AAIR

Original Article

Check for updates

Effects of Ser47-Point Mutation on Conformation Structure and Allergenicity of the Allergen of Der p 2, a Major House Dust Mite Allergen

Bhakkawarat Kulwanich [iD],[1] Sasipa Thanyaratsrisakul [iD],[2] Orathai Jirapongsananuruk [iD],[3] Belinda J. Hales [iD],[4] Wayne R. Thomas [iD],[4] Surapon Piboonpocanun [iD][1*]

[1]Institute of Molecular Biosciences, Mahidol University, Salaya, NakornPathom, Thailand
[2]Department of Medicine, National Jewish Health, Denver, CO, USA
[3]Department of Pediatrics, Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand
[4]Telethon Kids Institute, The University of Western Australia, Perth, WA Australia

OPEN ACCESS

Correspondence to
Surapon Piboonpocanun, PhD
Institute of Molecular Biosciences, Mahidol University, 999 Phutthamonthon Sai 4 Road, Tambon Salaya, Nakhon Pathom 73170, Thailand.
Tel: +66-2-441-9003 (ext. 1233)
E-mail: piboons@gmail.com

ORCID iDs
Bhakkawarat Kulwanich [iD]
https://orcid.org/0000-0003-0367-6500
Sasipa Thanyaratsrisakul [iD]
https://orcid.org/0000-0002-2188-5990
Orathai Jirapongsananuruk [iD]
https://orcid.org/0000-0002-1603-1106
Belinda J. Hales [iD]
https://orcid.org/0000-0003-2193-3996

ABSTRACT

Purpose: Hypoallergenic recombinant Der p 2 has been produced by various genetic manipulations, but mutation of a naturally polymorphic amino acid residue known to affect IgE binding has not been studied. This study aimed to determine the effect of a point mutation (S47W) of residue 47 of Der p 2 on its structure and immunoglobulin (Ig) E binding. Its ability to induce pro-inflammatory responses and to induce blocking IgG antibody was also determined.

Methods: S47 of recombinant Der p 2.0110, one of the predominant variants in Bangkok, was mutated to W (S47W). S47W secreted from *Pichia pastoris* was examined for secondary structure and for the formation of a hydrophobic cavity by 8-Anilino-1-naphthalenesulfonic acid (ANS) staining. Monoclonal and human IgE-antibody binding was determined by enzyme-linked immunosorbent assay. Allergen-induced degranulation by human epsilon receptor expressed-rat basophil was determined. Stimulation of the pro-inflammatory cytokine interleukin (IL)-8 release from human bronchial epithelial (BEAS2B) cells and inhibition of IgE binding to the wild type allergen by S47W-induced IgG were determined.

Results: S47W reduced secondary structure and failed to bind the hydrophobic ANS ligand as well as a monoclonal antibody known to be dependent on the nature of the side chain of residue 114 in an adjacent loop. It could also not stimulate IL-8 release from BEAS2B cells. IgE from house dust mite (HDM)-allergic Thais bound S47W with 100-fold weaker avidity, whereas IgE of HDM-allergic Australians did not. S47W still induced basophil degranulation, although requiring higher concentrations for some subjects. Anti-S47W antiserum-immunized mice blocked the binding of human IgE to wild type Der p 2.

Conclusions: The mutant S47W had altered structure and reduced ability to stimulate pro-inflammatory responses and to bind IgE, but retained its ability to induce blocking antibodies. It thus represents a hypoallergen produced by a single mutation of a non-solvent-accessible amino acid.

Keywords: Airway allergic reaction; point mutated Der p 2; hypoallergenic allergen

Wayne R. Thomas [ID]
https://orcid.org/0000-0001-9939-7682
Surapon Piboonpocanun [ID]
https://orcid.org/0000-0003-4374-8003

INTRODUCTION

Genetically modified allergens with reduced immunoglobulin (Ig) E-binding activity have the potential to reduce allergic side effects in immunotherapy and to facilitate treatment with rapid up-dosing and new methods of delivery. Their therapeutic potential has been demonstrated by their ability to induce IgG-blocking antibodies and to modify Th2-cell-mediated hypersensitivity[1,2] and further modifications that reduce interactions with the innate immune system might also favor the stimulation of immune down-regulatory pathways instead of hypersensitivity pathways.

