

Prevention of allergic asthma by vaccination with transgenic rice seed expressing mite allergen: induction of allergen-specific oral tolerance without bystander suppression

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Received 24 November 2010;

revised 10 February 2011;

accepted 11 February 2011.

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Keywords: bronchial asthma, bystander suppression, edible vaccine, house dust mite, immunotherapy, transgenic plant.

Summary

This study tested the feasibility of oral immunotherapy for bronchial asthma using a newly developed subunit vaccine in which a fragment (p45–145) of mite allergen (Der p 1) containing immunodominant human and mouse T cell epitopes was encapsulated in endoplasmic reticulum-derived protein bodies of transgenic (Tg) rice seed. Allergen-specific serum immunoglobulin responses, T cell proliferation, Th1/Th2 cytokine production, airway inflammatory cell infiltration, bronchial hyper-responsiveness (BHR) and lung histology were investigated in allergen-immunized and -challenged mice. Prophylactic oral vaccination with the Tg rice seeds clearly reduced the serum levels of allergen-specific IgE and IgG. Allergen-induced CD4⁺ T cell proliferation and production of Th2 cytokines *in vitro*, infiltration of eosinophils, neutrophils and mononuclear cells into the airways and BHR were also inhibited by oral vaccination. The effects of the vaccine were antigen-specific immune response because the levels of specific IgE and IgG in mice immunized with Der f 2 or ovalbumin were not significantly suppressed by oral vaccination with the Der p 1 expressing Tg rice. Thus, the vaccine does not induce nonspecific bystander suppression, which has been a problem with many oral tolerance regimens. These results suggest that our novel vaccine strategy is a promising approach for allergen-specific oral immunotherapy against allergic diseases including bronchial asthma.

Introduction

The induction of oral tolerance by protein or peptide administration is a promising approach to immunotherapy (Nelson, 2007). Oral tolerance is a T cell-mediated phenomenon involving a specific decrease in immune responses to antigens previously encountered via the oral route (Richman *et al.*, 1978). Oral tolerance has been demonstrated in numerous species, including humans and mice, and can be achieved using many different antigens (Mowat, 1987). However, despite long-standing research into oral tolerance, it has not yet been applied to the treatment of human allergic diseases.

The house dust mites (HDM) *Dermatophagoides pteronyssinus* (Der p) and *Dermatophagoides farinae* (Der f) are a major source of inhalant allergens that cause chronic allergic diseases such as bronchial asthma, rhinitis and atopic dermatitis. In addition, 45%–80% of patients with allergic asthma are sensitized to HDM allergens, suggesting that exposure to HDM is crucial for the development of the allergic disease (Thomas *et al.*, 2002). The HDM group 1 and 2 allergens are major allergens

based on the frequency of patients sensitized, the levels of specific IgE in sensitized subjects and total amount of these allergens in HDM extract (Tovey *et al.*, 1981; Thomas *et al.*, 2002). The group 1 allergens Der p 1 and Der f 1 are heat-labile, 25 kDa acidic glycoproteins that are present in HDM faeces. These proteins have cysteine protease activity that enables them to cleave CD25 and inhibit α 1-antitrypsin (Chua *et al.*, 1988), and it has been suggested that their enzymatic activity contributes to the pathogenesis of allergic diseases including bronchial asthma (Gough *et al.*, 1999; Kikuchi *et al.*, 2006a; Shakib *et al.*, 2008).

Oral immunotherapy is superior to alternative routes of administration because it allows close control of dosage and frequency of administration and has less impact on the patient than other routes such as the parenteral route. The efficiency with which allergens are delivered to gut-associated lymphoid tissues (GALT) is likely to be crucial for the induction of an appropriate immune response. However, most orally administered allergens are digested by proteolytic enzymes in the gastrointestinal tract, resulting in a loss of immunogenicity.

Several plant seeds contain protein bodies (PBs), which function as sites of protein storage and are resistant to proteolytic digestion (Nochi *et al.*, 2007; Takagi *et al.*, 2010). Therefore, bioencapsulation of allergens into PBs is a promising strategy to increase the immunogenicity of allergens used for oral immunotherapy.

Despite its high therapeutic potential, the mechanisms underlying oral tolerance are not well understood. Immune tolerance can arise from immune deviation of T cells to an anergic or apoptotic state, or from the induction of regulatory T cells (Tr1, Th3, CD4⁺CD25⁺) and suppressive cytokines such as TGF- β and IL-10 (Mayer and Shao, 2004). It is generally assumed that oral tolerance is specific for the orally administered protein. However, the inclusion of an antigen for which oral tolerance has already been established leads to inhibition of responses to other, unrelated antigens that are included in the immunization. This phenomenon is known as 'bystander suppression' (Miller *et al.*, 1991; Chen *et al.*, 1994). The induction of bystander suppression has been demonstrated in multiple allergic diseases, but it has also been shown to enhance susceptibility to infection by inhibiting antibody production. Most studies of bystander suppression have involved oral administration of soluble protein in animal models (Miller *et al.*, 1991; Chen *et al.*, 1994, 1995; Friedman and Weiner, 1994; Carvalho *et al.*, 1997). In the present study, we aimed to determine whether bystander suppression occurs following oral administration of transgenic (Tg) rice seeds expressing unrelated antigens.

Here, we generated Tg rice plants expressing in seeds a fragment (residues 45–145) of Der p 1 that contains the major mouse and human T cell epitopes. Oral administration of the Tg rice seeds to mice prior to systemic challenge with Der p 1 induced oral tolerance, characterized by inhibition of allergen-specific T helper 2 (Th2) cytokine synthesis (IL-4, IL-5, and IL-13) and allergen-specific IgE responses. The induction of oral tolerance was associated with inhibition of bronchial hyper-responsiveness

(BHR) following exposure to Der p 1. On the other hand, the effect of vaccine was antigen-specific because the levels of specific IgE and IgG in mice secondary immunized with unrelated antigens were not affected by oral vaccination with Der p 1 Tg rice. These results demonstrate the ability of Der p 1 Tg rice T cell epitopes to induce oral tolerance without associated bystander suppression.

Results

Expression of Der p 1 (45–145) in transgenic rice seeds

The Der p 1 (45–145) expression cassette (Figure 1a) was introduced into the rice genome by *Agrobacterium*-mediated transformation. The expression of Der p 1 (45–145) in mature grains of 40 independent primary T0 Tg rice plants (at least four grains from each Tg line) was examined by immunoblotting using an anti-Der p 1 antibody. As shown in Figure 1b, the most highly expressing line (line 28) accumulated ~90 μ g of Der p 1 (45–145) per grain (~7.5% of total seed protein). A band of approximately 12 kDa, which corresponds to the molecular weight (12.2 kDa) estimated from the sequence of Der p 1 (45–145) containing KDEL ER retention signal, was clearly detectable in lysates from line 28 by SDS-PAGE with Coomassie Brilliant Blue (CBB) staining. The identity of this band as Der p 1 was confirmed by immunoblotting (Figure 1c, arrow head). The 52nd amino acid residue may be N-glycosylated according to the Der p1 sequence, because the Asn constitutes a consensus Asn-X-Thr/Ser N-glycosylation signal. When the Der p 1 peptide was digested with Endo H, two bands were shifted to lower one (Figure S1), indicating that the Der p 1 was glycosylated.

