Immunostimulatory activity of polysaccharide–poly(I:C) nanoparticles

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Abstract

Immunostimulatory properties of mushroom derived polysaccharides (PS) as stand-alone agents were tested. Next, PS were nanocomplexed with poly(I:C) (pIC) to yield stable nanoparticles around 200 nm in size evidenced by atomic force microscopy and dynamic light scattering analyses. PSs were selectively engaged by cells expressing TLR2 and initiated NFκB dependent signaling cascade leading to a Th1-biased cytokine/chemokine secretion in addition to bactericidal nitric oxide (NO) production from macrophages. Moreover, cells treated with nanoparticles led to synergistic IL6, production and upregulation of TNFα, MIP2α, IFNγ and IP10 transcript expression. In mice, PS-Ovalbumin-pIC formulation surpassed anti-OVA IgG responses when compared to either PS-OVA or pIC-OVA mediated immunity. Our results revealed that signal transduction initiated both by TLR2 and TLR3 via co-delivery of pIC by PS in nanoparticle depot delivery system is an effective immunization strategy. The present work implicate that the PS and nucleic acid based nanoparticle approach along with protein antigens can be harnessed to prevent infectious diseases.

1. Introduction

Toll-like receptors (TLRs) are the most extensively studied pathogen recognition receptors (PRRs) that recognizes specific microbial associated molecular patterns (MAMPs) including lipopolysaccharides, peptidoglycans, lipoproteins, flagellin, unmethylated CpG motifs or viral RNA/DNA that triggers innate immune response [1–3]. TLRs are subcategorized as endosomal or intracellular receptors depending on their site of expression. TLR3, 7/8 and 9 are specialized to sense pathogens via their nucleic acids moieties [4]. TLR3, a member of endosome-associated TLR is activated directly in response to dsRNA, or synthetic poly(riboinosinic:polyribocytidylic acid) (pIC) [5] and are harnessed as vaccine adjuvant, anti-cancer or anti-allergic therapeutic agents. When these ligands are given in vivo, they are rapidly cleared by nucleases, and could be adsorbed by serum proteins hampering their in vivo therapeutic applications [6]. In order to improve their immune stimulatory potential repeated high doses are necessary, however, in many instances this may cause undesirable side effects including septic shock [7]. Nucleic acid backbone modifications such as phosphorothioate linkages are another widely accepted approach but this not only induces undesirable side effects such as granuloma formation, temporary splenomegaly, and lymphadenopathy but also increases the cost of the final product [8–11].

Several strategies were proposed as carriers for labile nucleic acids attempting to increase their in vivo performances [12–14]. Among many, liposome encapsulation, biodegradable nano/micro carriers and soluble macromolecules with cationic moieties were widely studied [12–14]. We and others demonstrated that, uptake, duration and in vivo immunostimulatory activity of oligodeoxynucleotides enhanced when encapsulated in sterically stabilized cationic liposomes (SSCL) [15–17]. Although co-administration of pIC with liposomes induced anti-viral immunity and activated effective CD8+ T cells in vivo [18], still several obstacles prevent these depot delivery systems enter into clinic. Of note, batch to batch variation, limited shelf-life, difficulty in reproducible reconstitution and sterility are the major concerns from FDA stand point. Furthermore, organic solvents used during the production of certain formulations are of major concern [19–21].

In the present study, natural amphiphilic polysaccharides purified from mushrooms were studied to assess their immunostimulatory
potential. Later, a nanoparticle delivery system was formulated to efficiently harbour pIC. The TLR2 mediated PS targeting and subsequent initiation of signal transduction cascade and ability of the nanoparticles to induce synergistic immune activation was evaluated. Furthermore, in an immunization study in mice co-delivering a model protein antigen ovalbumin (OVA), immunogenicity of PS-OVA-pIC nanoparticles was compared to either PS-OVA or pIC-OVA mediated immune response.

