

Lectins Isolated from Mushroom *Fomitella fraxinea* Enhance MHC-restricted Exogenous Antigen Presentation

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ABSTRACT

Background: Immunomodulators enhancing MHC-restricted antigen presentation would affect many cellular immune reactions mediated by T cells or T cell products. However, modulation of MHC-restricted antigen presentation has received little attention as a target for therapeutic immunoregulation. Here, we report that lectins isolated from mushroom *Fomitella fraxinea* enhance MHC-restricted exogenous antigen presentation in professional antigen presenting cells (APCs). **Methods:** Lectins, termed FFrL, were isolated from the carpophores of *Fomitella fraxinea*, and its effects on the class I and class II MHC-restricted presentation of exogenous ovalbumin (OVA) were examined in mouse dendritic cells (DCs) and mouse peritoneal macrophages. The effects of FFrL on the expression of total MHC molecules and the phagocytic activity were also examined in mouse DCs. **Results:** DCs cultured in the presence of FFrL overnight exhibited enhanced capacity in presenting exogenous OVA in association with class I and class II MHC molecules. FFrL increased slightly the total expression levels of both class I (H-2K^b) and class II (I-A^b) MHC molecules and the phagocytic activity of DCs. Antigen presentation-enhancing activity of FFrL was also observed in macrophages isolated from mouse peritoneum. **Conclusion:** Lectins isolated from the carpophores of *Fomitella fraxinea* increase MHC-restricted exogenous antigen presentation by enhancing intracellular processing events of phagocytosed antigens. (*Immune Network* 2007;7(4):197-202)

Key Words: *Fomitella fraxinea*, lectin, MHC-restricted antigen presentation, dendritic cell, macrophage

Introduction

Two distinct pathways have been known for the MHC-restricted presentation of exogenous antigens (1). In the classical paradigm of exogenous antigen presentation by professional APCs, antigens internalized by phagocytosis or endocytosis are processed and loaded on class II MHC molecules in a post-Golgi compartment. Professional APCs, however, have been shown to process exogenous antigens for the presentation on class I MHC molecules. This process, termed cross-pre-

sentation, was first demonstrated in the generation of CTL responses to minor histocompatibility antigens (2). Cross-presentation is now recognized as a more general mechanism for the generation of CTL responses against tumor cells, transplanted cells, bacteria, and even viruses (3-7). In the absence of such a mechanism, viral or tumor antigens expressed in nonprofessional APCs could escape immunosurveillance because cytotoxic T cell (CTL) responses can only be induced efficiently for the antigens presented via class I MHC molecules on professional APCs (8-10).

Since T cells can only recognize antigens presented on MHC molecules, modulators of MHC-restricted antigen presentation would affect many cellular immune reactions mediated by T cells or T cell products. However, modulation of MHC-restricted antigen presentation has received little attention as a target for thera-

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peutic immunoregulation. Recently, we showed that cyclosporin A and tacrolimus, but not rapamycin, inhibit MHC-restricted exogenous antigen presentation in professional APCs *in vivo* (11) as well as *in vitro* (12). These results demonstrate that modulation of MHC-restricted antigen processing may provide novel pharmacological targets for the regulation of T cell responses.

In an effort to search for and characterize activators of MHC-restricted antigen presentation, we examined the effects of lectins and polysaccharides isolated from several species of *Basidiomycetes*, and found that the lectins isolated from mushroom *Fomitella fraxinea* enhances efficiently MHC-restricted exogenous antigen presentation in a dendritic cell line, DC2.4 cells, and also in normal macrophages. Our study demonstrates that modulation of MHC-restricted antigen presentation may provide novel pharmacological targets for the regulation of T cell responses.

Materials and Methods

Cells and cell lines. T cell hybridomas, B3Z86/90.14 (B3Z) and DOBW, were kindly provided by Dr. Nilabh Shastri (University of California, Berkeley, CA) and by Dr. Clifford V. Harding (Case Western Reserve University, Cleveland, OH), respectively (13,14). The DC cell line (DC2.4) was obtained from the Dana-Farber Cancer Institute, Boston, MA, USA (15). Thioglycollate-eli-

cited mouse peritoneal macrophages were obtained from C57BL/6 mice.

