

Non-allergenic antigen in allergic sensitization: responses to the mite ferritin heavy chain antigen by allergic and non-allergic subjects

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Summary

Background The majority of house dust mite proteins are non-allergenic. There is, however, no information on the type of immune responses produced to these proteins and if the responses are affected by allergic sensitization.

Objective To identify and produce a non-allergenic antigen of the house dust mite and compare antibody and T cell responses with the responses to allergens in sensitized and non-sensitized individuals.

Results Ferritin heavy chain was cloned from a cDNA library as a candidate non-allergen of the house dust mite. It bound IgG but not IgE in the sera of allergic and non-allergic subjects and induced high T cell proliferative responses that correlated highly with the responses to the major allergen Der p 2. The cytokine response to the non-allergen was characterized by the release of high levels of both Th1 and Th2 cytokines from the PBMC of both allergic and non-allergic subjects. In contrast, the response to Der p 2 showed the expected high level of Th2 cytokine release from the PBMC of allergic subjects, while the Th2 cytokine production from PBMC of non-allergic subjects was low and even lower than that induced by ferritin heavy chain. The levels of IFN- γ release were similar for all groups. Der p 2 induced significantly more IL-10 than ferritin in the non-allergic group.

Conclusion The T cell responses to a non-allergenic protein of the house dust mite were high and strongly correlated with the response to the major allergen. The non-allergenic protein induced high levels of Th1 and Th2 cytokine in both allergic and non-allergic subjects, while the allergen induced high levels of Th2 cytokine in allergic subjects and low levels in non-allergic subjects. The responses to the allergen were thus independently up- and down-regulated with no evidence of bystander regulation.

Keywords allergen, antigen cytokine, ferritin, house dust mite, non-allergen, T cell

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Introduction

Most people produce IgG antibodies to house dust mite (HDM) proteins. The levels are higher in allergic subjects but with a significant overlap with the levels of non-sensitized individuals [1, 2]. For allergic subjects there can be a correlation between IgE and IgG antibodies to single purified allergens, as shown for Der p 1 [3, 4], and for Der p 2 in some [5] but not all studies [6, 7]. The latter result may be due to the affinities and assay systems [8]. Western blotting has shown that the IgE and IgG4 antigen binding profiles are similar [9], while total IgG antibody binding has a different profile [9, 10], which is found for sera from allergic and non-allergic subjects [5, 9]. Little is known about

the nature of any responses to the non-allergenic proteins, how they differ from responses to allergens and if they are affected by allergic sensitization. It is possible that the cytokines induced by allergens and antigens may cross-regulate the responses of each other. Indeed it is possible that high prevalence of allergic sensitization in modern communities [11] is associated with a diminution of protective bystander responses.

cDNA cloning was undertaken to produce a recombinant antigen that could be used for a reproducible comparison with the responses to allergens and the investigation of possible interactions in allergic sensitization. A HDM cDNA library was screened for clones producing highly antigenic polypeptides. One clone described here produced mite ferritin heavy chain in high yield and was used for further investigation. It bound IgG but not IgE in the sera of allergic and non-allergic subjects and induced high T cell proliferative responses that correlated with the responses to Der p 2. The Th1 and Th2 cytokine responses were both high and were not affected by the up- and down-regulation of the responses to allergen.

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Methods

Cloning and sequencing

A *Dermatophagoides pteronyssinus* lambda gt11 cDNA library was screened with a hyperimmune rabbit anti-HDM serum [12]. Clones with high reactivity were purified and were tested for IgE binding with a panel of human allergic sera. The clone 17C7 that did not react with IgE was subcloned into pUC18 for automated DNA sequencing (ABI373 sequencer, Perkin Elmer, Foster City, CA, USA). cDNA encoding the ferritin heavy chain open reading frame was amplified by polymerase chain reaction using the high fidelity *Pfu* and cloned into the pET-11d expression vector (Novagen, Madison, WI, USA).

