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Laminarin-mediated targeting to Dectin-1 enhances antigen-specific immune responses

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ABSTRACT

It has immense potential for immunotherapy and vaccination to target antigens to antigen-presenting cells (APCs). Here we described a method for delivering whole protein antigens to APCs via carbohydrate-mediated targeting of Dectin-1, which is a C-type lectin and mainly expresses on subpopulations of dendritic cells and macrophages. Laminarin, which is a β-1-3 glucan and a typical ligand for Dectin-1, was chemically coupled to ovalbumin (OVA). Compared to OVA alone, the conjugate was effectively recognized and ingested by CHO cells stably expressing Dectin-1 and bound to bone marrow dendritic cells (BMDCs) via Dectin-1. Laminarin modification led to significant enhancement of OVA-specific $CD4^+$ T-cell response. Moreover, when used to immunize mice, the conjugate enhanced the primary IgG antibody response to OVA. Taken together, our data suggest that APCs targeting based on glucan–Dectin-1 interaction is a promising approach to improve vaccines.

Introduction

Antigen-presenting cells (APCs), particularly dendritic cells (DCs) and macrophages, play a crucial role in the initiation and regulation of antigen-specific immunity [1,2]. In general, the immune responses are the outcomes of the following events: foreign proteins or antigens are taken up by APCs, processed into small peptides and displayed on the surface of APCs as a major histocompatibility complex (MHC). The MHC:antigen complex is then recognized by naive T cells and this recognition eventually leads to T-cells activation and evokes adaptive immunity, including humoral immune response and cellular immune response.

Given the importance of APCs in triggering and regulating immune systems, strategies for targeting these cells *in vivo* will greatly advance immunotherapy and facilitate the design of more effective vaccines. One approach to accessing APCs *in vivo* is to target APC-specific surface receptors with ligand–antigen conjugates that deliver targeted antigens to the antigen-processing/presentation machinery of APCs via receptor-mediated endocytosis. This can be done by complexing antigenic proteins to antibodies that are specific for surface receptors on APCs. For example, antibody-mediated targeting of antigen to DCs via C-type lectin DC-SIGN effectively induced antigen-specific naive as well as recall T-cell responses [3]. On the other hand, targeting DEC-205, another C-type lectin, with anti-DEC-205–antigen conjugate led to T-cell tolerance [4]. These studies demonstrate that targeting APCs can induce defined immune responses and also suggest that choosing different target receptors may produce different outcomes.

Although antibodies can provide high specificity for their conjugated antigens, as therapeutic agents, they have some disadvantages. For example, antibodies are somewhat expensive to produce and may have potential immunogenicity in many patients. As a result, there is a need to develop new and useful methods of accessing APCs *in vivo*. In this study, we explored the use of laminarin, a β-1-3 glucan, as an alternative to the antibody-based targeting of APCs. Dectin-1 could mediate the recognition and internalization of laminarin-conjugated OVA. The ability of the conjugate to enhance antigen-specific T-cell activation and antibody response was also investigated.

Materials and methods

Reagents and animals. Laminarin, OVA, poly I:C and rabbit anti-OVA polyclonal antibody were purchased from Sigma–Aldrich. Anti-Dectin-1 antibody (2A11) and IgG isotype control was from Cellsciences and Santa Cruz Biotechnology, respectively. C57BL/6 mice were from Shanghai Experimental Animal Laboratory, Chinese Academy of Sciences.

Preparation of OVA/laminarin conjugate. Fifty milligrams laminarin was dissolved in 2.25 ml of DMSO at 45 °C and mixed with 0.25 ml of 2-(4-aminophenyl)-ethylamine (200 μM) in DMSO. After incubation at 45 °C for 16 h, 15 mg sodium borohydride in 2.5 ml of DMSO was added, and the mixture was incubated at room temperature for 12 h. An additional 15 mg of sodium borohydride