2. Materials and methods

2.1. Materials

All cell culture media components were from Hyclone (USA). Cytokine, IgG ELISA were from Endogen and Southern Biotech, respectively (USA). TLR ligands were obtained from several vendors: peptidoglycan (PGN) (Fluka, Switzerland), pIC (Amersham, UK), β-Glucan based polysaccharides were provided by Prof. Olaf Erhatur (Cukurova University, Chem Dept., Adana, Turkey). A high pressure/high temperature stainless steel reactor (Parr 4575 HT/HP) with 500 ml volume was used for extraction of polysaccharide at subcritical water conditions from different mushrooms. The sugar content of the extracts was determined by HPLC (Varian ProStar210, equipped with a RI detector) against monosaccharide standards and protein determination by Lowry assay. Monosaccharide analyses of the PS yielded mainly glucose, in addition to mannose and galactose. The purity was found to be over 97%. Molecular weight determination was carried out by high performance size exclusion chromatography (on a HPSEC-MALLS system). In this study, polysaccharides were abbreviated as PS1 to PS4. Their physicochemical characteristics were as follow: i) Ganoderma lucidum (Alata strain)-PS1 (Ave. MWt: 2.9 × 10^6 Da and pKa: 6.64), ii) G. lucidum (Balcari strain)-PS2 (Ave. MWt: 3.8 × 10^6 Da and pKa: 6.99), iii) Shiitake-PS3 (Ave. MWt: 1.6 × 10^6 Da and pKa: 6.67), and iv) G. lucidum (Alata strain)-PS4 (Ave. MWt: 5.2 × 10^6 Da and pKa: 6.62). Endotoxin levels for all PSs were checked by LAL assay and were found to be undetectable (minimum detection limit of the assay was 0.01 EU/ml). PS nano-complexes with pIC (1:1 w/w, PS:ligand ratio) were prepared overnight at 4°C. Low-endotoxin levels for all PSs were checked by LAL assay and were found to be undetectable (minimum detection limit of the assay was 0.01 EU/ml). PS nano-complexes with pIC (1:1 w/w, PS:ligand ratio) were prepared overnight at 4°C. Endotoxin levels for all PSs were checked by LAL assay and were found to be undetectable (minimum detection limit of the assay was 0.01 EU/ml). PS nano-complexes with pIC (1:1 w/w, PS:ligand ratio) were prepared overnight at 4°C. Endotoxin levels for all PSs were checked by LAL assay and were found to be undetectable (minimum detection limit of the assay was 0.01 EU/ml).

2.2. Mice

Adult C57BL/6 and BALB/C mice (female, 6–8 weeks old) were housed in Animal of Molecular Biology and Genetics, facility and were provided with unlimited access of food and water. All experimental procedures were approved by the animal ethical committee of Bilkent University (Bil-AEC/Protocol#: 2006/027). RAW 264.7 cells (ATCC) or splenocytes were cultured with RPMI 1640 supplemented with 5% FBS, 50 mg/ml penicillin/streptomycin, 2 mM L-glutamine, 10 mM HEPES buffer, 0.1 mg/ml sodium pyruvate, HEPES buffer, 0.1 mg/ml sodium pyruvate. HEK 293 or stably hTLR2 transfected cells (Invivogen, USA), were kept in high-glucose DMEM media with 10% FBS.

2.3. Immunization

Adult female C57/Bl6 mice (5/group) were injected ip with of PS, pIC (15 μg each) or PS-pIC nanocomplex combined with 7.5 μg of OVA. One day before booster injection (ød – 1) animals were bled and next day injected with the same formulations. On day 28 mice were bled. Sera from the primary and secondary bleedings were studied for total IgG, IgG1, and IgG2a antibodies as described elsewhere [17]. Nitric oxide detection by Griess method was conducted on RAW 264.7 cells (10^6/ml) after 6–48 h of incubation as described by the suppliers (Promega WI, USA).

2.4. ELISA and NO assays

Immuno 2 HB microtitre plates (Thermo Scientific, USA) were coated with anti-cytokine or anti-IgG antibodies and then blocked with PBS-BSA 1X [17,35]. Serially diluted standards and culture supernatants or mouse sera were added to these plates for 2 h. Cytokine was detected using biotinylated anti-cytokine Ab (TNFs: XET22 and 16G: 20F3 clones) followed by phosphatase-streptavidin (Promo Bio, USA) whereas bound IgG subclasses were detected using phosphatase-conjugated anti-IgG, IgG1, and IgG2a antibodies as described elsewhere [17]. Nitric oxide detection by Griess method was conducted on RAW 264.7 cells (10^6/ml) after 6–48 h of incubation as described by the suppliers (Promega WI, USA).