Antigens and allergens

Tuberculin purified protein derivative (PPD) was purchased from CSL-Ltd, Melbourne, Australia. Recombinant Der p 2, mite ferritin heavy chain and pigeon cytochrome c were expressed using the pET expression system as insoluble inclusion bodies in *Escherichia coli*. They were solubilized in urea, renatured and isolated by affinity chromatography as described by Mueller [13]. Der p 2 was further purified using a Sepharose G-75 column. Protein concentrations were determined by Folins assay.

Study population

Peripheral blood was obtained fresh from 23 adult volunteers (12 allergic and 11 non-allergic). Their allergic status was determined either by skin prick testing with commercial extracts or by measurement of specific IgE to HDM using the Pharmacia CAPS system. Allergic subjects had either skin prick wheal to HDM of ≥ 7 mm or specific IgE to HDM of 5 or greater. Normal subjects had either no significant skin prick response to a battery of common allergens, or undetectable IgE levels to both HDM and rye grass. The CAP analyses were performed by the Immunology Department, Princess Margaret Hospital, Perth, Western Australia. Inhaled and intranasal corticosteroids were permitted for subjects with asthma or allergic rhinitis, respectively. Oral corticosteroids were not permitted within the previous 6 weeks. All subjects provided written informed consent prior to the study. The study was approved by the Ethics Committee of Princess Margaret Hospital.

Peripheral blood mononuclear cell (PBMC) proliferation and cytokine assays

PBMC proliferation and cytokine assays were performed using AIM V medium [14]. Proteins were added to the cell cultures at 30 µg/mL, this optimum concentration being determined by preliminary studies and being the same as previous studies [14]. Inspection of the cultures at 24 h showed no toxicity as judged by trypan blue staining and the viable cell numbers were the same as control cultures. For proliferation and cytokine assays the cells were incubated at 37 °C in 5% CO₂ for 5 days (determined from preliminary experiments).

Cytokine measurement

Cytokines were measured by ELISA. IL-13 and IFN-γ ELISAs were performed using commercial kits (Pelikine, CLB Amsterdam, the Netherlands); IL-5 and IL-10 were measured using paired antibodies from Pharmingen (Becton Dickinson

Pharmingen, North Ryde, NSW, Australia). The sensitivity of the ELISA tests were as follows: IFN-γ, 2 pg/mL; IL-5, 3 pg/mL; IL-13, 2.5 pg/mL; IL-10, 4 pg/mL. Measurements below the sensitivity limit were given a value of 0.

IgG and IgE antibody measurements

EIA plates (Corning Costar, Acton, MA, USA) were coated with an anti-pentaHis monoclonal antibody (Qiagen, Valencia, CA, USA, Catalogue no. 34660) in carbonate buffer 0.1 M pH 9.6 and then blocked with 2% v/v full cream milk. This was to capture the his-tagged recombinant proteins produced by the pET vectors. After washing, 1.5 µg of recombinant protein in 50 µL was incubated in each well overnight at 4 °C. Following washing, serum preabsorbed with an *E. coli* lysate [15] was applied to each well. After washing, anti-human IgG conjugated to horseradish peroxidase (Silenus Laboratories, Boro-nia, Australia) was added to each well for 1 h then washed. The assay was developed with K-Blue tetramethylbenzidine (TMB) substrate (Neogen Corporation, Lansing, MI, USA) and stopped with 1 M H₂SO₄. Absorbance was read at 450 nm. A standard curve of pooled serum known to bind the recombinant protein was constructed and results expressed in arbitrary units. Non-specific IgG binding was assessed with human myeloma IgG.

IgE was measured by dot-blot enhanced chemiluminescence with 2 µg protein per dot, a technique used to test IgE binding at under 1 ng/mL [14].