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in 250 μl DMSO was added and the mixture was incubated at room temperature for a further 5 h. The reaction was terminated by adding 12.5 ml of water and lowering the pH to 4 with acetic acid. The aminated laminarin was purified by diafiltration on regenerated cellulose membrane (cut-off 3.0 kDa; Millipore) and lyophilized. Twenty milligrams of the aminated laminarin was dissolved in 1 ml PBS and reacted with 170 μl of 3,4-diethoxy-3-cyclobutene-1,2-dione (Sigma) and 1 ml ethanol. The mixture was incubated at 23 °C for 4 h. The activated laminarin was purified on Bio-Gel P2 fine gel (Bio-Rad) eluted by ammonium bicarbonate buffer and lyophilized. For conjugation to OVA, a 20-fold molar excess of activated laminarin was reacted with OVA in 0.05 M phosphate/borate buffer (pH 9.0) at room temperature for 48 h. The final conjugate was purified on Sephacryl S-200HR columns (Pharmacia), dialyzed and lyophilized. Molecular size of the conjugate and absence of native OVA was assessed by SDS–PAGE stained with Coomassie blue. The carbohydrate content of the conjugate was determined by the phenol–sulfuric acid method and the protein was verified with MicroBCA Protein assay (Bio-Rad). The endotoxin levels of OVA, laminarin and OVA/laminarin conjugate were less than 1 ng/mg as determined by Endospecy (Seikagaku Kogyo Ltd.).

Cell culture and transfection. Chinese hamster ovary (CHO) cells were cultured in Nutrient Mixture F-12 Ham (Sigma). The culture media was supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin at 37 °C with 5% CO_2. To obtain CHO cells stably expressing Dectin-1, the cDNA encoding mouse Dectin-1 was amplified by RT-PCR from the total mRNA of mouse spleen and were cloned in-frame into pcDNA3.1-myc/his vector (Invitrogen) to generate myc-tagged construct. The plasmid was transfected into cells using LipofectAMINE 2000 (Invitrogen) and cells resistant to G418 (600 μg/ml) were generated. Several clones expressing Dectin-1 were obtained and the surface expression of Dectin-1 was verified by flow cytometry with anti-myc antibody (Invitrogen). Bone marrow dendritic cells (BMDCs) were prepared with GM-CSF according to the method described previously [5].

Immunofluorescence and confocal assay. FITC-labeled OVA/laminarin (F-OVA/Lam) and FITC-labeled OVA (F-OVA) was prepared as described previously [6]. The FITC/antigen molar ratios were 1.53 and 1.75 for F-OVA/Lam and F-OVA, respectively. Cells seeded on coverslips were washed twice in PBS and treated with anti-myc antibody and 10 μg/ml FITC-labeled antigens at 4 °C for 45 min. For the uptake study, cells were washed extensively and subsequently incubated for 5 min at 37 °C before fixation. Cells were washed, fixed with 1% paraformaldehyde and permeabilized with PBS containing 0.1% Triton X-100. Cells were stained with rhodamine-conjugated secondary antibody to detect Dectin-1. Finally, cells were washed three times in PBS and mounted on glass slides using Fluoromount mounting medium (Sigma). Labeled cells were visualized using a Zeiss LSM 510 confocal microscope.

Flow cytometry analysis. BMDCs were pre-incubated or not with 50 μg/ml anti-Dectin-1 antibody (2A11) or control IgG for 30 min, then incubated with FITC-labeled antigens at 4 °C for 1 h, washed and subjected to flow cytometry.

T-cell response assay. Mice (aged 6–8 weeks, six mice per group) were injected subcutaneously with the indicated antigens plus 100 μg poly I:C as adjuvant. Eight days after immunization, splenocytes (5×10^5/well, 5 wells per sample) were restimulated *in vitro* with 2 μM MHC class II-specific peptide $OVA_{323-339}$ for 84 h with 1 μCi/well ^{3}H-thymidine added for the last 12 h. The isotopic incorporation was assayed by a scintillation counter (Beckman, Germany). To assess antigen-specific cytokines production, splenocytes were cultured with the peptide for 3 days and the supernatants of the cell culture were collected and subjected to ELISA assays (Roche).