The copy number of the Der p 1 (45–145) gene integrated in the genome of line 28 was determined by Southern blot. The genomic DNA extracted from leaves of line 28 was digested with *EcoRI* or *HindIII* and hybridized with ³²P-labelled Der p 1 (45–145) probe. As shown in Figure S2, we estimate that at

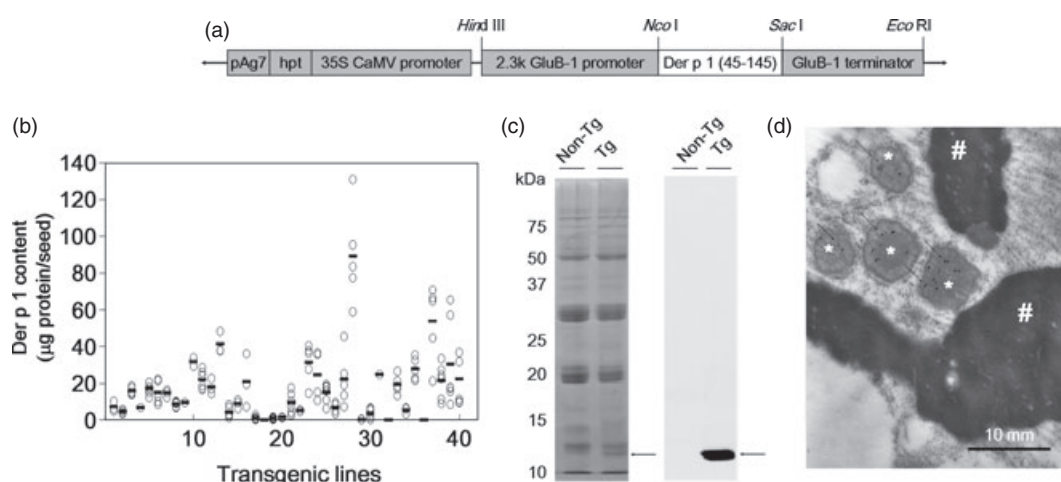


Figure 1 Expression of Der p 1 (45–145) in transgenic (Tg) rice. (a) Schematic representation of the transformation plasmid. Der p 1 (45–145)-coding sequence (white box) was placed under the control of the glutelin *GluB-1* promoter and *GluB-1* terminator in the plant transformation vector pGPTV-35S-HPT. (b) The expression of Der p 1 (45–145) protein in each Tg rice line (four or five seeds from each line) was analysed by immunoblotting. (c) Coomassie Brilliant Blue staining (left panel) and immunoblot analysis (right panel) were performed for the Tg Der p 1 (45–145) rice and non-Tg control rice seeds. Der p 1 (45–145)-specific signals (~12 kDa) are indicated by arrow heads. (d) Localization of Der p 1 (45–145) expressed in endosperm cells of the Tg rice seeds was examined by immunoelectron microscopy. *PB-I; #PB-II; bar = 1 μ m. Existence of Der p 1 (45–145) was indicated as black dots of ~20-nm gold particles.

least two copies of the Der p 1 expression cassette were integrated into line 28.

Intracellular localization of Der p 1 (45–145) in endosperm cells of line 28 was investigated by immunohistochemical electron microscopy with gold-labelled anti-Der p 1 antiserum. In rice endosperm cells, there are two types of PB: PB-I and PB-II (Tanaka *et al.*, 1980; Takaiwa *et al.*, 1999). PB-I is a spherical endoplasmic reticulum-derived and prolamin-containing PB with low electron density (Figure 1d, *). PB-II is an irregular-shaped protein storage vacuole with high electron density, which contains large amount of glutelin and globulin (Figure 1d, #). Der p 1 (45–145) synthesized in endosperm cells of line 28 was specifically deposited as aggregates in PB-I (Figure 1d, arrow). This result was further confirmed by observing that the Der p 1 fragment colocalized with prolamin in this PB (Figure S3).

Inhibition of allergen-specific immune responses by preventive oral vaccination with Tg rice seeds

Immunization of mice with purified rDer p 1 resulted in a significant increase in the serum level of Der p 1-specific IgE (Figure 2a). To investigate whether oral tolerance could be induced with Der p 1 Tg rice, mice were orally vaccinated with the rice daily for 7 days prior to immunization with rDer p 1. Oral administration of Tg but not non-Tg rice significantly inhibited the IgE response to subsequent immunization with rDer p 1 (Figure 2a). Following immunization with rDer p 1, the serum level of allergen-specific IgG, especially IgG₁ and IgG_{2b}, was also

significantly lower in mice that had consumed Tg rice (Figures 2a and 4g).

The suppressive effect of Der p 1 Tg rice on immunoglobulin responses was specific to Der p 1. Thus, the levels of specific IgE and IgG in mice immunized with Der f 2 or ovalbumin (OVA) were not affected by oral vaccination with Der p 1 Tg rice (Figure 2b,c).

Inhibition of allergen-induced airway inflammation by prophylactic oral vaccination with Tg rice seeds

We next investigated the effect of the Der p 1 subunit vaccine on allergen-induced airway inflammation in rDer p 1-immunized mice. Twenty-four hours after the last challenge with rDer p 1, bronchoalveolar lavage (BAL) was performed. A large number of inflammatory cells including eosinophils and neutrophils were recovered in the BAL fluid of rDer p 1-immunized mice following challenge with specific allergen. Prior vaccination with the Tg rice almost completely attenuated the infiltration of eosinophils and neutrophils (Figure 3a).

The Der p 1 (45–145)-mediated suppression of cellular infiltration was antigen-specific, because OVA or Der f 2-induced accumulation of inflammatory cells into the airways was not affected by administration of the Tg rice (Figure 3b,c).

Inhibition of allergen-specific CD4⁺ T cell responses by oral vaccination with Tg rice seeds

rDer p 1-specific *in vitro* proliferation of splenic CD4⁺ T cells was enhanced in mice immunized with rDer p 1. The proliferative

