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## Intramuscular immunization with DNA construct containing Der p 2 and signal peptide sequences primed strong IgE production

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### Abstract

**Background:** Previous studies demonstrated that allergen gene vaccination induced TH1-skewed responses and inhibited IgE production. This study evaluated and characterized the immune responses induced by three DNA constructs encoding different forms of Der p 2 for safe and efficacious vaccination against mite allergy.

**Methods:** Mice were immunized intramuscularly with DNA constructs encoding a major mite allergen, Der p 2, without a signal peptide (p2), with a signal peptide (p52), and with a signal peptide plus lysosomal-targeting sequence (p52-LA), respectively, followed by TH2-skewed protein challenge. Antibody and T-cell cytokine responses were assessed by ELISA. Primed dendritic cells (DCs) were adoptively transferred to naïve mice and humoral responses were examined after protein challenge. The circulating Der p 2 protein was detected by sandwich ELISA.

**Results:** Mice immunized with p52-LA showed strong and clear-cut TH1-type response, as evident by high IFN- $\gamma$  production and elevated levels of Der p 2-specific IgG2a production whereas construct p2 induced only moderate levels of TH1 response. In contrast, mice immunized with construct p52 showed a mixed TH1/TH2 phenotype and produced substantial circulating Der p 2 protein. Mice adoptively transferred with DCs primed by p52 construct, but not by the p2 or p52-LA constructs, were sensitized to produce high levels of Der p 2-specific IgE.

**Conclusions:** Immunization with DNA construct encoding a signal peptide could potentially prime TH2-skewed responses and IgE production. The additional inclusion of lysosomal-targeting sequences to such construct could improve the safety and efficacy of DNA vaccination against allergy.

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**Keywords:** Allergen vaccination; Der p 2; Signal peptide; LAMP-1; Dendritic cell

### 1. Introduction

The incidence of asthma, hay fever and other IgE mediated diseases has been steadily increasing over the past few years, despite the introduction of increasingly potent and effective drugs [1]. Thus, more effective therapies are necessary to modulate immune responses that could mitigate or prevent

allergic illness [2,3]. Allergen gene immunization has been shown to be effective in directing immune response towards a favorable TH1 phenotypic expression [4–6]. Mice receiving DNA vaccine encoding for T-cell epitopes of allergen genes showed abrogation of allergen-induced IgE synthesis [4] while the priming of TH1 cells as characterized by IFN- $\gamma$  production has been shown to counterbalance the progression of TH2-driven allergic processes [5,6].

The effectiveness of DNA vaccination is also dependent on the interaction between T-lymphocytes and antigen presenting cells (APCs). Dendritic cell (DC) is a potent and unique APC that has been shown to play a critical role in inducing protective immune responses of DNA vaccines [7–9]. The importance of DCs is underscored by the fact that DNA

**Abbreviations:** APCs, antigen presenting cells; DC, dendritic cell; ISS, immunostimulatory sequences; LAMP-1, lysosome associated membrane protein 1; MHC II, major histocompatibility complex class II

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transfected DCs could lead to general activation of DCs in the draining lymph nodes, which provide optimal conditions for effective T-cell activation and memory. Conversely, the pathogenic role of DCs in allergic disease has also been documented [10,11]. In vitro-generated DCs from patients sensitized to the Der p 1 antigen when adoptively transferred to human PBMC-reconstituted SCID mice were capable of inducing a marked increase in the production of specific IgE upon allergen challenged [10]. In another study, in vivo depletion of lung DCs during allergen challenge could abrogate typical features of asthma, and Th2 cytokine secretion were restored upon adoptive transfer of CD11c DCs [11].

Current approaches to optimizing the efficacy of DNA vaccines include incorporation of immunostimulatory sequences (ISS) in the plasmid backbone [12,13], utilizing appropriate delivery mechanisms [14,15], incorporating localization/secretory signals [16–18] and augmenting responses with adjuvants such as cytokines [19]. Increased gene expression has been reported to be essential for improving the efficacy of DNA vaccines [16–18]. The importance of signal sequence in the induction of protective immunity was illustrated when DNA plasmids without leader sequence were less immunogenic than their full-length counterparts. In addition, it has been proposed that poor antigen secretion may hold up the release of antigen from transfected cells and result in reduced circulating antigen available to achieve an optimal immune response [17,18].

Efficacy studies of DNA vaccines for allergic diseases have been previously reported, however, the immunological impact and potential risks of incorporating signal sequences in DNA vaccine constructs for allergic diseases have not been fully addressed to date. To address this issue, the major dust mite allergen from *Dermatophagoides pteronyssinus*, Der p 2, which has an IgE reactivity of up to 90% in mite-sensitized patients with allergic asthma and eczema [20–22], was used as a model allergen in this study. Mice immunized with DNA constructs containing Der p 2 without signal peptide sequence (p2), Der p 2 with signal peptide sequence (p52) or p52 plus a targeting sequence of lysosome associated membrane protein (LAMP-1) [p52-LA], respectively, were evaluated for their humoral and cellular responses. In addition, DCs adoptive transfer experiments were performed to further characterize the quality of the immune responses primed by the three DNA constructs.

