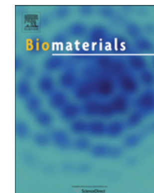
Biomaterials 32 (2011) 4275–4282

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials



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

Gizem Tincer [a], Seda Yerlikaya [a,1], Fuat C. Yagci [a], Tamer Kahraman [a], Osman M. Atanur [b], Oktay Erbatur [b], Ihsan Gursel [a,*]

[a] Biotherapeutic ODN Research Laboratory, Molecular Biology and Genetics Department, Faculty of Sciences, Bilkent University, Bilkent, Ankara 06800, Turkey
[b] Chemistry Department, Science Faculty, Çukurova University, Balcalı, Adana 01330, Turkey

ARTICLE INFO

Article history:
Received 27 November 2010
Accepted 12 January 2011
Available online 2 April 2011

Keywords:
Polysaccharides
Poly(I:C)
Nanoparticles
Targeted delivery system
TLR
Immunogenicity

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.

© 2011 Elsevier Ltd. All rights reserved.

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.

* Corresponding author. Tel.: +90 312 290 24 08; fax: +90 312 266 50 97.
E-mail address: ihsangursel@bilkent.edu.tr (I. Gursel).
[1] Present address: Department of Molecular Biology, University of Geneva, 30 quai Ernest-Ansermet, CH-1211 Geneva 4, Switzerland.

0142-9612/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biomaterials.2011.01.028

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

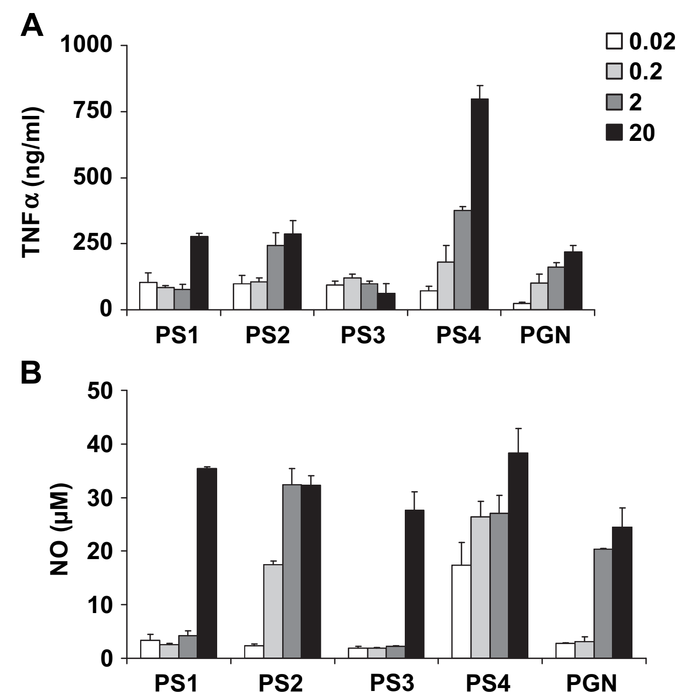


Fig. 1. Dose dependent TNFα, and NO, induction from RAW cells following 24 h post-stimulation with different β-Glucan polymers. A) TNFα and B) NO were detected by ELISA and Griess assay, respectively from cell supernatants. Result represents combination of at least two independent experiments (mean ±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