ELISA for anti-OVA IgG and IgG isotypes in mouse serum and for OVA glycoconjugate. Polystyrene microtiter plates were coated overnight with 50 μg/ml OVA. Plates were washed and blocked in 2% milk powder/PBS-0.05% Tween 20. Sera collected from the immunized mice were serially diluted (starting from 1:10) in 2% milk powder/PBS-0.05% Tween 20 and incubated overnight at 4 °C. Plates were then washed and antibody binding was detected using alkaline phosphatase-conjugated anti-mouse IgG antibody (Sigma–Aldrich). Serum titers were defined as the highest dilution of sera resulting in OD values of 405 nm twice that of background. To detect IgG isotypes, sera were diluted 1:200, and biotin-conjugated anti-mouse IgG1, IgG2a, IgG2b, IgG3 antibodies (BD Biosciences) and alkaline phosphatase-conjugated ExtrAvidin reagent (Sigma–Aldrich) were used. OVA glycoconjugate was also coated on the plate and subjected to ELISA using anti-OVA polyclonal antibody.

Statistical analysis. All data are expressed as mean ± SE. The experiments were carried out three times or as indicated. Statistical analysis was performed using one-way ANOVA. Post hoc comparisons were done with Scheffé F-procedure. A p value of less than 0.05 was taken as significant.

Results

Preparation of OVA-conjugated laminarin

We explored the use of 3,4-diethoxy-3-cyclobutene-1,2-dione (squarate) as a linking reagent to construct the OVA/laminarin conjugate [7]. As shown in Fig. 1A, by using squarate (a), aminated laminarin (b) can be attached first to squarate to form activated glycan (c), which can further react with the protein ovalbumin (OVA) to form a glycoconjugate (d). SDS–PAGE analysis shows that OVA has a molecular weight of about 43 kDa, while OVA/laminarin conjugate migrates as a retarded band with a molecular weight of about 66 kDa (Fig. 1B). The saccharide/protein ratio of OVA/laminarin conjugate was determined to be 0.53, meaning that about three laminarin molecules (average molecular weight of laminarin: 7.7 kDa) were attached to one OVA molecule. Both OVA and OVA/laminarin conjugate were recognized to the same extent by anti-OVA antibody in the ELISA assays (Fig. 1C), suggesting that OVA did not denature during the process of chemical modification. LPS, but not OVA or OVA/laminarin conjugate, could stimulate TNF-α secretion from murine BMDCs (Fig. 1D), ruling out the possibility of endotoxin contamination.

Dectin-1 mediates the binding and uptake of OVA/laminarin conjugate

Although laminarin is a well-defined ligand for Dectin-1 [8], it is not known whether the modification of laminarin with OVA could influence its recognition by Dectin-1. To address this question, OVA and OVA/laminarin conjugate were labeled with FITC (F-OVA and F-OVA/Lam, respectively) and immunofluorescence assay was conducted. CHO cells stably expressing Dectin-1 (CHO-Dectin-1 cells) were seeded on the coverslips and incubated with F-OVA or F-OVA/Lam at 4 °C. Dectin-1 was labeled with anti-myc antibody and rhodamine-conjugated secondary antibody. CHO-Dectin-1 cells could bind F-OVA/Lam, but not F-OVA (Fig. 2A upper panel and Supplementary data). Surface-labeled Dectin-1 could be co-localized well with F-OVA/Lam (Fig. 2A upper panel, merged). The internalization of F-OVA/Lam was subsequently investigated. When shifted to 37 °C for 5 min, the CHO-Dectin-1 cells took up F-OVA/Lam rapidly into Dectin-1-positive punctate structures (Fig. 2A lower panel, arrows). We next investigated the binding of F-OVA/Lam to murine BMDCs. BMDCs were incubated with F-OVA/Lam or F-OVA for 45 min at 4 °C and flow cytometry analysis was performed. As shown in Fig. 2B, F-OVA/Lam could bind to BMDCs, which express endogenous Dectin-1. In contrast, BMDCs did not recognize F-OVA.