A major house dust mite (HDM) allergen, Der p 2, is an ML-domain protein that consists of 2 β sheets folded over, with disulfide bonds, into a clam-like structure to create a lipid binding cavity.[3] Strategies for modifying Der p 2 have included fragmentation (4), fusion with other proteins,[4,5] abrogation of disulfide bonding[6,7] and the mutation of surface-exposed residues.[8,9] Much of the known natural amino acid sequence variation of Der p 2 has been found to be restricted to defined changes in amino acids at positions 40, 47, 111 and 114[10,11] that are located in or adjacent to the loop structures at one pole of the molecule.[2] Since the natural substitutions in these positions are known to affect antibody binding,[12,13] disruption in this region might also be effective. The substitution of D with N in amino acid 114 has been shown to markedly affect monoclonal antibody binding but with only a small effect on human IgE binding.[12] In comparison of titrations between Der p 2.0101 (D20101) and Der p 2.0107 (D20107) which only differ in position 47, substitution of S with T reduced the median IgE binding of patient's sera to half.[13] Large differences in IgE binding by different variants have also been demonstrated in Korea,[14] although the comparisons made did not pinpoint a single amino acid substitution.

The first described variant (D20101) of Der p 2 has been used internationally both as a reference sequence and for the production of genetically engineered allergens. However, it was obtained from a long-term culture of undocumented origin kept by the pharmaceutical company CSL Ltd. (Melbourne, Australia), so its relevance to naturally occurring variants is uncertain. Studies of HDM found in homes have shown that while the D20101 and similar sequences could readily be found in Australia[10] variants with even a resemblance to D20101 were absent in the homes examined in Bangkok.[11] Studies of domestic HDM from other geographical locations have not been reported, although a large study from a colony kept in Korea[15] found that only 1/60 variants characterised was the canonical D20101 and, from the amino acids at positions 40, 47, 111 and 114, a total of 10/60 would be D20101-like.[15] Comparisons of IgE binding by different variants with sera from subjects in Perth in Australia and from Bangkok in Thailand have not only revealed differences due to the variant used for measurement but also variations in titer that might be attributed to exposure to different variants.[16]

The study here reports the effects of making an unnatural substitution at the poorly solvent-accessible position 47 and describes its effects on secondary structure, lipid binding, IgE binding, inflammatory interactions with epithelial cells and its ability to induce IgG-blocking antibodies. The mutation of serine to the large hydrophobic tryptophan at position 47 was chosen for experimentation because position 47 was naturally buried in a hydrophobic environment and because although tryptophan has a high propensity to disrupt structure,[17] it does so by more local effects compared to the more global effects of the highly disruptive of proline and aspartic acid that directly impinge on the peptide backbone and change the charge.

MATERIALS AND METHODS

Sera

The use of sera from skin-prick positive HDM-allergic donors for IgE binding was approved by the Institutional Review Board at Siriraj Hospital (SiEc459/2008), Thailand and the Princess Margaret Hospital Ethics Committee, Perth, Western Australia (1347/EP). All donors gave written consent. Sera were selected on the basis of a positive skin prick test to *Dermatophagoides pteronyssinus* and/or *Dermatophagoides farinae* extracts with a wheal diameter >3 mm. Perth donors had specific anti-HDM IgE levels >10 kU/L as measured by ImmunoCAP. ImmunCAP data was not available for the Thai donors, but they showed IgE binding to rDer p 2 by enzyme-linked immunosorbent assay (ELISA) with absorbance values at 450 nm >0.2 optical density (OD) (**Tables 1** and **2**).