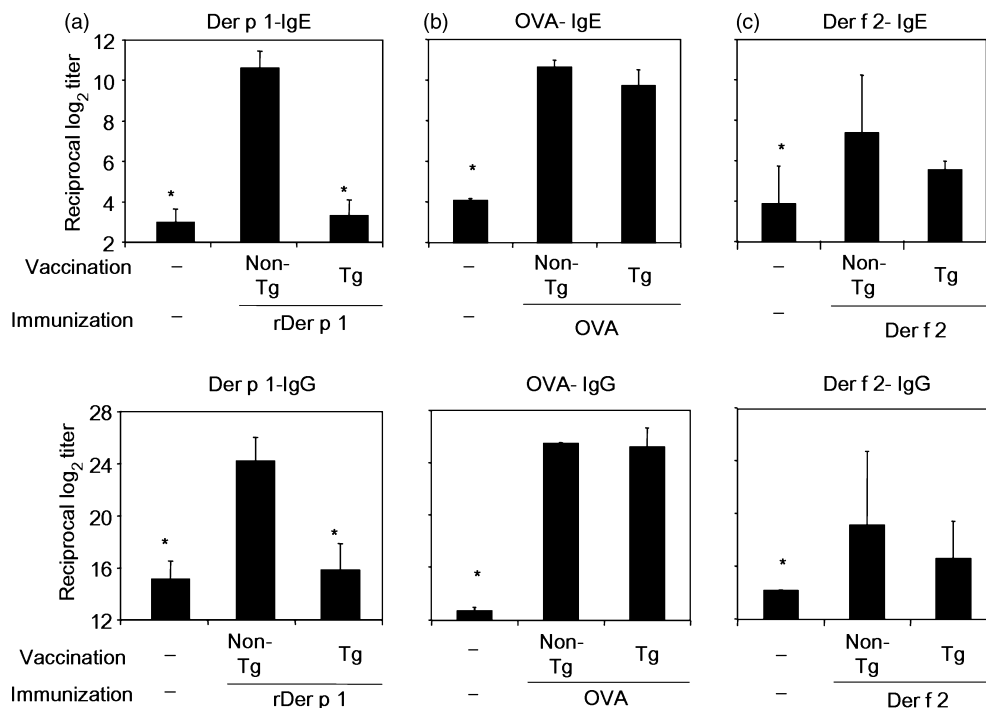


Figure 2 Reduction in Der p 1-specific immunoglobulin levels by prophylactic oral vaccination with Der p 1 transgenic (Tg) rice, with maintenance of OVA- and Der f 2-specific immunoglobulin. BALB/c mice were orally vaccinated by feeding *ad libitum* with a diet containing the Tg rice and a commercial mouse diet mixed in a 1 : 1 ratio. After oral vaccination for 1 week with the Tg rice grains, mice were given four intraperitoneal injections of 2 µg rDer p 1, 20 µg OVA or 10 µg Der f 2, adsorbed to alum on days 8, 15, 22 and 29. Blood was collected from the mice on day 36 and allergen [Der p 1 (a), OVA (b) or Der f 2 (c)]-specific serum IgE and IgG were assayed. Data are presented as the mean ± SD of five or six animals for each group (**P* < 0.05 vs. the non-Tg rice group).

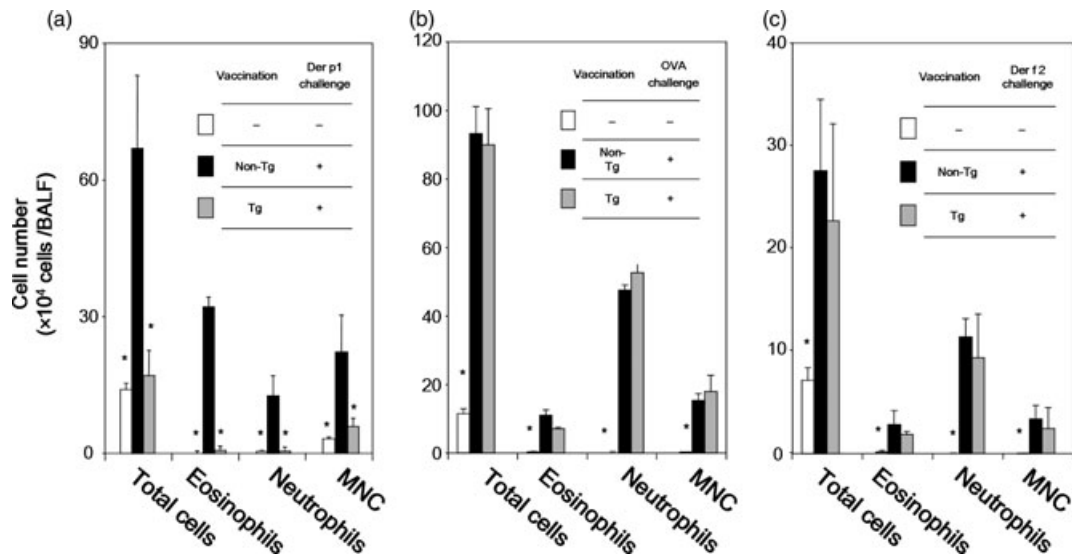


Figure 3 Inhibition of allergen-induced inflammatory cell infiltration by prophylactic oral vaccination with Der p 1 transgenic (Tg) rice. Mice were immunized with rDer p 1 (a), OVA (b) or Der f 2 (c) after oral vaccination and then challenged intranasally with 10 μ g of the corresponding allergen or left unchallenged on days 43 and 45. Twenty-four hours after the final allergen challenge, the total number of cells and the number of eosinophils, neutrophils and mononuclear cells (MNC) in the BAL fluid were measured. Data are presented as the mean $\pm$ SD of five or six animals for each group (* P < 0.05 vs. the control rice group).

response was significantly suppressed by prophylactic oral vaccination with Tg, but not non-Tg rice (Figure 4a). Upon stimulation with rDer p 1 *in vitro*, splenic CD4⁺ T cells from rDer p 1-immu-

nized mice produced significant amounts of the Th2 cytokines IL-4, IL-5 and IL-13, but not the Th1 cytokine IFN- γ (Figure 4b–d). Allergen-induced Th2 cytokine synthesis by T cells was