2.5. Luciferase assay

hTLR2 expressing HEK cells were transfected using FuGENE6 with pSduxNfoxB luciferase as described in manufacturer’s protocol. Following overnight stimulations, cells were further incubated for 24–36 h and were harvested and assayed for luciferase activity (Roche, Germany). Plots were generated from relative light units.

2.6. Cytokine and chemokine RT-PCR

Total RNA was extracted from the cells that were treated either with PS or with pIC nanocomplexes for 2–6 h. They were reverse-transcribed and amplified to obtain cDNA in a standard PCR reaction for 30 cycles using primers for murine specific target genes (Supplementary Table S2) as previously described [35,36]. PCR amplified material was separated on 2% agarose gels and visualized under UV light after ethidium bromide staining.

2.7. Atomic force microscopy (AFM) and size measurement studies of the nanocomplexes

pIC, PS4 and their nanocomplexes were diluted in DNase/RNase free H2O and were deposited on silicon wafer. Following complete drying images were taken by using non-contact mode NX100E model AFM (PSIA with XEI 1.6 software incorporated) with a 0.73–0.79 Hz scanning rate. The scanning area sizes were in 1 × 1 μm. Particle size analyses of the generated nanocomplexes were measured using dynamic light scattering method on a zetasizer (Model: Nano ZS, Malvern, UK).

2.8. Statistical analysis

Statistical significant differences between groups were determined using Student’s t-test analysis via SigmaStat software. P values < 0.05 were considered significant.

3. Results

3.1. TNFα and nitric oxide production by polysaccharides

Initial experiments were designed to understand the immunostimulatory potential of the four PS candidates in dose-titration (20–0.02 μg/ml) assays. A well established positive control peptidoglycan (PGN) was run in parallel to compare the response raised by β-Glucan polymers. Fig. 1 shows that when RAW cells were treated with different PSs for 24 h, a dose dependent TNFα (Fig. 1A) and NO (Fig. 1B) production were induced especially by PS2 and PS4. PS4 was the most active among other tested PSs at doses

Fig. 1. Dose dependent TNFα and NO, induction from RAW cells following 24 h post-stimulation with different β-Glucan polymers. A) TNFα and B) NO were detected by ELISA and Griess assay, respectively from cell supernatants. Result represents combination of at least two independent experiments (mean ± SEM) of triplicate samples treated with different stimulants (0.02–20 μg/ml). PS4 vs PS2 comparison gave a p < 0.001 for TNFα (at all doses) and p < 0.004 for NO (at 0.02 μg/ml).
greater or equal to 0.2 μg/ml (Fig. 1A). In the case of NO induction PS4 was superior (at all doses) than the rest of the stimulants including positive control PGN (Fig. 1B).

3.2. PS mediated upregulation of various cytokine/chemokine transcripts

To examine the kinetics of PS driven immune activation RAW cells were incubated with different β-Glucan stimulants. The culture fluids collected at different time intervals (6, 12, 24, 36, and 48 h after stimulation) after 2 μg/ml PS treatments were assayed for TNFα (Fig. 2A) and bactericidal mediator NO (Fig. 2B). As seen in Fig. 2A, as early as 6 h post-stimulation all PS types secreted substantial amount of TNFα. Among the tested groups, only PS4 type continued to increase TNFα level by 48 h. Similarly, nitric oxide secretion profiles of different PS types gave a time-dependent response. Again, consistent with Fig. 1, only PS2 and PS4 (Fig. 2B) displayed time-dependent NO production from macrophages. Also these β-Glucans’ effects on mRNA transcript upregulation of TLRs as well as various cytokines were assessed after 4 h incubation. When TLR transcript levels of an untreated mouse splenocytes were compared to that of PS treated cells’ transcript levels PS2 and PS4 treatment highly upregulated expression of tlr1, 2, 5, 7, and 9 genes (Supplementary Figure S1A). Moreover, compared to untreated vs PS treated cells, mRNA band intensities for il-15, il-18, and TNFα significantly increased only upon PS4 treatment (Supplementary Figure S1B). These results suggested that PS4 and then PS2 polysaccharides were the most potent stimulants.

