPCSEA), Ministry of Environment and Forests, Government of India" and Institutional Animal Ethics Committee, Indian Institute of Technology Kharagpur, India.

2.6. Cytotoxicity assay of conjugated glucan molecules

RAW 264.7 murine macrophage cells were seeded in a 96 well tissue culture plate (at a concentration of 2×10^4 cells/well) and kept for adherence (approximately 4 h). This was followed by the addition of 20 μl of different concentrations of conjugated or unconjugated glucans and incubated for 24 h in a humidified chamber at 37 °C and 5% CO_2 level (HERA cell). Cytotoxicity was assayed by incubating the treated cells with MTT (4 mg/ml) for 4 h at 37 °C. Formazon crystals formed were dissolved in DMSO after removing the media and absorbance was recorded at 595 nm using a microplate reader (Thermoscientific).

2.7. Quantification of NO and ROS production

Macrophage cell line (RAW 264.7) was seeded at a concentration of 5×10^5 cells/ml (180 μl of the cells/well) in a 96 well flat bottom plate and allowed to adhere for 4–7 h, followed by the addition of different concentrations of dendrimer conjugated as well as unconjugated glucan samples along with positive (LPS) and negative (PBS) controls. Nitric oxide (NO) production was quantified by measuring the nitrite concentration in cell culture supernatants after 48 h of incubation using Griess reagent. 50 μl of cell supernatant was mixed with Griess reagent and incubated for 10 min at room temperature following which absorbance was recorded at 540 nm.

ROS (reactive oxygen species) generation was computed on RAW 264.7 cells by staining with a fluorogenic dye (DCFDA) having emission spectra at 525 nm. PBS and H_2O_2 (1 μM) were taken as negative and positive controls. Briefly, cells (3×10^5cells/well) were incubated with samples for 12 h and then stained with 10 μM of DCFDA for 30 min, washed thoroughly with PBS (twice) to remove excess stain and acquisition done in a BD FACS Calibur flow cytometer. Fluorescent intensity was measured and interpreted in the form of increase or decrease in ROS production.

2.8. In vivo studies on S-180 tumor bearing mice models

2.8.1. Tumor inhibition studies

Study on regression/reduction of tumor volume on intraperitoneal treatment of dendrimer conjugated and unconjugated glucans were performed on five groups of Sarcoma 180 (S-180) ascitic tumor induced mice (8 mice/group). Control mice were placed in Group 1 while Groups 2 and 3 comprised 10 and 20 mg/kg mycelia glucan (soluble glucan) treated animals. Mice treated with dendrimer conjugated glucan (particulate glucan) at a dose of 10 and 20 mg/kg were placed in groups 4 and 5 respectively. After initial tumor injection in all groups of mice, treatments were given every alternate day for 5 dosages, thereafter which mice were sacrificed and reduction in tumor cell number or volume was noted. Fresh smears of the collected ascitic tumor from all groups were prepared in pre-cleaned slides and processed for live-dead analysis using Leishman staining.

2.8.2. Sarcoma 180 cell cycle analysis

S-180 ascitic tumor cells isolated from control group and particulate or soluble glucan treated groups were washed twice with PBS thoroughly, fixed in 70% ethanol and kept overnight at −20 °C. Cells were thawed and rewashed with 10 mM ice cold phosphate buffer the very next day, followed by incubation with RNase (250 μg/ml) and PI (50 μg/ml, a DNA intercalating dye) at 37 °C for 1 h. The various cell cycle phases of the isolated tumor cells were acquired in a BD FACS flow cytometer and quantified using CellQuest pro software generating an overall DNA histogram profile of the cells [24].

2.8.3. Immunophenotyping of splenocytes obtained from experimental mice

Single cell splenocytes were isolated from treated and untreated mice groups under aseptic conditions and depleted of RBC using a hypotonic lysis buffer. Cells were then thoroughly washed in phosphate buffer saline, adjusted to a concentration of about 10^6 cells/100 μl and finally incubated with fluorescent tagged monoclonal antibodies (Per CP-anti CD4, PE-anti CD8, FITC- anti CD19 and PE-anti MHC-II) for 30 min at 4 °C in dark conditions. After incubation, cells were washed two times in PBS containing 0.1% BSA and 0.01% sodium azide followed by the acquisition of data in BD FACS Calibur (USA). Lymphocyte subpopulations were assessed by the level of fluorescence intensity measured using CellQuest prosoftware.

2.8.4. Cytokine analysis of splenocyte culture supernatant

Splenocytes were aseptically isolated from each group of experimental mice (untreated as well as treated with particulate or soluble

glucan) and the contaminating RBCs were lysed with a hypotonic buffer. The isolated single cells were washed thoroughly with PBS and media. Splenocyte suspension (1×10^6 cells/ml) obtained from different groups of mice were seeded on a 96 well plate (180 μl/well) and cultured *in vitro* with a mitogen such as LPS (4 μg/ml) for 4 days. Cell culture supernatants were then collected after centrifugation and stored at −80 °C until further use. The level of cytokines such as IFN-γ and TNF-α in the culture supernatant of splenocytes was measured by cytometric bead array method. Preparation of samples for cytokine analysis was followed as given in the manual supplied with the kit and data was acquired using FCAP array software in flow cytometer. Cytokine quantification was done from a standard curve plotted using reference cytokine concentrations provided along with the kit.

2.9. Western Blotting of particulate and soluble glucan treated RAW 264.7 cell lysates

RAW 264.7 cells treated with particulate and soluble glucan (200 μg/ml) for different time intervals were lysed using a lysis buffer solution. Total cell lysates were collected and protein concentration was quantified by Bradford method (Bradford reagent, Sigma). Cellular extracts (containing equal protein concentration in all samples; approx. 30-50 mg) were then denatured by heating with the reducing dye and loaded in 10% SDS-PAGE for electrophoretic separation. Transfer of proteins to PVDF membrane (Millipore Corp., Billerica, MA, USA) was carried out overnight at 4 °C (25milliAmpere). Membranes were then removed and dipped in blocking buffer (2% BSA in PBS with 0.1% Tween20) for 2 h at room temperature followed by incubation with primary antibodies for another 2–4 h. Antibodies such as anti-MEK, anti-phospho-MEK, anti-p38, anti-phospho-p38 (Cell Signaling, Beverly, MA, USA) and β-actin (Sigma) were diluted at 1:1000 ratio. Following primary antibody incubation, blots were washed in PBST (phosphate buffered saline with Tween 20) three times and thereafter immersed in appropriate HRP conjugated secondary antibody (1:2500 dilution) for 1 h at room temperature. Membranes were intensively washed in PBST and bands were visualized using an enhanced chemiluminescence detection kit (Sigma, USA) after a brief exposure on hyperfilm ECL sheets (GE Healthcare Lifesciences).