## 2. Materials and methods

### 2.1. Mice

Female CBA/CaH mice (6–8 weeks old) used in this study were purchased from the Sembawang Laboratory, Singapore and housed under conventional conditions in the Animal Holding Unit at The National University of Singapore. All experiments were performed according to Institutional Guidelines for Animal Care and Handling (IACUC).

### 2.2. Preparation of recombinant Der p2

The cDNA of mature Der p 2 was amplified by polymerase chain reaction (PCR) from the plasmid pGEX-2T-Der p 2 [21] using the forward primer 5'-GCCTCGAGAAAAGAGATCAAGTCGATGTCAAA-3' and reverse primer 5'-GCGAATTCTTAATCGCGGATTTAGC-3'. The 5' *Xho*I and 3' *Eco*RI sites were used for directional in-frame cloning with the  $\alpha$ -factor signal sequence driven by the alcohol oxidase gene promoter in pPIC9 vector. The PCR reaction was carried out at 94 °C for 30 s, 50 °C for 30 s, 72 °C for 1 min for 30 cycles with pfu DNA polymerase (Promega, Madison, WI, USA) and the insert in pPIC9 was completely sequenced. The pPIC9-Der p 2 was linearized with BglII and transformed into *P. pastoris* strain GS115 by lithium chloride method as described in the *Pichia* expression manual (version E) (Invitrogen Corporation, Carlsbad, CA, USA). The His<sup>+</sup>Mut<sup>s</sup> clones were selected for Der p 2 protein expression. The yeast media containing the rDer p 2 were applied to a Sephadex G25 molecular sieving column (5 cm × 20 cm; Pharmacia, Uppsala, Sweden) equilibrated with 20 mM sodium acetate, pH 5.5. rDer p 2-containing fractions were pooled and applied to a SP-Sepharose fast flow column (2.6 cm × 15 cm; Pharmacia, Uppsala, Sweden) equilibrated with the previous buffer. Bound proteins were eluted with 600 ml of a linear sodium chloride gradient from 0 to 500 mM. The purity of recombinant Der p 2 was examined by SDS-PAGE followed by silver staining.

### 2.3. DNA plasmids construction

The p52 construct was generated by cloning cDNA coding for the mature Der p 2 (387 nucleotides, accession number AF276239) into a construct of pCI (Promega, Madison, WI, USA) containing the Der p 5 leader sequence (p5) as previously described [23]. The Der p 2 coding sequence was amplified using primers 5'-TTCCGGAGATCAAGTCGATGTCAAAG-3' and 5'-CGCTTAAGAATTAGCGCCTAAATCG-3', and cloned in-frame with p5. The second construct p52-LA was generated by cloning a region of the LAMP-1 (1305–1412 nucleotides, accession number AY069968) and the mature region of the Der p 2 gene into p5. The LAMP-1 targeting sequence was amplified using 5'-CATAAAATCCGCGATTGATCCCCATTG-3' and 5'-CTATCTAGACTAGATGGTCTGATAGCCGGCGTG-3'. The mature Der p 2 coding sequence was amplified using 5'-GTTTCCGGAGATCAAGTCGATGTC-3' and 5'-GGAAGCGGCCGCTAGATGGTCTGATAG-3'. The LAMP-1 fragment was fused in-frame to the 5' end of Der p 2 by PCR using the forward primer for LAMP-1 and reverse primer for Der p 2, and subsequently cloned in the p5 construct. The third construct containing the mature Der p 2 coding region without a signal peptide was generated by amplifying the Der p 2 coding sequence using primers 5'-AGCCTCGAGCCACCATGGATCAAGTCGAT-3' and 5'-CGCTTAAGAATTAGCGCCTAAATCG-3', which was



The labeling of guinea pig IgG with biotin was performed using biotin hydroxysuccinimide ester (Sigma, St. Louis, MO, USA) according to the manufacturer's instructions.

### 2.7. Splenocyte culture

Splenocytes were cultured in a complete RPMI-1640 medium supplemented with 10% heat-inactivated bovine calf serum (StemCell Technologies, Vancouver, Canada), 1 mM sodium pyruvate (HyClone, South Logan, UT, USA), 2 mM L-glutamine, antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin),  $5.5 \times 10^{-2}$  mM 2-β mercaptoethanol (Gibco BRL, Life Technologies, Paisley, UK), and incubated at 37 °C in 5% CO<sub>2</sub>. To study the primary response, splenocytes were cultured in 96-well plates ( $4 \times 10^5$  cells/well) in the presence or absence of 10 µg/ml of rDer p 2 protein. The supernatants were harvested at 72 h and 96 h for cytokine assays. For the secondary culture, splenocytes were cultured in 6-well plates ( $2 \times 10^7$  cells/well) with rDer p2 protein. At days 3, 5 and 7, cells were supplemented with fresh medium containing 10 U/ml of recombination mouse IL-2 (R&D systems, Minneapolis, MN, USA). At day 10, the cultured cells were harvested and purified by Ficoll-Paque plus (Amersham Biosciences AB, Uppsala, Sweden) centrifugation. Cells were re-stimulated in the presence or absence of 10 µg/ml of rDer p 2 protein in a 96-well plate ( $1 \times 10^5$  cells/well) with APCs ( $3 \times 10^5$  cells/well). APCs were derived from the mitomycin C-treated splenocytes of naïve mice. Briefly, mitomycin C (Roche Diagnostic GmbH, Mannheim, Germany) was added to the cells at a final concentration of 50 µg/ml and incubated in the dark at 37 °C for 20 min. The cells were washed three times with 30 ml of 1× HBSS before used. Culture supernatants were harvested at 72 and 96 h for cytokine assays.

