

Characterization of an immunomodulatory Der p 2-FIP-*fve* fusion protein produced in transformed rice suspension cell culture

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Abstract Der p 2, a major allergen of *Dermatophagoides pteronyssinus* mites, is one of the most clinically relevant allergens to allergic patients worldwide. FIP-*fve* protein (Fve) from the golden needle mushroom (*Flammulina velutipes*) is an immunomodulatory protein with potential Th1-

skewed adjuvant properties. Here, we produced and immunologically evaluated a Der p 2-Fve fusion protein as a potential immunotherapeutic for allergic diseases. Using an inducible expression system in cultured rice suspension cells, the recombinant Der p 2-Fve fusion protein (designated as OsDp2Fve) was expressed in rice cells under the control of an α -amylase gene (α Amy8) promoter and secreted under sucrose starvation. OsDp2Fve was partially purified from the cultured medium. The conformation of Der p 2 in OsDp2Fve remains intact as reflected by its unaltered allergenicity, as assessed by human IgE ELISA and histamine release assays, compared to non-fusion Der p 2 protein. Furthermore, the Fve protein expressed in OsDp2Fve retains its in vitro lymphoproliferative activity but loses its hemagglutination and lymphoagglutination effects compared to the native protein. Notably, in vivo evaluation showed that mice administered with OsDp2Fve possessed an enhanced production of Der p 2-specific IgG antibodies without potentiating the production of Der p 2-specific IgE and Th2 effector cytokines in comparison with mice co-administered with native Fve and Der p 2 proteins. These results suggest that the recombinant Der p 2-Fve fusion protein produced in rice suspension cell cultures has a great potential for allergy immunotherapy.

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Introduction

A new family of fungal immunomodulatory proteins (FIP) comprising LZ-8, FIP-*vvo*, FIP-*gts* and FIP-*fve*, with similar molecular weight, amino acid composition and biological activities, were identified and isolated from *Ganoderma lucidum* (Kino et al. 1989), *Volvariella volvacea* (Hsu et al. 1997), *Ganoderma tsugae* (Lin et al. 1997) and *Flammulina velutipes* (Ko et al. 1995), respectively. FIP-*fve* protein (Fve) from the edible mushroom *Flammulina velutipes* is a homodimeric protein under native conditions (Paaventhana et al. 2003). Each monomer possesses an N-terminal α -helix dimerization domain and an adjoining immunoglobulin (Ig) superfamily-like domain, which is similar to the fibronectin type III fold. The monomeric Fve polypeptide consists of 114 amino acid residues, has a the molecular weight of 12.7 kDa, and lacks a disulfide bond (Ko et al. 1995). Alignment of FIP showed that Fve shares 63% sequence identity with LZ-8 and FIP-*gts* and 54% sequence identity with FIP-*vvo*. Fve was able to induce mitogenic proliferation of human peripheral mononuclear cells (PBMCs) and enhance mRNA transcription of interleukin (IL)-2 and interferon (IFN)- γ in vitro (Ko et al. 1995; Wang et al. 2004). Administration of Fve with allergens either intraperitoneally, orally, or intranasally inhibited allergen-induced systemic anaphylactic responses (Ko et al. 1995), food allergic reactions (Hsieh et al. 2003), or allergic airway inflammation (Liu et al. 2003; Liu and Tsai 2005; Hsieh et al. 2007) in vivo, respectively. Recently, Fve has also been shown to exhibit potent adjuvant properties that enhanced the immunogenicity of the human papillomavirus type-16 (HPV-16) E7 oncoprotein and conferred increased anti-tumor effects against the inoculation of HPV-16 E7-expressing TC-1 cells in a mouse model (Ding et al. 2009). Taken together, these studies support the notion that Fve is a mitogenic protein with immunomodulatory or immunostimulatory activities that can be exploited for some clinical applications.

The cDNA encoding Fve has been cloned and expressed in *E. coli*, and the recombinant Fve was shown to retain its mitogenic immunostimulatory activity (Ko et al. 1997). This finding suggested that, the immunostimulatory activity of Fve can be mediated by the protein component without posttranslational modification. However, the *E. coli* expression

system is not a robust system for large-scale production of recombinant Fve due to the low solubility of Fve produced. Many natural products from mushrooms have been regarded as potential immunoregulatory agents that induce modulatory effects by enhancing the resistance against infectious agents (Hirasawa et al. 1999; Piraino and Brandt 1999), by increasing the rejection of malignant cells, and by suppressing systemic anaphylactic reactions (Ko et al. 1995; Hsu et al. 1997). Among those natural compounds, Fve is unique because its active component is the protein itself as opposed to a polysaccharide or a glycoprotein. As mentioned above, the production of large amounts of soluble Fve in *E. coli*-based expression systems is problematic (Ko et al. 1997). Insect cells expression system was explored and it was found that the recombinant Fve protein was highly glycosylated as the Fve polypeptide contained two potential N-linked glycosylation sites (Wu et al. 2008). Over-glycosylated recombinant Fve protein is not suitable for mechanistic and functional studies of Fve since the carbohydrate components could induce undesirable confounding immunological effects. Therefore, it is necessary and worthwhile exploring other alternative expression systems to improve the yield and quality of recombinant Fve protein to pave the way for future basic and clinical studies of this immunomodulatory protein.

Many biological systems are available for large-scale production of foreign proteins, such as transgenic animals, transgenic plants, microbial cell cultures, animal cell cultures, and plant tissue cultures. Advantages of plant systems as compared to others include the ease to scale up production, the low cost of biomass production, the low risk of product contamination by mammalian viruses and bacterial toxins, correct folding of proteins, low downstream processing requirements for proteins, the ability to introduce additional or multiple transgenes, and acceptance with regard to ethical considerations (Doran 2000). Among the various plant expression systems, the suspension culture of plant cells offers a promising system for the production of recombinant pharmaceutical proteins. An advantage of plant cell culture is that the recombinant protein can be expressed as a fusion with a signal sequence, so that the protein is secreted into the culture medium and then recovered in the absence of large quantities of contaminating proteins. Hence, the downstream

processing of protein purification from the culture medium becomes less expensive. Transgenic plant cell suspension cultures have been used to produce various pharmaceutical proteins; however, a low expression level remains one of the limiting factors (Kieran et al. 1997; Fischer et al. 1999; Doran 2000). The productivity of recombinant proteins ranges from about 0.006 to 4% of total soluble protein (TSP) or from 0.5 to 200 mg/L, depending on proteins and the ratio of cell to medium volume (Hellwig et al. 2004). Protease activities in the plant cell culture medium as well as the promoter used for driving the expression of a transgene are important factors influencing the productivity of recombinant proteins (Shin et al. 2003). Consequently, seeking alternative promoters and plant cell culture systems that could significantly enhance the productivity of recombinant proteins are important when aiming to reduce the cost of protein production.

α -Amylase is an essential enzyme for hydrolysis of starch during germination of cereal grains. The expression of α -amylase genes in germinating cereal grains and cultured rice suspension cells is activated by sugar starvation and repressed by sugar provision (Yu et al. 1991; Yu et al. 1992). The α Amy8 promoter and signal peptide have been used for expression of a recombinant β -glucuronidase (GUS) with good yield in transformed rice suspension cells (Chan et al. 1994). In the present study, the α Amy8 promoter and signal peptide were also used for expression of a recombinant Der p 2-Fve fusion protein in transformed rice suspension cells. The fusion protein designated as OsDp2Fve was partially purified from the culture medium, and the conformational and immuno-functional characteristics of OsDp2Fve were evaluated by in vitro and in vivo studies.

Experimental procedures

Materials

The rice variety used in this study was *Oryza sativa* L. cv. Tainung 67. Native Fve protein was purified from fresh *Flammulina velutipes* (golden needle mushroom; Ko et al. 1995; Seow et al. 2003). Recombinant Der p 2 (14.1 kDa; Tan et al. 2006) and recombinant Blo t 5 (13.9 kDa; Kuo et al. 2003) were prepared using the *Pichia pastoris* expression system as described.