Statistical analysis

Statistical comparisons were performed using non-parametric testing. Paired data were analysed by Wilcoxon tests. Unpaired data were analysed by the Mann–Whitney *U*-test. Correlation was performed with Spearman's correlation. A two tailed *P*-value of <0.05 was considered significant. Analyses were performed using Prism statistical and graphing package (GraphPad Software Inc.).

Results

Clone 17C7 sequence analysis and expression

The nucleotide sequence of the 813 bp cDNA fragment from clone 17C7 is available from GenBank, accession number AY005807. An open reading frame (ORF) was identified from bases 97–639, starting with a methionine at 97–99 and ending with a stop codon at 637–639. The deduced amino acid sequence revealed a 180 amino acid polypeptide, with no signal peptide and a predicted molecular mass of 20.7 kDa. A comparison with sequences in the GenBank database showed 70% identity with the soft tick *Ornithodoros moubata* ferritin heavy chain, and 67% identity with the hard tick *Ixodes ricinus* ferritin heavy chain. There was also significant identity with ferritins from other species, including bovine heavy chain ferritin (61%) and chicken heavy chain ferritin (62%).

The alignment (Fig. 1) shows the conservation especially at residues 67–89 and 106–160 and the seven residues that form the ferroxidase centre. A conserved N-glycosylation site (NQS) is found at 117–119. There are two cysteine residues, at positions 22 and 164. The cysteine at position 22 is conserved across the tick species and *D. pteronyssinus*. The clone therefore encodes a

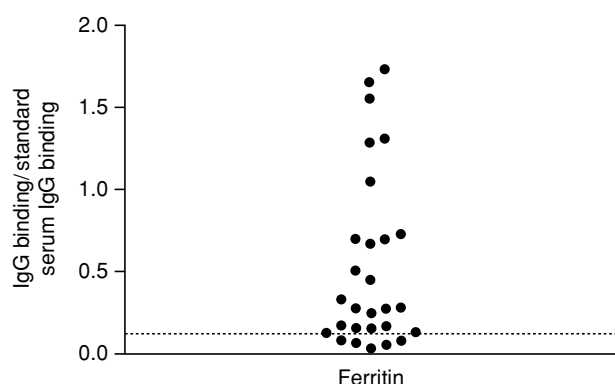


Fig. 2. Human IgG binding to recombinant ferritin. The results show the arbitrary binding units derived from dilutions of a standard reactive serum. The dotted line represents background binding measured using human IgG myeloma. Serum from both allergic and non-allergic subjects are included because there was no difference in their titres.

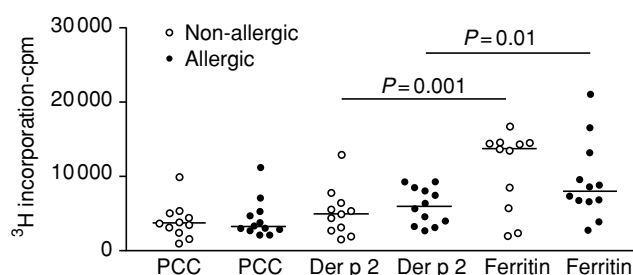


Fig. 3. Proliferative responses of allergic and non-allergic subjects to HDM ferritin heavy chain. Proliferation of PBMC from allergic and non-allergic people is shown by ^3H incorporation (cpm). Each point represents the response of an individual subject. The horizontal line indicates the median for each column. PBMC were stimulated with Der p 2, ferritin heavy chain (ferritin) or the control, pigeon cytochrome c (PCC). The responses of PBMC from allergic and non-allergic subjects were significantly higher to ferritin heavy chain than to Der p 2.

was 9210 cpm (Fig. 4), was significantly higher than the median response to Der p 2 of 5391 cpm ($P < 0.0002$). There was no significant difference in the response to ferritin heavy chain from allergic and non-allergic subjects. There was a very strong correlation between the proliferative responses to ferritin and Der p 2 ($P < 0.0001$) (Fig. 5). The response to PPD was consistently high even in individuals with low responses to the mite proteins (Fig. 4). As a control for the proliferation the ferritin heavy chain did not stimulate the proliferation of a human anti-cockroach T cell line (not shown).

