

Mucosal delivery of allergen peptides expressed by *Lactococcus lactis* inhibit allergic responses in a BALB/c mouse model

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Abstract Allergen-specific immunotherapy (SIT) is considered to be the only curative treatment of allergy, but its safety is always affected by immunologic properties and quality of allergen. Recombinant allergen derivative could be a potential therapeutic strategy, but clinical studies showed that macromolecular derivatives could not avoid T cell-mediated side effects. In this study, five Der p2-derived peptides (DPs) containing major T cell epitopes of Der p2 were first artificially synthesized. Compared with Der p2 macromolecular derivative DM, these DPs not only fully eliminated IgE-binding capacity but also reduced T cells reactivity, suggesting these DPs could be better therapeutic molecules. For their application in vivo, *Lactococcus lactis* was engineered to express these DPs, and their protective effects were evaluated in BALB/c mice models. Western blot showed that all DPs could be produced in the recombinant strains. Mucosal delivery of these strains could inhibit Der p2-induced allergic responses

in Der p2-sensitized mice, characterized by a reduction in specific IgE antibody and lung inflammatory responses. These protective effects were associated with an increase of specific IgG2a in serum and regulatory T cells in the mesenteric lymph nodes. On the whole, the suppressive effect induced by the DP mixture could be better than single DP, but a bit weaker than DM. These DPs could be promising candidate molecules for active vaccination and induction of tolerance, and thus promote the development of non-allergenic peptide in the treatment and prevention of allergy.

Keywords Allergen peptide · Allergy · Lactic acid bacteria · T cell epitopes

Introduction

Across the world, allergic asthma is becoming a public health problem that affects more than 300 million people (Gregory and Lloyd 2011). Among numerous allergens, house dust mite (HDM) is an important source of allergens responsible for the majority of allergic sensitization. One epidemiological study showed that approximately 70~80 % of asthmatic patients are allergic to HDM (Nelson et al. 1996). Allergen avoidance and pharmacotherapy form part of the primary treatment to manage allergic asthma, but changing the course of allergic diseases has proven to be difficult (Eifan et al. 2013). Only allergen-specific immunotherapy (SIT) can change this course and may lead to long-term improvement, which is sustained for years after the discontinuation of treatment (Niederberger 2009). However, traditional SIT is always associated with a number of problems, such as effectiveness, side effects, and renewed sensitization (Valenta and Niederberger 2007), which are primarily caused by the poor quality of allergen. To better advance the treatment and prevention of allergic diseases,

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some effective strategies to improve the safety of traditional SIT need to be sought.

Over the past decades, the introduction of molecular cloning technologies has allowed the cloning of a single allergen that can be produced as a defined molecule in unlimited amount from the corresponding DNA template (Valenta and Niederberger 2007). However, the inherent characteristics of wild allergen can lead to unavoidable cross-reaction with specific IgE of allergic patients, and thus trigger unacceptable side effects (Vrtala 2008). To solve this problem, some strategies have been performed to reduce IgE reactivity of allergen, while preserving certain advantageous immunologic properties (Gafvelin et al. 2007). It has been argued that the boundary of hypoallergenic derivative should be chosen to disrupt the three-dimensional (3D) structure of allergen and conformational IgE epitopes (Chen et al. 2012; Chen et al. 2008; Wang et al. 2010). Curin et al. (Curin et al. 2014) demonstrated that recombinant allergen derivative of Fel d1 (a major allergen of cat) with a disrupted 3D structure was suitable for vaccination and induction of tolerance in patients allergic to cats. However, the clinical trials performed with similar macromolecular derivatives showed that IgE-mediated side effects could be significantly reduced, but activation of T cell-mediated side effects could still be found (Purohit et al. 2008). A growing body of evidence indicates that immunotherapy with allergen T cell epitopes could be a promising treatment strategy with minimal side effects (Chen et al. 2012; Gupta et al. 2014). To date, allergen fragments used in previous studies were always obtained by chemical synthesis (Chen et al. 2012; Rupa and Mine 2012). and complex production process may affect their clinical application in future treatment and prevention of allergic diseases.

Lactic acid bacteria (LAB), an important member of the gut flora, has become the focus of some researches due to their prevalent use in traditional daily products and as probiotics (Gaggia et al. 2010). There has been increasing interests in the application of LAB as a mucosal vector to express functional molecules (Daniel et al. 2011; de Vos 2011). Recombinant LAB strains have proven to play beneficial roles in the treatment or prevention of some diseases, such as allergies (Ai et al. 2014; Daniel et al. 2006; Rigaux et al. 2009), pathogenic infections (Chen et al. 2011; Koo et al. 2012), inflammatory bowel disease (Motta et al. 2012), tumors (Ribelles et al. 2013), and other diseases (Mandal et al. 2014). The intrinsic advantages of LAB could not only achieve constitutive expression of protein on mucosal surface but also beneficially stimulate body's immune response. This novel strategy would become a competitive candidate for the treatment and prevention of some diseases, especially chronic diseases.