### 2.8. Cytokine assays

Supernatants were assayed for the presence of IFN-γ and IL-4 using antibodies pairs rat anti-mouse IFN-γ (R4-6A2) and IL-4 (BVD4-1D11), biotinylated rat anti-mouse-IFN-γ (XMG1.2) and anti-mouse-IL-4 (BVD6-24G2)], according to the manufacturer's instructions. The standard curves were generated with mouse recombinant IFN-γ and IL-4. All antibodies and cytokines were purchased from BD PharMingen, San Diego, CA, USA. The detection limits of IFN-γ and IL-4 assays were 20 and 10 pg/ml in respective. The results were deducted off from background readings of those without antigen stimulation.

### 2.9. Isolation of dendritic cells

Harvested lymph nodes were digested in PBS containing 100 U/ml of collagenase (Boehringer Mannheim, Mannheim, Germany) at 37 °C for 30 min. Cells were treated with 0.1 M EDTA for 5 min at room temperature to disrupt DC–T-cell complexes. Low-density cells were enriched by Optiprep

media (AXIS-SHIELD PoC AS, Oslo, Norway) centrifugation as recommended by the manufacturer. The cells were blocked with 5% fetal calf serum in PBS and anti-mouse FcR (CD16/CD32) (BD PharMingen, San Diego, CA, USA) for 20 min at 4 °C, washed with staining buffer, incubated for another 20 min at 4 °C with anti-CD11c microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) and subsequently CD11c<sup>+</sup> cells were sorted using AutoMacs (Miltenyi Biotec). The purified DCs of approximately 80% in purity were adoptively transferred to naïve mice via intravenous administration, at  $1 \times 10^5$  DCs/mouse.

### 2.10. Statistical analysis

The significance between experimental groups was calculated by the Mann–Whitney *U*-test (SPSS). A value of  $p < 0.05$  was considered significant.

## 3. Results

### 3.1. Der p 2 specific IgE and TH2 responses in mice immunized with rDer p 2 protein without adjuvant

The naïve mice receiving three subcutaneous injections of 10 µg of rDer p 2 protein at days 0, 4 and 8 showed detectable and significant Der p 2 specific IgE at day 14 ( $0.76 \pm 0.19$  ELISA units) and the IgE titers increased up to  $3.66 \pm 1.06$  ELISA units at day 28 (Fig. 2a). High levels of Der p 2 specific IgG1 (ranging from 1700 to 3000 ELISA units) were induced with the similar kinetics in these mice (Fig. 2b). There was no significant level of specific IgG2a detected in these mice (data not shown). Splenocytes from the protein immunized mice stimulated with rDer p 2 produced significantly high levels of IL-4 and low levels of IFN-γ as compared to PBS group ( $p < 0.05$ , Fig. 2c and d), indicating that a typical TH2-skewed phenotype was induced in these mice. This well established immunization protocol, which is a potent strategy to induce antigen specific TH2 responses without the use of any conventional adjuvant, was exploited in this study as a TH2 challenge to mice immunized with the various DNA constructs.

### 3.2. Der p 2 specific antibody responses in DNA immunized mice

The immunogenicity of the various DNA constructs expressing different forms of Der p 2 antigen was compared in mice immunized with construct p2, p52 and p52-LA, respectively. Mice immunized with construct p52 produced statistically significant and higher levels of IgG1 subclass as compared to other groups ( $p < 0.05$ , Fig. 3a). The IgG1 titer in these mice reached  $23,600 \pm 7300$  ELISA units on day 28, boosted at least five-fold at day 49, and subsequently maintained at an average of 80,000 ELISA units. In contrast, the IgG1 production for the p52-LA construct was at relatively

