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Conjugation of chitosan oligosaccharides enhances immune response to porcine circovirus vaccine by activating macrophages

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ABSTRACT

Porcine circovirus type 2 (PCV2)-associated diseases have led to great economic losses to the pig industry. Our lab previously found that conjugation of chitosan oligosaccharides (COS) or via a carrier protein enhanced the immunogenicity of PCV2 vaccine against infectious pathogens. However, precise mechanisms and signal transduction pathways underlying the efficacy of COS conjugation remains poorly defined. In this study, to better understand the effects and mechanism of COS conjugates maintain the adjuvant potential in vivo, we investigated its augmentation of macrophage function, including cell activation, NO production, cytokine production and phagocytosis. Additionally, the role of Toll-like receptors (TLR) proteins in this process was also assessed. The results indicate that, as compared to the PCV and PCV/COS, conjugation of COS effectively enhanced the NO production, cytokines generation and phagocytosis activity of macrophages. Noticeably, the generation of NO and proinflammatory cytokines was closely related to the TLR2/4 signaling pathways, strongly suggesting that conjugation of COS regulates innate and adaptive immunity by activation of macrophages, resulting in immune enhancement. In summary, the present study provides a potential mechanism of COS conjugation as a novel adjuvant to improve immune responses against various diseases.

1. Introduction

Recently, porcine circovirus type 2 (PCV2) has been associated with several disease syndromes collectively named porcine circovirus-associated disease (PCVAD) (Chae, 2005), which was estimated to cost around £88 million per year during the epidemic period (Alarcon et al., 2013). Several commercial vaccines have been available for the control of PCVAD, however, they failed to completely prevent PCV2 infection and transmission (Beach and Meng, 2012). Therefore, the use of adjuvant is an effective strategy to augment the efficacy of PCV2 vaccines. As a potential adjuvant, chitosan oligosaccharides (COS) was confirmed to have versatile biological functions such as immunostimulation, anti-inflammation and anti-infection (Qiao et al., 2010). To ensure the strong immunogenicity of COS while avoiding its possible toxicity, we previously explored the idea of covalently linking COS to PCV2 vaccine as an alternative to traditional mixing adjuvants. Our lab demonstrated that COS has significant adjuvant effects by covalently linking to PCV2 vaccine (Zhang et al., 2017a,b). Further, we also developed an effective adjuvant system by covalent conjugation of COS via a carrier protein (PCV-OVA-COS), which showed higher immunogenicity compared to the direct conjugation of COS (PCV-COS) or a carrier protein (PCV-OVA) alone (accepted). Thus, covalent conjugation of COS is an effective strategy to improve the immunogenicity of PCV2 vaccine.

Regretfully, until now the precise molecular mechanism underlying the efficacy of COS conjugation remains to be deeply defined. There are several reasons for which the conjugation increases the antigen immunogenicity. Recently, it was shown that the mechanisms of N-trimethyl chitosan-OVA conjugate were related to the induced dendritic cells (DCs) maturation, the increased antigen capturing and subsequent presenting (Slütter et al., 2010). In addition, the COS conjugates may be associated with the activation of macrophages and DCs via the mannose receptor or the Toll-like receptor 4 (TLR4) (Dang et al., 2011; Han et al., 2005). Results from many groups show that the enhancement of cellular immunity is associated with the antigen linkage to agonists targeting TLRs (Blander and Medzhitov, 2006; Khan et al., 2007). They found that a TLR agonist-antigen conjugate elicited cellular immunity based on the engagement of DC cross-presentation pathways and accumulation of DCs in regional lymph nodes (Oh and Kedl, 2010). Besides, the conjugation of antigen and adjuvant ensured their codelivery into a common endosomal compartment and subsequently promoted the

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presentation of antigen to T cells (Blander and Medzhitov, 2006; Burgdorf et al., 2008). Although the above factors may be essential to the vaccine activation, it remains unclear how they play the key role in initiating the immunoreactivity.

The antigen presenting cells (APC) like DCs and macrophages play a critical role in processing the exogenous antigen and priming specific immune response (Alatery et al., 2010; Rossi and Young, 2005). In our previous studies, we demonstrated that the conjugation of COS or via OVA induced the DC maturation and up-regulated the expression of surface markers, including MHC class II, CD40, CD80 and CD86 (accepted). Like DCs, macrophage is an integral part of the immune system which bridges the immune responses. Previous studies show that the treatment of macrophages with COS effectively increased the cell proliferation, phagocytosis function and cytokine production (Feng et al., 2004; Zhang et al., 2014). These results suggest that the adjuvant activity of COS was positively correlated with APC activation. However, the exact mechanism that mediates the activation of macrophages by COS conjugation are poorly defined. To better understand the mechanism of COS conjugation in vivo, we investigated the augmentation of macrophage function including cell activation, NO production, cytokine secretion and phagocytosis. Additionally, the role of TLRs in the presentation process was also explored.

2. Materials and methods

2.1. Reagents

COS was prepared as previously described with the deacetylation degree over 95% and the average molecular weight below 1 kDa (Supplementary Figs. 1–3), and no endotoxin was detected using limulus amebocyte lysate test (Zhang et al., 1999). Inactivated PCV2 vaccine was obtained from WINSUN PHARM, Inc. (Guangdong, China). 3-maleimidobenzoic acid N-hydroxysuccinimide ester (MBS), 2-iminothiolane, 3,3′,5,5′-tetramethylbenzidine (TMB) and methylthiazolyldiphenyl-tetrazolium bromide (MTT) were purchased from Sigma (MO, USA). Rabbit anti-TLR4, horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG and HRP-conjugated goat anti-mouse IgG were obtained from Cell Signaling Technology Inc (MA, USA). Rabbit anti-TLR2 and mouse anti-β-actin were purchased from Santa Cruz Biotechnology (CA, USA). Dulbecco's-modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Corning (VA, USA).