2.10. Statistical analysis

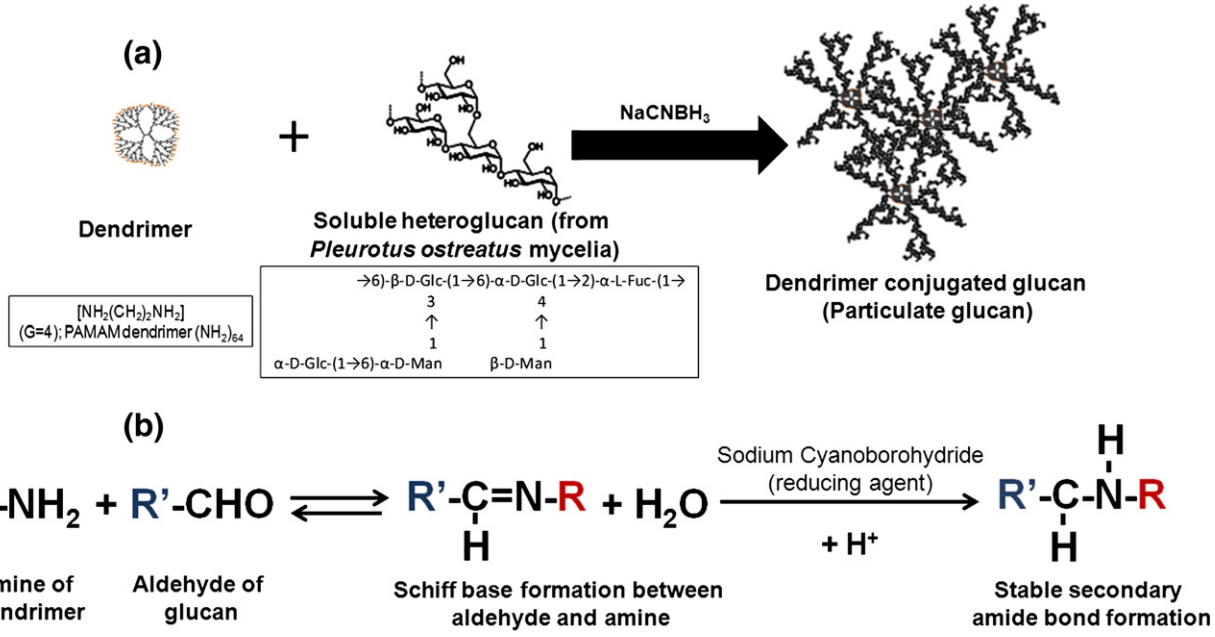
All statistical data unless otherwise specified were presented as mean ± SD/standard error of mean (SEM) using t-test statistics for unpaired observations as well as single one way ANOVA. P-values < 0.05 were considered to be statistically significant. GraphPad Prism software and MS Excel has been used for statistical analysis.

3. Results

In this study, we prepared particulate forms of the soluble heteroglucan and ascertained its higher immune enhancing capacity. A schematic formulation of the conjugation steps involved in the preparation of particulate glucans has been illustrated in Scheme 1. Concisely, soluble glucans were co-incubated with dendrimers at various ratios in a defined reaction medium containing a reducing agent such as sodium cyanoborohydride. This reducing agent effectively reduced any labile Schiff base intermediate formed between the aldehyde (from glucan) and amine group (from dendrimer) and led to the formation of a chemically stable covalent secondary amide linkage (-NH- bond) between the two target molecules. The formation of particulate glucans by conjugation generated a polytethered biomacromolecule with multiple glucans bound to the various amine groups of the dendrimer.

3.1. TNBS assay

Degree of conjugation between dendrimer and glucan was determined by estimating the amount of amine groups present before and after conjugation in the dendrimer molecule by TNBS assay. Varying ratios of glucan: dendrimer (25:1; 50:1; 75:1 and 100:1) were incubated with a reducing agent such as sodium cyanoborohydride to generate conjugated molecules which were bonded with secondary amide linkages. It has been noted that at high conjugating ratios of 25:1, the amount of amine groups not participating in the conjugation process were higher which may be probably due to excess availability of conjugating functional groups in the environs. On the other hand, conjugation at 100:1 ratio of glucan: dendrimer showed approximately 80% conjugation efficiency with an estimation of about 0.56 μg/ml of free amine



Scheme 1. (a) Schematic representation of conjugation of soluble glucan to hyperbranched dendrimers (PAMAM with 64 amine groups) to form a multi-tethered glucan molecule of particulate nature and (b) Chemistry behind the conjugation depicting the final formation of a stable secondary amide bond on reduction of Schiff base by sodium cyanoborohydride.

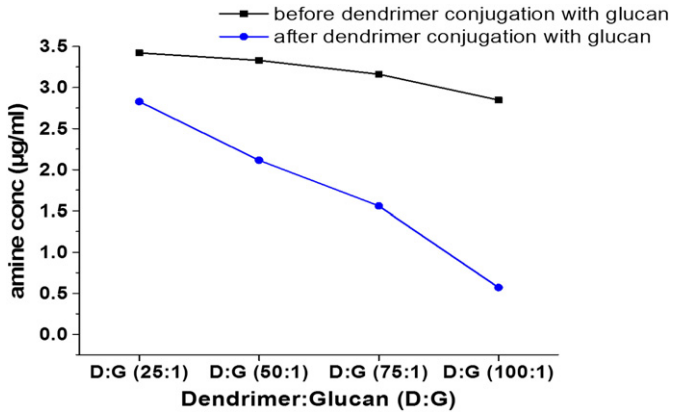


Fig. 1. TNBS assay of dendrimer: glucan conjugates at different ratios to determine the ratio with maximum degree of conjugation.

groups (Fig. 1). Unconjugated dendrimers at similar ratio showed 2.84 µg/ml of free amine. This confirmed that the ratio of 100:1 was appropriate for conjugation and therefore further characterization and biological studies were performed with multi-glucan conjugates of this ratio (100:1).