To examine the specificity of the proliferation, responses to horse apoferritin (Sigma, St Louis, MO, USA) were studied in 14 subjects. The proliferation (Fig. 6) was low, except for one subject who had a stimulation index of 7.3. The median stimulation index of 2.15 (25 to 75%, range 1.40 to 2.95) was not significantly different from the control PCC.

Cytokine responses

There was an expected significant difference in IL-5 release in the response to Der p 2 of PBMC from allergic and non-allergic donors ($P < 0.015$) (Fig. 7). The allergic group produced a median of 18.3 pg/mL (25 to 75%, range 5.2 to 26.0) compared with 4.8 pg/mL for the non-allergic group (25 to 75%, range 0 to 12.6). Ferritin induced significant IL-5 release that was similar from the PBMC of both non-allergic (8.7 pg/mL, 25 to 75%,

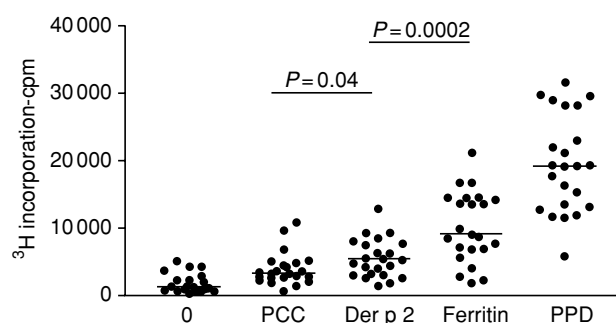


Fig. 4. Proliferative responses of all subjects to HDM ferritin heavy chain. Proliferation of PBMC from allergic and non-allergic people is shown by ^3H incorporation (cpm). Each point represents the response of an individual subject. The horizontal line indicates the median for each column. PBMC were stimulated with Der p 2, ferritin heavy chain (ferritin) or the control, pigeon cytochrome c (PCC). The median responses of PBMC calculated from all subjects were significantly higher to ferritin heavy chain than to Der p 2, although individuals exhibited a wide range of responsiveness. All subjects responded highly to PPD.

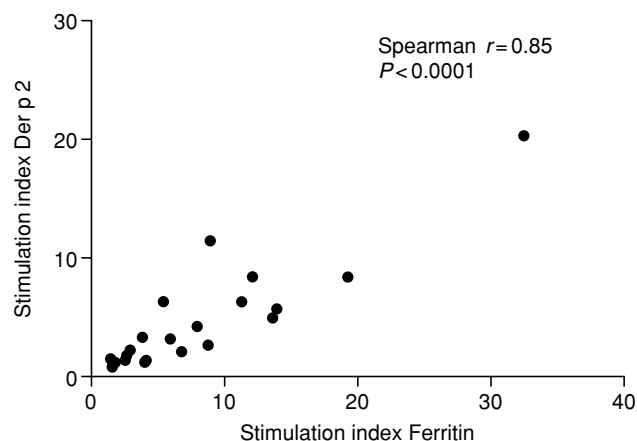


Fig. 5. Correlation of proliferative responses to Der p 2 and mite ferritin heavy chain. The correlation of the proliferation to Der p 2 and ferritin heavy chains is shown as a stimulation index. Both allergic and non-allergic subjects are included because no difference was found in their response. Each point represents an individual. The correlation was strong ($r = 0.85$) and highly significant ($P < 0.0001$).