Isolation of lectins. The lectins were isolated from the carpophores of *Fomitella fraxinea* as described previously (16). Briefly, two kilograms of the frozen fruiting bodies were homogenized in 20 liters of 50 mM Tris-HCl buffer (pH 8.0) with a blender and then extracted for 18 hours at 4°C with frequent gentle swirling. The resulting suspension was filtered and ammonium sulfate was added to the filtrate to 95% saturation. After standing overnight at 4°C, the resulting precipitate was separated by centrifugation at 8,000 ×g at 4°C for 20 min. This precipitate was dissolved in 50 mM NaCl, 50 mM Tris-HCl buffer (pH 8.0) and then dialyzed against distilled water for 5 days at 4°C with frequent changing of distilled water. After dialysis, the dialysate was freeze-dried to yield crude FFrL.

Preparation of microencapsulated OVA. Microspheres containing OVA were prepared using a solvent-evaporation method, as described previously (12), using OVA dissolved in 3% polyvinyl alcohol (4 mg/ml) and poly (DL-lactide-co-glycolide) (PLGA; lactide : glycolide = 50 : 50; Sigma-Aldrich, St. Louis, MO, USA) dissolved in a mixture of acetone and ethanol (9 : 1) (5%). The concentration of OVA was determined by micro-bicinchoninic acid assay kit (Pierce, Rockford, IL) according to the manufacturer's instructions after lysing the mi-

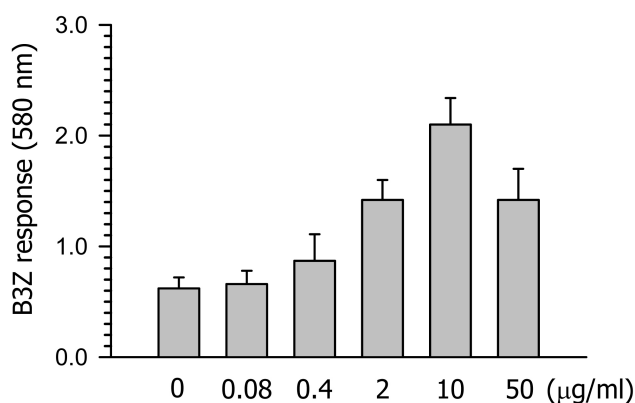


Figure 1. Effects of FFrL on the cross-presentation of exogenous OVA in DC2.4 cells. DC2.4 cells were incubated with the indicated amounts of FFrL for 18 h, and then added with OVA-microspheres. After 2 h incubation, the cells were fixed, and the amounts of OVA peptides presented on class I MHC molecules were assessed using B3Z cells, which expresses β -galactosidase upon recognition of the OVA peptide, SIINFEKL, complexed with H-2K^b molecule. The amount of β -galactosidase expressed in B3Z cells was determined by an enzymatic assay using chlorophenolred β -D-galactopyranoside as a substrate.

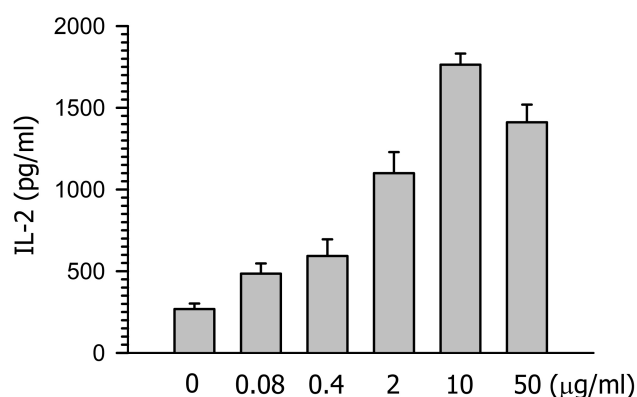


Figure 2. Effects of FFrL on the class II MHC-restricted presentation of exogenous OVA in DC2.4 cells. Indicated amounts of FFrL were added to cultures of DCs generated from mouse BM cells with GM-CSF for 18 h, and then added with OVA-microspheres. After 2 h incubation, the cells were fixed, and the amounts of OVA peptides presented on class II MHC molecules were assessed using OVA-specific CD4 T cell hybridoma, DOBW. The amounts of IL-2 produced from OVA-specific CD4 T cells were assayed by a commercial IL-2 ELISA kit.