Mouse IgG antibody against recombinant Der p 2 variant 2.0110 (D20110) and S47W were raised through an antibody production service by Biomedical Technology Research Center, Faculty of Associated Medical Sciences, ChiangMai University. Briefly, purified recombinant D20110 and S47W were mixed with complete Freud's adjuvant (Sigma-Aldrich, St. Louis, MO, USA) and injected subcutaneously into four 6-weeks-old BALB/c mice (100 μg per dose). The immunizations were repeated with the same antigen using incomplete Freud's adjuvant (Sigma-Aldrich) at 2-week intervals for 2 more immunizations. Blood samples were collected. Mouse sera were titrated for D20110 anti-serum and anti-S47W antiserum- by binding ELISA.

Allergens

Site-directed mutagenesis was performed based on the quick change protocol (Strategene, La Jolla, CA, USA) using pPICzα harboring D20110 cDNA as a template. Specific primers were designed to change TCA (S) to TTT (W) as shown below.

5′ CGAAGCCAACCAAAAC<u>TTT</u>AAAACCGCTAAAATTG 3′

Table 1. Serum specific IgE against HDM extract or Der p 20104. Specific IgE levels of Perth donors measured by ImmunoCAP

Perth donor	HDM (kU/L)
1	100
2	10.7
3	10.3
4	18.1
5	51.35
6	61.8

Ig, immunoglobulin; HDM, house dust mite.

Table 2. Serum specific IgE against HDM extract or Der p 20104. Specific IgE levels of Thai donors measured by direct binding to Der p 20104 (values are mean ± SE from 4 independent assays)

Thai donor	OD 650 nm
1	0.62245 ± 0.066
2	1.6478 ± 0.038
3	1.186525 ± 0.139
4	1.471075 ± 0.005
5	1.375 ± 0.12
6	1.795 ± 0.008
7	0.936175 ± 0.054
8	1.0037 ± 0.018
9	0.684875 ± 0.005
10	0.854 ± 0.145

Ig, immunoglobulin; HDM, house dust mite; SE, standard error; OD, optical density.

Circular dichroism (CD spectroscopy)

Protein samples were prepared at a final concentration of 10 μM in phosphate-buffered saline (PBS). CD spectra were recorded from 260 to 190 nm at 0.02-cm path length, 1-nm resolution, with a scan speed of 50 nm/min on a Jasco J-815-150S spectropolarimeter. The CD data were used in calculations of secondary structure using the *CDPro* suite of programs.

Hydrophobic staining of Der p 2 with 1,8-ANS

Staining Der p 2 with 1-anilinonaphthalene 8-sulfonic acid (ANS; Sigma-Aldrich) to measure the hydrophobic binding capacity was conducted as before.[11,16] Briefly, ANS was dissolved in methanol at a concentration predetermined from an absorbance value at 372 nm using a molar extinction coefficient of 8,000 M^{-1}.[11,16] The mixture of 200 μM ANS and 3.5 μM D20110 or S47W was incubated for 10 minutes at 25℃ before determining the emission spectra of ANS on a Jasco FP-6300 fluorometer. ANS was excited at 390 nm with a 5-nm slit width, and the emission spectra were measured from 400–600 nm with a 5-nm slit width.

Inhibition of human IgE binding to Der p 2 by D20110 and S47W

The inhibition of IgE-antibody binding to D20110 coated on ELISA plates by D20110 and the S47W was performed as follows: 500 ng of D20110 in PBS were added per well on 96-well Maxisorb plates (Nunc, Rochester, NY, USA) and incubated at 4℃ overnight. Sera of HDM-allergic donors were diluted 1:8 to 1:32 based on pre-determined levels of IgE binding to D20110. Diluted sera were incubated with serially diluted D20110 or S47W in PBS-A (PBS containing 3% skim milk, 0.05% Tween 20) at 4℃ overnight. D20110-coated 96-well plate was washed with PBS-A. The absorbed sera were centrifuged at 17,210 g for 10 minutes before the supernatant was added to D20110-coated 96-well plate and incubated at room temperature for 2 hours. For the assays conducted with T,hai sera the IgE binding was developed with horseradish peroxidase (HRP)-labelled goat IgG anti-human IgE antibodies as previously described,[16] and for the assays conducted with sera from Perth donors the binding was developed with monoclonal biotinylated anti-IgE and europium-conjugated streptavidin as previously described.[13]

The results were calculated as mean and standard error of the percent inhibition obtained from each of the different sera.