Der p2, one of the most potent and common HDM allergens, can cause allergic response in most patients allergic to HDM (Thomas et al. 2002). Der p2-derived fragments can induce specific IgG antibodies response upon immunization

that block allergic patients' IgE recognition and allergen-induced basophil degranulation equally well as antibodies against Der p2 (Chen et al. 2012). Our previous study demonstrated that Der p2 macromolecular derivative DM could inhibit Der p2-induced allergic responses in mice models (Ai et al. 2015). In this study, five Der p2 peptides (DPs) containing major T cell epitopes of Der p2 were produced to reduce IgE-binding capacity and T cell response. For their application in vivo, *Lactococcus lactis* was engineered to express these DPs, and their protective effects and mechanisms involved were evaluated in mice models.

Materials and methods

Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *L. lactis* was grown in M17 medium supplemented with 0.5 % (w/v) glucose at 30 °C without shaking, and *Escherichia coli* was cultured in Luria Broth at 37 °C. When required, the antibiotics and inducers were used at the following concentrations: kanamycin (50 µg/ml) and isopropyl-β-D-thiogalactoside (IPTG; 0.1 mM) for *E. coli* and chloramphenicol (10 µg/ml) and nisin (10 ng/ml) for *L. lactis*.

Preparation of the DPs

Five DPs containing major T cell epitopes were synthesized by Shanghai Apeptide Co., Ltd. These peptides were purified by preparative high-performance liquid chromatography (HPLC), and their identities were confirmed by mass spectrometry (MS).

Analysis of the IgE reactivity to DPs

The IgE reactivity of synthetic DPs were evaluated using the sera from 27 HDM-allergic patients as described previously (Chen et al. 2008) with minor modifications. A solution of single DP (0.1 mg/ml) was sprayed onto a nitrocellulose membrane using a Bio-Dot airbrush equipment. For control purposes, the same concentrations of BSA (Sigma, USA) and purified Der p2 were respectively used as positive and negative controls, respectively. After drying at 37 °C overnight, the nitrocellulose strips were blocked with confining liquid and incubated with a 1:1 dilution of the sera from HDM-allergic patients, sera from a non-allergic person, or confining liquid. After incubation, the bound IgE antibodies were detected using alkaline phosphatase-labeled anti-human IgE antibodies and visualized via the addition of an alkaline phosphatase substrate.

Table 1 Bacterial strains and plasmids used in this study

Strains/plasmids	Characteristics	Reference
Strains		
<i>Escherichia coli</i> BL21D	Recombinant Der p2-expressing <i>E. coli</i> BL21(DE3)	Ai et al. (2014)
<i>Lactococcus lactis</i> NZ9000	<i>L. lactis</i> strain derived from <i>L. lactis</i> MG1363	Ai et al. (2014)
<i>L. lactis</i> 8148	<i>L. lactis</i> NZ9000 containing plasmid pNZ8148	Ai et al. (2014)
LP1	<i>L. lactis</i> NZ9000 containing plasmid P1	This study
LP2	<i>L. lactis</i> NZ9000 containing plasmid P2	This study
LP3	<i>L. lactis</i> NZ9000 containing plasmid P3	This study
LP4	<i>L. lactis</i> NZ9000 containing plasmid P4	This study
LP5	<i>L. lactis</i> NZ9000 containing plasmid P5	This study
LL-DM	<i>L. lactis</i> NZ9000 containing plasmid pNZ8148-DM	Ai et al. (2015)
Plasmids		
pUC57D	Amp ^r ; plasmid pUC57 carrying Der p2 gene	Ai et al. (2014)
pNZ8148	Cm ^r ; expression plasmid; <i>nisA</i> promoter	Ai et al. (2015)
pNZ8148-DM	pNZ8148 carrying SPusp45::Der p2 derivative DM	Ai et al. (2015)
P1	pNZ8148 carrying SPusp45::Der p2 peptide 1	This study
P2	pNZ8148 carrying SPusp45::Der p2 peptide 2	This study
P3	pNZ8148 carrying SPusp45::Der p2 peptide 3	This study
P4	pNZ8148 carrying SPusp45::Der p2 peptide 4	This study
P5	pNZ8148 carrying SPusp45::Der p2 peptide 5	This study

Immune characteristics of DPs in vitro

The stimulation of spleen cells by synthesized DPs was performed as described by Ren et al. (2014). Briefly, spleen was isolated from Der p2-sensitized mice (positive mice in Fig. 1), and a single cell suspension was obtained by mechanical disruption. After red blood cell lysis, spleen cells were cultured in RPMI1640 medium and stimulated with single DP or a mixture of DPs (10 µg/well) in 96-well plates (Corning, USA) at 37 °C. After incubation for 72 h, the supernatants were respectively collected and stored at −80 °C until further analysis. The levels of interleukin (IL)-4, IL-10, interferon-γ (IFN-γ), and IL-12 in the supernatants were measured by ELISA.

Construction of recombinant *L. lactis* strains

Based on Der p2-coding sequence (KJ933442.1), five specific fragments expressing DPs (Table 2) were amplified by PCR using the respective primers (Table S1). The resulting

fragments digested by *KpnI* and *XbaI* were cloned into the *KpnI*-*XbaI*-digested pNZ8148-DM plasmid containing signal peptide gene *Usp45*. The resulting plasmids (P1, P2, P3, P4, and P5) were respectively introduced into *L. lactis* NZ9000 by electroporation, yielding corresponding recombinant strains (Table 1).