crosspheres in a lysis buffer containing 0.1 % SDS and 0.1 N NaOH. For phagocytosis assays, microspheres containing both OVA and fluorescein isothiocyanate (FITC) were prepared by adding FITC (final, 5 mg/ml) to a mixture of acetone and ethanol (9 : 1) together with PLGA (final, 5%).

Cross-presentation assay. *LacZ* T cell activation assays were used to assess the amounts of cross-presented OVA peptides, as previously described (12). Briefly, APCs were cultured in the presence of different concentrations of FFrL for 18 h in 96-well plates (1×10^5 /well), and then added with OVA-microspheres (50 μ g/ml as OVA). After 2 h incubation at 37°C, the plate was washed twice with 300 μ l/well of pre-warmed PBS, and then fixed with 100 μ l/well of ice-cold 1.0% paraformaldehyde for 5 min at room temperature. The plate was washed 3 times with 300 μ l/well of PBS, and B3Z cells were added (2×10^5 /well). After incubating for 4 h at 37°C, *lacZ* activity was measured either by colorimetric analysis after incubating freeze-thaw lysed cells with β -galactosidase substrate, chlorophenol red β -D-galactopyranoside (Calbiochem, Darmstadt, Germany), as described previously (12).

Generation of bone marrow-derived DCs (BM-DCs). DCs were generated from total BM cells as described previously (17). Briefly, BM cells obtained from femurs of BALB/c mouse were cultured in a 6-well plate ($5 \times$

10^6 /well) in a culture medium supplemented with 200 U/ml rmGM-CSF. At days 3 and 4 from the initiation of the culture, nonadherent cells were discarded by replacing the culture medium with fresh medium containing the cytokines after gentle shaking. DCs were harvested by gentle pipetting at day 6.

MHC class II-restricted presentation assay. BM-DCs were cultured in the presence of different concentrations of FFrL for 18 h in 96-well plates (1×10^5 /well), and then added with OVA-microspheres (50 μ g/ml as OVA). After 2 h incubation at 37°C, unphagocytized OVA-microspheres were removed by suction, and then fixed with ice-cold 1.0% paraformaldehyde for 5 min at room temperature. The plate was then washed twice with 300 μ l/well of pre-warmed media, and added with DOBW cells (1×10^5 /well). After 24 h incubation at 37°C, the plate was centrifuged at 1,800 rpm, and the culture supernatant was collected and assayed for IL-2 content using an IL-2 ELISA kit (BD Biosciences).

Phagocytosis assay. DCs were cultured in the presence of different concentrations of FFrL for 18 h in 6-well plates (2×10^6 cells/well), and then added with microspheres (average diameter, 300 nm) containing both ovalbumin (OVA) and fluorescein isothiocyanate (FITC). After 2 h, unphagocytized microspheres were removed by washing with pre-warmed PBS. The plate was chilled on ice for 20 min, then the cells were harvested