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

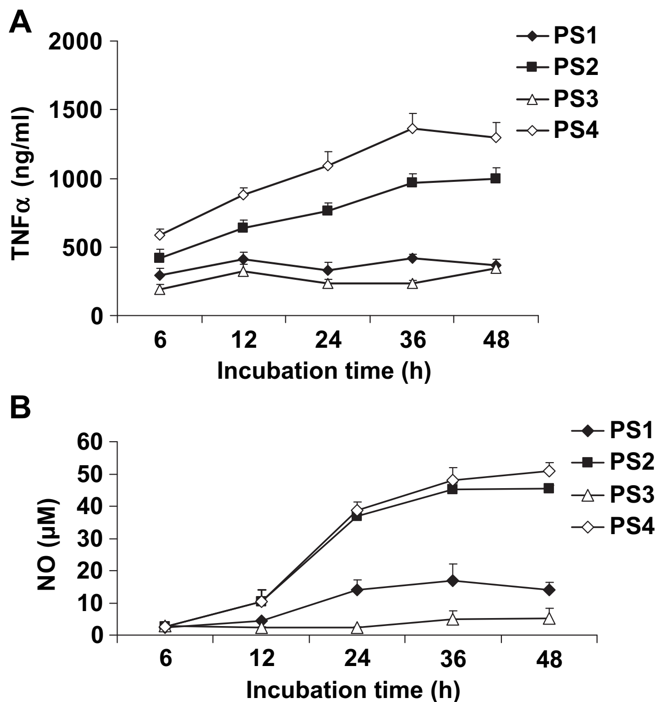


Fig. 2. Time dependent TNFα, and NO release by RAW cells following different PS treatments at 2 μg/ml. A) TNFα and B) NO levels were assessed by ELISA and Griess assay respectively. Result represents combination of at least three independent experiments (mean ± SEM) of triplicate samples treated at different 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).

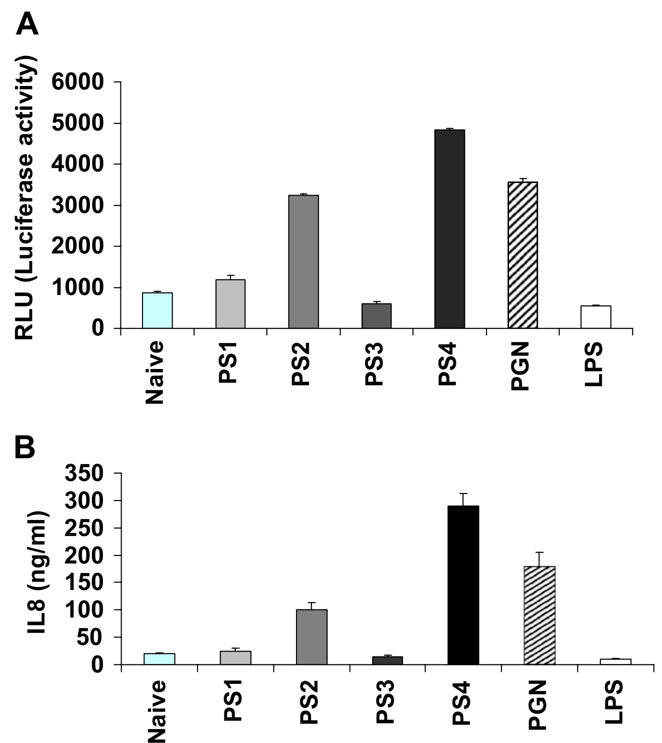


Fig. 3. PS mediated promoter assay and IL8 production by *hTLR2*-HEK cells. A) Relative luciferase activity after PS treatment of stable *hTLR2*-HEK cells. Cells were transfected with p5xlucNFκB plasmid for 24 h and stimulated with 5 μg/ml of each PS for 12 h. B) IL8 production following PS treatment. 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 $p < 0.001$.