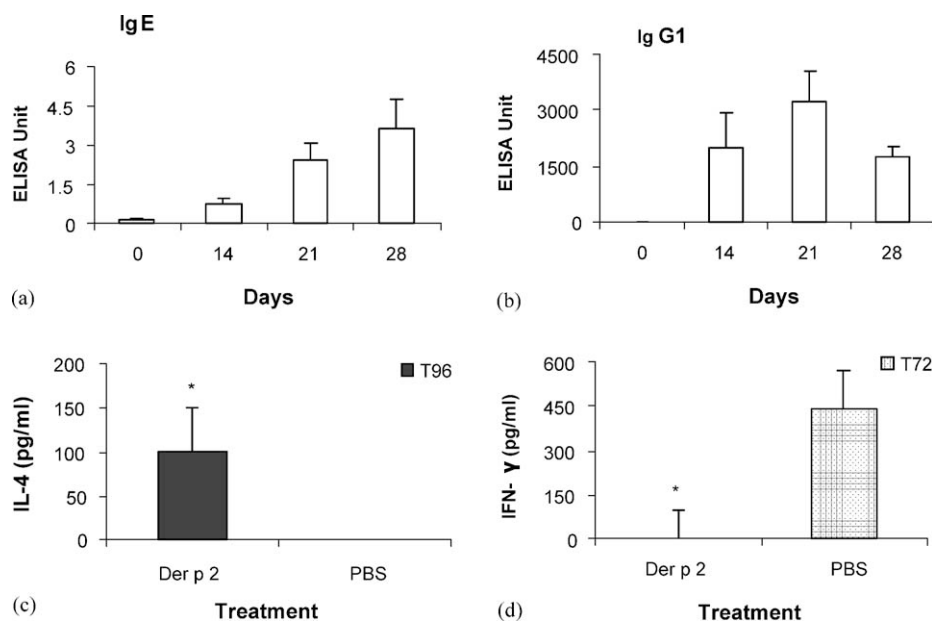


Fig. 2. Specific immunoglobulin responses and splenic cytokine profile of mice immunized with Der p 2 protein. Mice were administered with 10  $\mu$ g of rDer p 2 protein on days 0, 4, and 8 via subcutaneous route and monitored for Der p 2 specific IgE (a) and IgG1 (b) production. To assay for splenocytes cytokine profile, mice were similarly immunized and spleens were harvested on day 10. Splenocytes were cultured with 10  $\mu$ g/ml of rDer p 2 and supernatants were collected at 72 and 96 h for IL-4 (c) and IFN- $\gamma$  (d) assays. Results shown are mean  $\pm$  S.E.M.,  $n = 4-5$  per group, \* compared with PBS treated controls,  $p < 0.05$ . T: time (h).

low levels of 1200 ELISA units until day 42, and rose to an average level of 20,000 ELISA units after protein challenge. The IgG1 production for p2 immunized mice was consistently maintained at a range of 500–3000 ELISA units. Mice immunized with the pCI vector DNA did not produce any Der p 2 specific antibodies and showed a background reading of about 20 ELISA units up to day 42. However, the three subcutaneous injections of rDer p 2 protein into the vector immunized control mice induced significant levels of specific IgG1 (about 300 ELISA units) from day 49 onwards, the kinetics of IgG1 production correlated well with the kinetics of the specific IgE production in these mice as shown in Fig. 3a and c.

For the specific IgG2a production, the initial profiles were almost similar in all DNA immunized mice, ranging from 1000 to 2000 ELISA units (Fig. 3b). Upon Der p 2 protein challenge, mice primed with p2 or p52 showed an approximate 10-fold increase in IgG2a titer (about 24,000 ELISA units) at day 56. The p2 immunized mice subsequently displayed an average IgG2a titer of 15,000 ELISA units. Mice primed with p52-LA were found to generate predominantly higher titers of IgG2a than the rest ( $p < 0.05$ ), with an approximate 15-fold increase at day 56, and subsequently maintained at an average of 80,000 ELISA units. Mice immunized with the pCI vector responded with an average background level of approximately 50 ELISA units after the protein challenge.

Prior to the subcutaneous protein challenge, there were some detectable levels of Der p 2 specific IgE titres in p52 immunized mice ( $0.25 \pm 0.05$  ELISA units), and the spe-

cific IgE levels in p2 and p52-LA immunized mice were below the detection limit of 0.2 ELISA units in our IgE ELISA assays (Fig. 3c). After the protein challenge, mice that were immunized with construct p2 had a slight elevation in specific IgE levels ( $0.32 \pm 0.15$  ELISA units) while the control mice immunized with pCI vector DNA displayed IgE titers of about 1.5 ELISA units. The kinetics and profiles of the specific IgE and IgG1 production induced by Der p 2 protein in the pCI vector control mice were very similar, but with reduced levels of antibodies, as compared to that of protein immunized control mice (Fig. 2a and b). The reduced levels of IgE and IgG1 seen in these pCI vector control mice were probably due to the TH1 adjuvant effects of the CpG motif present in the pCI vector backbone DNA. It is well established that specific IgE levels are generally much lower than that of total IgE and the low but significant levels of specific IgE detectable by ELISA usually represent a reliable and meaningful indicator for TH2 responses. Nevertheless, T-cells studies were then carried out to further characterize the phenotypes of the immune responses in these mice.