2.2. Cell lines

A murine macrophage cell line, RAW 264.7, was obtained from the American Type Culture Collection (VA, USA). The cells were grown in DMEM containing 10% FBS, 100 units/mL penicillin and 100 μg/mL streptomycin at 37 °C under a 5% CO_2 atmosphere.

2.3. Preparation of PCV conjugates

To prepare the PCV conjugates (COS-PCV, OVA-PCV and COS-OVA-PCV), primary amine of reactants was reacted with 2-iminothiolane or MBS, followed by coupling to each other by using the thiol-maleimide reaction (Fig. 1), as detailed in Supplementary Methods.

2.4. Cell viability assay

The effect of PCV, the mixture of PCV and COS (PCV/COS), and the PCV conjugates on cell viability was analyzed by the MTT assay (Liu et al., 2015). Briefly, RAW 264.7 macrophages were incubated in 96-well plates (Corning 3599, USA) with different concentrations of PCV samples (5, 10, 15, 20, 30, 40 and 50 μg/mL) or LPS (10 μg/mL) for 24 h or 48 h, and the amount of formazan salt was measured at 570 nm using an automated ELISA plate reader.

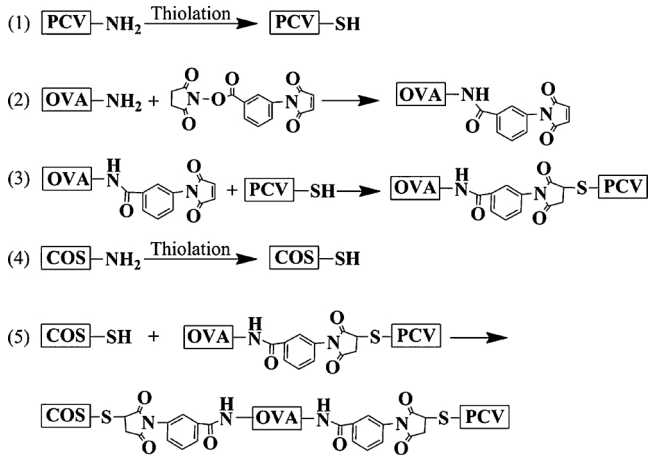

Fig. 1. PCV conjugates prepared for evaluating the effect of covalently linked COS or via OVA. COS: chitosan oligosaccharides, OVA: Ovalbumin.

2.5. Nitric oxide (NO) secretion

The released level of NO in the medium was measured using a colorimetric assay with Griess reagent (Cao et al., 2011). Briefly, RAW 264.7 cells (3×10^4 cells/mL) were cultured with PCV samples or LPS in 96-well plates (Corning 3599, USA) for 24 h. Then 100 μL culture supernatant was mixed with an equal volume of Griess reagent (1% sulfanilamide and 0.1% *N*-[1-naphthyl]-ethylenendiamine dihydrochloride in 5% phosphoric acid) and treated the cells for 10 min with continuous shaking, before measuring the optical density at 540 nm by an automated ELISA plate reader. Sodium nitrite ($NaNO_2$) was used to generate a standard curve.

2.6. Identification of signaling pathways involved in NO secretion

To identify the signaling pathways responsible for mediating PCV conjugates effect on RAW 264.7 cell activation, cells were pre-treated with the p38 MAPK inhibitor SB203580 (10 μM), the JNK inhibitor SP600125 (25 μM), or the JAK2 inhibitor SD1008 (10 μM) for 1 h, followed by incubating with PCV conjugates or LPS (10 μg/mL) for 24 h and NO was measured as described above.

2.7. Reverse transcription PCR (RT-PCR) analysis

RAW 264.7 in 6-well plates (Corning 3516, USA) were treated with PCV, PCV conjugates or LPS (10 μg/mL) for 24 h, respectively. Total RNAs were isolated using Trizol (Invitrogen, USA) and the cDNA was synthesized using RT-PCR kit (TaKaRa, Japan) according to the manufacturer's instruction. The cDNA was amplified by PCR using specific primers set for mouse iNOS, TNF-α, IFN-γ, TLR-2, TLR-4, IL-1β, IL-6, IL-8, Capcase-1 and Actin as an internal control. The primers used were listed in Table 1 (Supplementary). The quantitative real-time PCR (qRT-PCR) was performed using SYBR Select Master Mix and 7500 Fast Real Time PCR system (Applied Biosystems, Foster City, CA, USA). PCR analysis was carried out under the following conditions: denaturation at 95 °C for 10 min, and then 40 cycles of denaturation for 30 s at 95 °C, annealing for 30 s at 60 °C, extension for 30 s at 72 °C. Relative quantification was done using the $2^{-\Delta\Delta CT}$ method, and the expression levels of genes were normalized to that of housekeeping gene β-actin.

2.8. Phagocytosis activity

For fluorescent staining, the FITC-bound antigen was prepared as described previously (Yue et al., 2012). The RAW 264.7 cells were cultured in 24-well chamber slides to 50% confluence and then exposed to FITC, FITC-PCV, FITC-PCV plus COS, FITC-PCV-COS, FITC-PCV-OVA

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