3.3. Engagement of PS by TLR2

Several β-Glucan polymers were reported to trigger signaling cascade either using cell surface expressed TLR2 (alone or engaging TLR1 or TLR6), TLR4 or even cytosolic sensors such as NOD1 and NOD2 [22,31]. To differentiate between these alternatives HEK cells stably expressing hTLR2, hTLR4 or TLR2/6 genes were transfected with p5xLucNFκB and in subsequent experiments co-transfected with pcDNA3NOD1 or pcDNA3NOD2 or mock plasmids to check whether these PS polymers either engages only through TLRs or additionally interact with the cytosolic NOD sensors. In Fig. 3, NFκB-mediated luciferase activity studies demonstrated that PS triggers via TLR2 receptor (Fig. 3A) and furthermore regulates IL8 production (Fig. 3B). Expression of TLR6 did not improve TLR2 mediated promoter activation (data not shown). Consistent with previous results, PS4 is the most potent inducer. Of note, other TLRs or NODs were not contributing to this immune cascade since subsequent experiments demonstrated that there was no further significant increase (either luciferase activity or IL8 production) mediated by PS treatment of cells expressing TLR4, or NOD1 or NOD2 (data not shown).

3.4. PS4 activity upon in vivo administration to mice

These four PSs were given to mice (2 μg/ml) and 6 h later PEC and spleen cells were recovered and incubated in culture without further stimulation to evaluate cytokine production or chemokine message transcript upregulation by ELISA and RT-PCR, respectively. Consistent with previous observations, PS4 was the most active stimulant among the tested polysaccharides. It yielded the highest IL6 production from PECs after 48 h ex-vivo incubation (Fig. 4A). The spleen cells TNFα and IP10 transcript levels were significantly upregulated when PS1, 2, and 3 vs PS4 band intensities were

![Fig. 2. Time dependent TNFα, and NO release by RAW cells following different PS treatments at 2 μg/ml. A) TNFα and B) NO levels were assessed by ELISA and Griess assay respectively. Result represents combination of at least three independent experiments (mean ± SEM) of triplicate samples treated at different time points (6 h, 12 h, 24 h, 36 h & 48 h). PS4 vs PS1, PS2 (except 12 h) or PS3 comparison gave a p < 0.01 for TNFα (at all time points) and for NO, PS4 vs PS2 is NS, and PS4 vs PS1 or PS3 is p < 0.001 (at all time points except 6 h).](image)

![Fig. 3. PS mediated promoter assay and IL8 production by hTLR2-HEK cells. A) Relative luciferase activity after PS treatment of stable hTLR2-HEK cells. Cells were transfected with p5xLucNFκB plasmid for 24 h and stimulated with 5 μg/ml of each PS for 12 h. B) IL8 production following PS treatment. Stable hTLR2-HEK cells were stimulated with 5 μg/ml of each PS for 24 h. IL8 ELISA was studied from the culture supernatants. Data represents combination of at least two independent experiments (mean ± SEM) of triplicate samples treated with different stimulants (PS1-4, PGN and LPS). PS4 vs PS1, 2, 3 and LPS gave a p < 0.001.](image)
compared. (Fig. 4B). Although previously PS2 activity in culture was similar to PS4, surprisingly it failed to reproduce this trend when administered in vivo. When *in vitro* and *in vivo* data are collectively considered, among four tested PS candidates, PS4 consistently displayed the highest immunostimulatory performance, and was selected to develop PS4-pIC nanoparticles.