### 3.3. T-cell responses of DNA immunized mice

We have shown that the p52 construct could induce remarkably high levels of specific IgG1, moderate levels of IgG2a, accompanied by some detectable levels of specific IgE, suggesting that the immune responses primed by construct p52 could be of a mixed TH1/TH2 phenotype. This had prompted us to perform further studies to characterize the

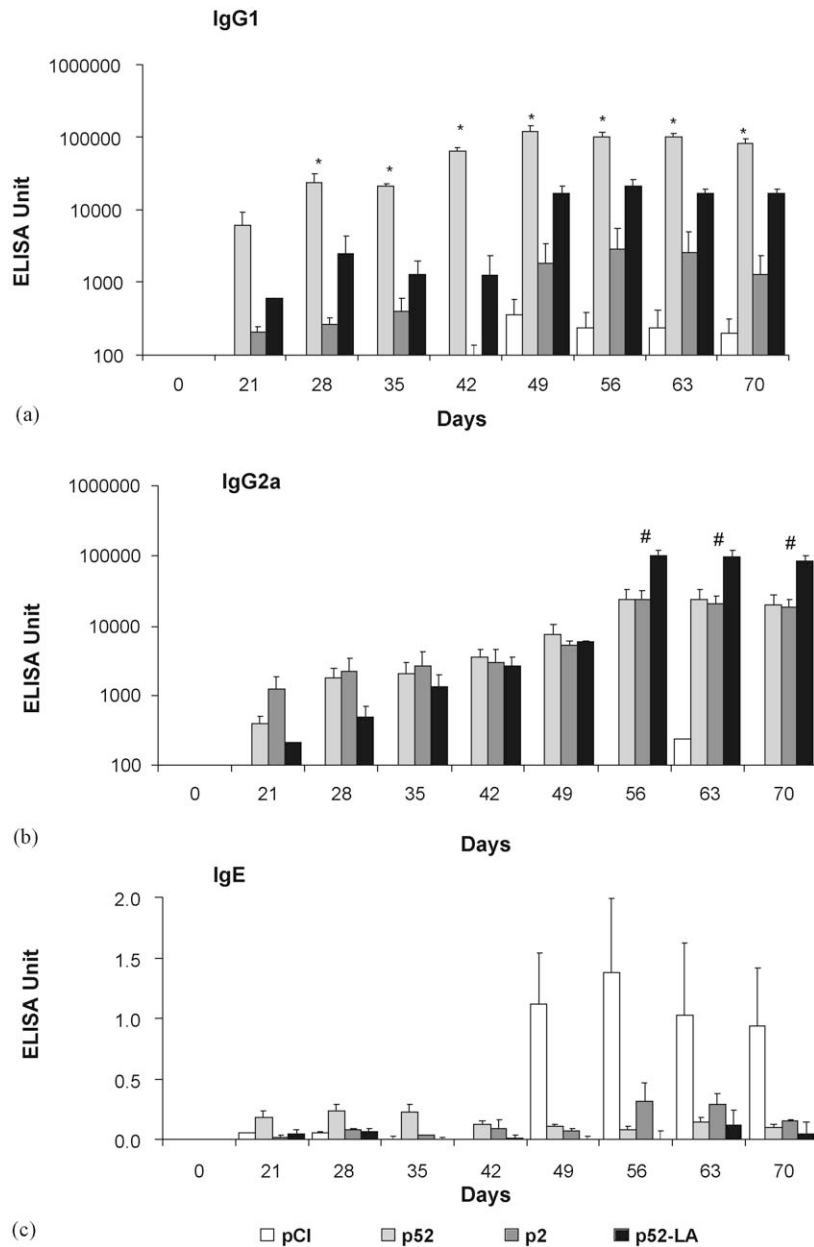


Fig. 3. Specific immunoglobulin responses of mice immunized with DNA constructs and challenged with Der p 2 protein. Mice were immunized with 50  $\mu$ g of p52, p2, p52-LA or pCI vector on days 0 and 14, and challenged with 10  $\mu$ g of rDer p 2 protein on days 35, 39 and 43. Mice were bled and sera were monitored for Der p 2 specific IgG1 (a), IgG2a (b) and IgE (c) production. Results shown are mean  $\pm$  S.E.M.,  $n = 5$  per group, \* compared with pCI, p2 or p52-LA,  $p < 0.05$ ; # compared with pCI, p2 or p52,  $p < 0.05$ .

386 T-helper type response by examining the T-cell cytokine profiles. Splenocytes from the immunized and control mice were  
 387 stimulated with rDer p 2 for 10 days and then the enriched  
 388 Der p 2 specific T-cells were re-stimulated with rDer p 2 protein. Culture supernatants were collected at 72 and 96 h for  
 389 IL-4 and IFN- $\gamma$  analysis by ELISA. As shown in Fig. 4a,  
 390 upon secondary stimulation with rDer p 2, IFN- $\gamma$  in the culture  
 391 supernatants of cells from p52 primed mice was only  
 392 detectable at 96 h time point ( $1.5 \pm 0.3$  ng/ml). Cells from  
 393 p52-LA primed mice showed about three-fold increase in  
 394 IFN- $\gamma$  production ( $4.9 \pm 0.4$  ng/ml) as compared to that of

397 p52 and pCI primed mice ( $p < 0.05$ ) while cells from mice  
 398 primed by p2 construct produced an intermediate levels of  
 399 IFN- $\gamma$  ( $2.8 \pm 1.3$  ng/ml). Some low levels of IL-4 (approx-  
 400 imately 20 pg/ml, Fig. 4b) were detected in cell cultures of  
 401 p52, pCI and PBS primed mice. Induction of this basal level  
 402 of IL-4 production was non-antigen specific and the cell type  
 403 responsible for the IL-4 production was unclear. The IL-4  
 404 levels were much lower than that produced by the typical  
 405 TH2-skewed cells (100–150 pg/ml; Fig. 2c). Nevertheless,  
 406 this basal IL-4 production was greatly attenuated in cells cul-  
 tures of the p52-LA and p2 primed mice ( $p < 0.05$ ).