3.2. Characterization of dendrimer conjugated glucan (particulate glucan) by FTIR and NMR spectroscopic studies

The multi-glucan construct generated by conjugating manifold glucan molecules with the amine groups of the PAMAM dendrimer was physiochemically characterized by spectroscopic techniques. The FTIR spectra of soluble glucan, dendrimer and dendrimer conjugated glucan have been shown in Fig. 2. Spectral bands at positions of 3404 (having broad and strong intensity), 2923, 1042 and 1075 cm^{-1} corresponded to O-H stretching, C-H stretching, C-O stretching and C-C stretching vibrations in the soluble glucan molecule. Additionally, peaks at 1200–950 cm^{-1} marks as a probable fingerprint for every polysaccharide while a sharp and strong peak at 1646 cm^{-1} denotes C = O stretch vibrations [21]. On the other hand, PAMAM dendrimers with ethylenediamine core and amine functional groups generated infrared absorption of medium intensity at 3448, 2925, 1649 and 1541 cm^{-1} designating N-H stretches, C-H stretches, N-H bends (amide I) and C-N stretch (amide II) vibrations. In the glucan -dendrimer conjugates, secondary amide linkage at 1651 cm^{-1} (amide I) and 1546 cm^{-1} (amide II) demonstrates the successful conjugation of multiple glucans with the surface amino groups of the dendrimer. An average intensity

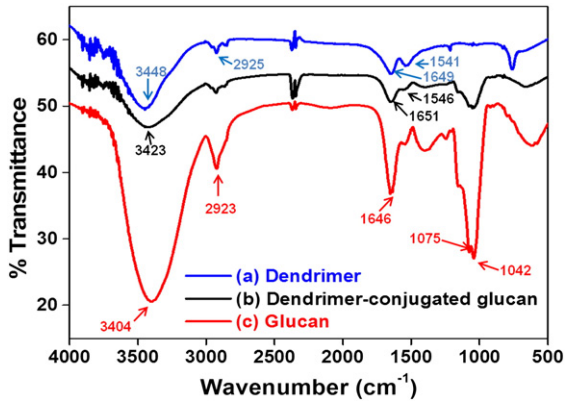


Fig. 2. FTIR spectral analysis of (a) dendrimer, (b) particulate (dendrimer-conjugated) glucan and (c) soluble glucan.

peak at 3423 cm^{-1} may be assigned to the N-H stretch vibrations in the conjugated molecule. Other FTIR spectral bands signifying characteristic polysaccharide molecule were also present in the construct.

^{1}H NMR studies of the glycoconjugates further corroborated the FTIR analysis. An intense sharp proton signal at 7.5 ppm in the dendrimer conjugated multi- glucan construct (Fig. 3c) served as a signature peak to unambiguously confirm the secondary amide bond linkage (-NHCH) between the two conjugating biopolymers (glucan and dendrimer). Dendrimer molecules on the other hand showed peak intensities at 2.4, 2.5, 2.6 and 3.1 ppm signifying the presence of $-NH_2$ molecules on the surface. Protons bound to nitrogen of NH_2 undergo chemical shift at lower frequency due to higher electron density whereas the same protons after a secondary amide bond formation (-NH) approaches a higher chemical shift due to lesser deshielding of the protons. Proton NMR of only glucan showed absence of characteristic signal for ^{1}H associated with the nitrogen of an amide group. Resonance signals at other positions such as 1.20, 4.91, 4.68 and 5.14 ppm were attributed to various linkages of fucose, mannose and glucose, the detailed structure of which has already been reported earlier [21,22].

3.3. TEM and hydrodynamic size measurements by DLS

The size and shape of the multi-construct was examined by electron microscopic studies. Fig. 4 depicts the TEM representative pictures and the hydrodynamic sizes of dendrimer as well as conjugated and unconjugated glucan. Samples subjected to TEM have been negatively stained with 2% uranyl acetate to prevent the polymers from damage caused by electron beams. On close examination, the TEM micrographs of soluble glucan confer the molecule to be fibrillar, elongated strands which were twisted to each other with an average width of about 20-25 nm. On the contrary, amine funcionalized dendrimers (PAMAM) were microscopic structures with a mean diameter of 1–2 nm. A higher magnification of dendrimer and glucan threads has been shown in the inset of Fig. 4a and c to clearly distinguish their microscopic structures. After conjugation with dendrimers, the average width or diameter of the multi-tethered glucan construct expanded to about 800–1000 nm and may be thereby represented as particulate glucan. Furthermore in aqueous medium, polymeric molecules tend to swell and increase their overall size and diameter. This was substantiated by dynamic light scattering measurements in which the average hydrodynamic diameter of dendrimer, soluble glucan and particulate glucan were estimated to be 1.25 nm, 110 nm and 2 µm respectively.

3.4. In vitro cytotoxicity assay in RAW 264.7 cells

The cytotoxic effects of particulate and soluble glucan tested in RAW 264.7 macrophage cell lines affirmed the non-toxicity of the glycoconjugates as well as their soluble format. Fig. 5a represents the MTT results of particulate and soluble glucan treated cells at concentrations of 50, 100 and 200 µg/ml. All replicates showed no inhibition in the proliferation of macrophage cells after treatment with glucan for 24 hrs.

3.5. Nitric oxide (NO) and ROS secretion studies in macrophages

The role of nitric oxide (NO) and reactive oxygen species (ROS) in macrophage mediated cytotoxicity has led to tumor cell obliteration of diverse histogenetic origins [25,26]. Polysaccharides or glucans in this respect may be regarded as an effective macrophage stimulator for the biological system. In our investigation, we have observed a pronounced enhancement in NO and ROS secretion from RAW 264.7 macrophages after treatment with the multi-tethered glucan (represented as particulate glucan). Approximately, two fold increase in the level of NO production was noted in macrophages incubated with particulate glucan at 12.5 µg/ml concentration than its soluble form at similar conditions (Fig. 5b). At higher concentrations, however the difference in the level

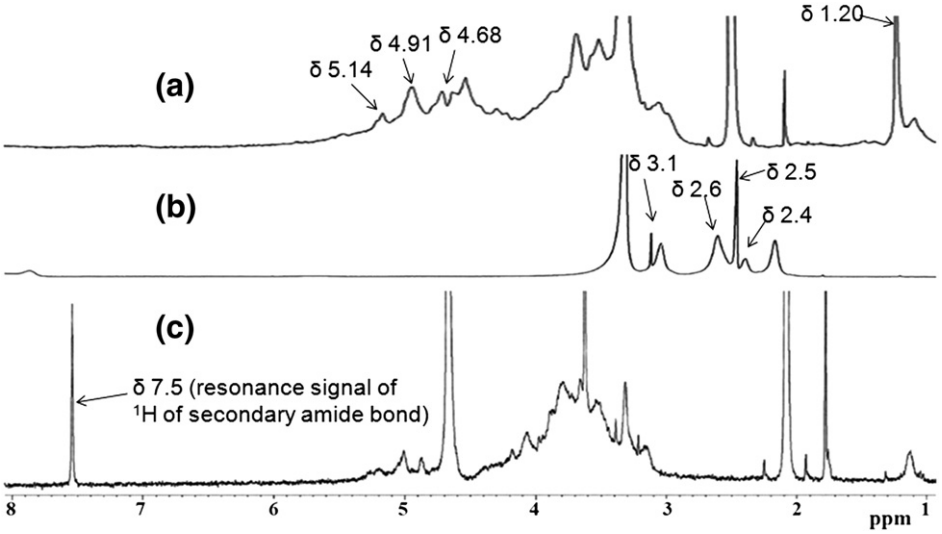


Fig. 3. NMR spectral analysis of (a) unconjugated soluble glucan, (b) dendrimer and (c) dendrimer conjugated glucan dissolved in DMSO-d_6/D_2O.