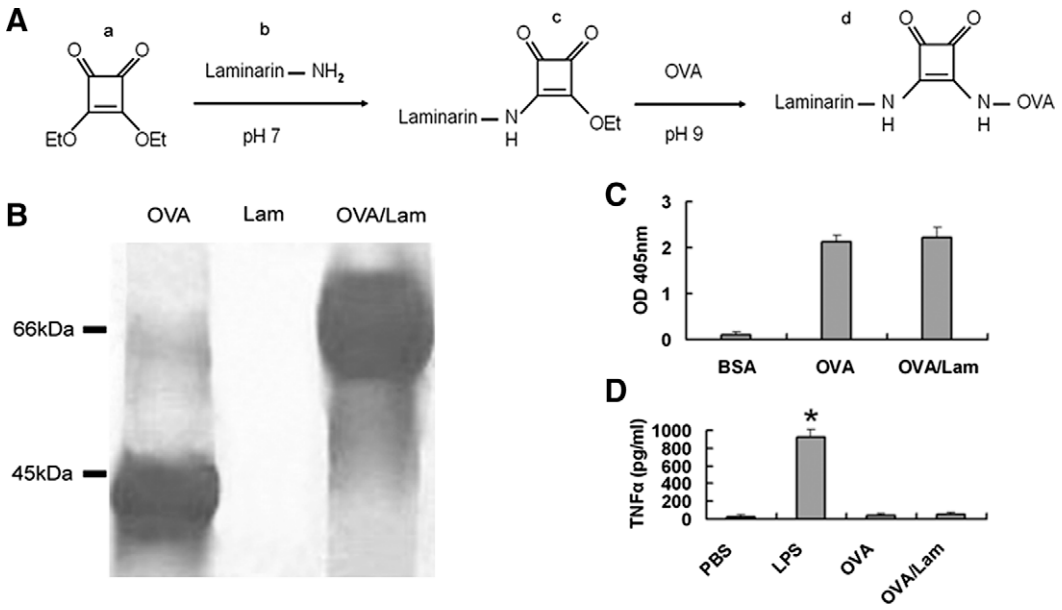

Fig. 1. (A) Schematic representation of laminarin conjugation to OVA. Et: $-C_2H_5$. (B) Analysis of OVA conjugate by 10% SDS–PAGE followed by Coomassie blue staining: lane 1, OVA only; lane 2, laminarin (Lam); lane 3, the OVA/laminarin conjugate (OVA/Lam). (C) BSA, OVA and OVA/laminarin were coated on polystyrene microtiter plates and ELISA was conducted as described in Materials and methods. (D) BMDCs were treated with PBS, 0.2 μg/ml LPS, 50 μg/ml OVA or OVA/laminarin conjugate for 24 h. TNF-α secretion was measured by ELISA. $^*p < 0.05$.

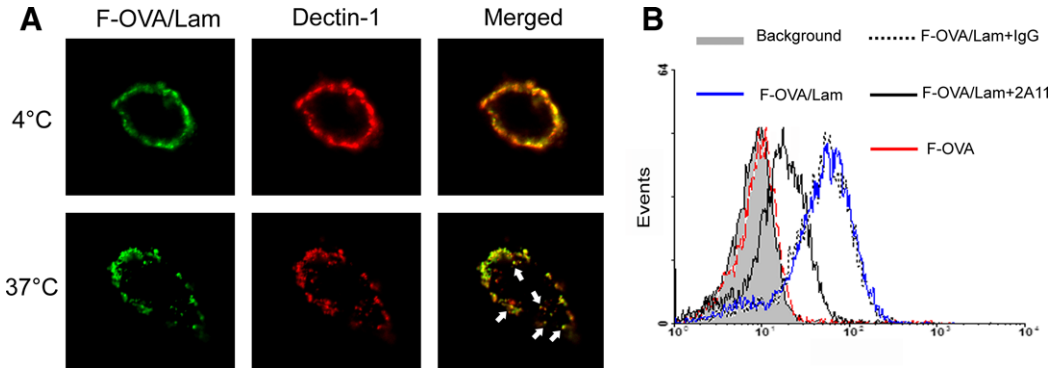

Fig. 2. (A) CHO cells stably expressing Dectin-1 (CHO-Dectin-1) were seeded on the coverslips and incubated with anti-myc antibody and 10 μg/ml FITC-labeled OVA/laminarin (F-OVA/Lam) at 4 °C for 45 min. Cells were washed, shifted or not to 37 °C for 5 min before fixation and permeabilization. Cells were labeled with rhodamine-conjugated secondary antibody to detect Dectin-1. After extensive washing, cells were subjected to immunofluorescence and confocal assays. Arrows: F-OVA/Lam was internalized into Dectin-1-positive punctate structures. (B) BMDCs were pre-incubated or not with 50 μg/ml anti-Dectin-1 antibody (2A11) or control IgG for 30 min. Then the cells were further incubated with 10 μg/ml F-OVA or F-OVA/Lam at 4 °C for 1 h. Cells were washed and analyzed by flow cytometry.