Primers

Primers used for the cloning of plasmid constructs are listed in Table S1 online.

Plasmid construction

The 900-bp α Amy8 promoter and the 90-bp α Amy8 signal peptide (SP) DNA sequence were PCR-amplified using primers pro-F and sp-R and pAG8 (Chan et al. 1994) as the DNA template. The *Der p 2-Fve* fusion gene was generated by the two-step PCR. The *Der p 2* (Dp2) coding sequence was amplified from pGEX-2T-Der p 2 (Chua et al. 1990) with pGEX 5' sequencing primer and Dp2Fve-R primers. The Fve coding sequence was amplified from pGEX-2T-Fve (Ko et al. 1997) with pGEX 3' sequencing primer and Dp2Fve-F primers. These two PCR products were mixed in 1:1 ratio, amplified with pGEX 5' and 3' sequencing primers and ligated into *Bam*HI and *Eco*RI sites of pGEX-4T-1 vector to generate the pGEX-4T-Dp2Fve. The 732-bp *Der p 2-Fve* fusion gene was PCR-amplified with Dp2-F and Fve-R primers using pGEX4T-Dp2Fve as the DNA template. DNA fragments containing α Amy8, α Amy8 signal peptide sequence, and *Der p 2-Fve* were ligated sequentially and inserted upstream of the nopaline synthase gene (*Nos*) terminator in pBlue-script SK⁺, generating pBAmy8-SP-Dp2FveN. The binary vector pSMY1H containing the *CaMV35S* RNA (35S) promoter fused to hygromycin phosphotransferase (*Hph*) cDNA (Ho et al. 2000) was digested with *Hind*III and blunt-ended. pBAmy8-SP-Dp2FveN was linearized with *Xho*I, blunt-ended, and inserted into the truncated pSMY1H, generating pAmy8-SP-Dp2FveN. The accuracy of all junction sites throughout the plasmid construction was verified by DNA sequencing.

Transformation of rice

Immature rice seeds were dehulled, sterilized with 3% NaOCl for 30 min, washed extensively with sterile water, and placed on N6D agar medium (Toki 1997) for callus induction. After 1 month, calli derived from scutellum were sub-cultured to fresh N6D medium for transformation. Plasmid pAmy8-SP-Dp2FveN was introduced into *Agrobacterium tumefaciens* strain EHA101 (Hood et al. 1986) with an electroporator

(BTX, San Diego, CA) according to the manufacturer's instructions. Rice transformation was performed with a modified method (Chen et al. 2002) based on previously described methods (Hiei et al. 1994; Toki 1997).

Establishment of suspension culture cells

Rice suspension cell cultures were established from transgenic seeds that express high levels of Der p 2-Fve and maintained in the Murashige and Skoog (MS) liquid medium containing 2 mg/L of 2,4 D and 50 mL/L of hygromycin. Cultures were maintained on a rotary shaker (110 rpm) under continuous dark at 28°C. Suspension cells (0.5 mL) were sub-cultured into 25 mL of fresh medium every 7 days in a 125 mL flask. To induce Der p 2-Fve expression, rice suspension cells were transferred from the sucrose-containing MS medium to fresh sucrose-free MS medium at 40% (v/v) density.

Southern blot analysis

Genomic DNA was extracted from embryos of T1 transgenic seeds, digested with *Bgl*II, and subjected to DNA gel blot analysis as described (Lu et al. 2002). The Der p 2-Fve cDNA (732 bp) was PCR-amplified and random-primer labeled with ³²P, which was then hybridized to the membrane containing the DNA sample.

Northern blot analysis

Transgenic rice seeds were germinated for 5 days. Total RNA was isolated from seedlings using a Trizol RNA isolation kit (Invitrogen, Carlsbad, USA). Aliquots of 10 µg total RNA were fractionated on a 1.2% agarose gel and blotted onto a Hybone-N⁺ nylon membrane (Amersham Biosciences, Buckinghamshire, England). Ethidium bromide staining of ribosomal RNA was used as an internal control. The ³²P-labeled Der p 2-Fve cDNA was then hybridized to the membrane containing the RNA sample.

Purification of OsDer p 2-Fve from the culture medium

Forty milliliter of rice suspension cells were cultured in 100 mL of sucrose-free MS medium for 3 days.

Cells were removed from the culture medium by filtration through a 400-mesh nylon sieve, and total proteins in the culture medium were concentrated approximately 15-fold using a 10 k Pellicon cassette (Millipore, Bedford, MA, USA). Ammonium sulfate was added to the concentrated cultured medium to 50% saturation. The mixture was stirred for 30 min at 4°C and centrifuged at 10,000g for 15 min. The supernatant was collected and ammonium sulfate was added to 80% saturation. The mixture was stirred for 30 min at 4°C and centrifuged at 10,000g for 15 min. The pellet was reconstituted in phosphate buffered saline (PBS), and dialyzed against three changes of 500 mL of 50 mM NaOAc buffer (pH 5.0). The dialysate was applied to a 5-mL Hi-trap SP-Sepharose FF column (Amersham Biosciences AB, Uppsala, Sweden) that had been pre-equilibrated with 50 mM sodium acetate (pH 5.0). OsDp2Fve was eluted with a linear gradient of 0 to 1 M sodium chloride in 50 mM sodium acetate (pH 5.0). Fractions containing the fusion protein OsDp2Fve were dialyzed against PBS and concentrated with the Centricon Plus-20 PL10 (Millipore). The purity of OsDp2Fve was determined by densitometry of Coomassie blue stained SDS-PAGE.

Western blot analysis

Protein samples were resolved in 12% SDS-PAGE and transferred to nitrocellulose membrane by electroblotting. Blots were probed with a Der p 2-specific monoclonal antibody (clone C1; Tsai et al. 2000) followed with peroxidase conjugated anti-mouse IgG (Amersham Biosciences). Signals were developed with chemiluminescence substrate (Amersham Biosciences) and autoradiography.

Enzyme-linked immunosorbent assay for Der p 2-specific human IgE

All ELISA procedures were carried out for 1 h at room temperature in a volume of 50 µL unless specified otherwise (Kuo et al. 2003). ELISA plates (Costar Co., Cambridge, MA, USA) were coated with 0.5 µM of OsDp2Fve (13.4 µg/mL) or recombinant Der p 2 (7 µg/mL) produced in yeast in 0.1 M NaHCO₃, pH 8.2. After washing three times with 10 mM Tris, 150 mM NaCl, and 0.05% Tween-20 (TBST), plates were blocked with 100 µL of 1%

bovine serum albumin (Sigma, St.Louis, MO, USA) in TBST. Sera of asthmatic children ($n = 24$) sensitized to Der p 2 were diluted (ranging from 1:10 to 1:100) in blocking buffer and incubated in OsDp2Fve- or Der p 2-coated wells in duplicate for 16 h at 4°C. Plates were washed and incubated with biotin-conjugated monoclonal mouse anti-human IgE (250 ng/mL in blocking buffer; Southern Biotechnology, Birmingham, CA). After washing with TBST, ExtrAvidin-alkaline phosphatase (Sigma) diluted 1:2,000 in blocking buffer, was added. Signals were developed with *p*-nitrophenylphosphate substrate (Sigma). Absorbance was measured at 405 nm using a microtiter plate reader (Tecan Austria GmbH). The human IgE titer was defined as OD_{405nm} value $\times$ serum dilution factor. To determine Der p 2-specific IgE and IgG1 from mice, Der p 2-coated wells were incubated at 4°C overnight with serially diluted mouse sera and biotinylated rat anti-mouse IgE (R19-15) and IgG1 (G1-1.5; Serotec, Oxford, England) as detection antibodies (250 μ g/mL), respectively. For comparisons of mouse immunoglobulin responses, a similar protocol was performed to generate a standard curve except for the following changes. Monoclonal anti-mouse Ig κ light chain (187.1; Pharmingen, San Diego, CA) was coated in duplicate wells, and twofold serial dilutions of mouse IgE (C38.2) and IgG1 (107.3; Pharmingen) were used as standards starting from 50 ng/mL. Antibody titers were converted from the OD_{405nm} value by using a standard curve on the same plate. One ELISA unit was defined as 1 ng/mL of immunoglobulin standards. Der p 2-specific IgG2c was determined by an ELISA kit for mouse IgG2c detection (Bethyl Laboratories, Montgomery, TX, USA) according to the manufacturer's instructions.