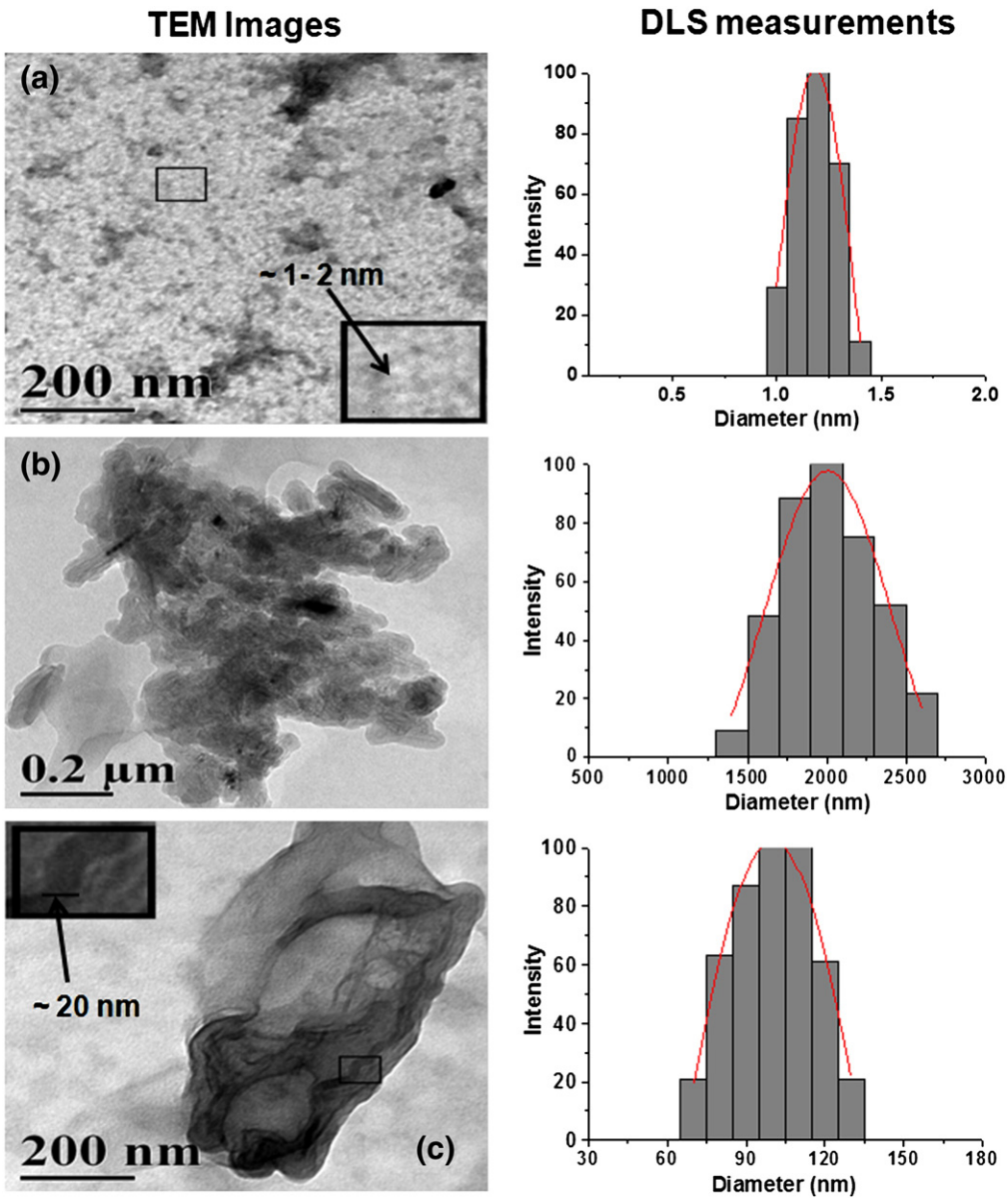


Fig. 4. TEM micrograph images and hydrodynamic particle size distribution (corresponding bar graphs) of (a) dendrimer (b) dendrimer-conjugated glucan and (c) glucan.

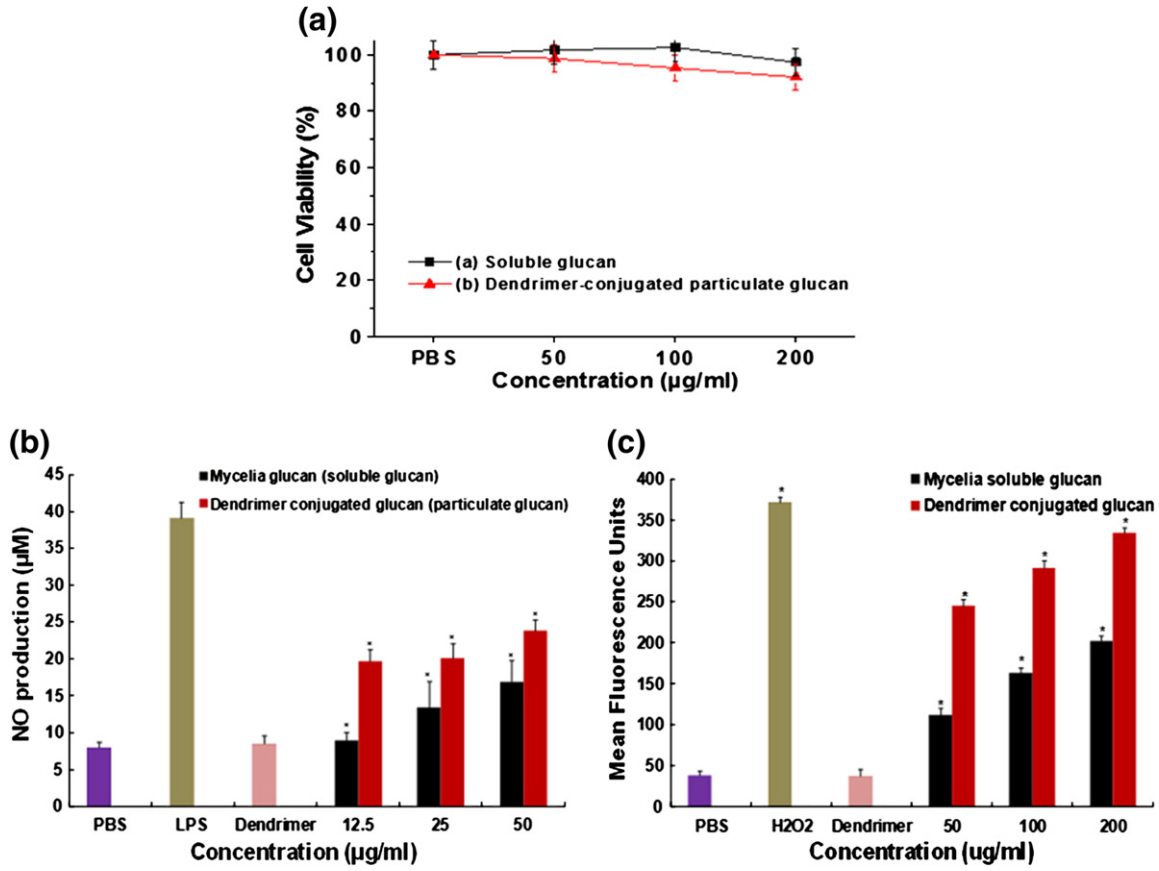


Fig. 5. Detection of (a) cell viability, (b) NO and (c) ROS production on treatment with dendrimer-conjugated and unconjugated glucans in RAW 264.7 macrophage cells. To confirm the multivalency effect, free radical generation with only dendrimer challenged cells have also been performed. Values were presented as mean ± SD and significance level evaluated using Student's t-test statistics and ANOVA: $^{*}P < 0.05$.