Furthermore, the binding of F-OVA/Lam was significantly inhibited by anti-Dectin-1 antibody (2A11), but not control IgG. These results suggest that the OVA/laminarin conjugate efficiently targets Dectin-1 to be recognized and internalized by the cells.

Injection of OVA/laminarin conjugate enhanced CD4$^+$ T-cell response

We next investigated the efficiency of this conjugate to enhance immune responses. C57BL/6 mice were immunized subcutaneously by a single injection of the indicated doses of PBS, native OVA, a mixture of OVA and laminarin, OVA/laminarin conjugate with or without excessive amount of laminarin, together with 100 μg poly I:C as adjuvant. Eight days later, splenocytes were obtained and the T-cell responses were monitored. The MHC class II-specific peptide $OVA_{323-339}$ was used to gauge CD4$^+$ T-cell responses. As shown in Fig. 3A, the T-cell proliferation was induced only slightly by native OVA. In contrast, treatment of OVA/laminarin conjugate stimulated significantly higher level of CD4$^+$ T-cell proliferation response than that of native OVA. OVA/laminarin conjugate also more potently induced cytokines production, including IFN-γ and IL-2 (Fig. 3B and C). Additionally, co-injection of excessive amount of laminarin impaired the enhancement of T-cell response mediated by OVA/laminarin, which verified that the enhancing effect was due to the carbohydrate moiety of laminarin that is attached to OVA. Unlike OVA/laminarin, the mixture of OVA and laminarin failed to enhance T-cell response which underscored the requirement of the conjugation of OVA to laminarin for the enhancing effect. Taken together, these data suggest that laminarin modification of OVA led to significant enhancement of presentation of antigenic peptide to CD4$^+$ T cells *in vivo*.

OVA/laminarin conjugate immunization increases antibody response to OVA

Because the OVA/laminarin conjugate could generate potent CD4$^+$ T-cell response, which is important for antibody production [9], the efficiency of this conjugate to induce antibody response was tested. Five groups of six mice were immunized subcutane-