Histamine release assay

Histamine release assay was performed with heparinized whole blood using the histamine release kit (IBL International GmbH, Hamburg Germany) according to the manufacturer's instructions. One hundred and fifty micro-liter of heparinized whole-blood samples from Der p 2-sensitized adults were incubated in duplicate for 1 h at 37°C with an equal volume of recombinant protein at various concentrations. OsDp2Fve and allergens, Der p 2 and Blo t 5, were fivefold serially diluted in the histamine release

buffer to concentrations ranging from 3.2 nM to 1.0×10^{-3} nM. Histamine release occurred upon allergen stimulation of basophilic granulocytes depending on their sensitivity to the allergen. The resultant mixture was centrifuged at 700g for 10 min. After centrifugation, 100 μ L of the supernatant was mixed with 100 μ L of indicator buffer (provided in the kit) and 20 μ L of acylation reagent, and incubated for 30 min at room temperature. Then, 750 μ L of assay buffer was added and the samples were vortexed. The resultant mixtures were introduced into the respective wells (50 μ L per well) of the microtiter plate. Another 50 μ L of freshly prepared enzyme conjugate was added into each well, followed by 50 μ L of histamine antiserum. The plate was covered with adhesive foil and incubated for 3 h at room temperature on a reciprocal shaker at 80 rpm. The plate was washed 4 times with 250 μ L of wash buffer and dried. Subsequently, 100 μ L of 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution was added into each well. Samples were incubated further for 40 min at room temperature on an orbital shaker at 80 rpm. The reaction was stopped by adding 100 μ L of TMB stop solution into each well and gentle mixing. The optical density of each well was measured at 405 nm 15 min after the addition of the TMB stop solution. The histamine concentrations were determined by using the standard curve in the same plate.

Lymphoagglutination and lymphoproliferation assays

Mouse splenocytes (1×10^6 per well) were seeded into a 96-well flat-bottom plate in triplicate. Cells were stimulated with 0.01–10 μ M of OsDp2Fve, Fve or Der p 2 for 24 h in RPMI-1640 medium supplemented with 10% of heat inactivated FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 55 μ M of β -mercaptoethanol, antibiotics (100 U/mL Penicillin and 100 μ g/mL Streptomycin) in a 5% CO₂ incubator at 37°C. Photos of cultured cells were taken at 200 $\times$ magnification with a phase-contrast microscope. For proliferation assays, cells were cultured as above and pulsed with [³H]-thymidine for the last 18 h of culture. The cells were harvested after 72 h and [³H]-thymidine incorporation was measured by liquid scintillation counting. Concanavalin A and the wild type rice culture medium treated by the same

purification steps (designated as Os medium) were used as positive and negative controls, respectively.

Mice and experimental protocol

The C57BL/6 J mice were housed under conventional conditions in the Animal Holding Unit at The National University of Singapore. Animal experiments were performed according to Institutional Guidelines for Animal Care and Handling. Groups of 6 female mice (6–8 weeks old) were administered subcutaneous injections of Der p 2 (10 µg/dose), Der p 2 plus Fve (10 µg + 10 µg/dose), OsDp2Fve (20 µg/dose) or Der p 2 (10 µg/dose) plus Os medium on days 0, 4, 8, and 14. Sera were collected weekly and analyzed for Der p 2-specific immunoglobulins.

Splenic T-cell cultures

Mice were sacrificed on day 28 and splenocyte culture was prepared. Cells were cultured with 10 µg/mL of Der p 2 at 2×10^6 cells/mL in complete RPMI-1640 medium in a 5% CO₂ incubator at 37°C. Mouse recombinant IL-2 (10 U/mL) was added on days 3 and 5. Viable cells were recovered by using Ficoll-Paque PLUS (GE Healthcare, Piscataway, NJ) gradient centrifugation on day 7. Der p 2-specific T cells were reactivated with 10 µg/mL of Der p 2 by culturing 1×10^5 viable cells with 3×10^5 of mytomycin C-treated splenocytes cells in 96-well plates. Culture supernatants were harvested at 24, 48, and 72 h and assayed for secreted cytokines IL-4, IL-5, and IFN-γ by ELISA as previously described (Tan et al. 2006).

Relative quantitative PCR

Total RNA from primary splenic T-cell culture was isolated at 24 h using the RNeasy mini kit (QIAGEN Inc., Valencia, CA). The first-strand cDNA was generated from 0.5 µg of total RNA using d(T)₂₀ oligonucleotide and the SuperScript III first-strand synthesis super-mix (Invitrogen, Carlsbad, CA) in 20 µL reaction mixture. IL-4, IL-5, IL-13, and IFN-γ gene expression were assessed with the TaqMan PCR master mix and gene expression assays Mm00445259_m1, Mm00439646_m1, Mm00434204_m1, and Mm00801778_m1 (Applied Biosystems, Foster City, CA), respectively. Real-time

PCR reactions were carried out in 20 µL amplification mixture with 0.9 µM of each primer and 0.25 µM of probe on the ABI 7300 real-time PCR system (Applied Biosystems). The PCR conditions included an incubation step at 50°C for 2 min, a polymerase activation step at 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. Quantitation of the relative cytokine mRNA expression were determined by the comparative cycling threshold (Ct) method by the $2^{-\Delta\Delta C_t}$ formula (Pfaffl 2001). Results were normalized to β-actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression determined with probes labeled with the VIC dye.

Statistical analysis

The significance between experimental groups was calculated by the two-tailed Mann–Whitney *U* test except for the in vitro lymphoproliferation assay where the two-tailed student's *t*-test was used. A *P* value less than 0.05 was considered significant.

Results

Generation of transgenic rice expressing *Der p 2-Fve*

A *Der p 2-Fve* fusion gene placed downstream of the αAmy8 promoter-signal peptide (Fig. 1a) was introduced into the rice genome. Sixty-four transgenic lines were obtained and further analyzed. T1 seeds of each transgenic line were germinated for 5 days under hygromycin selection, and embryos were collected for analysis of mRNA expression. The accumulation of *Der p 2-Fve* mRNA varied significantly in the different lines (Fig. S1). Four transgenic lines (lines 7, 36, 51, and 63) that express a high level of the *Der p 2-Fve* fusion protein (OsDp2Fve) were found to contain various copy numbers (ranging from 2 to 5) of the *Der p 2-Fve* gene (Fig. S2).