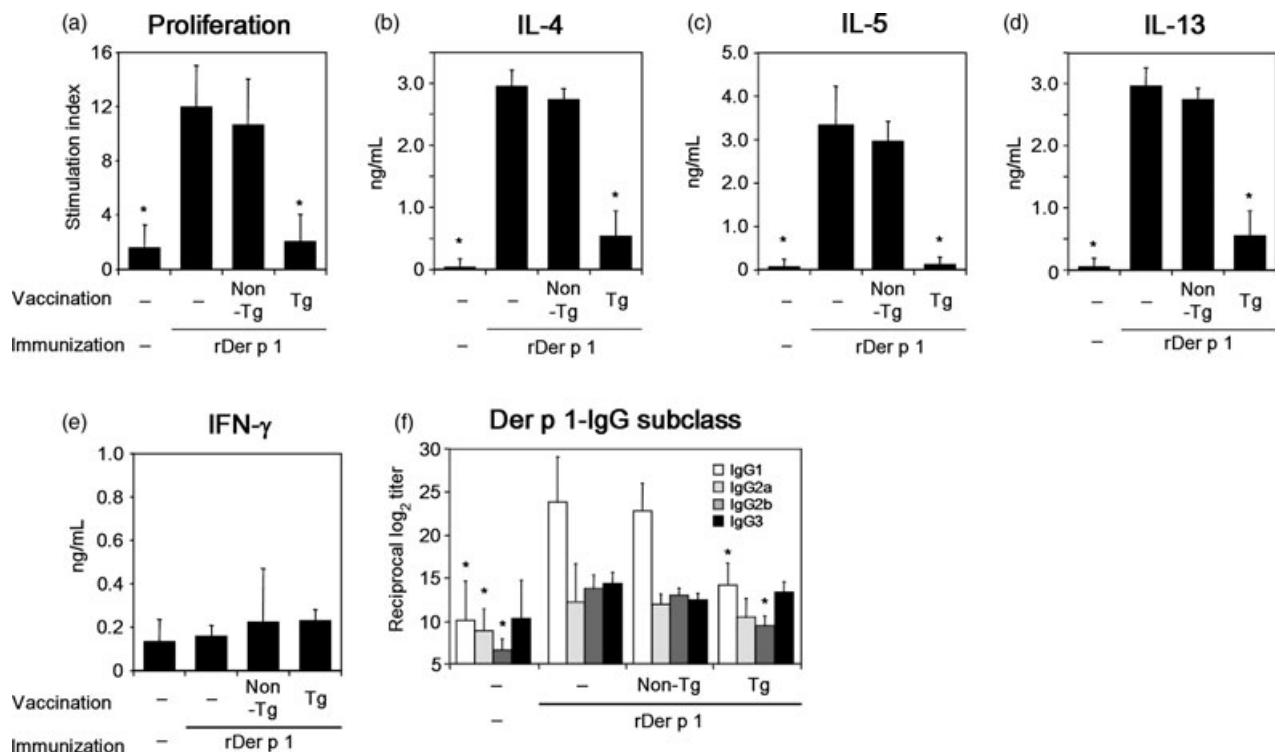


Figure 4 Inhibition of allergen-specific T cell responses by prophylactic oral vaccination with transgenic (Tg) rice. Splenic CD4⁺ T cells prepared from negative control mice, non-Tg rice or Tg-rice-fed mice were cultured with irradiated normal splenocytes and allergen for 7 days, respectively. The proliferative response (a) and the concentration of cytokines (IL-4, IL-5, IL-13 and IFN- γ) released into the culture supernatant (b–e) were assayed. Der p 1-specific subclasses (f) in the serum of mice were measured after immunization with rDer p 1. Data are presented as the mean $\pm$ SD of five or six animals for each group (* P < 0.05 vs. the non-Tg rice group).

significantly diminished by oral vaccination with Tg, but not non-Tg rice (Figure 4b–d). In contrast, Der p 1-specific immune suppression was not induced even by oral administration of about 20-fold higher amounts of the purified rDer p 1 (5 mg) than that of Tg rice (about 225 µg), indicating an advantage of Tg rice over the purified rDer p 1 (Figure S4). It is notable that immunosuppressive cytokines such as IL-10 and TGF-β were not affected by Tg rice-induced oral tolerance, when their levels analysed in the splenic T cell culture assay were compared between Tg and non-Tg rice-fed mice (Figure S5). Furthermore, the profile of CD4⁺CD25⁺Foxp3⁺ regulatory T cells in spleen was not changed even after the induction of immune suppression by oral vaccination with Tg rice, when compared with naive and control mice inoculated with non-Tg rice (Figure S6). These results suggest that IL-10 and TGF-β producing regulatory T cells were not essential for induction of oral tolerance in the prophylactic treatment.

Histological analysis of the inhibition of allergen-induced airway inflammation

Haematoxylin and eosin (HE)-stained lung specimens from mice that were immunized and challenged with Der p 1 exhibited extensive infiltration of inflammatory cells including eosinophils, oedematous changes in the airway submucosa and epithelium, and derangement of the epithelial lining (Figure 5b,e). These changes were not observed in untreated control mice (Figure 5a,d). Goblet cell hyperplasia was observed in the lungs of allergen-challenged mice based on periodic acid-Schiff (PAS) staining (Figure 5g,h). These pathological features, which closely resemble those observed in human asthma, were significantly reduced by oral vaccination with Tg rice (Figure 5c,f,i). Thus, cellular infiltration, oedema and goblet cell hyperplasia in the lungs of mice vaccinated with the Tg rice (Figure 5c,f,i) were less extensive than that in challenged mice that received non-Tg rice (Figure 5b,e,h).

Improvement of BHR by oral vaccination with Tg rice

The effect of the Tg rice on allergen-induced BHR in rDer p 1-immunized mice was also evaluated. Twenty-four hours after the final allergen challenge, a marked enhancement in bronchial responsiveness to inhaled methacholine (MCh) was observed in rDer p 1-immunized and -challenged mice by measuring R_L under anaesthesia and artificial ventilation (Figure 6). Oral vaccination with the Tg rice significantly inhibited the development of allergen-induced BHR (Figure 6).

Discussion

Seed-based oral vaccines have great advantages for immunotherapy (Streatfield, 2006; Hiroi and Takaiwa, 2006; Takaiwa, 2007a). They do not require special techniques or instruments such as needle and syringe for their administration. They are also safer than vaccines produced by fermentation systems using animal cells or microorganisms because there is little chance of contamination with potentially harmful agents such as viruses, prions or endotoxins when the antigen is produced in plants. Furthermore, storage and transport of these vaccines is economical and straightforward because antigens that accumulate in seeds are stable for 2–3 years at room temperature. The production scale of seed-based oral vaccines is also easy to control by changing the acreage depending on demand rather than the escalation of new plant, because large scale production of antigens (tolerogens) is required in the case of pollinosis or HDM allergy. All of these factors indicate that the production and use of plant-based vaccines is highly cost-effective.

Most Der p 1-dominant epitopes for both human and murine T cells are located within the sequence from amino acids 45–145 of the mature Der p 1 protein (Yssel *et al.*, 1992; O'Brien *et al.*, 1994). Therefore, we selected this region for oral

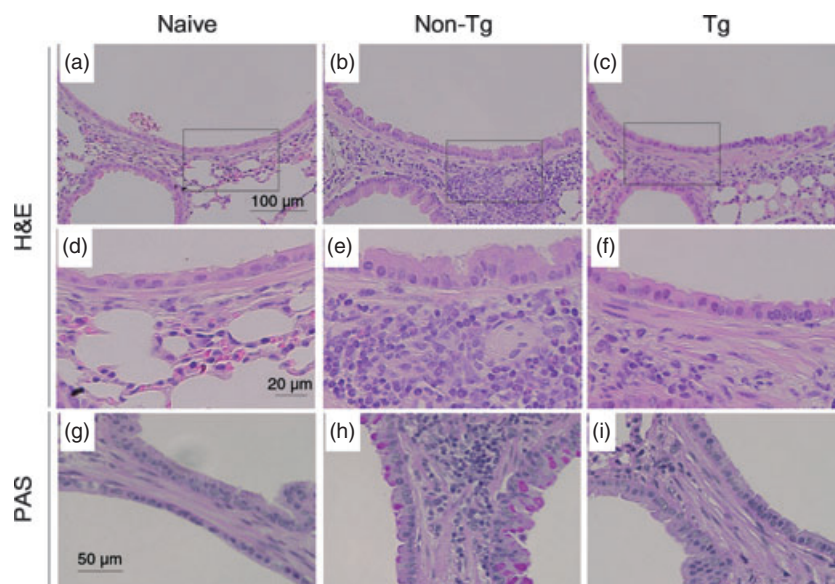


Figure 5 Inhibition of allergen-induced inflammatory cell infiltration in the lung by oral vaccination with Der p 1 transgenic (Tg) rice. Lung specimens from unchallenged mice (a, d and g), control non-Tg rice-fed and rDer p 1-challenged mice (b, e and h) and Tg rice-fed and rDer p 1-challenged mice (c, f and i) were collected 24 h after the final allergen challenge and stained with HE (a–f) or PAS (g–i) (goblet cell hyperplasia was indicated by arrows). High power magnifications of the regions surrounded by the squares in panels a, b and c are presented in panels d, e and f, respectively. The results shown are representative of at least three or four animals for each group.