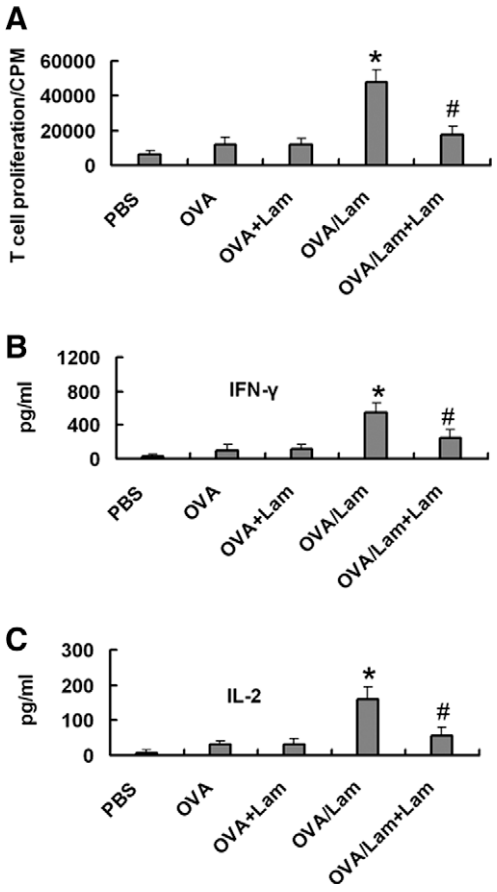

Fig. 3. Stimulation of OVA-specific $CD4^+$ T-cell responses by OVA/laminarin conjugate *in vivo*. C57BL/6 mice were subcutaneously injected with PBS, 1 μg OVA, 1 μg OVA with 600 ng laminarin (OVA + Lam) or OVA/laminarin conjugate (containing 1 μg OVA) in the absence (OVA/Lam) or presence (OVA/Lam + Lam) of 50 μg laminarin, plus 100 μg poly I:C as adjuvant. Eight days after immunization, 5×10^5 splenocytes from the mice were transferred to a 96-well plate and stimulated with $OVA_{323-339}$ (A) for 84 h with 1 μCi/well ^{3}H-thymidine added for the last 12 h. Then the cells were collected and the isotopic incorporation was assayed. The supernatants from the cell culture of $OVA_{323-339}$-stimulated splenocytes for 3 days were subjected to ELISA assays to detect IFN-γ (B) and IL-2 (C) production. $^*p < 0.05$ vs. OVA-treated group. $^\#p < 0.05$ vs. OVA/Lam-treated group. Results are from a total of six mice per group from two independent experiments.

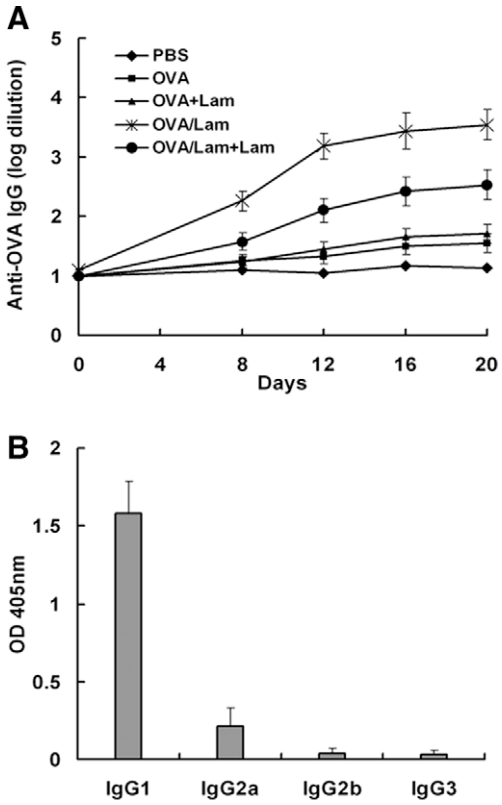

Fig. 4. Induction of antibody response by OVA/laminarin conjugate. C57BL/6 mice were immunized as described in Fig. 3. (A) By the indicated day post-immunization, the sera anti-OVA IgG titers were measured by ELISA as described in Materials and methods. Statistical significance: OVA/Lam group vs. OVA group, OVA/Lam + Lam group vs. OVA/Lam group, $p < 0.05$ at all time points except 0 day. (B) Isotypes of anti-OVA IgG in mice that received the targeted immunization. Sera were collected by 16-days post-immunization from the mice injected with OVA/Lam conjugate. ELISA was conducted as described in Materials and methods. Data were plotted as OD values of 405 nm. Results are from a total of six mice per group from two independent experiments.

ously with the indicated reagents as described above. Serum anti-OVA IgG titers were determined by ELISA at different times of immunization. As shown in Fig. 4A, native OVA and the mixture of OVA and laminarin stimulated low level of antibody production. On the contrary, OVA/laminarin conjugate significantly enhanced antibody response. By 8 days the OVA/laminarin-treated group had mounted response to antigen. From then on, the IgG titers continued to rise and stabilized by 20 days. As expected, co-injection of excessive laminarin significantly inhibited OVA/laminarin-induced antibody production. The isotypes of anti-OVA IgG produced by OVA/laminarin administration were determined by 16-days post-immunization (Fig. 4B). IgG1 was the predominate isotype, in agreement with previous finding for adjuvant-dependent protein antigens [10]. IgG2a was also present in the sera, while IgG2b and IgG3 could hardly be detected. These results suggest that OVA/laminarin conjugate, at a low dose of antigen, induced efficient antibody response after primary immunization.

Discussion

Targeting of antigen to APCs has been shown to be an efficient strategy for vaccine enhancement [9]. Usually, this has been achieved using complexes of complete antigenic proteins and antibodies specific for the surface molecules on APCs. In this study, we have investigated the use of laminarin, a β-1-3 glucan, as a mean of delivering antigens to APCs. An OVA/laminarin conjugate was synthesized and the efficiency of this antigen–carbohydrate complex to potentiate immune responses was demonstrated.