Sucrose starvation induced accumulation of OsDp2Fve in the culture medium

T1 seeds of transgenic line 7 expressing a high level of *Der p 2-Fve* was selected for establishment of suspension cell cultures (Fig. S1 and Fig. S2). For

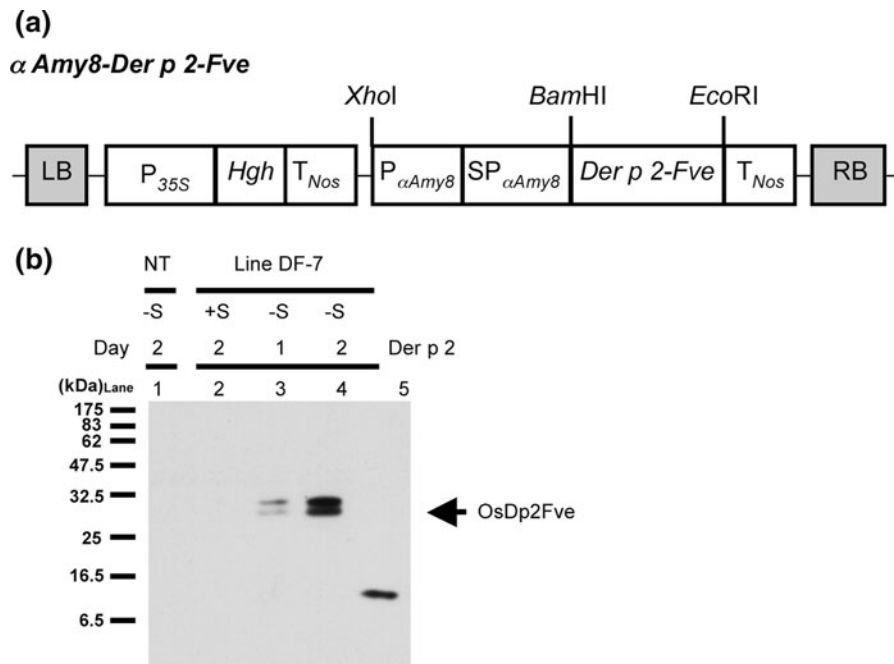


Fig. 1 Expression of OsDp2Fve in cultured transgenic rice suspension cells is induced by sucrose starvation. **a** Expression cassette for rice transformation. P_{AI} , AI promoter; Hgh , hygromycin phosphotransferase; T_{Nos} , Nopal synthase gene terminator; P_{Ubi} , Ubi promoter; LB and RB, left and right borders of T-DNA, respectively. **b** Western blot analysis of transformed rice cell line DF-7 expressing OsDp2Fve fusion protein. NT non-transformed control. One milliliter of suspension cells generated from T1 transgenic seeds were cultured in

25 mL sucrose-containing medium for 3 days, and 1 mL of fresh cells were transferred to 25 mL sucrose-containing (+S) or sucrose-free (–S) MS medium for 1 and 2 days. Proteins were collected from the medium, and OsDp2Fve fusion protein was detected by western blot analysis using an anti-Der p 2 monoclonal antibody. A total of 300 ng of proteins from the medium was loaded in each lane. Lane 5 containing 30 ng of Der p 2 protein was used as a control

induction of OsDp2Fve expression, cells were cultured in sucrose-containing medium for 2 days and then transferred to sucrose-free medium for 1 or 2 days. The production of OsDp2Fve into the culture medium was analyzed by western blotting using an anti-Der p 2 monoclonal antibody. As shown in Fig. 1b, the accumulation of OsDp2Fve increased with time under sucrose starvation. Two protein bands were detected with the monoclonal anti-Der p 2 antibody and migrated between 32.5 and 25 kDa molecular weight markers. The estimated molecular weight of these two proteins was similar to the calculated molecular weight of 26.8 kDa for OsDp2Fve. As Fve sequence contains two potential N-glycosylation sites, the appearance of two bands could be due to the existence of different glycoform. However, the possibility of protein degradation derivatives cannot be ruled out and further study is required to verify such possibilities. The yield of

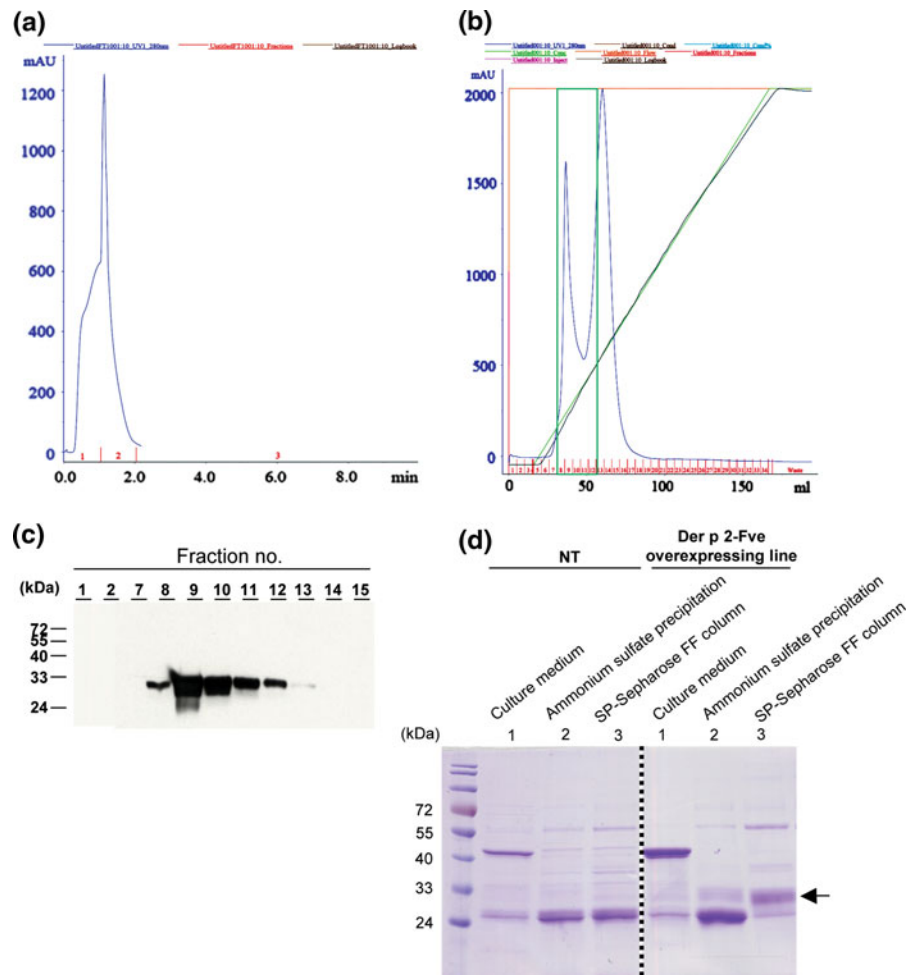
OsDp2Fve fusion protein in the culture medium was estimated to be 7.5 mg/L, which is equivalent to 10.5% of total secreted proteins at day 2.

Purification of the OsDp2Fve fusion protein from the culture medium

OsDp2Fve was purified from the culture medium by concentration, ammonium sulfate precipitation, and cation chromatography methods. The culture medium containing OsDp2Fve was concentrated approximately 15-fold by using a 10 k Pellicon cassette (Millipore). The protein fraction obtained at precipitation in 50–80% ammonium sulfate was then separated on a Hi-trap SP column. The OsDp2Fve protein was detected in fractions 8–13 eluted with a linear gradient of 0–25% NaCl (Figs. 2a–c). The purity of the protein was estimated as 75% based on desitometry (Fig. 2d).