3.5. Physical characterization of PS4-pIC nanocomplexes

When nucleic acids are injected *in vivo* they are either subjected to premature digestion by nucleases or adsorbed by serum proteins, therefore, hampering their bioavailability. Accumulating evidence strongly suggested that their *in vivo* applications are limited due to their labile nature. Strategies to improve their stability as well as retaining activity and facilitate their cellular internalization without premature clearance are of great importance to pursue these ligands into clinical use\[15,17\]. To establish whether PS4-pIC nanocomplexes led to a significantly high IL6 production (Fig. 6 and data not shown). It is important to note that pIC alone, when given at this concentration could not produce any detectable IL6, however, the nanocomplexes yielded $1250 \pm 57$ ng/ml of IL6 secretion. This amount is >6x fold more cytokine production to that of PS4 alone stimulation (Fig. 6A left panel). Furthermore, macrophages stimulated by these nanocomplexes induced very strong bactericidal mediator, NO compared to either of the PS4 alone or pIC alone treatments (Supplementary Figure S3). When spleen cells were incubated with PS4-pIC nanocomplexes, the gene message levels of *tnfa*, *il15*, *il18*, *mip3a*, and *ip10* were significantly upregulated (compared to either PS4 alone or pIC alone treatments, Fig. 6B). The activity of these nanocomplexes were further analyzed following ip injection of mice (3/group) and checking IFNγ transcript level from recovered spleen cells (Fig. 6C). Consistently, PS4-pIC nanoparticle treated animals displayed the highest *ifng* message. These results strongly support the synergistic activation mediated by the nanocomplex.

3.7. Immunization with PS4-pIC nanoparticles

To examine whether the PS4-pIC nanoparticles could act as an effective immune adjuvant/delivery system, ovalbumin (OVA) was loaded as the model antigen and C57BL/6 mice were immunized. Primary and secondary bleeding sera were analyzed to detect the levels of total IgG, IgG1 and IgG2a. As seen in Fig. 7, total IgG and anti-OVA specific IgG subtypes were significantly boosted by the PS4-OVA-pIC nanoparticles when compared to dual combination groups (i.e. pIC + OVA or PS + OVA). This was evident even after primary immunization, since there was 2–3-fold more anti-OVA Ab induction at 1/2000 antibody titre. (Fig. 7A). As expected, significantly higher magnitude of total IgG, IgG1 as well as IgG2a titers were obtained after booster injection. In Fig. 7B, at titre 1/8000, all IgG types were improved at least 2-fold and up when mice were immunized with the nanocomplex. These data implied that generated stable nanoparticles ensured simultaneous internalization of the adjuvant (signal 1) and antigen (signal 2) by antigen presenting cells (APCs), thereby leading to stronger anti-OVA immunity.

4. Discussion

Natural polysaccharides extracted from mushrooms was shown to activate host immune system leading to production of various Th1-biased cytokines [23]. One approach to induce an improved immune response is to ensure that these formulations achieve simultaneous presentation of antigen and adjuvant to relevant APCs. Developing such systems that act in synergy with the loaded adjuvant may further improve the success of the therapy and help host defence [14,24–26].

In this study, PS/Nucleic acid ligand nanocomplexes were designed to solve several key problems associated with poor in vivo performance. Polysaccharides used in this study are: i) naturally occurring, readily available, easily extracted in bulk ii) cheap iii) does not require sophisticated formulation technology as it is water soluble iv) readily undergoes stable interaction with charged....
macromolecules due to their amphiphilic nature v) effectively engage by cells expressing TLR2 on their surface (i.e. natural targeting), vi) the labile cargo is protected from premature degradation/elimination and finally vii) act as a co-adjuvant in vaccine formulations along with the adjuvant to augment Ag-dependent immune activation. Our findings strongly suggest that all these key features are fulfilled by the developed formulation.

Recent studies showed that a natural β-glucan polysaccharide isolated from *Schizophyllan* modified with peptides and cholesterol formed a complex with TLR9 ligand, CpG ODNs and significantly elevated secretion of cytokines (IL12, IL6 and TNFα) from BMDCs, and murine macrophages [27,28]. These compounds were proposed to trigger several immune cells through various receptors including complement receptor (CR3), scavenger receptors, as well as TLR4 or TLR2/6 [29–32]. In other study, *G. lucidum* polysaccharides enhanced CD14 mediated endocytosis of LPS and promoted TLR2/4 signal transduction of cytokine expression [31].