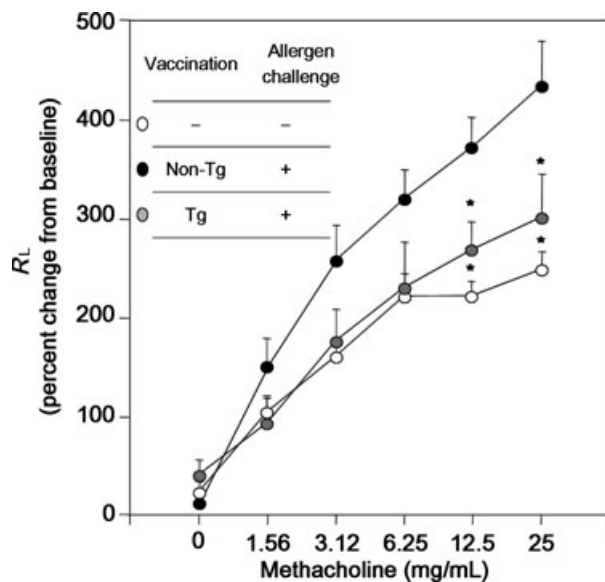


Figure 6 Prophylactic oral vaccination with transgenic rice alleviated bronchial hyper-responsiveness (BHR). BHR to aerosolized methacholine was measured 24 h after the second allergen challenge. Data are presented as the mean $\pm$ SD of eight to ten animals for each group (* $P < 0.05$ vs. the control rice group).

vaccination against allergen-induced airway inflammation. It should be noted that the Der p 1 (45–145) fragment, in which the tertiary structure of full-length Der p 1 is disrupted by loss of disulfide bonds, cannot cross-link Der p 1-reactive IgE. In addition, the Der p 1 (45–145) fragment is predicted to lack cysteine protease activity because of the absence of the N-terminal region, which contains the Cys34 residue that is indispensable for Der p 1 enzymatic activity (Chua *et al.*, 1988).

The hypoallergenic Der p 1 (45–145) was specifically localized within ER-derived PB-Is that are resistant to proteolytic digestion by proteases in the gastrointestinal tract (Figure 1d). Der p 1-specific immune responses such as antigen-specific IgE production and CD4⁺ T cell proliferation were not induced even by oral administration of higher amounts of purified rDer p 1 (Figure S4). Recent studies using recombinant proteins from Japanese cedar pollen allergen indicate that the dose of T cell epitope required for suppression of allergen-specific IgE in mice was about 20-fold lower when administered in the form of PB-I compared to purified peptide (Takagi *et al.*, 2010). Therefore, in addition to being a safe vaccine for allergic disease caused by Der p 1, Tg rice may also be very effective for the induction of mucosal immunity.

As shown in Figure 4, the Der p 1 (45–145) fragment was able to prevent IgG and IgE responses, the production of Th2 cytokines (IL-4, IL-5 and IL-13) and the development of allergic airway inflammation resulting from immunization and challenge with Der p 1 (Figure 4). Based on previous studies demonstrating the requirement of IL-4 to IgE synthesis (Geha *et al.*, 2003), the inhibition of IgE production by prophylactic vaccination was likely to result from the reduction in IL-4 production.

To investigate whether plant-based oral immunotherapy prevents allergic airway inflammation, the effect of Tg rice on allergen-induced infiltration of inflammatory cells into the airways and BHR was examined. The infiltration of inflammatory cells, especially eosinophils and neutrophils, into the airways (Figures 3 and 5) and the induction of BHR (Figure 6) were

also significantly reduced by oral vaccination with the Tg rice. Therefore, our plant-based oral immunotherapy strategy may be a promising new approach for the treatment of bronchial asthma.

The ability of Der p 1 (45–145) to suppress immune responses was specific for Der p 1. Serum IgE and IgG responses and airway inflammatory cell infiltration induced by the unrelated antigens Der f 2 and OVA were not affected by administration of the Tg rice seed containing Der p 1 (Figures 2 and 3). Furthermore, specific IgE and IgG responses in mice immunized with another major HDM allergen were not suppressed by oral vaccination with the Tg rice expressing Der p 1 (data not shown). Collectively, these findings indicate that the Der p 1 Tg rice oral vaccine does not induce bystander immune suppression.

Bystander immune suppression is thought to be because of regulatory T cells. Transforming growth factor-beta (TGF- β)-secreting Th3 cells have been described during the induction of oral tolerance and are thought to be triggered by specific antigen but to act in an antigen-nonspecific fashion after they encounter antigen (Weiner, 2001; Faria and Weiner, 2006). Another subtype of regulatory T cell is characterized as CD4⁺ CD25⁺ Foxp3⁺ (Nagatani *et al.*, 2004) and is thought to act by suppressing naïve bystander T cells and by preventing antigen-presenting cells from priming naïve T cells (Tang and Bluestone, 2008). However, the levels of suppressive cytokines IL-10 and TGF- β were not increased in orally tolerized mice fed with Tg rice when compared with mice fed on non-Tg rice or mice that were not treated. Furthermore, there was little difference in the profile of CD4⁺ CD25⁺ Foxp3⁺ regulatory T cells between mice fed with Tg and non-Tg rice. These findings may be related to the absence of bystander immune suppression in mice observed with nonspecific OVA treatment or Der f 2. The cellular mechanisms for bystander suppression and the inhibition of naïve T cells specific for the bystander antigen remain unclear (Tang and Bluestone, 2008). In this study, an antigen expressed in PB-I of Tg rice was used for the induction of antigen-specific immune suppression. The pathway by which this antigen in PB-I was digested, taken up by antigen-presenting cells and presented to naïve T cells is not known. In future studies, it will be interesting to investigate whether differences in the shape or conformation of antigens in solution influence the induction of Treg cells by comparing soluble antigen and antigen expressed in PB-I of Tg rice.

The amount of Der p 1 (45–145) peptide expressed in the Tg rice seeds was about 4.5 μ g/mg seed. Mice were fed $\sim$ 1.5 g rice grains/day, which equates to approximately 50 mg Der p 1 (45–145) over 7 days ($\sim$ 6.8 mg per day). Based on a mouse weight of 30 g, extrapolating these data indicates that an effective dose for induction of immunological suppression in a 60-kg human would be approximately 3 kg of Der p 1 rice seeds per day, containing $\sim$ 14 g Der p 1 (45–145). However, the minimal amount of rice for induction of tolerance in mice or humans still remains to be established.