It has long been appreciated to use lectin–carbohydrate interactions for tissue-specific delivery of drugs, nucleic acids and proteins. For example, the asialoglycoprotein receptor and the mannose receptor in the liver have been used as drug delivery targets based on this approach [11]. Carbohydrates of low molecular weight have the distinct advantage of being non-immunogenic and thus unlikely to elicit undesired immune responses during the course of clinical medication [12]. Additionally, it costs not too much to generate carbohydrates. Therefore, carbohydrates provide a promising alternative to antibody-mediated targeting methods. DCs and macrophages express a number of cell surface lectins of C-type class that recognize specific carbohydrate moieties. These lectins, such as DC-SIGN, DEC-205, Clec9A and mannose receptor (MR) [3,13–15], represent a particularly interesting class of surface receptors to target for shaping the immune responses owing to their restricted expression patterns and potential abilities to mediate the adsorptive uptake of bound ligands.

Dectin-1 is a C-type lectin-like receptor mainly expressed on DCs and macrophages subpopulations [16]. It has been shown to be a major receptor for β-1-3 and β-1-6 linked glucans found on yeast cell walls [17,18] and to play an important role in anti-fungal immunity [19]. Upon binding β-glucan-containing particles derived from

yeast, Dectin-1 mediates the internalization of these ligands. Meanwhile, the crosslinking of Dectin-1 by its ligands triggers intracellular signals via its cytoplasmic immunoreceptor tyrosine-based activation motif (ITAM), leading to various cell-specific responses, such as the production of cytokines and chemokines, generation of reactive oxygen and regulation of T helper cell responses [20,21]. Therefore, Dectin-1 is not only an endocytic receptor for antigen uptake, but also a signaling receptor to modulate immune responses [16]. In fact, Dectin-1 has been chosen to be a targeting molecule for an antigen/anti-Dectin-1 conjugate and this strategy has been proved to be effective to enhance immune responses [22].

Previous studies show that $CD4^+$ T-cell response plays an important role in promoting humoral immunity [9]. In this study, we demonstrated that OVA/laminarin was capable of inducing effective $CD4^+$ T-cell activation. In agreement with this effect, the OVA/laminarin conjugate elicited efficient antibody response. It is assumed that targeting antigens to Dectin-1 could enhance $CD4^+$ T-cell response and indirectly improve antibody production. Using MHC class I-specific peptide $OVA_{257-264}$, we also detected the enhancing effect of OVA/laminarin on $CD8^+$ T-cell response, but the effect on $CD8^+$ T-cell response was not so obvious as that on $CD4^+$ T-cell response (data not shown). This result implies that Dectin-1 could mediate cross-presentation of antigens which is in line with previous report [23]. It also indicates that the OVA/laminarin conjugate preferentially enhances antigen presentation via MHC class II pathway.

The immunofluorescence assay and flow cytometry analysis showed that Dectin-1 could effectively recognize and internalize OVA/laminarin conjugate, but not native OVA. These results suggest that the OVA/laminarin conjugate enhanced immune responses via targeting Dectin-1-expressing cells, which are mainly DCs and macrophages *in vivo*. This conjugate was shown to be more efficient than unconjugated OVA to induce OVA-specific T-cell activation and antibody response. Our study, together with previous report [22], confirmed the efficiency to target Dectin-1 for antigen delivery and enhancing immunity. Because Dectin-1 contains an ITAM motif, binding of OVA/laminarin conjugate to Dectin-1 might trigger signals in APCs to modify immune responses. However, in the preliminary experiments, the conjugate failed to induce cytokine secretion from BMDCs (Fig. 1D). Also, it should be noted that no response was detected when OVA/laminarin conjugate was injected in the absence of adjuvant (data not shown). These observations suggest that Dectin-1 mediated efficient adsorptive uptake of the OVA/laminarin conjugate, but it may not induce signals to promote immune responses possibly because the conjugate failed to crosslink Dectin-1 on the cell surface.

Taken together, we manipulated laminarin–Dectin-1 interaction for antigen delivery to APCs. The carbohydrate–lectin-interaction approach broadens the scope of methods for targeting antigens to APCs and may be promising to be developed for treating individual diseases in the future.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.11.173.

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