Contrary to previous reports, our findings suggested that mushroom derived PS polymers recognized only via TLR2 but not via TLR4 or TLR2/6. Furthermore, to date, there is no document reporting the collaborative activation of TLR3 ligand with PS obtained from edible mushrooms. Moreover, in the present study plain PS compounds were used with no further modification (i.e cholesterol or peptide). The cytokine secretion panels (dose-dependent and time-dependent findings) presented in Figs. 1 and 2 clearly suggested that PS4 is the most potent stimulant. Moreover, in HEK cell system we established that these PS types only interact with TLR2 (Fig. 3) but not with other cell surface or cytosolic receptors (NOD1 or 2). In the literature, receptor usage of PS is still elusive. This is probably due to the problem associated with the extraction of PS from different sources. Similar compounds were reported to trigger a group of immune cells including macrophages, neutrophils, monocytes, natural killer cells and dendritic cells through various receptors including Dectin-1 and scavenger receptors [31,32]. We have attempted to delineate the contribution of PS mediated activation via the scavenger receptor CXCL16. It was found that it does not involve in PS mediated cytokine production, since there was no up or down regulation of the *cxcl16* gene transcript (Supplementary Figure S2). However, Dectin-1 has not been studied here, and must be checked to resolve its involvement.

We further assessed the nitric oxide production mediated by different PS compounds. Since NO is an important regulator and mediator of macrophage directed cytotoxicity against microbes and tumour cells, detecting the most potent NO inducer as well as establishing the breadth of activation pattern of PS4 was very critical [33,34]. The results of in vitro findings were reproduced in vivo (Figs. 1, 2 and 4). Among all tested PSs, consistently PS4 triggered the highest IL6 production in addition to NO secretion as well as enhanced several key cytokine/chemokine gene expression in PECs and splenocytes. This is the first example to demonstrate that a polysaccharide extracted from mushrooms induced strong NO production from immune cells.

PS4-pIC nanoparticles supported the added effect of formulating them as a carrier depot system. In addition to IL6 production by
Fig. 6. PS-pIC nanoparticles induced higher IL-6 production and strong gene upregulation of various cytokine and chemokines after in vitro and in vivo stimulations. A) RAW cell supernatants from 36 h culture were analyzed by ELISA. Result represents combination of at least two independent experiments (mean ± SEM) of triplicate samples. $p < 0.002$ nanocomplexes vs nucleic acid alone treated groups. B) After stimulation with PS4 (0.016 μg/ml) or PS4-pI:C (0.16 μg/ml each) for 4 h in culture, total RNA from spleen cells were subjected to RT-PCR and cDNA were amplified against gene-specific primers. C) PS4-pIC nanoparticles (2 μg each) was injected to mice and spleen cells were recovered 18 h post-treatment for IFN-γ transcript analyses (inlet; representative band intensities from a single animal). Band intensities were reported as average of 3 mice/group.
immune cells compared to their alone treatments (Fig. 6A), data also demonstrated that several cardinal chemokine and cytokine mRNA messages were upregulated (Fig. 6B). The in vivo benefit of PS4-pIC nanocomplexes were documented in ex vivo study along with model antigen immunization study. Data suggested that these formulations could improve primary and booster immunity against OVA antigen (Fig. 7). This is primarily due to the fact that, the present formulation helped avoiding premature clearance and digestion of its cargo as well as increased stability and efficient delivery of OVA + adjuvant simultaneously to APCs, thereby resulting more pronounced immune response.

5. Conclusions

The present work established that combination of mushroom derived polysaccharide and pIC forms stable nanocomplexes and triggered upregulation of inflammatory cytokines, and bacterialid mediators as evidenced by dose and time-dependent kinetic profiles of, IL6, IL18, TNFz and NO productions. Immune enhancement of PS is dependent on TLR3 signaling, compounded by the pIC mediated TLR3 signaling resulted stronger pro-inflammatory effect of the formulation. When used in vivo as a protein vaccine carrier, nanocomplex led to elevated IgG titers against ovalbumin antigen in mice, compared to either PS + OVA or pIC + OVA immunizations. In conclusion, present system can be formulated with other clinically valuable bioactive agents such as siRNA, plasmids, and different nucleic acid based TLR ligands, along with peptides or proteins as vaccine carriers to control infectious pathogenic insults.

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Appendix. Supplementary data

Supplementary data associated with this article can be found in online version at doi:10.1016/j.biomaterials.2011.01.028.

References