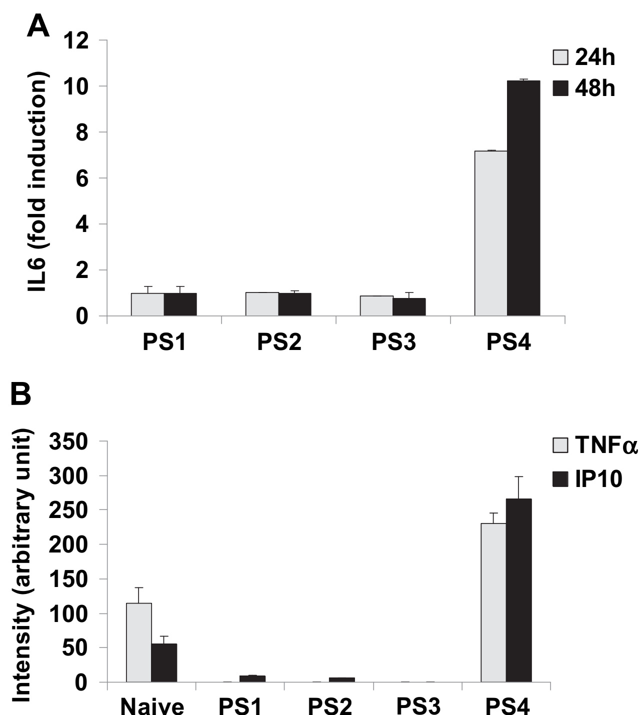


Fig. 4. *In vivo* immunostimulatory activity of polysaccharides. A) Six hours after i.p. PS injection (2 μg/ml), PECs were isolated (3 mice/group). Cells (4×10^6/ml) were incubated *ex-vivo* for further 24 h or 48 h, IL6 levels from supernatants were measured by cytokine ELISA. Fold induction of IL6 production over naive cells was plotted (naive cells induced 56 ± 13 @ 24 h and 164 ± 23 @ 48 h pg/ml IL6). B) mRNA levels of TNFα and IP10 were assessed by RT-PCR from spleen cells. Densitometric measurements (obtained from 3 independent mice) for the gel band intensities were plotted. PS4 vs PS1, 2 and 3 gave a $p < 0.001$.

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 complexes form stable nanoparticles their physical characteristics by i) AFM and by ii) dynamic light scattering techniques (Zetasizer Nano, ZS™) were studied. AFM photomicrographs revealed that PS4 appeared as individual nanoparticles around 50–70 nm in size (Fig. 5). This was further confirmed by Zetasizer and was found to be ca. 40 ± 20 nm (Ave ± SEM) (Supplementary Figure S2). PS4 nanocomplex with pIC led to an increase in the nanoparticle size from 40 nm to 165 nm (Supplementary Figure S2). The stability of these nanocomplexes was also studied by dynamic light scattering method using Zetasizer. There was no significant change in the size of the generated nanocomplexes over a period of two weeks indicating that there was no spontaneous fusion (or disintegration) of the individual particles when suspended either in PBS or in FBS supplemented media (Supplementary Table S1, and data not shown).

3.6. Synergistic immune activation by PS4-pIC nanoparticles

Next we analyzed the immunostimulatory effect of PS4 nanocomplexes harbouring one of the labile nucleic acid ligands, pIC. Whether it improves pIC dependent immune activation to that of its free form is of great interest. As presented in Fig. 6, PS4-pIC nanoparticles induced synergistic activation on both RAW 264.7 or mouse spleen cells upon *in vitro* or *in vivo* treatments. 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 nanoparticles yielded 1250 ± 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, mip3α, 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 nanocomplex treated animals displayed the highest *ifnγ* 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 led 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