Expression and identification of DPs in recombinant strains

For induction of the DPs, recombinant strains were grown to an OD₆₀₀ of 0.5, and then incubated with 10 ng/ml of nisin (Sigma, USA). After incubation of 6 h, the supernatants were collected by centrifugation and treated as previous description (Nandakumar et al. 2003). The supernatants were separated by Tricine-SDS-PAGE and transferred to a PVDF membrane (Millipore, USA) by electroblotting. The bounded peptides were detected using rabbit polyclonal anti-Der p2 antibodies followed by a HRP-conjugated goat anti-rabbit antibody and visualized by addition of a HRP substrate.



Fig. 1 The establishment of mice models. The BALB/c mice were daily receiving PBS, recombinant or control strain via the oral route for 4 weeks. After the last immunization, mice were sensitized by injection

of a mixture of Der p2/Alum or sterile PBS for 3 weeks at weekly intervals. To induce airway inflammation, all mice were challenged by exposure to HDM allergen over a 45-min period

Table 2 The sequence of synthetic DPs

Peptide	Sequence	Location in Der p2
P1	QVDVKDCANHEIKKVLVPGCHGSEP	AA: 2–25
P2	CIIHRGKPFQLEAVFEANQNTKTA	AA: 28–50
P3	KIEIKASIDGLEVDVPGIDPNACH	AA: 52–73
P4	HYMKCPLVKGQQYDIKYTWNVPKIAPKSE	AA: 75–102
P5	NVVVTVKVMGDDGVLACAIATHAKIRD	AA: 104–129

Bacteria suspensions used in mice models

The recombinant and control strains used in mice were cultured and induced as mentioned above. Subsequently, the bacterial cells were collected by centrifugation and resuspended in sterile phosphate-buffered saline (PBS) to a final concentration of 1×10^{10} colony forming units (cfu)/ml.

Mice

Female BALB/c mice (SPF, 4 weeks old) were purchased from Shanghai SLAC Laboratory Animals Co., Ltd. (China), and housed at the Laboratory Animals Center of Jiangnan University in a barrier environment. Mice were kept in an automatic light/dark cycle (light periods of 12 h, 23 ± 1 °C, 55 ± 5 %) and given food and water ad libitum. All mice were randomly divided into ten groups, and each group was kept in two cages ($n = 5$ /cage). All mice were acclimatized to the environment for 1 week before the formal mice experiment.

Vaccination protocol and measurement of allergic-specific response

The establishment of mice models was performed as described previously (Ai et al. 2014). BALB/c mice were administered with 2×10^9 cfu (200 µl) of either recombinant or control strain or PBS by means of gavage for weeks 1–4. Seven days after final vaccination, mice were sensitized intraperitoneally with 10 µg of purified Der p2 formulated with 2 mg of Alum (Pierce, USA) at weekly intervals for 3 weeks. Mice in the naive group were fed with the same volume of PBS within same intervals. To induce airway inflammation, all mice were challenged by exposure to 0.1 mg/ml of HDM allergen over a 45-min period for 7 days (Fig. 1). The levels of specific antibodies in serum, cytokines in bronchoalveolar lavage fluid (BALF), and histological analysis of lung tissues were performed as described previously (Ai et al. 2014). Mice in the positive group were sensitized to Der p2, expressing significant allergic responses, and the naive mice with PBS were used as a negative control.

FACS analysis

Fresh mesenteric lymph nodes (MLN) were isolated from the respective groups, and single cell suspensions were obtained by mechanical disruption. After red blood cells lysis, single cell suspensions were incubated with FITC- and APC-labeled mAbs to mouse CD4 and CD25 at 4 °C for 30 min. For analysis of intracellular Foxp3, the strained cells were fixed and permeabilized with the fixation/permeabilization solution, and incubated with PE-labeled anti-Foxp3 antibody at 4 °C for 30 min. Finally, the stained cells were washed and analyzed by flow cytometry (FACSCalibur; BD, USA).

Statistical analysis

The data are expressed as means $\pm$ the standard errors of the means (SEM). All data were analyzed using one-way analysis of variance (ANOVA) followed by Duncan's multiple comparison test. $P < 0.05$ were considered to be significantly different.

Results

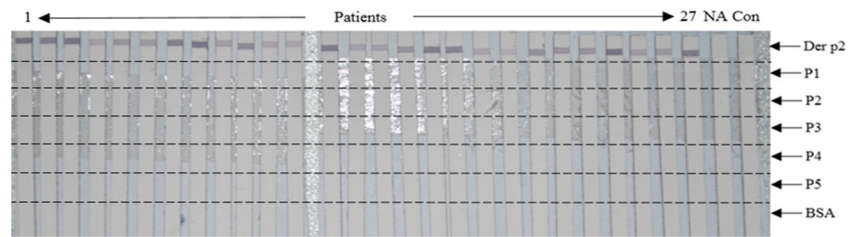
No relevant IgE reactivity could be detected in different DPs

The IgE-binding capacity of different DPs was compared with that of Der p2 in a non-denaturing dot-blot assay using sera from 27 HDM-allergic patients (Fig. 2). Each of the HDM-allergic patients showed IgE reactivity of varying intensity to Der p2 (Fig. 2, 1–27). In contrast, no relevant IgE reactivity to any DPs could be detected. When sera from non-allergic person or buffer solution without sera were used, no IgE reactivity to Der p2 or DPs was observed (Fig. 2, NA and Con). In addition, no IgE reactivity to BSA was found in various groups.

