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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 polyI: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, MIP3α, 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

Abbreviations: AFM, Atomic Force Microscopy; BMDC, Bone marrow derived dendritic cell; HEK, Human embryonic kidney; NOD, Nucleotide-binding oligomerization domain; ODN, Oligodeoxynucleotide; PEC, Peritoneal exudate cells; PGN, Peptidoglycan; pIC, Poly(riboinosinic:ribocytidylic acid); PS, Polysaccharide.

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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. Oktay Erbatur (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 >93%. Following protease digestion to eliminate protein contamination purity reached 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* (Balcalı 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: 6.69). 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 nanocomplexes with pIC (1:1 w/w, PS:ligand ratio) were prepared overnight at 4 °C. Unbound pIC was filtered (Microspin G-25 column) free nucleic acid concentration was measured by Nanodrop™ from the eluent. pIC incorporation was over 90% in all preliminary trials.

2.2. Mice

Adult C57BL/6 and BALB/C mice (female, 6–8 weeks old) were housed in Department 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, 0,11 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 ($@d = 13$) 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 by ELISA.

2.4. ELISA and NO assays

Immulon 2 HB microtitre plates (Thermo Scientific, USA) were coated with anti-cytokine or anti-IgG antibodies and then blocked with PBS-BSA 1% [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 (TNFα: XT22 and IL6: 2OF3 clones) followed by phosphatase-streptavidin (Perbio Pierce, 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 (10^5/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 p5xlucNFκB 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 H_2O and were deposited on silicon wafer. Following complete drying images were taken by using non-contact mode XE-100E 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

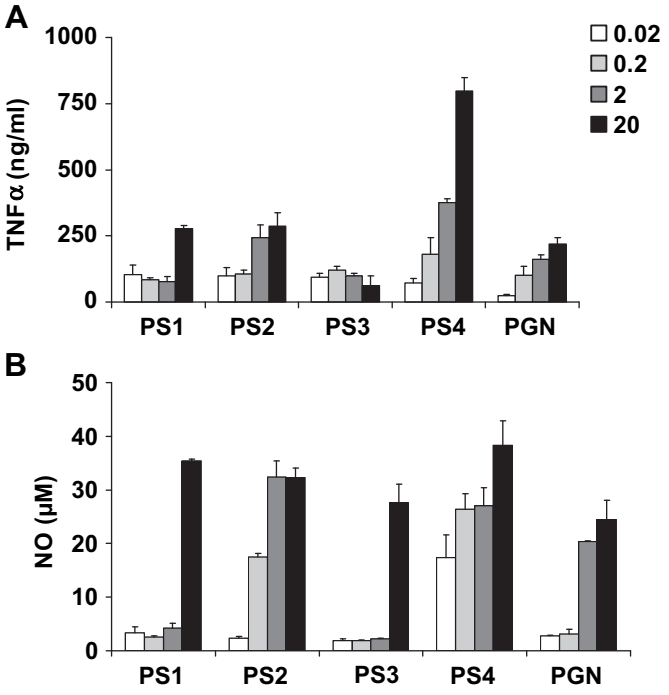

Fig. 1. Dose dependent TNFα, and NO, induction from RAW cells following 24 h post-stimulation with different β-Glucan polymers. A) TNFα and B) NO were detected by ELISA and Griess assay, respectively from cell supernatants. Result represents combination of at least two independent experiments (mean ± 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).