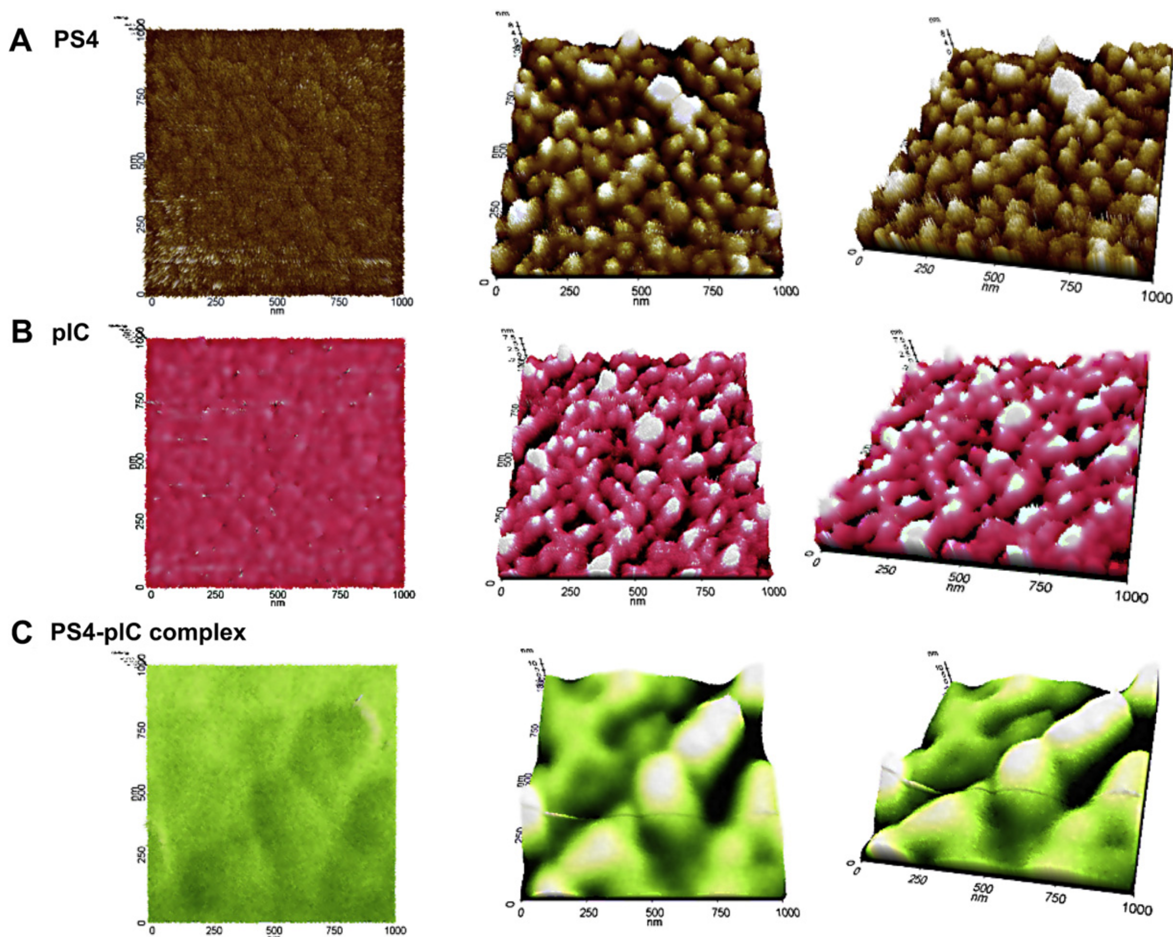


Fig. 5. AFM photomicrographs of phase, 2D phase (left) and 3D topography (middle and right) images. A) PS4, B) pIC and C) PS4-pIC nanoparticles at 1000 nm^2 area. Images were recorded under contact mode.