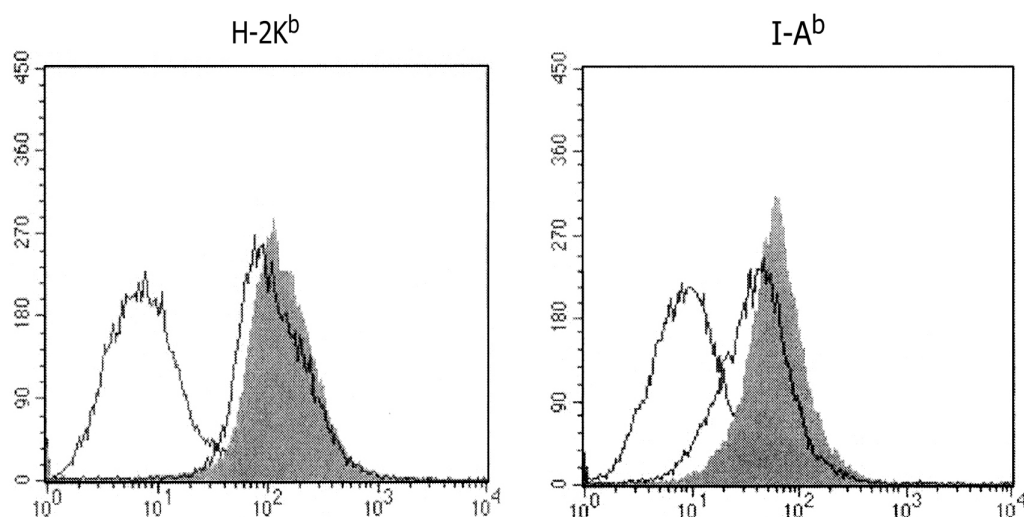


Figure 3. Effects of FFrL on the expression of MHC molecules. DC2.4 cells were cultured with 10 μ g/ml of FFrL for 18 h, and then the cells were harvested by gentle pipetting after cooling on ice. The expression levels of class I and class II MHC molecules were assessed using anti-H-2K^b and anti-I-A^b monoclonal antibodies. Shaded histogram represents the expression levels of H-2K^b and I-A^b molecules in DC2.4 cells cultured in the presence of FFrL, and thin line histogram represents the expression levels of H-2K^b and I-A^b molecules in DC2.4 cells cultured in the absence of FFrL. Isotype control was shown as thin histograms (left).

by treating with Cell stripper solution (Cellgro Mediatech, Herndon, VA) as suggested in the manufacturer's instruction, fixed in 1% paraformaldehyde in PBS, and flow cytometric analysis was performed on a FACS Caliber flow cytometer (Becton Dickinson).

Phenotypic analysis. DCs were cultured in the presence of different concentrations of FFrL for 18 h in 6-well plates (2×10^6 cells/well). The plate was then chilled on ice for 20 min, and the cells were harvested by treating with Cell stripper solution (Cellgro Mediatech). The cells were stained with monoclonal antibodies recognizing murine MHC molecules after blocking of FcR-binding anti-CD16/CD32 monoclonal antibody (clone 2.4G2) as described previously (17), and flow cytometric analysis was performed on a FACS Caliber (Becton-Dickinson). The monoclonal antibodies, H-2K^b (clone AF6-88.5) and I-A^b (clone AF6-120.1, and an isotype-matched control antibody were purchased from BD Biosciences.

Results

FFrL enhances cross-presentation of exogenous antigen in DCs. To examine the effects of the lectins, FFrL, on the presentation of exogenous OVA in association with class I MHC molecules, DC2.4 cells were cultured in the presence of FFrL for 18 h, and then allowed to phagocytose OVA-microspheres for 2 h. The DC2.4 cells were then washed, fixed with paraformaldehyde, and the amount of OVA peptide-class I MHC complexes was measured using a T cell hybridoma, B3Z, which recognizes OVA peptide (SIINFEKL)-H-2K^b complex and expresses β -galactosidase [14]. As shown in Fig. 1, DC2.4 cells stimulated with FFrL exhibited enhanced capability in cross-presenting exogenous OVA. The optimal dose of FFrL for maximum augmentation of cross-presentation capability appeared to be 10 μ g/ml in DC2.4 cells.