DPs induced less cytokines production than Der p2 in vitro

To further assay the immune characteristics of different DPs, cytokine production in spleen cells from Der p2-sensitized mice were measured after stimulation with Der p2 and

Fig. 2 IgE reactivity of five DPs. Nitrocellulose-dotted Der p2, DPs, and BSA were exposed to sera from HDM-allergic patients (1–27), individual without allergy (NA), and a solution without sera (Con)



different DPs (Fig. 3). Der p2 induced much higher levels of IFN- γ and IL-12 in the spleen cell culture than different DPs (Fig. 3a, 3c). The high IFN- γ and IL-12 production was accompanied by higher IL-10 production in the Der p2-stimulated culture relative to DP-stimulated cultures (Fig. 3b). The level of IL-4 in the Der p2-exposed culture was higher than that in the DP-exposed cultures (Fig. 3d). Apart from IL-12, there seemed to be no significant difference in cytokine production between single DP and DP15 (Fig. 3).

Identification of DPs expressed in recombinant strains

The expression of five DPs in the supernatants of recombinant strains were identified by Western blotting (Fig. 4). Nisin induction resulted in the production of peptide P1 with an

expected molecular mass that can be recognized by rabbit polyclonal antibody directed to Der p2 (Fig. 4a, LP1). The molecular size of peptide P1 expressed in strain LP1 was a bit greater than that of synthetic peptide P1 due to the expression of plasmid fragments. No signal was observed in the supernatant of control strain (Fig. 4a). In addition, the expression of other DPs can also be detected in the supernatants of corresponding strains (Fig. 4b–e).

DP-expressing strains inhibited Der p2-specific IgE responses

To evaluate the prophylactic effect of DP-expressing strains, the levels of Der p2-specific antibodies in serum were measured (Fig. 5). In contrast to the naive mice, an apparent Th2-

Fig. 3 Cytokine profiles induced by DPs in spleen cells cultures. The levels of IFN- γ (a), IL-10 (b), IL-12 (c), and IL-4 (d) were measured and expressed as mean $\pm$ SEM. The mean values followed by different letters differ significantly ($P < 0.05$)

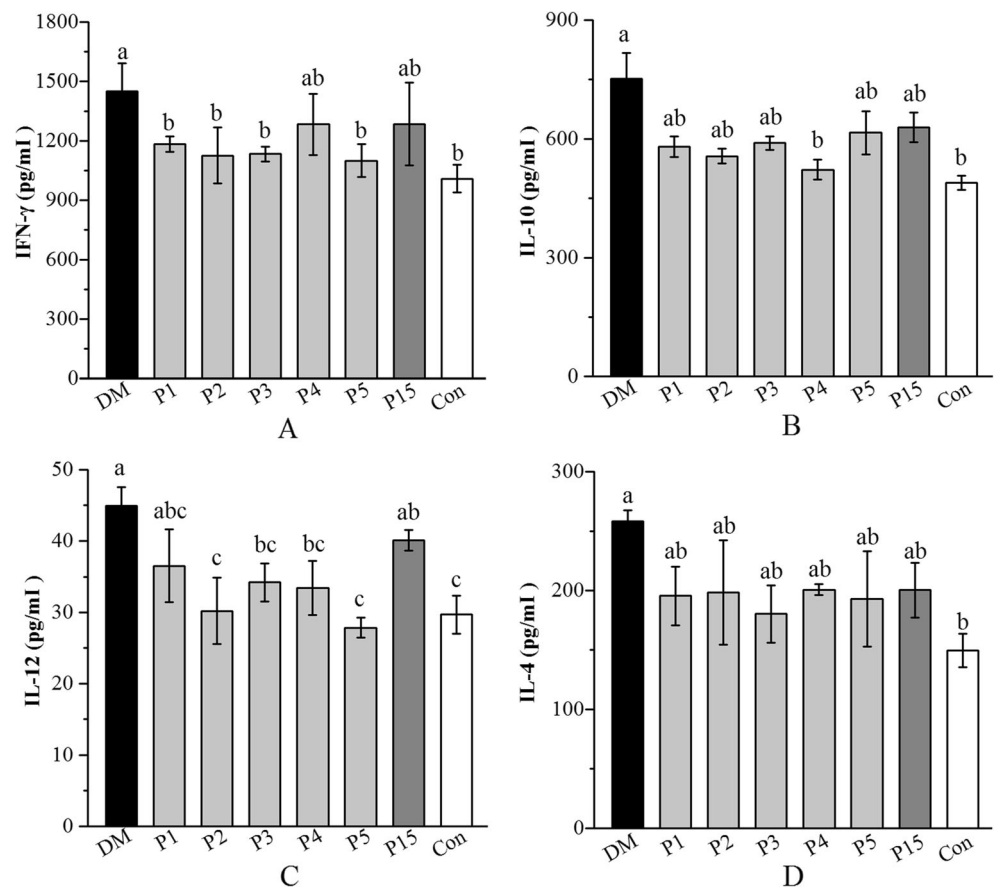
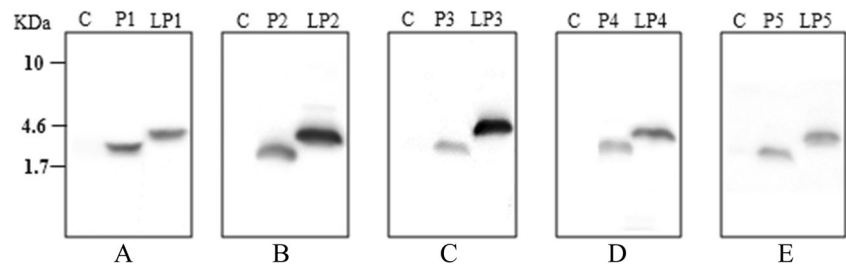