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

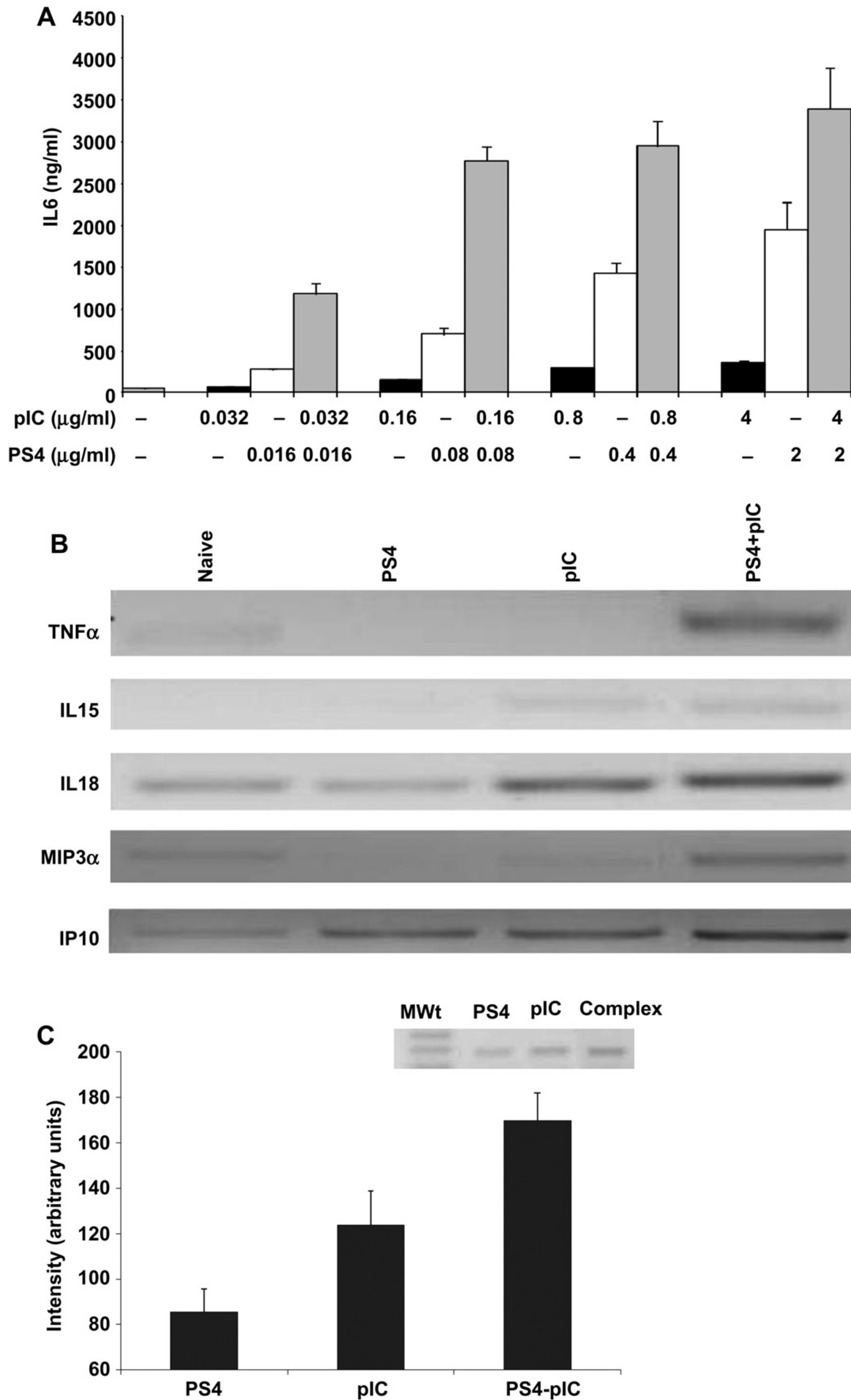


Fig. 6. PS-pIC nanoparticles induced higher IL6 production and strong gene upregulation of various cytokine and chemokines after *in vitro* and *in vivo* stimulations. A) RAW cell supernatants from 36 h culture were analyzed by ELISA. Result represents combination of at least two independent experiments (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 IFNγ transcript analyses (inlet; representative band intensities from a single animal). Band intensities were reported as average of 3 mice/group.

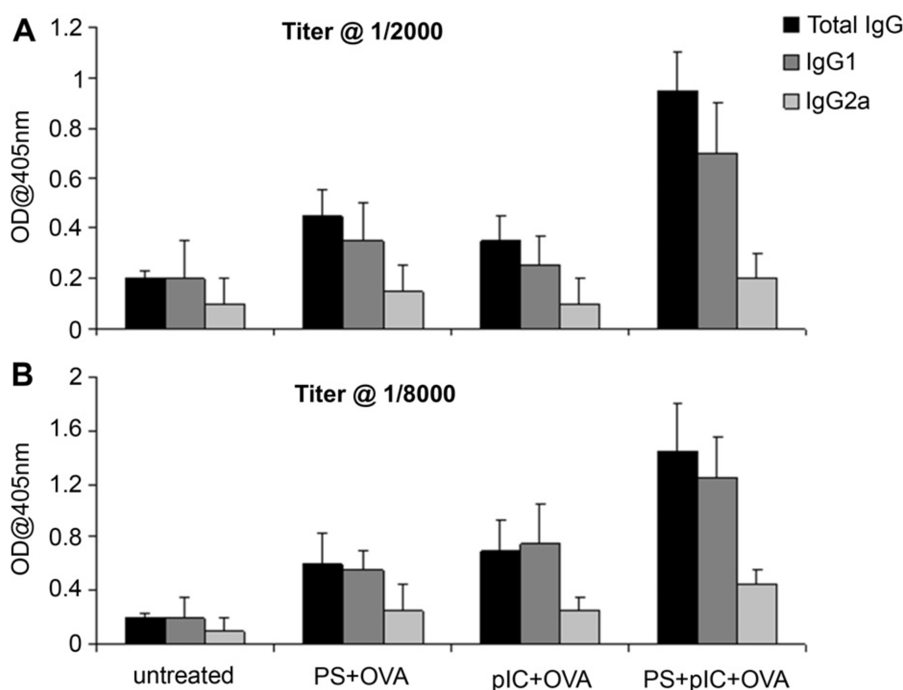


Fig. 7. IgG anti-OVA response of immunized mice. Animals were injected i.p with 15 μg of PS4 + pIC combination or PS4-pIC nanoparticles incorporating 10 μg OVA. Serum anti-OVA titers at day 13 (A) and day 28 (B) were determined by Total IgG, IgG1 and IgG2a ELISA. Data represents the average ± SD serum IgG anti-OVA response (five mice per 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 bactericidal mediators as evidenced by dose and time-dependent kinetic profiles of, IL6, IL18, TNFα and NO productions. Immune enhancement of PS is dependent on TLR2 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.