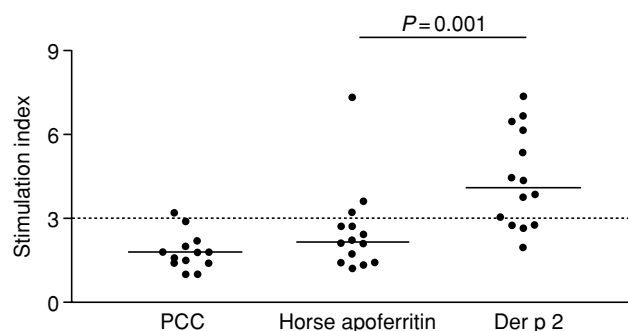


Fig. 6. Proliferative responses to horse apoferritin. Each point represents the stimulation index of the proliferative response of an individual subject. The horizontal line indicates the column median. The median response to horse apoferritin was numerically and statistically similar to the control pigeon cytochrome c (PCC), while Der p 2 induce positive stimulation. The individual with the SI of 7.3 to horse apoferritin has a history of allergy to horses.

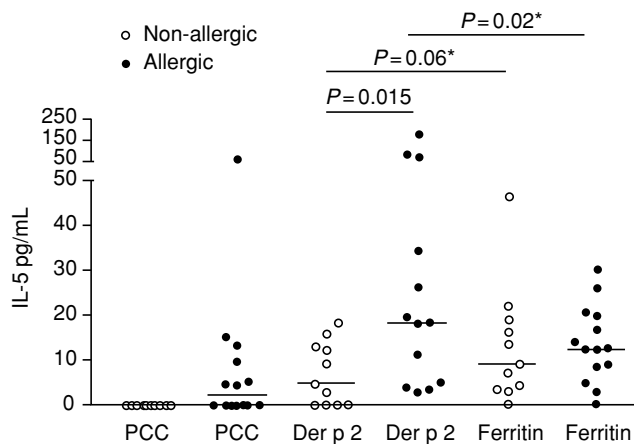


Fig. 7. Production of IL-5 in response to HDM ferritin. IL-5 release was measured in the supernatants of PBMC from allergic and non-allergic subjects stimulated with Der p 2, ferritin heavy chain (ferritin) or the control, pigeon cytochrome c (PCC). Each point represents the response of an individual subject. The horizontal line indicates the median for each column. Significant release was found for all groups stimulated by the mite proteins. The IL-5 release from PBMC from allergic subjects stimulated with Der p 2 was significantly higher than the release of all other groups.

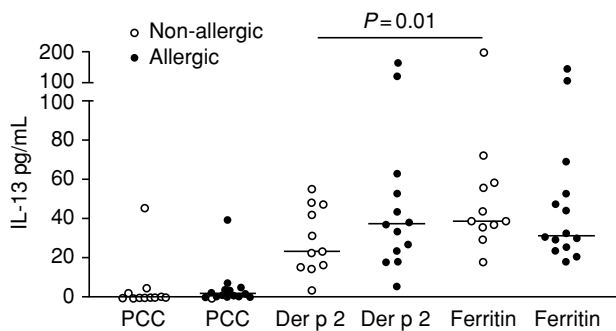


Fig. 8. Production of IL-13 in response to HDM ferritin. IL-13 release was measured in the supernatants of PBMC from allergic and non-allergic subjects stimulated with Der p 2, ferritin heavy chain (ferritin) or the control, pigeon cytochrome c (PCC). Each point represents the response of an individual subject. The horizontal line indicates the median for each column. Significant release was found for all groups stimulated by the mite proteins. The median release of all groups was similar, although the IL-13 produced by non-allergic PBMC to ferritin heavy chain was significantly higher than that induced by Der p 2.

range 3.7 to 17.3) and allergic subjects (12.1 pg/mL, 25 to 75%, range 8.2 to 13.9). The IL-5 release from PBMC of non-allergic subjects tended to be higher when stimulated with ferritin heavy chain than with Der p 2 ($P < 0.06$).