In conclusion, we have demonstrated the prophylactic efficacy of oral vaccination with Tg rice seeds accumulating Der p 1 (45–145) in a mouse model of asthma, with a reduction in allergic airway inflammation and reduced BHR. Immunotherapy using this PB-I-based allergen delivery system may be applicable to bronchial asthma in humans. Taken together with our previous demonstration of the prophylactic efficacy of a rice seed-based oral vaccine for Japanese cedar pollen allergy, our

results indicate that this unique approach to immunotherapy has considerable potential for the prevention and treatment of chronic allergic diseases.

Experimental procedures

Plasmid construction and rice transformation

The dominant human and murine T cell epitopes in Der p 1 are mostly located within amino acids 45–145 of the mature Der p 1 protein (Yssel *et al.*, 1992; O'Brien *et al.*, 1994). Therefore, this region was selected for oral vaccination against allergen-induced airway inflammation. A DNA fragment coding for Der p 1 (45–145) was synthesized using optimized codons preferentially used in genes encoding rice seed storage proteins (Takaiwa, 2007b). The signal peptide of glutelin, a major rice seed storage protein, and the ER retention signal (tetrapeptide KDEL) were attached to the N- and C-terminus of the Der p 1 (45–145) fragment, respectively, to enhance its accumulation level in the seed (Takagi *et al.*, 2005a,b). To direct expression to the edible endosperm tissue of rice seed, 2.3 kb of the glutelin *GluB-1* promoter (Qu and Takaiwa, 2004) was linked to the Der p 1 fragment. The expression cassette was inserted into the binary vector pPGTV-35S-HPT (Goto *et al.*, 1999) (Figure 1a). The resulting expression plasmid was introduced into the genome of *Oryza sativa* L. cv. Kitaake by *Agrobacterium*-mediated transformation, as described previously (Goto *et al.*, 1999).

Detection of Der p 1 (45–145) expression in Tg rice

Total mature seed proteins from Der p 1 (45–145) Tg rice or non-Tg rice were analysed by SDS-PAGE and immunoblotting as described previously (Yang *et al.*, 2008). The amount of Der p 1 (45–145) was calculated by comparing the intensity of the immunoblot band with a standard Der p 1 (45–145) synthesized in and purified from *E. coli*. Detection of *N*-glycosylation was carried out using endoglycosidase H (Endo H) as described previously (Yang *et al.*, 2008).

Southern blot analysis

Southern blot analysis was performed as described previously (Takagi *et al.*, 2005a). Integration of the Der p 1 (45–145) fragment into the rice genome was detected with a ³²P-labelled Der p 1 (45–145) gene probe and genomic DNA blotted on to a nylon membrane.

Immunohistochemical electron microscopy

Electron microscopy of Der p 1 (45–145) Tg rice seed was performed as described previously (Takaiwa *et al.*, 2009).

Oral vaccination of mice with Tg rice or purified recombinant Der P1 dissolved in PBS

All experimental animal protocols were performed in accordance with guidelines approved by the animal use committee at the Tokyo Metropolitan Institute of Medical Science. Six- to eight-week-old female BALB/c mice (Japan Clea, Tokyo, Japan) were orally vaccinated by feeding *ad libitum* with a diet containing the Tg rice and a commercial mouse diet mixed in a 1 : 1 ratio, as described previously (Takagi *et al.*, 2005a). For the experiment of recombinant antigen, 6–8 week-old female BALB/c mice were orally vaccinated by feeding 0.5 or 5 mg purified recombinant Der P1 (Indoor Biotechnologies Inc., Charlottesville, VA) dissolved in PBS on day 1. Mice were given

four intraperitoneal injections of 2 µg of recombinant Der p 1 (rDer p 1) (Takai *et al.*, 2005, 2006) adsorbed to alum (Imject Alum; Pierce, Rockford, IL) on days 8, 15, 22 and 29, after oral vaccination for 1 week with the Tg rice grains. The mice were fed with ~1.5 g of fine powder of the rice seeds containing approximately 6.8 mg of Der p 1 (45–145) peptide per day for 7 days. Blood was collected from the mice on day 36, and allergen-specific serum IgE and IgG were assayed (Takai *et al.*, 2009). On days 43 and 45, rDer p 1-immunized mice were lightly anesthetized with pentobarbital (62.5 mg/kg, intraperitoneally) and challenged by intranasal injection of 20 µL of 0.5 mg/mL rDer p 1.

Preparation of whole recombinant Der p 1

Wild-type Der p 1 is hyper-glycosylated during translation in yeast (Jacquet *et al.*, 2002). To avoid confounding immune responses against yeast-derived glycosylated moieties, we prepared a less-glycosylated form of rDer p 1 in which the 52nd asparagine residue was replaced with glutamine (Takai *et al.*, 2006). Before being used for immunization, rDer p 1 was activated by regenerating its cysteine protease activity with L-cysteine as described previously (Takai *et al.*, 2005).

Detection of allergen-specific IgE, IgG and IgG isotypes

The Der p 1-induced antibody response was quantified by ELISA using HRP-conjugated monoclonal anti-mouse IgE (clone LO-ME-2; Serotech, Oxford, UK) and goat anti-mouse IgG, IgG₁, IgG_{2a}, IgG_{2b} and IgG₃ (Southern Biotechnology Associates, Birmingham, AL) antibodies for detection, as described previously (Takagi *et al.*, 2005a).

Allergen-induced T cell responses *in vitro*

CD4⁺ T cells were prepared from spleens of immunized mice on days 36 and 46 by positive selection with anti-mouse CD4 antibody-conjugated magnetic beads (Miltenyi Biotec, Bergisch-Gladbach, Germany). The purity of the resulting CD4⁺ T cells was >95% as determined by flow cytometry. For induction of T cell proliferation, 1 × 10⁵ CD4⁺ T cells were incubated for 7 days with 5 × 10⁵ irradiated spleen cells from normal mice together with 10 µg/mL rDer p 1. Cell proliferation was assayed using the Cell Titer 96[™] Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI). Data are presented as stimulation indexes calculated as the OD 490 nm ratio of [(wells cultured with allergen)–(background wells containing medium alone)]/[(wells cultured without allergen)–(background wells containing medium alone)]. The concentration of IL-4, IL-5, IL-10, IL-13, TGF-β and IFN-γ in the culture supernatants was assayed using Quantikine ELISA kits (R&D systems, Minneapolis, MN).

Analysis by flow cytometry

Mononuclear cells were isolated from spleen and were stained with the FITC-CD25 and PE-CD4 mAbs (BD Biosciences, Franklin Lakes, NJ) in PBS containing 5 mM EDTA, 10% FBS and 5 µg/mL Fc block (BD Biosciences) (Michell *et al.*, 2009). Dead cells positive for 7-aminoactinomycin (7-AAD; BD Biosciences) staining and auto-fluorescent cells were excluded using the FL3 channel. For intracellular FoxP3 expression, cells were stained as mentioned earlier, then permeabilized and fixed using the eBioscience Fixation/Permeabilization kit (eBioscience, San Diego, CA) and stained with allophycocyanin (APC)-conjugated

anti-FoxP3 (FJK-16a) (Michell *et al.*, 2009). Flow cytometric analysis and cell separation were performed using FACS Cant™ II (BD Biosciences).