Fig. 2 Steps for purification of Der p 2-Fve.

a. Elution profiles of OsDp2Fve after SP-Sepharose FF column fractionation. **b** flow-through fractions and **b** bound fractions eluted with a linear gradient of 0–25% NaCl. **c** Western blot analysis of OsDp2Fve purified with SP-Sepharose FF column using an anti-Der p 2 monoclonal antibody. **d** Analysis of protein samples at different purification steps. Two μ g of total proteins obtained at different steps in the purification process were analyzed by Coomassie blue stained 12% SDS-PAGE. *Lane 1* culture medium; *lane 2* pellet obtained after ammonium sulfate precipitation (50–80%); *lane 3* eluted fractions from the SP-Sepharose FF column. *NT* non-transformed control. The *arrowhead* indicates the position of OsDp2Fve



Recombinant OsDp2Fve retains the conformation-dependent functional integrity of native Der p 2

To determine the conformational integrity of recombinant Der p 2 expressed as a fusion protein with Fve (i.e. OsDp2Fve) produced by the rice cell suspension culture system, we first assessed its reactivity with human IgE by direct ELISA. Plate-bound native Der p 2 or OsDp2Fve were reacted with a total of 24 Der p 2-positive allergic sera with various specific IgE titers to Der p 2. The Der p 2-specific IgE reactivity of OsDp2Fve was comparable to that of native Der p 2 as there was no significant difference in the ELISA readout ($P = 0.89$, Fig. 3a). Furthermore, the cross-inhibition of soluble OsDp2Fve or Der p 2 on immobilized Der p 2, as assessed by competitive ELISA with six individual sera, resulted in similar

inhibition curves (Fig. S3). The human IgE reactivity was subsequently examined by measuring the allergen-triggered histamine release from basophils. The blood sample from an adult sensitized to Der p 2 but not to Blo t 5 (another common mite allergen), was used to test the bioreactivity of OsDp2Fve on cell membrane-bound Der p 2 specific IgE. The dose-dependent histamine release profiles were similar for OsDp2Fve and Der p 2 in the 0.5 pM to 1.6 nM concentration range (Fig. 3b). The histamine release profiles were highly specific to Der p 2 as no histamine release induced by the Blo t 5 control allergen was observed. Since it is well-known that the human IgE reactivity of Der p 2 is highly dependent on the conformational integrity of the Der p 2 protein, these data imply that the conformation of the recombinant Der p 2 fused with Fve (OsDp2Fve) is similar to that of Der p 2.

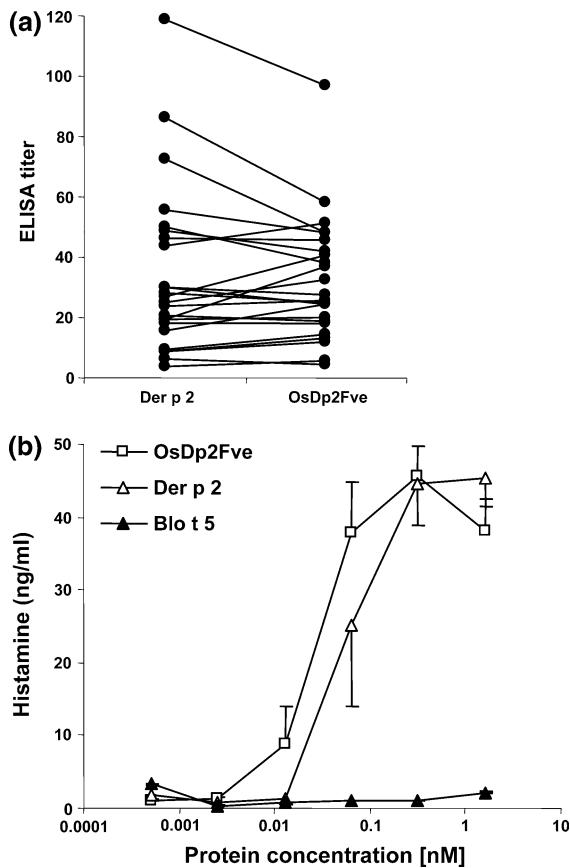


Fig. 3 Comparisons of the IgE reactivity in human sera regarding OsDp2Fve and Der p 2. The conformational integrity and function of Der p 2 in the OsDp2Fve fusion protein was evaluated by the human IgE reactivity. **a** Plate-bound OsDp2Fve or Der p 2 was reacted with 24 sera from Der p 2-positive allergic children by direct human IgE ELISA. The y-axis represents ELISA titer as determined by OD_{405nm} reading $\times$ serum dilution factor. Each dot represents one individual serum; the Der p 2-specific IgE reactivity to Der p 2 and OsDp2Fve from each serum is linked by a line. **b** The whole blood sample of a Der p 2-sensitized adult was stimulated with OsDp2Fve or Der p 2 at concentration ranging from 1.6 nM to 0.51 pM (6 dosage points), and concentrations of histamine released from basophils were determined. The recombinant Blo t 5 was included as a negative control. Results are expressed as the mean $\pm$ sem from two independent experiments

OsDp2Fve retains its *in vitro* lymphoproliferative activity but loses its hemagglutination and lymphoagglutination effects

To analyze the functional integrity of the recombinant Fve in OsDp2Fve fusion protein, mouse splenic lymphocytes were cocultured with OsDp2Fve or

native Fve, and the agglutination and the proliferation of lymphocytes were examined. Native Fve induced lymphoagglutination of mouse lymphocytes after about 20 min of coculture at concentrations higher than 1 μ M. Lymphoagglutination was observed to occur in a dose-dependent manner, starting at a concentration of 0.2 μ M, with native Fve after 24 h of coculture (Fig. 4a, upper). Massive aggregation of mouse lymphocytes could be seen at concentration higher than 5 μ M. By contrast, OsDp2Fve did not induce lymphoagglutination of mouse lymphocytes at 20 min and only induced observable agglutination at concentrations higher than 1 μ M at 24 h. The most obvious agglutination was seen at concentrations between 2 and 5 μ M (Fig. 4a, middle). This degree of cellular agglutination was similar to 0.5–1 μ M of Fve induced lymphoagglutination. However, the lymphoagglutinating activity of OsDp2Fve was not observed as the concentration increased to 10 μ M. The mechanism is yet to be studied. Der p 2 alone, as expected, could not induce any lymphoagglutination.

To determine the lymphoproliferative activity of OsDp2Fve, mouse splenic lymphocytes were cocultured with OsDp2Fve, native Fve, or Der p 2 in a concentration range between 0.01 and 10 μ M. To rule out a possible contamination of OsDp2Fve with non-protein cellular components, which have been attributed to possess lymphostimulatory activity (Liao et al. 2006; Kim et al. 2007), cultured Os medium obtained from wild-type cells purified by the same processing protocol as OsDp2Fve was included as a control. Both the OsDp2Fve fusion protein and native Fve could induce bell-shape dose-dependent proliferative response curves from mouse splenocytes and the maximum proliferative responses peaked at concentrations between 1 and 3 μ M. The OsDp2Fve could induce a higher magnitude of [^{3} H]-thymidine incorporation as compared to native Fve. By contrast, Der p 2 and Os medium control did not stimulate any [^{3} H]-thymidine incorporation (Fig. 4b). Compared with the classical mitogen concanavalin A induced sigmoid-shape dose-dependent proliferative curve, the proliferation of mouse splenocytes reached plateau at around 1 μ g/mL. Assuming that concanavalin A was all in a tetramer form in culture media with a homogeneous molecular weight of 100 kDa, the molar concentration of 1 μ g/mL was roughly equivalent to 0.01 μ M. At this concentration both Fve and OsDp2Fve could not induce a significant