Both Der p 2 and ferritin heavy chain induced significant and substantial IL-13 release compared with the blank and PCC controls (Fig. 8). There was no significant difference in the release induced by ferritin or Der p 2 in allergic subjects, or a difference between the allergic and non-allergic groups. In the non-allergic group, however, the production of IL-13 was higher in response to ferritin than in response to Der p 2 ($P < 0.01$).

The PBMC of both allergic and non-allergic subjects produced large quantities of IFN- γ when stimulated by ferritin heavy chain (Fig. 9). This was not statistically significantly different from the IFN- γ induced by Der p 2. The PBMC from several allergic subjects released low levels in response to Der p 2 but the titres were variable.

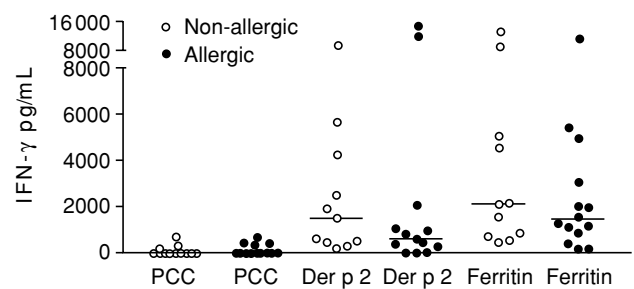


Fig. 9. Production of interferon- γ in response to HDM ferritin. Interferon- γ release was measured in the supernatants of PBMC from allergic and non-allergic subjects stimulated with Der p 2, ferritin heavy chain (ferritin) or the control, pigeon cytochrome c (PCC). Each point represents the response of an individual subject. The horizontal line indicates the median for each column. Significant release was found for all groups stimulated by the mite proteins but there were no significant differences between the groups.

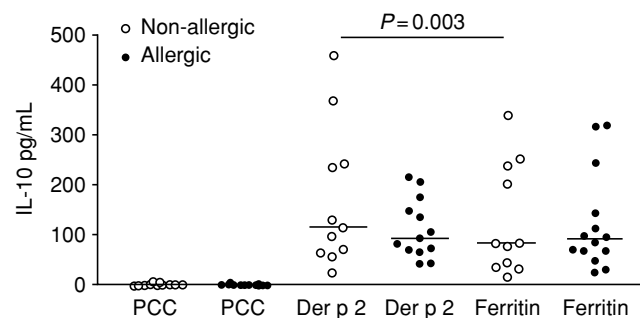


Fig. 10. Production of IL-10 in response to HDM ferritin. IL-10 release was measured in the supernatants of PBMC from allergic and non-allergic subjects stimulated with Der p 2, ferritin heavy chain (ferritin) or the control, pigeon cytochrome c (PCC). Each point represents the response of an individual subject. The horizontal line indicates the median for each column. Significant release was found for all groups stimulated by the mite proteins. The median release of all groups was similar, although the IL-10 produced by non-allergic PBMC to Der p 2 was significantly higher than that induced by ferritin heavy chain.

The IL-10 release induced by Der p 2 and ferritin heavy chain did not show any differences when the responses between PBMC from allergic and non-allergic subjects were compared for each stimulus (Fig. 10). The PBMC of non-allergic individuals, however, released significantly more IL-10 in the response to Der p 2 than the response to ferritin heavy chain.

Discussion

Immunoblotting studies have demonstrated that HDM extracts contain antigens that bind IgG but not IgE antibodies [9]. The immunostaining of these protein bands had a similar pattern and intensity with sera from allergic and non-allergic subjects. This differs from the responses to known allergens, including Der p 1, where IgG antibodies were predominantly found in allergic subjects [3, 4, 16]. There are data showing that Der p 2 induces IgG in allergic and non-allergic subjects [6, 7] but the affinity may differ [8]. Studies with birch have shown that minor allergens bind more IgG antibody than the major Bet v 1 allergen [17] and the IgG binding was similar for sera from allergic and non-allergic subjects. There is therefore evidence that both mite and pollens have antigens that induce IgG antibody but

are usually not allergenic. The T cell responses to these non-allergens have not been investigated.