Fig. 4 The identification of different DPs in recombinant strains. Molecular weight was displayed in the left margins in kilodaltons



biased specific response was observed in Der p2-sensitized mice characterized by a high level of specific IgE, whereas oral administration of any DP-expressing strains significantly inhibited specific IgE production (Fig. 5a). It should be noted that all DP-expressing strains had a weakly inhibitory effect on the production of specific IgE relative to LL-DM, and no significant difference was observed between different strains (Fig. 5a). With regard to the protective antibodies, any DP-expressing strains induced less production of specific IgG2a than the mixture of five strains which had a similar result with LL-DM (Fig. 5b). The level of specific IgG1 was not affected by any strains (Fig. 5c). In addition, no significant specific responses could be detected in the naïve mice.

DP-expressing strains attenuated airway inflammation

In contrast to the naïve mice, airway challenge of Der p2-sensitized mice led to a significant Th2-biased airway inflammatory response determined by an increase in Th2 cytokines IL-4 and IL-5 (Table 3). The development of airway response was significantly attenuated by treatment with any DP-expressing strains. Surprisingly, no significant difference in Th2 cytokines was observed between DP-expressing strains and LL-DM. No obvious difference in the level of IL-10 was observed in different groups (Table 3). Furthermore, histological analysis showed that Der p2-sensitized mice in positive group had a significant airway inflammation characterized by extensive infiltration of inflammatory cells and histologic

changes relative to the naïve mice (Fig. 6). Oral application of any DP-expressing strains or the mixture significantly inhibited inflammatory cells infiltration, and their prophylactic effect did not seem to be significantly different from LL-DM (Fig. 6; Fig. S1).

DP-expressing strains increased the level of regulatory T cells in MLN

To investigate whether the protective effect induced by DP-expressing strains was associated with Tregs, the percentage of CD4⁺CD25⁺Foxp3⁺-expressing T cells in the MLN was measured by FACS (Fig. 7). When mice were sensitized to Der p2, the level of Tregs was significantly increased in mice fed with any DP-expressing strains relative to those fed with PBS or control strain, and no obvious difference was observed between different strains (Fig. 7). This increase was greater in mice fed with the mixture than single strain. It was noteworthy that the level of Tregs was a bit lower in mice fed with the mixture than those in LL-DM group (Fig. 7). After airway challenge, the levels of Tregs in different groups were similar to that in the naïve mice (data not shown).

Discussion

Recombinant LAB strain as a mucosal bio-therapeutic agent has demonstrated to play a positive role in the treatment or

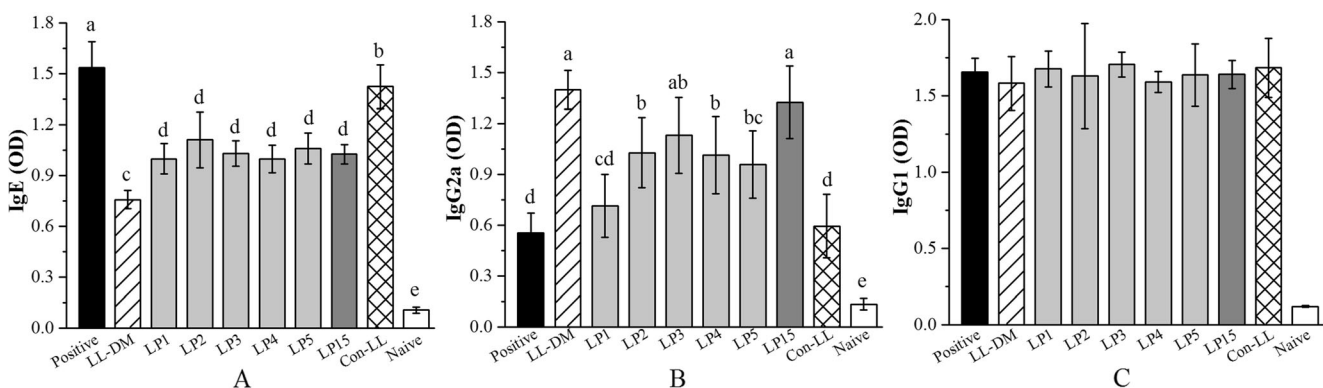


Fig. 5 Detection of specific antibodies IgE (a), IgG2a (b), and IgG1 (c) in serum from respective groups. Data was expressed as mean ± SEM. The mean values followed by different superscript letters differ significantly ($P < 0.05$)