Analysis of allergen-induced airway inflammation

Twenty-four hours after the last allergen challenge, airway inflammation was evaluated by measuring inflammatory cells in BAL fluid, BHR and histopathological changes in the lungs as described previously (Nakata *et al.*, 2001) with modifications. Lung sections were stained with HE or PAS.

Nonspecific airway responsiveness was assessed by restrained whole-body plethysmography (Buxco Electronics, Wilmington, NC) according to procedures recommended by the manufacturer. Briefly, mice were anesthetized with intraperitoneal injection of 5 mg/kg pentobarbital, 0.2 mg/kg xylazine and 0.02 mg/kg pancuronium bromide and tracheally-cannulated and ventilated (175 breaths/min, tidal volume: 6 mL/kg). Lung resistance (R_L) was measured after exposing animals to aerosolized PBS containing increasing concentrations of Mch (1.56–25 mg/mL).

Statistical analysis

Results are expressed as arithmetic means $\pm$ standard deviation (SD). Statistical analysis was performed by one-way analysis of variance and Dunnett's method. $P < 0.05$ was considered to be statistically significant.

Acknowledgements

We thank Takeshi Kato (Juntendo University) for technical assistance in the preparation of rDer p 1. This work was supported by the research grants 'Functional analysis of genes relevant to agriculturally important traits in the rice genome IP2001', 'Genomic for Agriculture Innovation GMC009' and 'Agri-health Translational Research Project' from the Ministry of Agriculture, Forestry and Fisheries of Japan, to F. Takaiwa and T. Hiroi, and a Grant-in-Aid for SPSR from MECST 2008-12 in Japan the Ministry of Education, Science, Sports, and Culture of Japan, to T. Hiroi.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 Analysis of Der P1 (45–145) glycoprotein derived from rice seed.

Figure S2 Southern blot analysis of Der p 1 (45–145) was performed in *EcoRI* (E)- and *HindIII* (H)-digested genomic DNA of the non-transgenic control and transgenic rice seeds.

Figure S3 The localization of 13 kD prolamin in the Tg rice seeds was determined by immunohistochemical electron microscopy.

Figure S4 Potential of antigen-specific immune suppression by fed with purified rDer P1 dissolved in PBS.

Figure S5 The production of suppressive cytokine TGF- β and IL-10.

Figure S6 The frequency of CD4⁺CD25⁺Foxp3⁺ regulatory T cells.

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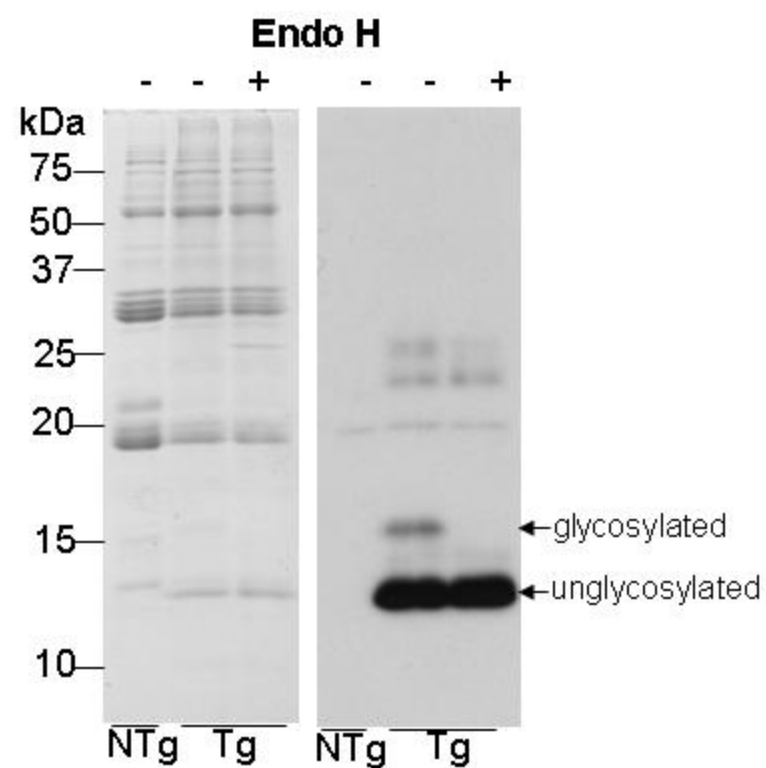
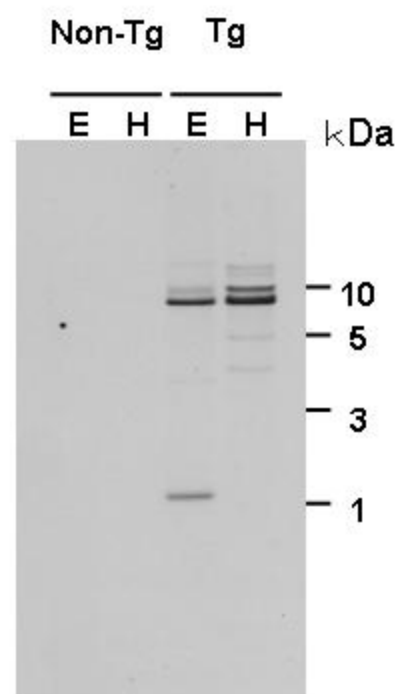


Figure S2

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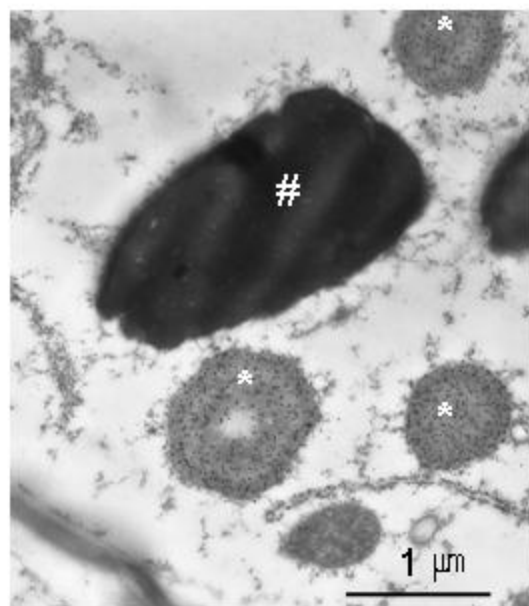