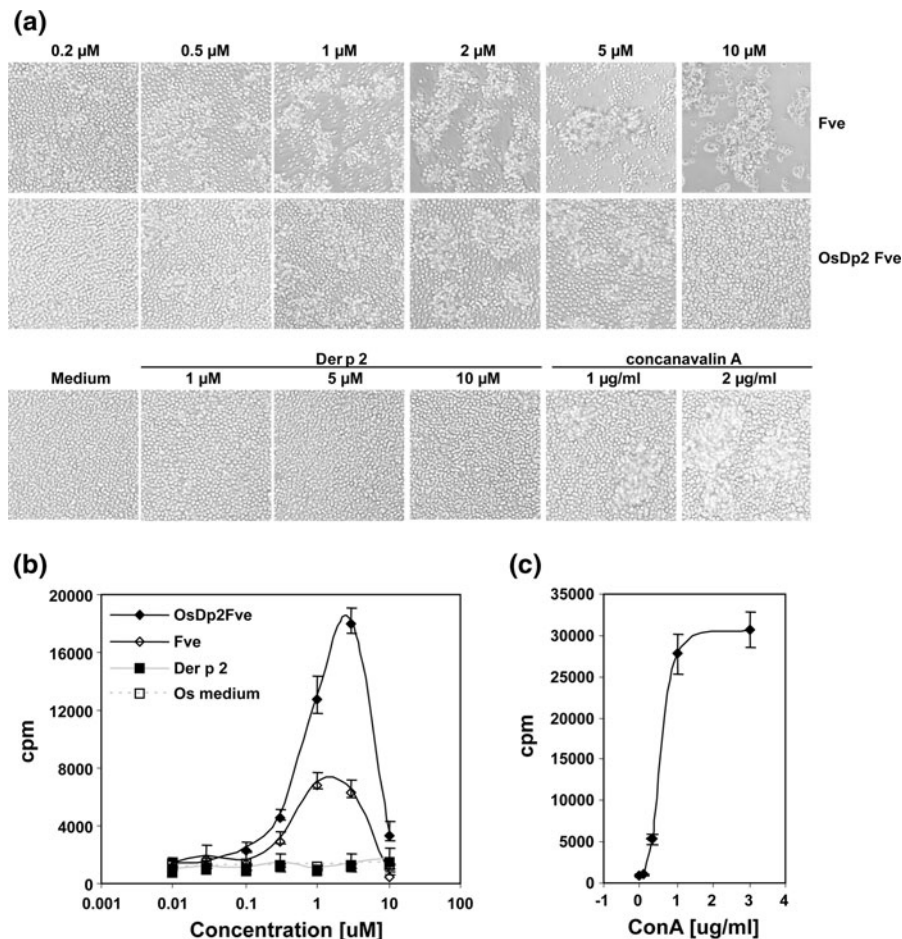


Fig. 4 Comparisons of lymphoagglutination and lymphoproliferation in mouse splenocytes co-cultured with OsDp2Fve and Der p 2. **a** Mouse splenocytes were co-cultured with various concentrations of OsDp2Fve, Fve, or Der p 2 in the complete RPMI-1640 medium. The degree of lymphoagglutination was examined under phase-contrast microscopy (original magnification 200 $\times$) after 24 h. Medium alone and concanavalin A (*ConA*) were included as negative and positive

control, respectively, for comparison. **b** The Fve-stimulated lymphoproliferation in mouse splenocytes co-cultured with various concentrations of OsDp2Fve, Fve, or Der p 2 was determined by [^{3} H]-thymidine incorporation assay after 72 h. Purified medium from the wild-type rice cell culture (Os medium) was included in the assay. **c** Concanavalin A-stimulated lymphoproliferative response in mouse splenocytes was used as a positive control. *cpm* counts per minute

lymphoproliferative activity as compared to concanavalin A (Fig. 4b, c). However, the maximum cellular agglutination induced by concanavalin A was far more less than that induced with Fve although the active concentration range was higher for Fve (Fig. 4a). Taken together, there was no correlation between lymphoagglutinating and lymphoproliferative activities. The Fve protein in OsDp2Fve showed a decreased potency in inducing agglutination of mouse lymphocytes, yet retained its activity in inducing proliferation of mouse lymphocytes when compared with native Fve.

OsDp2Fve enhanced Der p 2-specific IgG1 and IgG2c production in vivo

To explore the in vivo immunogenicity of OsDp2Fve by using Der p 2-specific immune responses as readouts, we immunized C57BL/6 J mice subcutaneously with Der p 2 alone, Der p 2 plus native Fve (Der p 2 + Fve), OsDp2Fve, or Der p 2 plus purified Os medium (Der p 2 + Os medium) on days 0, 4, 8 and 14. A low-dose subcutaneous immunization of Der p 2 without any adjuvant could elicit a low level of Der p 2-specific IgE and IgG1 but not IgG2c,

showing the intrinsic Th2-skewing property of the major dust mite allergen Der p 2. The Der p 2-specific IgE production was significantly increased when mice were received Der p 2 + Fve immunization. Mice received OsDp2Fve injection showed reduced Der p 2-specific IgE as compared to that elicited by Der p 2 + Fve (Fig. 5a). Der p 2 + Os medium immunization did not boost the Der p 2-specific IgE or IgG1 significantly. The immunization with OsDp2Fve greatly enhanced the production of Der p 2-specific IgG1 and IgG2c in terms of both magnitude and kinetics. Although the production of Der p 2-specific IgG1 and IgG2c was significantly enhanced from day 14 onwards following immunization with Der p 2 + Fve, OsDp2Fve-immunized mice produced significantly higher levels of Der p 2-specific IgG1 and IgG2c already on day 8 ($P < 0.005$ as compared with the other 3 groups; Fig. 5b and Fig. 5c). On day 14, the Der p 2-specific IgG2c from OsDp2Fve-immunized mice (291.4 ± 80.2 EU) was also significantly higher than that of mice immunized with Der p 2 + Fve (63.0 ± 30.8 EU; $P = 0.025$). The OsDp2Fve-immunized mice consistently produced higher Der p 2-specific IgG2c on day 21 (693.3 ± 377.1 EU vs 125.2 ± 58.3 EU, $P = 0.054$) and day 28 (1524.7 ± 627.0 EU vs 328.0 ± 150.3 EU, $P = 0.093$) as compared to Der p 2 + Fve immunized mice, although the differences did not reach statistical significance. Taken together, these results demonstrate that the Fve protein expressed in fusion with Der p 2 exhibits a different adjuvanticity. The differential immunomodulatory effect was manifested by suppressing the Der p 2-specific IgE production while enhancing the Der p 2-specific IgG1 and IgG2c productions.

Mice immunized with OsDp2Fve produced similar levels of Th2 effector cytokines as compared to the Der p 2 immunized mice

To investigate profiles of Der p 2-specific T cell cytokines, spleens from the four groups of immunized mice were harvested on day 28, and splenocytes were stimulated with $10 \mu\text{g/mL}$ of Der p 2 protein in vitro. Total RNA from cultures was isolated 24 h post-stimulation and the expression of IFN- γ , IL-4, IL-5, and IL-13 from primary cultures was determined by relative quantitative RT-PCR using β -actin as the reference. Notably, the splenocytes from mice