Table 3 The levels of cytokines in BALF after challenge

Group	IL-10 (pg/ml)	IL-4 (pg/ml)	IL-5 (pg/ml)
Positive	411 ± 55	102 ± 7a	279 ± 7a
LL-DM	430 ± 27	57 ± 8b	81 ± 12b
LP1	452 ± 29	60 ± 6b	109 ± 14b
LP2	426 ± 21	53 ± 13b	104 ± 17b
LP3	413 ± 20	59 ± 5b	113 ± 19b
LP4	440 ± 38	56 ± 10b	103 ± 23b
LP5	463 ± 14	59 ± 4b	109 ± 26b
LP15	468 ± 25	51 ± 5b	94 ± 18b
Con-LL	404 ± 29	82 ± 3c	156 ± 15c
Naive	438 ± 91	28 ± 5d	45 ± 17d

The data with different letters in the same column was significantly different ($P < 0.05$)

prevention of some diseases (Daniel et al. 2011; Tsai et al. 2012). Our previous study indicated that recombinant Der p2-expressing strain could attenuate HDM-induced airway inflammation in mice models (Ai et al. 2014). However, wild allergen always cause adverse side effects owing to their inherent allergenic activity (Akdis and Blaser 2000; Chen et al. 2008; Vrtala et al. 2004). In contrast, hypoallergenic derivatives are considered an effective alternative in SIT to avoid wild allergen-induced side effects (Chen et al. 2008; Vrtala et al. 2004). Here, five DPs containing major T cell epitopes were produced to reduce or eliminate IgE reactivity and T cells responses, and then their protective effects and mechanisms involved were evaluated in mice models.

Der p2 contains primarily conformational IgE epitopes, and no relevant sequential IgE epitopes have been found (Chen et al. 2012; Takai et al. 1997). Some Der p2 derivatives have proven to reduce IgE reactivity compared with Der p2, which could be attributed to the disruption of 3D structure (Ai et al. 2015; Campana et al. 2010; Chen et al. 2008). In this

study, any DPs were demonstrated to eliminate IgE reactivity, and thus can avoid IgE-mediated side effects. However, Chen et al. demonstrated that side effects was not only associated with IgE reactivity, but also with specific T cells responses (2012). In contrast to DM, both single DP and DP15 induced less cytokines production, indicating that DPs have a decreased responses with T cells. Recent study indicated that Der p2 fragments could induce lower lymphoproliferative response and less cytokines secretion in PBMCs from HDM-allergic patients, but still could stimulate the production of specific antibodies against Der p2 in vivo (Chen et al. 2012). Rupa and Mine (Rupa and Mine 2012) found that egg white allergen T cell epitope peptides could have a significant effect on specific antibodies and immune responses in mice models. These results suggested that DPs could have a potential modulatory effect on specific antibodies production and immune responses in vivo.

Mucosal vaccine is considered a promising candidate for immunization and vaccination against some diseases (Daniel et al. 2011). Recombinant LAB strains expressing allergen were demonstrated to have a significant effect on the suppression of allergic responses, but few studies report the expression and application of allergen T cell epitope in recombinant strain (Rigaux et al. 2009; Schwarzer et al. 2011). In this study, low molecular weight DPs can be expressed in recombinant strains, suggesting these strains could have a prophylactic effect in Der p2-induced allergic responses.

Allergic asthma caused by HDM is a common Type I allergy, in which allergen-specific IgE plays an important role (Djukanovic et al. 2004). Clinical trials have shown that successful SIT in allergic diseases is always associated with the induction of allergen-specific IgG antibodies (Jutel et al. 2005; Moingeon et al. 2006). The induction of IgG can play a protective role not only through the inhibition of allergen-induced, IgE-mediated release of inflammatory mediators from mast cells and basophils but also through the inhibition of IgE-

Fig. 6 Histological analysis of lung tissues. Representative image ($\times 150$) from the positive group (a), control group (b) and groups treated with control strain (c), LL-DM (d), a mixture of five strains (e), and LP1 (f). The size of scale bars within the figure was 100 μ m. The inflammatory cells were indicated with arrows