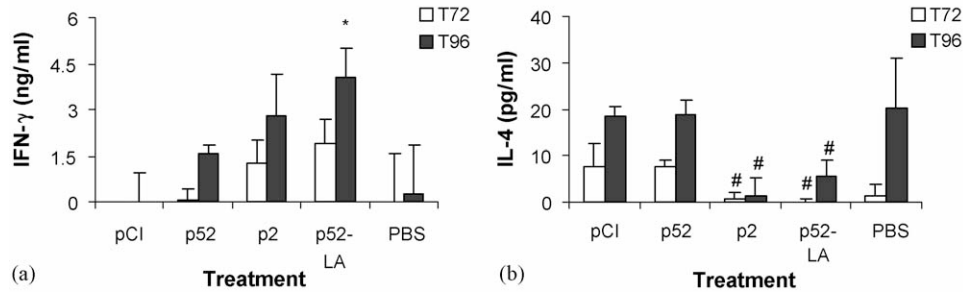


Fig. 4. Splenocytes cytokine production of DNA immunized mice. Mice were immunized with DNA plasmids or PBS on days 0 and 14. Spleens were harvested on day 21 and splenocytes were cultured with 10  $\mu$ g/ml of rDer p 2. Der p 2-specific cells were harvested on day 10 and re-stimulated with rDer p 2 protein. Culture supernatants were collected and assayed for IFN- $\gamma$  (a) and IL-4 (b) production. Results shown are mean  $\pm$  S.E.M.,  $n=4-5$  per group, \* compared with pCI, p2 or p52,  $p<0.05$ ; # compared with p52,  $p<0.05$ . T: time (h).

#### 3.4. Adoptive transfer of DCs from p52 vaccinated mice primed for IgE production

The T-cell cytokine data implied that the p52 construct could induce immune responses of a mixed TH1/TH2 phenotype, but there was some ambiguity in the data obtained. This had prompted us to further address the question by carrying out the DCs transfer experiments. DCs from the various groups of DNA immunized mice were adoptively transferred to the naïve mice, which were subsequently challenged with three subcutaneous injections of rDer p 2 protein. Recipients of DCs from p52 immunized mice showed a significant increase in Der p 2 specific IgE production, which peaked at  $10.3 \pm 3.7$  ELISA units on day 35 ( $p<0.05$ , Fig. 5a), with a corresponding increase in Der p 2 specific IgG1 production

( $2380 \pm 1030$  ELISA units, Fig. 5b). IgE titres were maintained at an average of about  $7 \pm 2$  ELISA units ( $p<0.05$ ) up to day 49. The induction profiles and kinetics of Der p 2 specific IgE and IgG1 production were similar to the humoral responses of mice that only received three subcutaneous injections of rDer p 2 protein described earlier (as shown in Fig. 2a and b). However, a higher magnitude of IgE production was observed in mice receiving DCs from p52-primed mice after the TH2 protein challenge as compared to the solely protein immunized control mice. The data suggested that there was some degree of augmentation of IgE production in mice primed by DCs from p52-immunized mice.

In contrast, such significant priming for specific IgE production was not observed in other groups of mice receiving