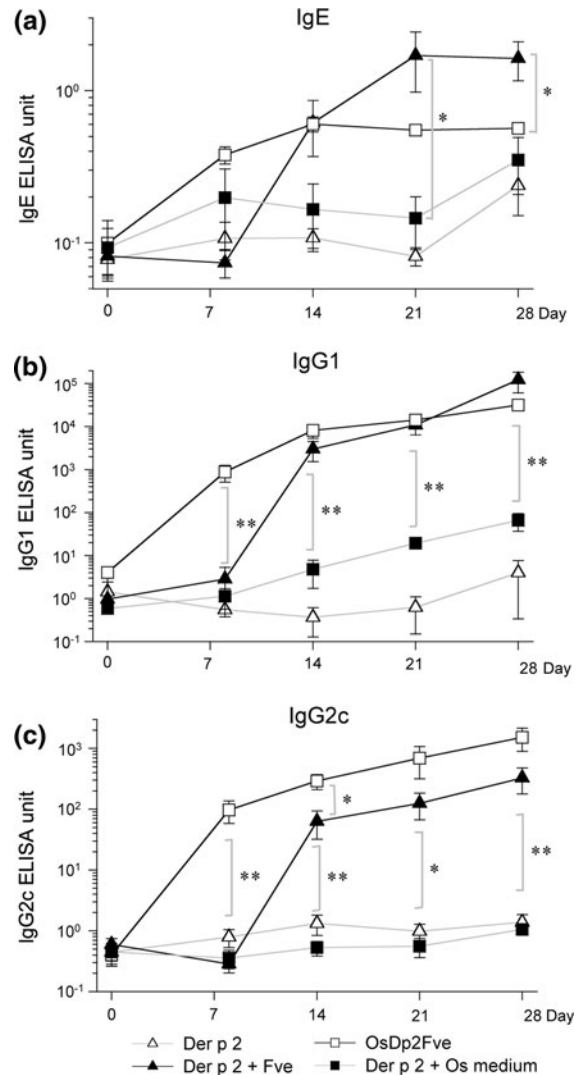
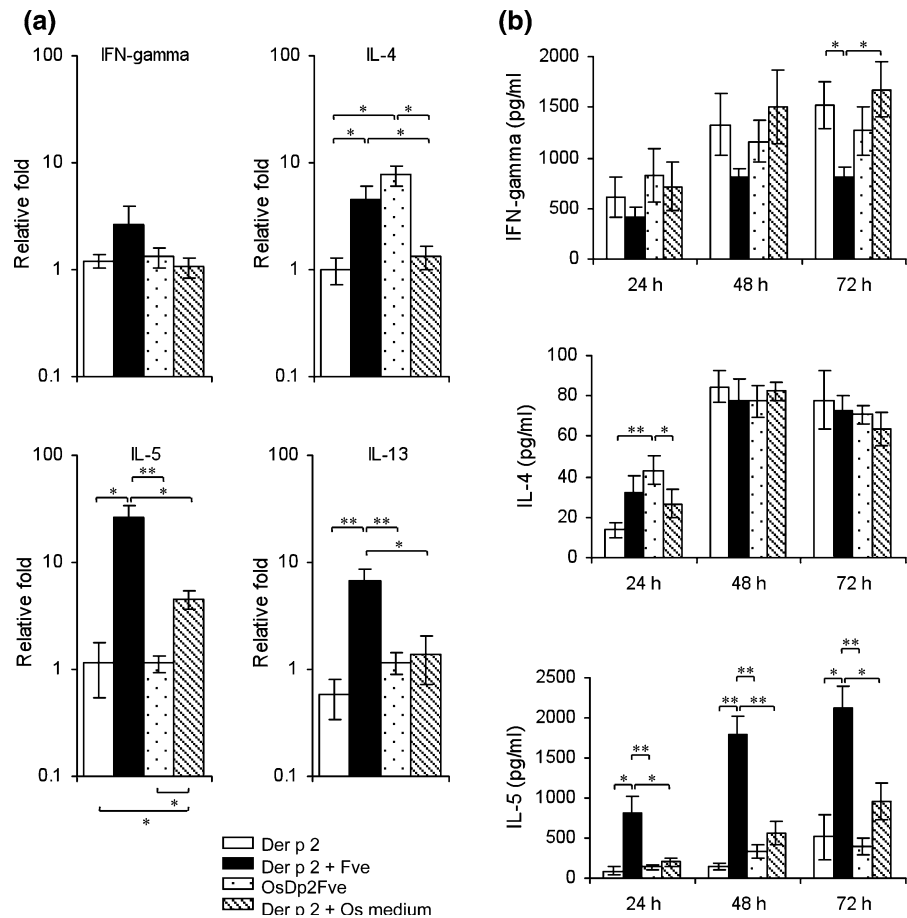


Fig. 5 Kinetics of Der p 2-specific humoral responses. Mice were immunized by subcutaneous injections with Der p 2, Der p 2 + Fve, OsDp2Fve, or Der p 2 + Os medium in phosphate buffered saline in the absence of other adjuvants on days 0, 4, 8, and 14. Sera were collected at the indicated time points for determination of the Der p 2-specific IgE (a), IgG1 (b), and IgG2c (c). The titer of antibodies was determined by ELISA. One ELISA unit corresponds to the OD_{405nm} reading for 1 ng/mL of the standard. IgG1 and IgG2c titers are presented on a logarithmic scale. Data are expressed as mean $\pm$ sem ($n = 6$). * $P < 0.05$; ** $P < 0.005$

immunized with Der p 2 + Fve showed a significantly enhanced expression of Th2-type cytokine IL-5 and IL-13 transcripts as compared to those from the other 3 groups. The level of IL-4 transcripts in splenocytes from mice immunized with OsDp2Fve or Der p 2 + Fve is comparable and significantly higher

Fig. 6 Splenocyte cytokine profiles from immunized mice. **a** Mice were sacrificed on day 28 and splenocyte were cultured with 10 $\mu\text{g/mL}$ of Der p 2 for 24 h. The expression of IFN- γ , IL-4, IL-5, and IL-13 transcripts from primary cultures was determined by relative quantitative RT-PCR with reference to the expression level of β -actin. **b** T cells were continued to grow for 1 cycle and re-stimulated with Der p 2 for various periods of time in the presence of mitomycin C-treated syngeneic splenocytes. Culture supernatants were collected for determination of IFN- γ , IL-4, and IL-5 by cytokine ELISA. A Results shown are mean $\pm$ sem (n = 6). * P < 0.05; ** P < 0.005



than that from the other two groups (Fig. 6a). Expression of IL-5 was higher in the splenocytes from mice immunized with Der p 2 + Os medium as compared to that from the groups immunized with Der p 2 or OsDp2Fve. No difference was detected in the expression of IFN- γ among the four groups of mice. Similar cytokine expression profiles were obtained with GAPDH as the reference (Fig. S4).

To further investigate the secreted cytokine profiles of Der p 2-specific T cells, primary splenocyte cultures at day 7 were re-stimulated with 10 $\mu\text{g/mL}$ of Der p 2 protein in the presence of mitomycin C-treated syngeneic splenocytes as antigen-presenting cells, and the levels of cytokine proteins in the culture supernatants were measured by ELISA. The results showed that Der p 2-specific T cells from the group immunized with Der p 2 + Fve produced the highest level of Th2 effector cytokine

IL-5 as compared to the other 3 groups at 24, 48, and 72 h (Fig. 6b). Consistent with the determined levels of RNA transcripts, the level of IL-4 produced in T cells of OsDp2Fve-immunized mice at 24 h was higher than that from the groups immunized with Der p 2 or Der p 2 + Os medium. However, T cells from all four groups produced similar levels of IL-4 at 48 and 72 h. The IFN- γ production was also comparable in T cells of all 4 groups of mice, although it was somewhat reduced in the group immunized with Der p 2 + Fve. In summary, mice receiving subcutaneous immunization with Der p 2 + Fve produced significant higher levels of Th2 effector cytokines IL-5 and IL-13 as compared to mice receiving immunization with Der p 2 alone. By contrast, T cells from mice immunized with fusion protein OsDp2Fve produced similar levels of Th2 effector cytokines as compared to T cells from Der p 2-immunized mice.

Discussion

In the present study, the α My8 promoter was used to produce a Der p 2-Fve fusion protein in sucrose-starved rice suspension cell cultures. The yield of OsDp2Fve in the culture medium was estimated to be 7.5 mg/L, which is equivalent to 10.5% of total secreted proteins. The fusion protein was enriched to 75% purity after a few steps of purification, and then tested for its efficacy as an allergy immunotherapy agent based on several parameters.