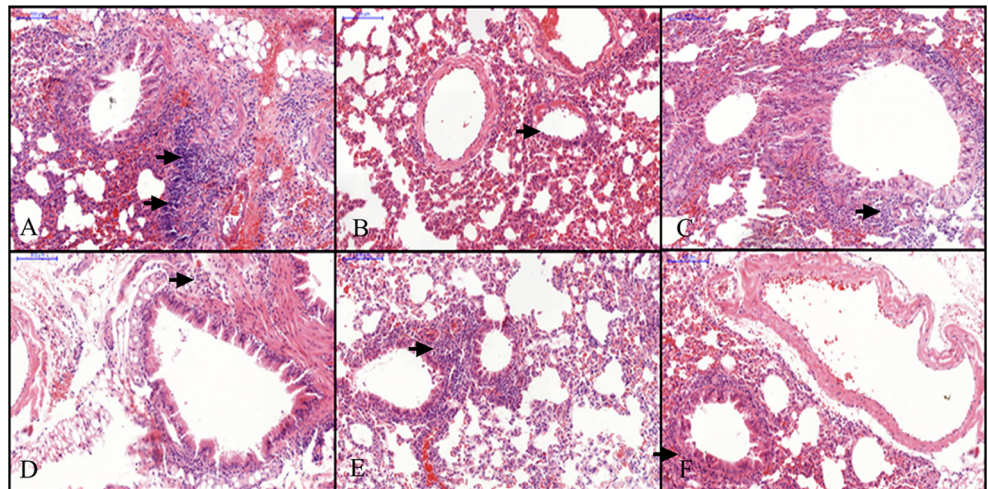
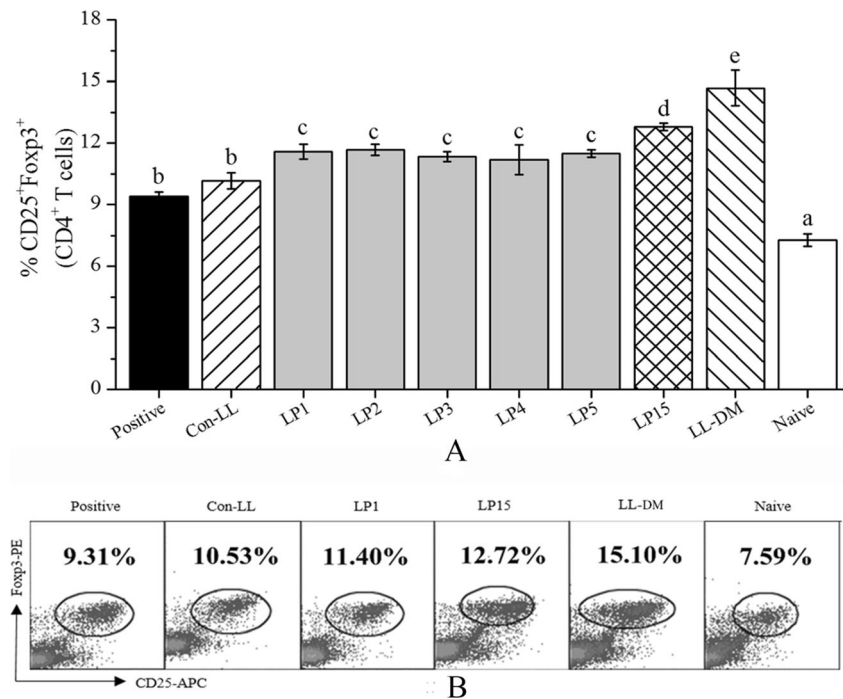


Fig. 7 Analysis of CD4⁺CD25⁺Foxp3⁺ expressing T cells in the MLN (**a**). Representative images from respective groups were shown (**b**). Data was expressed as mean $\pm$ SEM. The mean values followed by different superscript letters differ significantly ($P < 0.05$)



facilitated allergen presentation to T cells (Moingeon et al. 2006; Wachholz and Durham 2004). Mucosal delivery of allergen which could impair allergic responses as significant specific IgG2a production together with a drastic reduction of specific IgE was elicited (Daniel et al. 2006; Rigaux et al. 2009; Schwarzer et al. 2011). Similar to LL-DM, LP15 increased the production of specific IgG2a, which was higher than single strain. This result was similar to specific IgG responses induced with Der p2, which were higher and detected earlier than those fed with an individual fragment (Chen et al. 2012). Vaccination with allergen can induce specific immune deviation via T cell anergy (Cao et al. 1997), implying that a mixture of DPs could play a similar role as Der p2 in vivo.

Allergic asthma is caused by the aberrant expansion in lung of T-helper cells that produce Th2 cytokines and is characterized by infiltration of inflammatory cells. Th2 cytokines have been recognized to play an important role in the infiltration of inflammatory cells (Nonaka et al. 1995). Mucosal delivery of single DP or DP15 showed a significant inhibitory effect on airway inflammation together with a reduction in the levels of IL-4 and IL-5. These findings concur with those showing that oral application of recombinant LAB producing the major birch pollen allergen Bet v1 reduced inflammatory cells infiltration and the level of IL-4 in BALF (Daniel et al. 2006). Surprisingly, no significant difference in the suppression of airway inflammation was observed between different strains, which may be attributed to lower degree of airway inflammation. IL-10 is considered to be involved in several regulatory mechanisms, including the down-regulation of mast cell function, a decrease in eosinophil-sensitive chemokine production

and reduced eosinophil survival (Saraiva and O'Garra 2010). However, no significant difference was found in IL-10 between various groups. Taken together, mucosal delivery of DPs could attenuate airway inflammation primarily via a reduction in Th2 cytokines in BALF.