of NO production decreased after treatment with particulate and soluble glucan. This suggests that at higher concentrations, a cell receptor saturation point may be the limiting factor beyond which interaction with ligands cease to increase.

Parallel results were also obtained in ROS generation studies evaluated by DCFDA dye staining of treated macrophages. Particulate glucan treated cells exhibited a double increase in ROS secretion with respect to cells incubated with soluble glucan at 50 µg/ml (Fig. 5c). There was a dose-dependent increase in the production of free radicals symptomatic of an increase in receptor-glucan interactions.

3.6. In vivo S-180 tumor inhibition after particulate and soluble glucan administration to murine models

To further ascertain the multivalent effect of conjugated or particulate glucans, *in vivo* studies on tumor induced murine models have been carried out. Sarcoma 180 bearing mice were intraperitoneally administered with particulate or soluble glucans at 10 and 20 mg/kg concentration and thereafter sacrificed to assess the degree of tumor inhibition. It was noted that particulate or multivalent glucan treated mice groups augmented the tumor inhibitory effects to a plausible grade as compared to soluble glucan treated groups. While soluble unconjugated glucans caused approximately 43.3% of tumor inhibition at 20 mg/kg, particulate glucans upgraded the inhibition gain to 76.67% in similar circumstances (Table 1). Morphological analysis of S-180 smears by Leishman staining as illustrated in Fig. 6 clearly demonstrated the apoptotic cells with membrane blebs and nuclear fragmentations.

Cell cycle analysis of S-180 cells collected from the peritoneal exudates of treated and untreated mice groups indicated the different stages of DNA of the tumor cells. On analysis, it was revealed that sub G0/G1 hypodiploid population (marked as apoptotic phase) was higher in particulate glucan treated groups. Particulate and soluble glucans at 20 mg/kg treatment in mice models showed 30.74 and 8.59% of G0/G1 stages respectively (Fig. 7).

3.7. Immunophenotyping and cytokine analysis of splenocytes isolated from treated mice groups

Immunophenotyping of splenocytes showed a marked escalation in B-cells, T-cells and macrophage markers in treated groups with respect to control. However, the percentage of these immune cells was found to be higher in splenocytes derived from particulate glucan treated mice groups (Fig. 8). Average lymphocyte subpopulations of CD4, CD8, CD19 and MHC-II bearing cells were marked to be 16.29, 14.49, 35.70

Table 1
Percentage of ascitic S-180 tumor inhibition in dendrimer-conjugated and soluble glucan treated mice models (Group 1–5).

	Group	Packed cell volume (ml)	Cell number (cells/ml)	Inhibitory %
Untreated mice	1	4.2 ± 0.22	$12 \times 10^7 \pm 0.41$	0
Soluble or unconjugated glucan treated mice	2	2.7 ± 1.15	$7.8 \times 10^7 \pm 0.59$	35[a]
	3	2.5 ± 0.87	$6.8 \times 10^7 \pm 1.04$	43.3[a]
Particulate or dendrimer conjugated glucan treated mice	4	1.3 ± 0.35	$4.7 \times 10^7 \pm 0.96$	60.84[a]
	5	0.7 ± 0.81	$2.8 \times 10^7 \pm 0.74$	76.67[a]

Each value designates mean ± SEM of eight independent data points (N = 8)
[a] Implies significance level from untreated or control groups with $P < 0.05$.

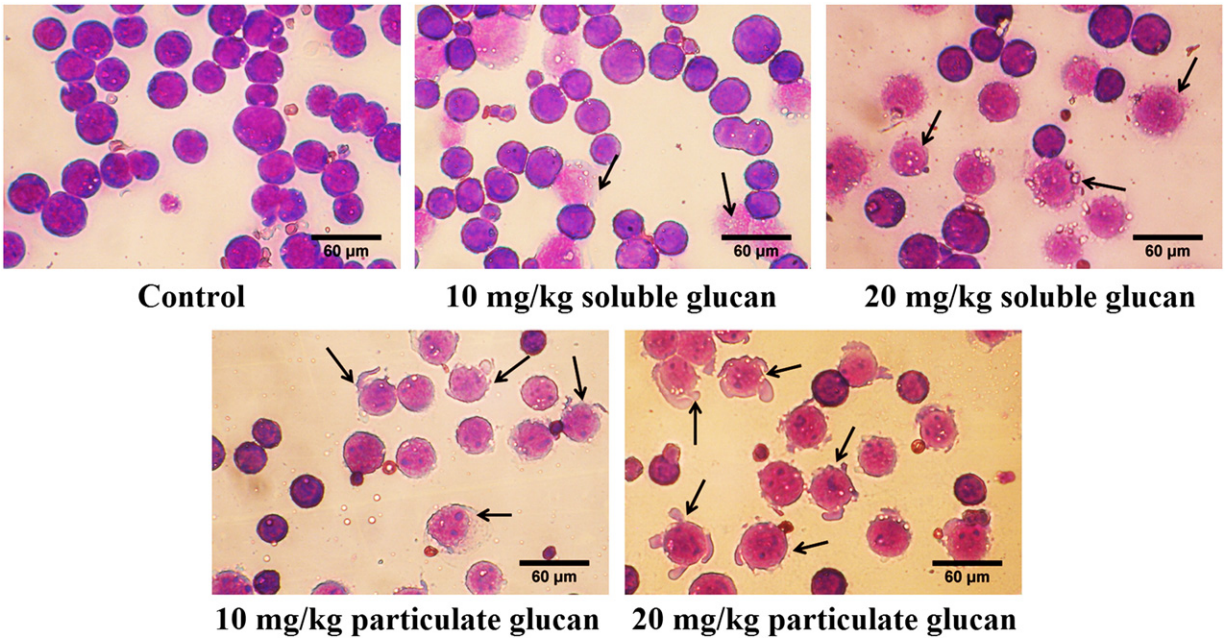


Fig. 6. Leishman staining of Sarcoma 180 cells isolated from particulate and soluble glucan treated mice groups. The arrows indicate apoptotic cells with membrane blebbings and disintegration. Violet colored cells represent live cells which still retained membrane integrity.

and 35.79% on particulate glucan administration (20 mg/kg treatment). Comparatively, soluble glucans at similar conditions revealed a percentage of 13.18, 11.95, 23.33 and 20.97% for CD4, CD8, CD19 and MHC-II respectively, confirming that glucans with multiple ligand binding sites possess greater efficiency to cause potent activation of innate and adaptive immune cells to act against tumorogenic conditions.