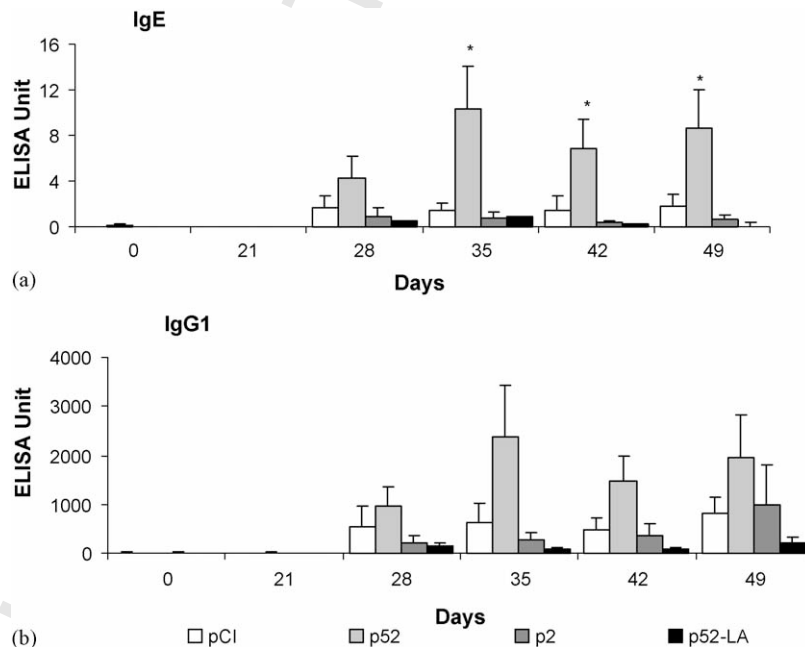


Fig. 5. Humoral response of mice adoptively transferred with DNA primed DCs. Mice were immunized with DNA constructs on days 0 and 14. DCs isolated from the lymph nodes of DNA primed mice were adoptively transferred into the recipient mice at  $1 \times 10^5$  DCs/mouse. Recipient mice were challenged with 10  $\mu$ g of Der p 2 protein on days 13, 17 and 21. Mice were bled and sera were analyzed for Der p 2 specific IgE (a) and IgG1 (b) production. Results shown are mean  $\pm$  S.E.M.,  $n=5-8$  per group, \* compared with pCI, p2 and p52-LA mice,  $p<0.05$ .

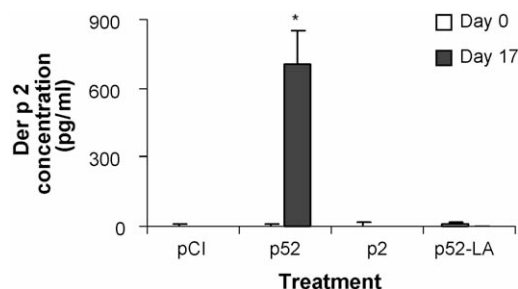


Fig. 6. Quantitation of circulating Der p 2 protein in immunized mice. Mice were immunized with 50  $\mu$ g of pCI, p52, p2, p52-LA constructs on days 0 and 14. Sera were collected on days 0 and 17 for the detection of Der p 2 protein by sandwich ELISA. Results shown are mean  $\pm$  S.E.M.,  $n=7-11$  per group, \*compared with results of pCI, p2 or p52-LA groups,  $p<0.001$ .

DCs from p52-LA and p2 immunized mice upon similar Der p 2 protein challenge. It is also worth noting that mice receiving DCs of pCI immunized mice elicited relatively low levels of specific IgE (1.1–1.8 ELISA units) and specific IgG1 (approximately 500 ELISA units). The magnitude of the TH2-skewed humoral responses in these mice was significantly lower than that seen in mice receiving the p52-primed DCs and the control mice immunized with Der p 2 protein only (Fig. 2a and b). This observation can probably be explained by the non-antigen specific suppressive effects on TH2 responses that were conferred by the CpG motifs of the pCI vector backbone DNA. Only low and insignificant levels of IgG2a were induced in all groups of mice (data not shown).

### 3.5. Circulating Der p 2 protein detected in mice primed with p52

The results from the DCs adoptive transfer experiments strongly suggested that construct p52 could prime a potent TH2-skewed response and induced strong specific IgE production. We postulated that DCs in these mice could be sensitized by the significant levels of secretory Der p 2 produced endogeneously. To confirm this notion, detection of Der p 2 in mouse serum after DNA immunization was performed using Der p 2 sandwich ELISA. As shown in Fig. 6, a significantly high level of circulating Der p 2 protein ( $700 \pm 150$  pg/ml,  $p<0.001$ ) was detected in the sera of mice immunized with construct p52, but not in sera from mice immunized with constructs p2 and p52-LA.

## 4. Discussion

We have evaluated the effects of DNA immunization with the Der p 2 gene alone, Der p 2 gene fused with Der p 5-derived signal peptide sequence, and Der p 2 gene fused with the signal peptide plus the LAMP-1 targeting sequences on the basis of both humoral and cellular responses. The signal peptide sequence of the Der p 2 gene was replaced with that of the Der p 5, as it has been previously shown that the Der p

5 gene vaccination led to induction of high levels of antigen specific TH1 skewed protective immunity [5]. Furthermore, incorporation of the Der p 5 signal peptide sequence in the Der p 1 gene immunization resulted in an enhanced in vivo expression of Der p 1 protein in transfected muscle cells [23], thus making it an ideal candidate signal peptide sequence for this study.