Tregs are considered to play an indispensable role in maintaining immunological unresponsiveness to harmless antigen and in suppressing excessive immune response deleterious to host (Sakaguchi et al. 2008). Oral tolerance is considered to originate in the intestinal immune system, in which induction of Tregs in MLN plays a decisive role (Worbs et al. 2006). The level of Tregs was significantly increased in any DP-expressing strain groups relative to the naïve mice, whereas the mixture had a greater effect than single strain. Karimi et al. (Karimi et al. 2009) demonstrated that Tregs isolated from *Lactobacillus reuteri*-fed mice had a greater capacity to suppress T-effector cell proliferation and may effectively attenuate airway hyper-responsiveness and inflammation in ovalbumin-sensitized mice model. After challenge, the levels of Tregs in recombinant strain-fed groups were returned to a normal state that was similar to that in the naïve mice, which can be explained by the detection of Tregs at the sites of inflammation; in many situations, their ability to migrate to and remain in inflamed tissue is important for their function in vivo (Larché 2007; Tang and Bluestone 2008). These results indicated that mucosal delivery of DP15 could inhibit allergic response via an increase of Tregs to a greater extent than a single DP.

Compared with Der p2 macromolecular derivative DM, DPs could be promising candidate molecules for an active

vaccination and the induction of tolerance in the treatment or prevention of allergy. Recombinant LAB strain combines the advantages of LAB and non-allergenic peptides, which would promote the development of mucosal vaccine. Allergen peptides were expressed for the first time in a LAB vector used as mucosal vaccine, and thus greater efforts are needed to evaluate their availability.

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Compliance with ethical standards All of the mice experiments were approved by the Ethics Committee of Jiangnan University, China. The study procedures were performed according to the European Community guidelines (Directive 2010/63/EU) for the care and use of experimental animals. All mice were killed by cervical dislocation under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering. The sera of HDM-allergic patients were provided by HOB Biothch group, which is mainly involved in the study and development of allergen detection kit (Ai et al. 2015).

Conflict of interest All authors declare no conflict of interest.

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Mucosal delivery of allergen peptides expressed by *Lactococcus lactis* inhibit allergic responses in a BALB/c mouse model

(Applied Microbiology and Biotechnology)

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1 **Table S1** Primers used in the amplification of five DPs

Primers	Sequence (5'-3')	Restriction
P1-F	<u>ggggtacc</u> GATCAAGTTGATGTTAAAGA	<i>KpnI</i>
P1-R	<u>gctctaga</u> TGGTTCTGAACCATGACA	<i>XbaI</i>
P2-F	<u>ggggtacc</u> TGTATTATTCATCGTGG	<i>KpnI</i>
P2-R	<u>gctctaga</u> AGCAGTTTTTGTATTTTG	<i>XbaI</i>
P3-F	<u>ggggtacc</u> AAAATTGAAATTAAAGCTTC	<i>KpnI</i>
P3-R	<u>gctctaga</u> ATGACATGCATTTGGATC	<i>XbaI</i>
P4-F	<u>ggggtacc</u> CATTATATGAAATGTCCATT	<i>KpnI</i>
P4-R	<u>gctctaga</u> TTCAGATTTTGGTGCAAT	<i>XbaI</i>
P5-F	<u>ggggtacc</u> AATGTTGTTGTTACTGTT	<i>KpnI</i>
P5-R	<u>gctctaga</u> ATCACGAATTTTAGCATG	<i>XbaI</i>

2 Restrictions used in cloning are underlined.

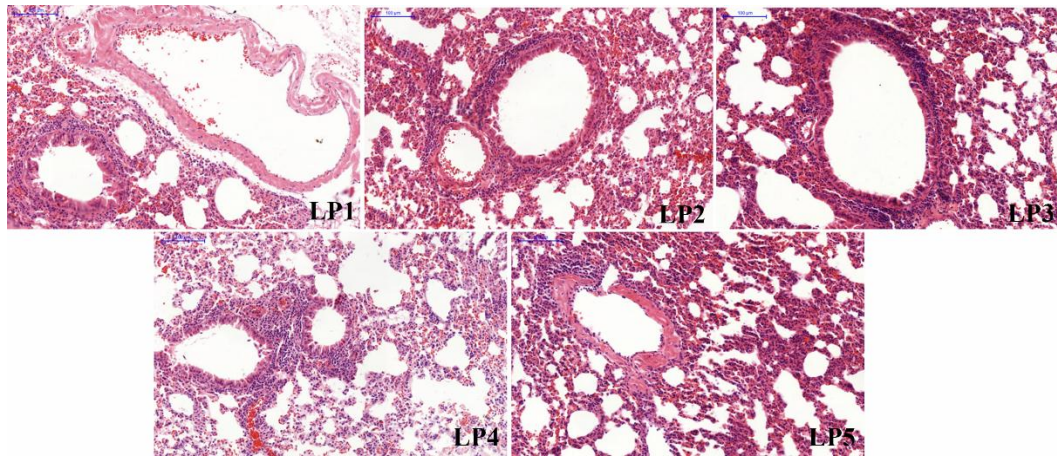


Fig. S1 Histological analysis of lung tissues. Representative image (150×) from different DP-expressing strain treatment groups (LP1-LP5). The size of scale bars within the figure was 100 μm.