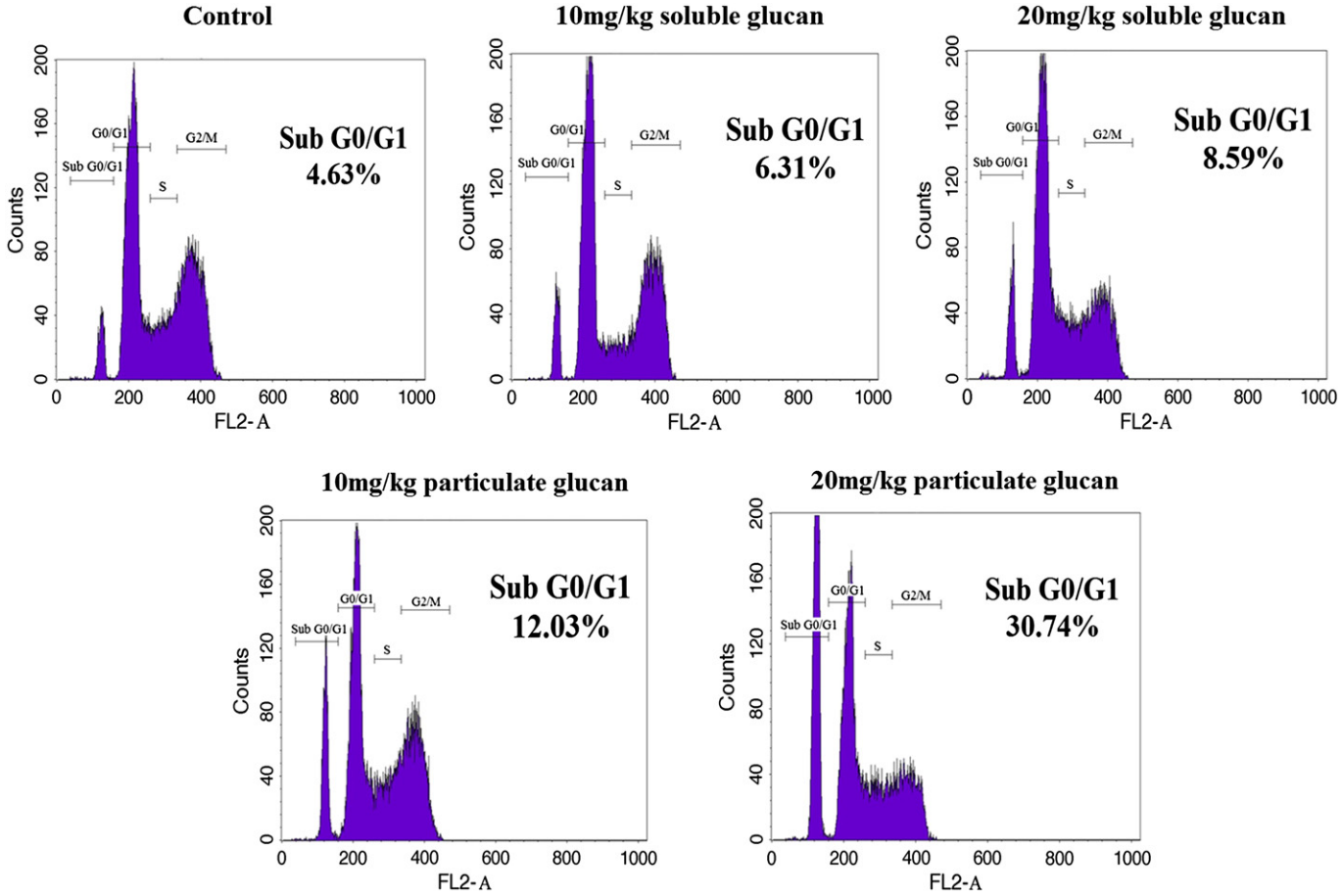


Fig. 7. Cell cycle analysis of particulate (dendrimer conjugated glucan) and soluble glucan treated S-180 tumor cells isolated from the peritoneal lavage of tumor bearing mice.

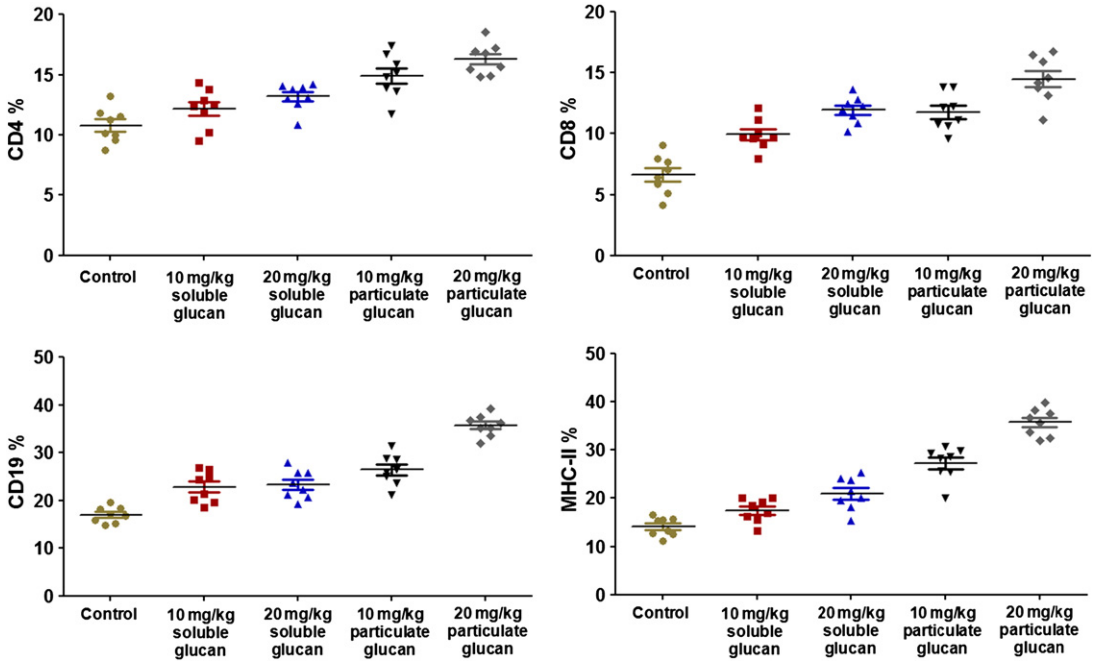


Fig. 8. Immunophenotypic analysis of splenocytes isolated from experimental mice groups. Antibodies against cell surface markers of B-cells (CD19), T-cells (CD4 and CD8) and macrophages (MHC-II) were used to determine the lymphocyte sub-populations. Values were presented as mean ± SEM of eight independent data points.