Acknowledgements

This work was supported by the Scientific and Technological Research Council of Turkey (TÜBİTAK, Grant No: 108S316). IG received grant support from EU/FP6/MC IRG (Grant # 036615) and EU 7th Framework Project UNAM-Regpot (Grant # 203953). GT and FCY received scholarship grants from SANTEZ (0448-STZ-2009-2) and TUBITAK. We thank to Dr. Aykutlu Dana and his group members for their invaluable support during AFM studies. Drs. Can K. Akcali and Mayda Gursel are sincerely acknowledged for their critical reading of the manuscript.

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

[1] Janeway Jr CA, Medzhitov R. Innate immune recognition. Annu Rev Immunol 2002;20:197–216.
[2] Medzhitov R. Toll-like receptors and innate immunity. Nat Rev Immunol 2001;1:135–45.
[3] Takeda K, Akira S. Toll-like receptors in innate immunity. Int Immunol 2005;17:1–14.
[4] Ishii KJ, Koyama S, Nakagawa A, Coban C, Akira S. Host innate immune receptors and beyond: making sense of microbial infections. Cell Host Microbe 2008;3:352–63.
[5] Schmidt KN, Leung B, Kwong M, Zarember KA, Satyal S, Navas TA, et al. APC-independent activation of NK cells by the Toll-like receptor 3 agonist double-stranded RNA. J Immunol 2004;172:138–43.
[6] Barry ME, Pinto-Gonzalez D, Orson FM, McKenzie GJ, Petry GR, Barry MA. Role of endogenous endonucleases and tissue site in transfection and CpG-mediated immune activation after naked DNA injection. Hum Gene Ther 1999;10:2461–80.
[7] Sparwasser T, Miethke T, Lipford G, Borschert K, Hacker H, Heeg K, et al. Bacterial DNA causes septic shock. Nature 1997;386:336–7.
[8] Krieg AM, Efler SM, Wittpoth M, Adhami AI, M.J., Davis HL. Induction of systemic TH1-like innate immunity in normal volunteers following subcutaneous but not intravenous administration of CPG 7909, a synthetic B-class CpG oligodeoxynucleotide TLR9 agonist. J Immunother 2004;27:460–71.
[9] Monteith DK, Henry SP, Howard RB, Flournoy S, Levin AA, Bennett CF, et al. Immune stimulation–a class effect of phosphorothioate oligodeoxynucleotides in rodents. Anticancer Drug Des 1997;12:421–32.
[10] Sparwasser T, Hultner L, Koch ES, Luz A, Lipford GB, Wagner H. Immunostimulatory CpG-oligodeoxynucleotides cause extramedullary murine hemopoiesis. J Immunol 1999;162:2368–74.
[11] Lipford GB, Sparwasser T, Zimmermann S, Heeg K, Wagner H. CpG-DNA-mediated transient lymphadenopathy is associated with a state of Th1 predisposition to antigen-driven responses. J Immunol 2000;165:1228–35.