cDNA cloning was used to isolate a non-allergenic mite antigen that could be used for detailed and reproducible studies. The mite ferritin heavy chain induced *in vitro* proliferative responses in PBMC, which were larger than that found to the major allergen Der p 2. IgG antibodies could readily be detected in the sera of most people irrespective of their allergic status. The PBMC response was not the result of mitogenic stimulation because a significant number of people responded strongly to PPD but not to ferritin heavy chain and it did not stimulate a control T cell line. The magnitude of the proliferative response to ferritin heavy chain correlated strongly with the magnitude of response to the major allergen Der p 2, indicating a common source of sensitization. The sequence of mite ferritin heavy chain had 70% identity to the tick homologue and 60% identity to mammalian homologues. This degree of identity is not indicative of a high level of cross-reactivity, but the ferritin heavy chains do contain some large blocks of conserved sequence that could contain common epitopes. The poor response to horse apoferritin, however, shows that the conserved residues do not induce T cell cross-reactivity. The sequence outside these blocks is highly variable, even between mites and ticks, so the responses are not cross-reactivities to other sources of ferritin such as meat.

The cytokine release data showed that the non-allergenic nature of ferritin heavy chain is not associated with poor T cell responses or strong Th1-biased responses, but rather that it induced a balance of both Th1 and Th2 cytokines. As found by others, it was not possible to measure IL-4 release in PBMC cultures, even to Der p 2, but the Th2 cytokines IL-5 and IL-13 could be readily measured. The balanced cytokine response was the same in both allergic and non-allergic subjects, even though the allergen induced high Th2 responses in allergic subjects. This was also evident when the results of individuals were calculated as Th1/Th2 cytokine ratios (not shown). Der p 2 induced the expected high Th2 responses in PBMC of allergic subjects and interestingly the Th2 response from the PBMC of non-allergic subjects was, as shown for IL-13 and IL-5, even lower than that induced by ferritin heavy chain. This reduction suggests that there may be an allergen-specific down-regulation of Th2 responses in non-allergic subjects. A possible mechanism for non-allergenicity of the ferritin heavy chain is that it could induce the regulatory cytokine IL-10, which could modify responses. This has been predicted to occur for high-dose exposure to cat allergens [18]. The level of IL-10 induced by ferritin was, however, similar to that induced by Der p 2 and there was no difference in the release from PBMC from allergic and non-allergic subjects. The PBMC from non-allergic subjects did, however, release more IL-10 in response to Der p 2 than to ferritin heavy chain, which could be related to reduced Th2 responses to the allergen.

The ferritin heavy chain has provided a HDM antigen that was easily produced and readily induced T cell stimulation. The fact that the mite polypeptide is a heavy chain distinguishes it from the secretory ferritins, which are abundant proteins in insect haemolymph and fat bodies, and hence may be found in the environment [19]. The antigenicity of the recombinant ferritin heavy chain here is in keeping with X-ray crystallographic [20] and antigenic studies [21] showing recombinant ferritin heavy chain can be readily produced with the natural folding

and antigenic conformation. The ferritin heavy chain has indeed been used as partner for expressing recombinant fusion polypeptides [22].

The data here provide the first information on responses to a non-allergenic protein in an allergen source. By analogy with responses to other complex antigens, some interaction between the cytokines induced by allergens and non-allergens could be anticipated. For example, the Th2 responses of susceptible BALB/c mice to *Leishmania major* are the result of one antigen, LACK, inducing high levels of IL-4 that cross-regulate the responses to the protective antigens of the parasite [23]. Mice that do not respond to the LACK antigen make Th1 protective responses and survive infection. Here, the proliferative responses to ferritin heavy chain were highly correlated with the response to Der p 2, but there was no evidence of an interaction of the cytokine responses with the up- and down-regulation of the responses to the allergen during sensitization and protection.

Acknowledgements

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