Quantification of proinflammatory cytokines such as tumor necrosis factor–alpha (TNF-α) and interferon-gamma (IFN-γ) from splenocytes isolated from experimental mice groups was estimated by cytometric bead array method using flow cytometry. It was observed that splenocytes isolated from conjugated glucan treated mice showed an inflated cytokine level on mitogen (LPS) stimulation for 4 days. Quantitatively an average of about 2950 and 110 pg/ml of TNF-α and IFN-γ were estimated in splenocyte supernatants obtained from mice injected with 20 mg/kg of dendrimer conjugated glucans. Splenocytes with soluble glucan treatment at similar doses showed approximately 1674 and 4 pg/ml secretion of the above mentioned cytokines i.e., TNF-α and IFN-γ respectively (Fig. 9).

3.8. Molecular signalling studies

Upstream protein kinases such as molecules of the MAP kinase pathway have been reported to be involved in regulating effector functions of immune cells [27,28]. p38 and MEK are two of the most important molecules of this pathway. This led us to appraise the phosphorylated or activated status of MEK and p38 signalling molecules in macrophages on stimulation with particulate or soluble glucans. RAW 264.7 cells were challenged for 5, 15, 30 and 60 min with 200 μg/ml concentration of glucan molecules. MEK phosphorylation was observed from 5 min with maximal activity attained at 30 min. It was noted that cells stimulated with particulate or dendrimer-conjugated glucans boosted the phosphorylative activity of MEK (45 KDa) about two times higher than soluble glucan (Fig. 10). The kinase activity was however transient and was abridged after 30 min of treatment. Immunoblots of phosphorylated p38 after particulate glucan stimulation also showed highest kinase activity at 30 min while soluble glucan on the contrary exhibited its maximal activity at 60 min. The degree of phosphorylation of p38 was about 54% higher in particulate glucan treatment with respect to soluble form at 30 min challenge. All the other home cell molecules (MEK, p38 and β-actin) were detected to be at basal levels in all treatment conditions.

4. Discussion

Glucans have remained as a potent alternative medicine for centuries and have also played a remarkable role in curing many malignancies. Their immunomodulatory and antitumor properties have been

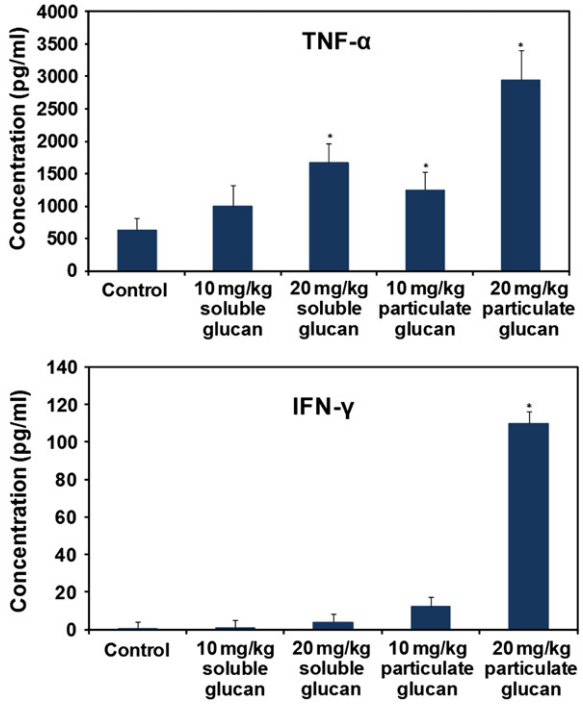


Fig. 9. Cytokine levels (TNF-α and IFN-γ) in the culture supernatant of splenocytes obtained from control mice as well as mice treated with particulate or soluble glucan. Splenocytes were subjected to 4 days mitogen (LPS, 4 μg/ml) stimulation before measuring the cytokine levels. All values were represented as mean ± SD (n = 8) and significance level was calculated using Student's t-test statistics and ANOVA: $^{*}P < 0.05$.