It is well established that the backbone sequences of plasmid DNA vectors containing ISS or CpG motifs that enhance TH1-skewed responses [25]. Previous studies also demonstrated that allergen gene vaccination by the intramuscular route induced TH1-skewed responses and inhibited IgE production [4,5,23]. Our study revealed that mice immunized intramuscularly with the p52 construct exhibited a mixture of TH1 and TH2 responses, an unexpected observation. Priming of TH2 responses in p52 primed mice was first indicated by production of low but detectable levels of Der p 2 specific IgE and significant levels of specific IgG1. It was further confirmed by DC adoptive transfer experiments and the detection of circulating Der p 2 protein in the mouse sera. We propose that the Der p 5 derived signal peptide facilitated a high level expression of Der p 2 in vivo, resulting in leakage or secretion of Der p 2 into systemic circulation, creating a microenvironment with high level of circulatory Der p 2 protein. The APCs such as DCs could then be exogenously primed and conferred TH2 sensitizing effects mimicking an exposure to aeroallergen. Such APCs could be endowed with the TH2 triggering capability but their effects could be partly suppressed by the CpG mediated-TH1 response resulting in a mixed TH1/TH2 phenotype in the p52 immunized mice. We have also ruled out the possibility of interfering effects of T-cells and B-cells in our study, as adoptively transferred T-cells or B-cells failed to induce any significant IgE production upon TH2-skewed protein challenge (data not shown), indicating that the IgE response was induced by the transferred DCs only. It has been reported that antigen-pulsed DCs could exacerbate asthmatic features [26–28] and play an important role in the pathogenesis of allergic disease [18,19]. The administration of OVA-pulsed myeloid DCs to the airways of naive animals induced sensitization to OVA, leading to a vigorous TH2 response after re-challenge of the airways with OVA aerosol [26–28]. Furthermore, vaccination with OVA-pulsed bone marrow DCs or Langerhan cells did not reduce allergen-specific TH2 responses in a murine model of severe atopic asthma [29]. Taken together, our data clearly indicated that the use of a strong signal peptide in DNA construct is highly unfavorable for allergen DNA vaccine design as this priming could potentially lead to undesirable TH2 sensitization.

This study is the first to demonstrate that DNA vaccines that encode the Der p 2 allergen gene fused with a N-terminal signal peptide and a C-terminal LAMP-1 targeting sequence could enhance a strong antigen-specific TH1 response. It has been well established that the LAMP-1 targeting sequence could direct the targeting of the endogenously synthesized protein antigen to an endosomal/lysosomal vesicular pathway

in the transfected APCs and enhanced the delivery of the antigen to the major histocompatibility complex class II (MHC II) compartment of APCs such as DCs [30]. In our study, contrary to p52 and when compared to p2 and pCI vector control, p52-LA DNA priming displayed the highest IFN- $\gamma$  and IgG2a production upon receiving a protein challenge. This strong TH1 response has also been reported in the efficacy study of DNA vaccines containing LAMP [31–33]. Using LAMP for targeting human papillomavirus E7 to the subcellular compartments has led to enhanced vaccine potency by stimulating E7-specific CD4<sup>+</sup> TH1 cell response [31]. The incorporation of the lysosome-targeting signal in HIV gag DNA vaccines has been shown to promote long-term CD4<sup>+</sup> and CD8<sup>+</sup> T-cells memory response and strong antigen-specific TH1 response, as compared to constructs lacking LAMP [32,33]. In our study, p52-LA construct was capable of cellular retention of Der p 2 expressed in vivo, and triggered a strong TH1-skewed response. Although the p2 construct lacking secretory signal peptide might also resulted in cellular retention of Der p 2, a lower magnitude of TH1 response was induced. Specific targeting of the expressed Der p 2 to the lysosomal compartment conferred by the p52-LA construct greatly enhanced the magnitude of TH1 responses after the protein boost. Furthermore, our data revealed that the p2 construct immunization may not induced fully protective immunity in view of the fact that some IgE was produced and the magnitude of TH1 responses were relatively lower than that induced by p52-LA construct upon the protein challenge. This is probably due to the specific MHC II targeted compartment of transfected APCs that enhanced the CD4<sup>+</sup> mediated response, whereas cytoplasmic protein that lacks targeted signaling will be processed and present to MHC I and, thus, less effective in eliciting MHC II responses [33].

This study demonstrated that the use of a strong signal peptide could lead to an enhanced level of circulating allergen that primed TH2-skewed responses, and such risk could be eliminated by the incorporation of the intracellular targeting sequences to direct the allergen into the lysosomal compartment to induce strong TH1 responses without the concurrent induction of TH2 responses. It is worth noting that the priming of the masked TH2 responses by p52 was not clearcut in the primary responses seen in mice, rather our DC adoptive transfer experiments clearly revealed that such DNA vaccination approaches could sensitize mice to produce specific IgE upon subsequent encounters with allergen. Previous proof of concept studies showed that DNA construct encoding the full length Der p 5 gene could induce strong and protective TH1-skewed responses, which could inhibit IgE production and TH2-mediated physiopathologies [5]. However, the potential risk of such construct concurrently priming for low levels of TH2-skewed responses, which could be easily masked by the much stronger TH1-skewed responses, has not been fully addressed using the DCs adoptive transfer approach describes in the current study. The findings derived from the current study caution against the use of signal peptide sequences in the design of DNA constructs for vaccinations against aller-

gic diseases. In addition, this study has shown for the first time that the incorporation of an intracellular targeting signal sequences in DNA vaccine construct could promote a safer and more efficacious responses against allergic reactions. Furthermore, the use of a DNA-prime and protein boost strategy, as shown in our study, could potentially enhance TH1 responses, representing a useful strategy to augment the efficacy of DNA vaccines for allergic diseases in general.

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