FFrL enhances class II MHC-restricted presentation of exogenous antigen in DCs. To examine the effects of FFrL on class II MHC-restricted presentation of exogenous antigen, DCs generated from bone marrow cells of Balb/c mice (H-2^d) were cultured with different concentrations of FFrL for 18 h, and then allowed to phagocytize OVA-microspheres for 2 h. The DCs were fixed with paraformaldehyde, and then the amount of OVA peptide-class II MHC complexes was measured using OVA-specific CD4 T cell hybridoma, DOBW cells. As shown

in Fig. 2, FFrL also enhanced class II MHC restricted presentation of exogenous OVA significantly and dose dependently in BM-DCs.

FFrL enhances slightly the total expression levels of both class I and class II MHC molecules. To examine whether the enhanced capacity of FFrL-stimulated DCs to present OVA peptides in association with MHC molecules was due to increased expression of MHC molecules on the cell surface, DC2.4 cells were cultured with 10 μ g/ml FFrL for 18 h, harvested, and then the expression levels of class I and class II MHC molecules were determined by anti-H-2K^b and anti-I-A^b monoclonal antibodies. FFrL increased slightly the total expression levels of both class I and class II MHC molecules (Fig. 3).

FFrL slightly enhances the phagocytic activity. To examine whether the enhanced capacity of FFrL-stimulated DCs to present OVA peptides in association with MHC molecules was due to increased phagocytic activity, DC2.4 cells were cultured with 10 μ g/ml FFrL for 18 h, and then added with microspheres containing both OVA and FITC. After 2 h incubation, unphagocytized microspheres were washed, and the cells were harvested by gentle pipetting after cooling on ice. Flow cytometric analysis of the harvested cells showed that

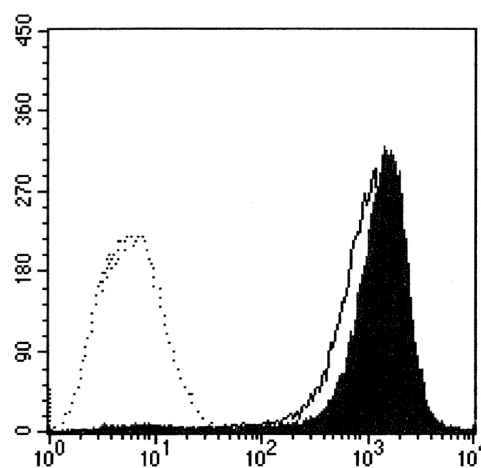


Figure 4. Effects of FFrL on the phagocytic activity. DC2.4 cells were cultured with 10 μ g/ml of FFrL for 18 h, and then added with microspheres containing both OVA and FITC. After 2 h incubation, unphagocytized microspheres were washed, and the cells were harvested by gentle pipetting after cooling on ice, and then analyzed by flow cytometry. Shaded histogram represents the phagocytic activity of DCs cultured in the presence of FFrL, and thin line histogram represents the phagocytic activity of DCs cultured in the absence of FFrL. DCs that were not incubated with FITC-labeled microspheres were shown as dotted histograms (left).

FFrL slightly increased the phagocytic activity of DC2.4 cells (Fig. 4).

FFrL also enhances cross-presentation of exogenous antigen in peritoneal macrophages. Cross presentation-enhancing activity of FFrL was also examined in macrophages. In this experiment, peritoneal macrophages were obtained from thioglycollate-injected mouse peritoneum, and were cultured in the presence of FFrL for 18 h, fixed with paraformaldehyde, and the amount of OVA peptide-class I MHC complexes was measured using a T cell hybridoma, B3Z. As shown in Fig. 5, macrophages stimulated with FFrL also exhibited enhanced capability in cross-presenting exogenous OVA. The optimal dose of FFrL for maximum augmentation of cross-presentation capability appeared again to be 10 μ g/ml.

Discussion

The present study demonstrates that the lectins isolated from the carpophores of *Fomitella fraxinea* increase MHC-restricted exogenous antigen presentation. FFrL enhanced both class I MHC and class II MHC-restricted presentation of exogenous antigen in professional APCs. FFrL appeared to increase MHC-restricted exogenous antigen presentation by activating intracellular processing events of the phagocytosed antigens, because treatment of DCs with FFrL increased only slightly the total level of expression of H-2K^b and I-A^b molecules and the phagocytic activity.