[12] Borges O, Silva M, de Sousa A, Borchard G, Junginger HE, Cordeiro-da-Silva A. Alginate coated chitosan nanoparticles are an effective subcutaneous adjuvant for hepatitis B surface antigen. Int Immunopharmacol 2008;8:1773–80.
[13] Fischer S, Schlosser E, Mueller M, Csaba N, Merkle HP, Groettrup M, et al. Concomitant delivery of a CTL-restricted peptide antigen and CpG ODN by PLGA microparticles induces cellular immune response. J Drug Target 2009;17:652–61.
[14] Schlosser E, Mueller M, Fischer S, Basta S, Busch DH, Gander B, et al. TLR ligands and antigen need to be coencapsulated into the same biodegradable microsphere for the generation of potent cytotoxic T lymphocyte responses. Vaccine 2008;26:1626–37.
[15] Gursel I, Gursel M, Ishii KJ, Klinman DM. Sterically stabilized cationic liposomes improve the uptake and immunostimulatory activity of CpG oligonucleotides. J Immunol 2001;167:3324–8.
[16] Suzuki Y, Wakita D, Chamoto K, Narita Y, Tsuji T, Takeshima T, et al. Liposome-encapsulated CpG oligodeoxynucleotides as a potent adjuvant for inducing type 1 innate immunity. Cancer Res 2004;64:8754–60.
[17] Xie H, Gursel I, Ivins BE, Singh M, O'Hagan DT, Ulmer JB, et al. CpG oligodeoxynucleotides adsorbed onto polylactide-co-glycolide microparticles improve the immunogenicity and protective activity of the licensed anthrax vaccine. Infect Immun 2005;73:828–33.
[18] Zaks K, Jordan M, Guth A, Sellins K, Kedl R, Izzo A, et al. Efficient immunization and cross-priming by vaccine adjuvants containing TLR3 or TLR9 agonists complexed to cationic liposomes. J Immunol 2006;176:7335–45.
[19] Mozafari MR. Liposomes: an overview of manufacturing techniques. Cell Mol Biol Lett 2005;10:711–9.
[20] Crommelin DJ, Storm G. Liposomes: from the bench to the bed. J Liposome Res 2003;13:33–6.
[21] Chirila TV, Rakoczy PE, Garrett KL, Lou X, Constable IJ. The use of synthetic polymers for delivery of therapeutic antisense oligodeoxynucleotides. Biomaterials 2002;23:321–42.
[22] Kim YG, Park JH, Shaw MH, Franchi L, Inohara N, Nunez G. The cytosolic sensors Nod1 and Nod2 are critical for bacterial recognition and host defense after exposure to Toll-like receptor ligands. Immunity 2008;28:246–57.
[23] Lin ZB, Zhang HN. Anti-tumor and immunoregulatory activities of Ganoderma lucidum and its possible mechanisms. Acta Pharmacol Sin 2004;25:1387–95.
[24] Bourquin C, Anz D, Zwiorek K, Lanz AL, Fuchs S, Weigel S, et al. Targeting CpG oligonucleotides to the lymph node by nanoparticles elicits efficient antitumoral immunity. J Immunol 2008;181:2990–8.
[25] Wilson KD, de Jong SD, Tam YK. Lipid-based delivery of CpG oligonucleotides enhances immunotherapeutic efficacy. Adv Drug Deliv Rev 2009;61:233–42.
[26] Mizu M, Koumoto K, Anada T, Matsumoto T, Numata M, Shinkai S, et al. A polysaccharide carrier for immunostimulatory CpG DNAs to enhance cytokine secretion. J Am Chem Soc 2004;126:8372–3.
[27] Shimada N, Coban C, Takeda Y, Mizu M, Minari J, Anada T, et al. A polysaccharide carrier to effectively deliver native phosphodiester CpG DNA to antigen-presenting cells. Bioconjug Chem 2007;18:1280–6.
[28] Mizu M, Koumoto K, Kimura T, Sakurai K, Shinkai S. Protection of polynucleotides against nuclease-mediated hydrolysis by complexation with schizophyllan. Biomaterials 2004;25:3109–16.
[29] Chan GC, Chan WK, Sze DM. The effects of beta-glucan on human immune and cancer cells. J Hematol Oncol 2009;2:25.
[30] Zhu XL, Lin ZB. Effects of Ganoderma lucidum polysaccharides on proliferation and cytotoxicity of cytokine-induced killer cells. Acta Pharmacol Sin 2005;26:1130–7.
[31] Hua KF, Hsu HY, Chao LK, Chen ST, Yang WB, Hsu J, et al. Ganoderma lucidum polysaccharides enhance CD14 endocytosis of LPS and promote TLR4 signal transduction of cytokine expression. J Cell Physiol 2007;212:537–50.
[32] Lin YL, Liang YC, Lee SS, Chiang BL. Polysaccharide purified from Ganoderma lucidum induced activation and maturation of human monocyte-derived dendritic cells by the NF-kappaB and p38 mitogen-activated protein kinase pathways. J Leukoc Biol 2005;78:533–43.
[33] Nathan C. Nitric oxide as a secretory product of mammalian cells. FASEB J 1992;6:3051–64.
[34] Chin, M. P., Schauer, D. B. and Deen, W. M., Nitric oxide, oxygen, and superoxide formation and consumption in macrophages and colonic epithelial cells. Chem Res Toxicol.
[35] Gursel I, Gursel M, Yamada H, Ishii KJ, Takeshita F, Klinman DM. Repetitive elements in mammalian telomeres suppress bacterial DNA-induced immune activation. J Immunol 2003;171:1393–400.
[36] Giulietti A, Overbergh L, Valckx D, Decallonne B, Bouillon R, Mathieu C. An overview of real-time quantitative PCR: applications to quantify cytokine gene expression. Methods 2001;25:386–401.