Figure S 4

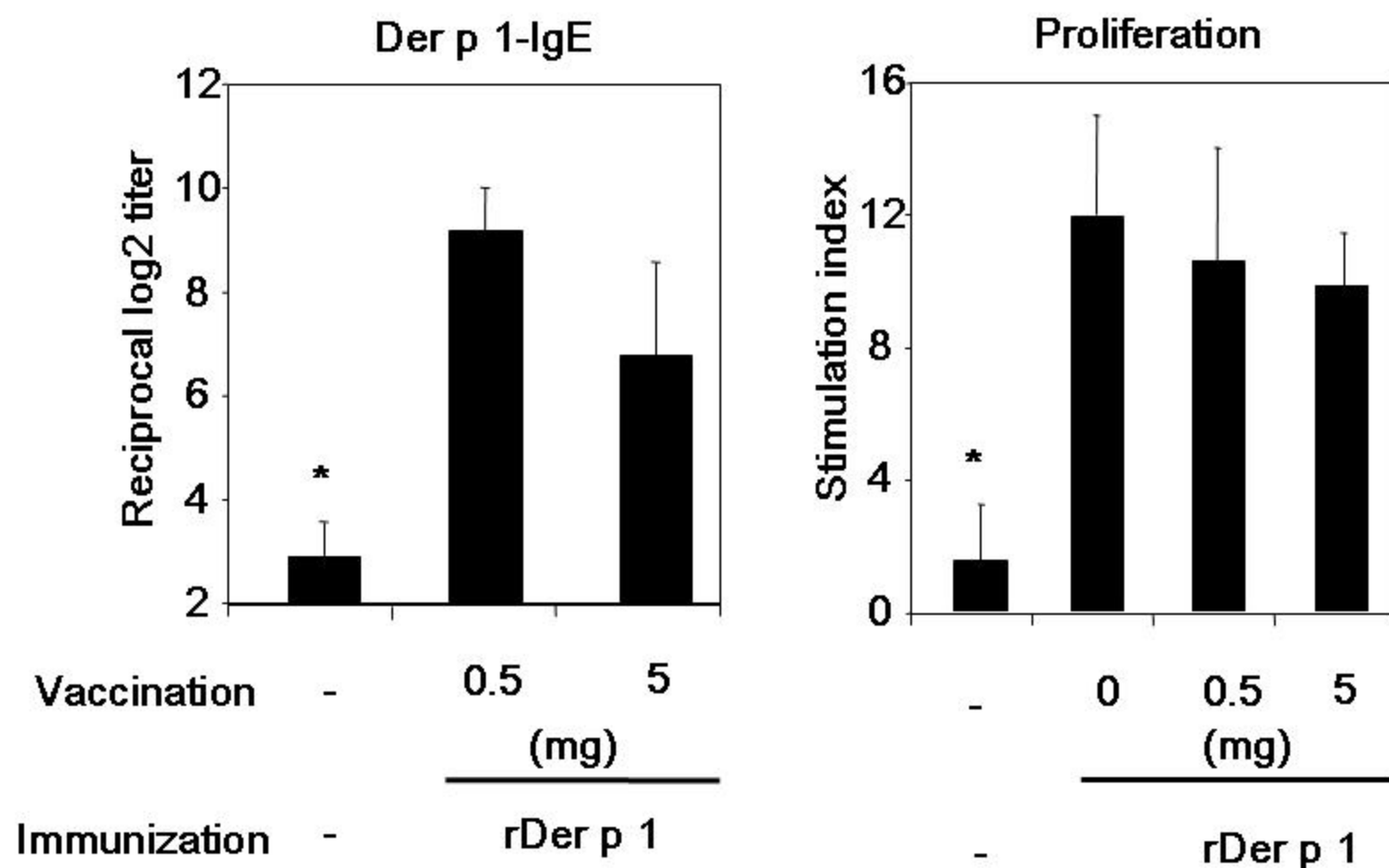


Figure S5

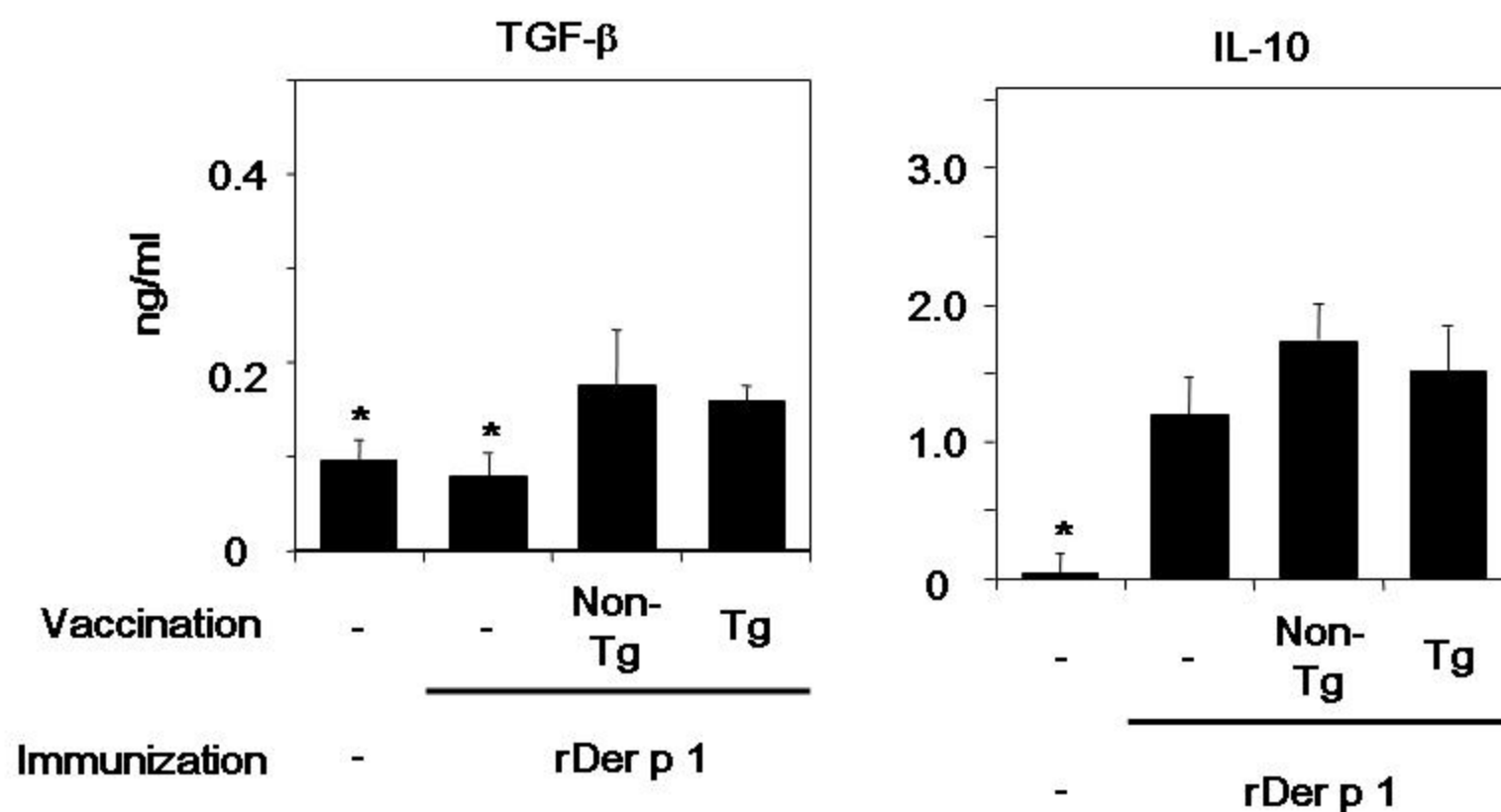


Figure 6