The house dust mite allergen Der p 2 has been expressed in bacteria, yeast, and tobacco systems (Chua et al. 1990; Lynch et al. 1994; Hakkaart et al. 1998a; Hakkaart et al. 1998b; Lienard et al. 2007). The recombinant Der p 2 produced in prokaryotic systems were insoluble and therefore required denaturing and renaturing processes to recover a portion of protein with correct conformation (Derewenda et al. 2002). The problem of poor protein solubility was solved by expressing recombinant Der p 2 in eukaryotic systems such as in *Pichia pastoris* with high yields (Tan et al. 2006). The recombinant Der p 2 (rDer p 2) fused with a glutathion-S-transferase expressed in *Escherichia coli* was shown to be as allergenic as the native Der p 2, and 22 of 24 patient sera tested showed IgE reactivity (Lynch et al. 1994). A non-fusion rDer p 2 protein expressed in baker's yeast, *Saccharomyces cerevisiae*, showed biological activity and immunological characteristics similar to the natural allergen, although 5 out of 19 nDer p 2-specific human sera failed to react with the recombinant protein (Hakkaart et al. 1998a). Recombinant proteins rDer p 2 and rDer p 2 fused with a (His)₆ tag (rDer p 2-His) at the C-terminus have also been expressed in tobacco plants and suspension-cultured BY-2 tobacco cells (Lienard et al. 2007). The amount of rDer p 2-His produced in BY-2 tobacco cells was about 5 times higher than rDer p 2 produced in the crude protein extract from tobacco plants or BY-2 tobacco cells, suggesting that the presence of the C-terminal extension stabilized the recombinant allergen (Lienard et al. 2007). The allergenicity of an allergen was, to some extent, dependent on the binding of human IgE to the correct conformational epitopes (Ball et al. 2009; Melanie et al. 2009; Padavattan et al. 2009). Der p 2 of the OsDp2Fve fusion protein was reactive to human IgE with similar potency as the non-fused Der p 2 protein,

as determined by direct ELISA, inhibition ELISA and the basophilic histamine release assay (Fig. 3 and Fig. S3). Taken together, our results thus indicate that the conformation of Der p 2 in the OsDp2Fve fusion protein is close to the conformation of Der p 2 produced in *Pichia pastoris*.

Proteins of the FIP family are mitogens for human PBMCs and mouse splenocytes with differential hemagglutinating and/or lymphoagglutinating activities in vitro (Kino et al. 1989; Ko et al. 1995; Hsu et al. 1997). In our experiments, mouse splenocytes agglutinated in a Fve dose-dependent manner, and more than 90% of cells were agglutinated at concentrations above 5 μ M within 20 min of coculture. By contrast, the potency of OsDp2Fve in the induction of the agglutination of mouse splenocytes was lower as compared to native Fve. A possible explanation for the reduced agglutinating activity of OsDp2Fve is a disturbance of the N-terminal α -helix, which comprises the dimerization domain of Fve, in the fusion protein (Paaventhian et al. 2003). It is worth noting that OsDp2Fve, unlike its native counterpart, did not agglutinate human red blood cells after 24 h of incubation (results not shown). The loss of hemagglutinating and lymphoagglutinating activities of OsDp2Fve is beneficial for the potential use of Fve as immunotherapeutics or as food supplement because it will significantly reduce the potential side effects and therefore enhance the safety of Fve.

The mitogenic and INF- γ -inducing activities of Fve have led to the speculation of its immunopotentiating function as an adjuvant. Indeed, coadministration of Fve with the oncoprotein HPV-16 E7 effectively enhanced the HPV-16 E7-specific Th1 immune responses and protected mice from a subsequent challenge with HPV-16 E7-expressing tumor cells or prolonged the survival of tumor-bearing mice. This protective effect was INF- γ -, CD4- and CD8-dependent. Interestingly, CD8⁺ dendritic cells from mice injected intravenously with Fve showed a phenotypic maturation manifested by the upregulation of major histocompatibility complex class II and CD86 (Ding et al. 2009). These results indicated that Fve might not only act on lymphocytes but also on other cell types such as dendritic cells to enhance the antigenicity of inert antigens. Thus, it appears that Fve could act as an immunopotentiator in the innate compartment as well as an immunoregulator in the adaptive compartment of the immune system.

Other previous studies have shown that Fve can induce a strong production of IFN- γ by mouse splenocytes and human PBMCs (Ko et al. 1995; Wang et al. 2004; Ou et al. 2009). In the mouse model for food allergy, elevated IFN- γ levels in the sera of mice fed with Fve were accompanied with a suppression of ovalbumin-induced anaphylaxis, plasma histamine levels, and pathological changes in the jejunum (Hsieh et al. 2003). The Fve-stimulated IFN- γ production can be mediated by the p38 mitogen-activated protein kinase pathway (Wang et al. 2004) and by Ca^{2+} mobilization and activation of protein kinase C- α (Ou et al. 2009). It has been reported that treatment with IFN- γ can induce the conversion of $\text{CD4}^+\text{CD25}^-$ T cells into Foxp3 expressing regulatory T (Treg) cells and enhancement of regulatory properties. The authors proposed that the high concentration of IFN- γ produced either locally or systemically might be regarded as a danger signal to the immune system and enable it to call for a controlling mechanism (Wang et al. 2006). An increase of Foxp3 $^+$ Treg cells in the $\text{CD4}^+\text{CD25}^+$ T cell population and concomitant increases of IL-10 $^+$ and transforming growth factor β^+ cells among $\text{CD4}^+\text{CD25}^+\text{Foxp3}^+$ Treg cells were observed from stimulating PBMCs of *Dermatophagoides farinae*-sensitized allergic rhinitis patients with a peptide containing the N-terminal 63 residues of Der f 2 in combination with Fve (Wu et al. 2009). Furthermore, in a mouse model for local nasal immunotherapy, Der p 2-sensitized mice treated with either Der p 2 peptide or Der p 2 plus Fve could downregulate not only Der p 2-specific IgE and Th2 immune responses but also Der p 2-induced airway inflammation and airway hypersensitivity (Liu et al. 2003; Liu and Tsai 2005). These results suggested that Fve could execute its immunomodulatory function on the established adaptive immunity by enhancing the number and the function of allergen-specific Treg cells, possibly via its potent activity in stimulating IFN- γ production.

In this study, our data show that the administration of OsDp2Fve and coadministration of Der p 2 and Fve enhances Der p 2-specific IgG1 and IgG2c responses (Fig. 5). However, OsDp2Fve exhibits a more robust immunopotentiating effect in terms of kinetics and magnitude as compared to native Fve administered as a mixture with Der p 2. Our results show that the enhanced production of Der p 2-specific IgG2c (the signature antibody commonly associated

with Th1-skewed responses) in mice injected with OsDp2Fve is not directly linked to the pathway involving the upregulation of the Th1 cytokine IFN- γ (Figs. 5c and 6a). It has been well documented that, in addition to antigen specific CD4 Th1 cells, NK cells can help B cells for IgG2a/IgG2c class switching (Wilder et al. 1996; Satoskar et al. 1999) in both IFN- γ dependent and independent ways (Gao et al. 2001). In addition IL-17 from Th17 cells may drive the IgG2a/IgG2c isotype class switch in B cells (Mitsdoerffer et al. 2010). Therefore the immunopotentiating effects of Fve on the Der p 2 allergen cannot be explained simply by the Th1-Th2 paradigm; thus, the underlying mechanisms remain to be elucidated in further studies. Intriguingly and unexpectedly, the Der p 2-specific immune responses showed that coadministration of Der p 2 and Fve proteins enhanced the production of Der p 2-specific IgE and Th2 effector cytokines such as IL-5 and IL-13. It appears that the native Fve enhanced the Der p 2-specific Th2 immune responses like an amplifier.

In summary, we have successfully produced the soluble fusion protein OsDp2Fve in a rice cell suspension culture system. The conformation of Der p 2 in the fusion protein remains intact as reflected by its unaltered allergenicity. The Fve in the fusion protein retained its in vitro lymphoproliferative activity and exhibited enhanced in vivo immunomodulatory functions. Mice administered with OsDp2Fve showed an enhanced production of Der p 2-specific IgG antibodies without an increase in the production of Der p 2-specific IgE and Th2 effector cytokines. The reduced lymphoagglutinative and hemagglutinative activities of OsDp2Fve will likely make it a safer reagent for human use. More importantly, our mouse study showed that OsDp2Fve could skew the Der p 2-specific adaptive immune responses that possibly downregulate the allergen-specific Th2 phenotype. Taken together, our data support the notion that OsDp2Fve is a promising candidate for further developments into a prophylactic or therapeutic reagent for allergy immunotherapy.

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