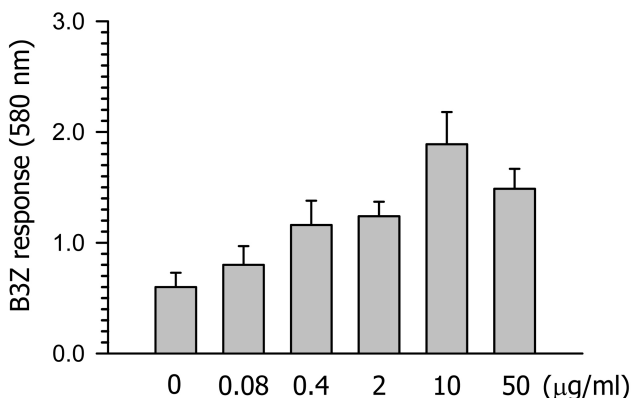


Figure 5. Effects of FFrL on the cross-presentation of exogenous OVA in macrophages. Thioglycollate-elicited macrophages were incubated with the indicated amounts of FFrL for 18 h, and then added with OVA-microspheres. After 2 h incubation, the cells were fixed, and the amounts of OVA peptides presented on class I MHC molecules were assessed using B3Z cells, as described in Fig. 1.

Lectins are glycoproteins which have been known to induce cell agglutination (18). Lectins have been found from diverse living organisms including animals, plants and microorganisms (19,20). Some of the lectins isolated from mushrooms have been shown to possess immunomodulatory and antitumor activity (21-25), although the mechanism of action is not clear. Our results suggest that immunomodulatory and antitumor activity activities of mushroom lectins may at least in part due to activation of antigen presenting capability of professional APCs. Since T cells can only recognize antigens presented on MHC molecules, the impact of the augmentation of MHC-restricted antigen presentation must be far-reaching.

In all of the experiments described in the present study, APCs were exposed with FFrL for 18 h, and then allowed to phagocytose OVA-microspheres for 2 h. The APCs were washed to remove unphagocytosed OVA-microspheres, fixed with paraformaldehyde, and then washed thoroughly again to remove paraformaldehyde, before functional assays with OVA-specific CD4 or CD8 T cells. Thus, the activation of OVA-specific T cells must be due to enhanced expression of OVA peptides on APCs, and not due to the carry-over of the lectins to T cell cultures.

The precise mechanisms by which FFrL enhances MHC-restricted antigen processing pathways remain to be determined. Because FFrL was shown to exert mitogenic activity when added to splenic lymphocytes, we examined the effects of FFrL on the proliferation of DCs and peritoneal macrophages. We found that FFrL did not exert mitogenic activity on these cells (data not shown). Recently, we showed that DCs efficiently phagocytose OVA-microspheres encapsulated with PLGA, and then process the phagocytosed OVA in a vacuolar alternate mechanism to present OVA peptides in association with class I MHC molecules to T lymphocytes (26). The vacuolar alternate pathway involves partial digestion of phagocytosed antigens within the acidic phagolysosomes and binding of the peptides to class I MHC molecules in the same compartment, much like the classical class II MHC pathway of exogenous antigen (27). Because FFrL increased both class I and class II MHC-restricted exogenous antigen presentation pathways, we are tempted to speculate that FFrL activates molecular machinery in the vacuolar alternate pathway of antigen processing.

Although the precise mechanism of action is not elucidated, the present study demonstrates that the lectins isolated from mushroom *Fomitella fraxinea* enhances MHC-restricted exogenous antigen presentation in professional APCs. Based on the present study as well as our recent studies which demonstrated that MHC-restricted antigen presentation can be blocked by immunosuppressive calcineurin inhibitors (11,12), we would like to propose that modulation of MHC-restricted antigen presentation could be a novel means for the regulation of T cell responses.

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