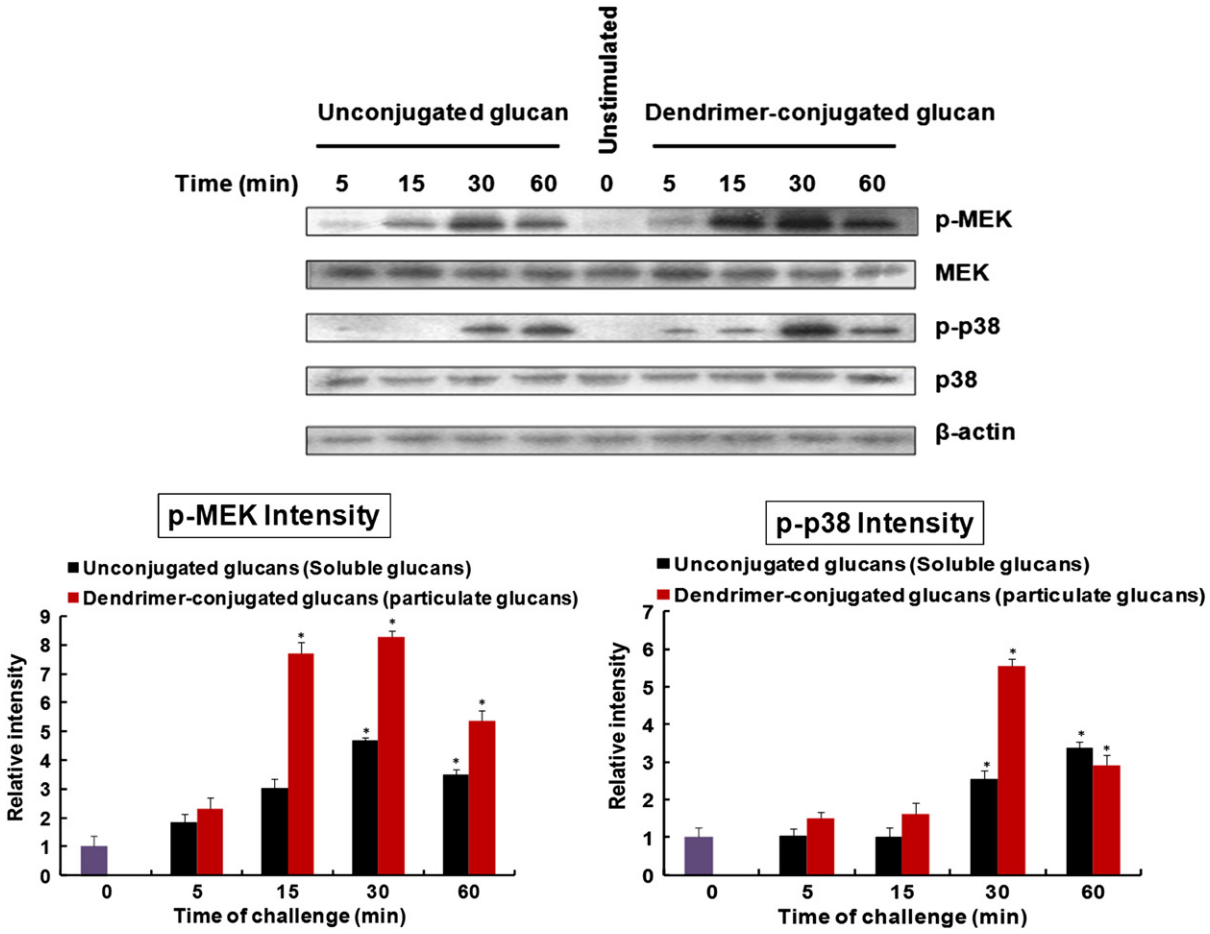


Fig. 10. Western blotting of dendrimer conjugated (particulate) and unconjugated (soluble) glucan challenged RAW 264.7 cell lysates. Blots were probed with phosphorylated and unphosphorylated MEK and p38 antibodies for detection of kinase activity. The intensity of the phosphorylated signalling molecules were analysed in Image J software and represented as histogram. Red bars designate conjugated particulate glucans while black bars signify the unconjugated soluble forms.

proved to be therapeutically beneficial for boosting the immune system and making it competent for many anomalies [21,29–31]. However, enhancing the immune stimulatory properties of these soluble glucans to a more effective level remains as a crucial challenge. There are also reports which reveal that chemical modification of particulate glucans can make them water soluble in nature. The insoluble nature was reported to be mainly due to intramolecular linkages within the molecule (by various forces of attraction) which shielded hydroxyl groups from forming bonds with water [32,33]. Chemical modification such as carboxymethylation, sulfation or phosphorylation disrupted the intramolecular forces and introduced charged groups into the glucan thereby increasing their interaction with water molecules and making them soluble in nature [34,35]. Such modifications did not alter the functional binding unit of the molecule nor led to any degradation of the compound and hence there was no significant loss of biological activity. However in our reported work, the particulate nature was achieved because of the aggregation of a number of heteroglucan molecules to PAMAM dendrimers leading to increase in the molecular size and diameter. Unlike the previous literature reports where particulate/insoluble glucans were single functional molecules, the polytethered heteroglucan carried multiple functional glucan molecules which augmented their biological potency and thereby provided a newer approach to study the effects of glucan. The size of these particulate glucans ranged from 2-3 μm which is similar to the particle size of zymosan (~3 μm) [36]. Zymosan (reported as a β-glucan standard in many reports) however may not be considered as a pure glucan as it contains mixtures of mannans, mannoproteins, glucans and chitin [37]. On the contrary, multi-tethered heteroglucan construct generated by conjugation based methods may be represented as particulate forms of pure glucan with minimal contaminants. Various spectroscopic and electron microscopic analysis have confirmed the successful conjugation of glucan with dendrimer. These particulate glucans were able to enhance free radical production (NO and ROS) in macrophages than the soluble forms. Reactive oxygen species (ROS) contribute to processes leading to tumor cell apoptosis and are also involved in transcriptional activation and receptor-mediated signalling pathways [38,39]. Nitric oxide on the other hand mediates its function mostly by inhibiting DNA synthase and damaging the mithochondrial electron transport chain [40].

Numerous reports have suggested that different forms of glucan (particulate or soluble) exhibited antitumor efficacies in murine models [41–43]. *In vivo* studies with the multi-glucan construct on ascitic S-180 tumor models also led to regression in the tumor volume supporting the multivalent effect of the conjugated glucan. Furthermore, cell cycle analysis and leishman staining clearly depicted the apoptotic tumor cells in the sub G0/G1 phase. Administration of particulate glucans to tumor bearing mice showed about 34% higher tumor inhibition than soluble glucans. When immune cells get activated, they secrete a repertoire of inflammatory molecules which ultimately lead to death of the tumor cells [44–46]. This phenomenon of immune cell mediated tumor cytotoxicity was supported by immunophenotyping of splenocytes from particulate/soluble glucan treated mice groups which revealed the presence of an activated immune system in the host mice. It would be noteworthy to mention here that the percentage of immune cell surface markers and level of Th1 cytokine such as TNF-α and IFN-γ were predominantly high in splenocytes isolated from mice

groups treated with dendrimer glucan conjugates. TNF-α and IFN-γ are two major pro-inflammatory cytokines which have been associated with cancer immunotherapy [46,47]. Mice deficient in IFN-γ have been reported to be less proficient in suppressing tumors such as mammary or prostrate carcinomas as well as lack the ability to avert metastasis of tumor cells to other healthy tissues [48].

Activation of the mitogen activated protein kinase (MAPK) cascade which includes p38 and MEK molecules has been well documented to be associated with the accumulation of inducible nitric oxide synthase (iNOS) in macrophages and regulates the intracellular NO release [49]. Reports have also revealed that one of the mechanisms by which β-glucans mediate their immune enhancing properties was by upregulating the MAP kinase pathway [50,51]. In this context, our hypothesis proves that exposure to these dendrimer conjugates over expresses the MAP kinase molecules in macrophages which further augments the intracellular NO secretion as well as other immunologic phenomena. The phosphorylation activity of MEK or p38 on glucan challenge is a downstream mechanism which is regulated after receptor-ligand binding interactions on the cellular surface. Higher availability of ligands for glucan receptors in immune cells (in case of particulate glucans) may have amplified downstream signalling mechanisms and led to elevated immune reactions as compared to univalent glucan molecules. The degree of immunoaugmentative responses elicited by various forms of glucan preparations propounds its differential receptor binding capacity in phagocytic cells. Various reports have revealed that different forms of glucan (particulate or soluble) bind to discrete receptors or varying combination of receptors [13,43,52,53]. The question now lies whether particulate molecules (such as multi-tethered glucans) follow the same receptor fate as other normally isolated particulate glucans such as zymosan. This may be thus aimed as a futuristic study which needs in depth investigation to complete the mechanistic cellular story of varied forms of glucan induced stimulation.

5. Conclusion

The multi-tethered dendrimer-glucan constructs exhibited higher immune stimulation in both *in vitro* and murine models as compared to the parent soluble heteroglucans suggesting that multivalency serve to be an important factor in receptor-ligand interactions for enhanced immune activation. This was further corroborated by an upregulated downstream signalling (MAPK pathway) in macrophages on challenge with particulate glucans. Generating a higher activated form of glucan by conjugating it with branched dendrimers may thus give decisive leads in developing better tumor therapies.

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