Binding of mouse anti-D20110, mouse anti-S47W and monoclonal anti-Der p 2 to D20110 and S47W

Recombinant D20110 and S47W in PBS were added at 500 ng per well on 96-well Maxisorb plates (Nunc) and incubated at 4℃ overnight. Sera from D20110 or S47W immunized mice were diluted 1:50 in PBS-A. The allergen-coated 96-well plates were washed with PBS-A. Diluted sera were added and incubated at room temperature for 2 hours. The allergen 96-well plates were then washed with PBS-A before 1:1,000 diluted HRP-labelled goat IgG anti-mouse IgG (H+L) antibodies)KPL, Milford, MA, USA) in PBS-A was added and incubated at room temperature for 1 hour. Bound antibody was detected with 3,3′,5,5′-Tetramethylbenzidine (TMB) by measuring absorbance at 650 nm. The results were calculated as mean and standard error of the percent inhibitions obtained from 4-6 experiments.

The same sandwich ELISA was used to measure the binding of the 1D8 monoclonal anti-Der p 2 antibody (Indoor Biotechnologies, Cardiff, UK) which was added at 100 ng/mL. The variants 20101 and 20104 were included as negative and positive controls respectively based on their known binding characteristics.

Inhibition of human IgE binding to Der p 2 by mouse anti-D20110 or S47W IgG

Recombinant D20110 in PBS was added at 500 ng per well on 96-well Maxisorb plates (Nunc) and incubated at 4°C overnight. Sera from D20110 or S47W immunized mice were added at dilutions of 1:50, 1:100, and 1:1,000 in PBS-A and incubated at room temperature for 2 hours. Plates were washed 3 times with PBS-A and 1:8 to 1:32 dilutions of sera from 9 HDM-allergic donors based on predetermined levels of direct IgE binding to D20110 were added and incubated at room temperature for 2 hours. HRP-labelled goat IgG anti-human IgE antibody (KPL) diluted 1:1,000 in PBS-A was added and incubated at room temperature for 2 hours. Bound antibody was detected with TMB by measuring absorbance at 650 nm. The results were calculated as mean and standard error of the percent inhibitions obtained from each of the different sera.

Bronchial epithelial cell culture and stimulation with Der p 2

The human bronchial epithelial cell line BEAS-2B immortalized by a replication defective hybrid of adenovirus and SV40 exhibits squamous cell differentiation. It was purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained in DMEM/F12-1 (DMEM/F12 supplemented with 15 mM HEPES, 2.85 mM L-glutamine (Millipore, Bedford, MA, USA), 5% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin and 1.25 µg/mL amphotericin B. Cells were grown in humidified atmosphere with 5% CO_2 at 37°C until 80%–90% confluence was reached. To examine their response to inflammatory stimuli, the DMEM/F12-1 was replaced with LHC-9 (Gibco, ThermoFisher Scientific) containing 5% heat-inactivated FBS and the cells were seeded on 24-well culture plates at 1×10^5 cells/well and incubated overnight. The attached BEAS-2B cells were washed twice with 20 mM HEPES-BSS then incubated in RPMI-1640-1 (RPMI-1640 supplemented with 25 mM HEPES, 2 mM L-glutamine, 5% heat-inactivated FBS) overnight. They were then washed twice with 20 mM HEPES-BSS and further incubated with D20110 and the S47W diluted in serum-free RPMI-1640 for 24 hours under 5% CO_2 at 37°C. Initial experiments showed that a dose of 25 µg/mL allergen was required to achieve sufficient IL-8 release for the inhibition assays and that lower doses induced proportionally less cytokines. The synthetic triacylated lipoprotein-TLR1/2 ligand, Pam3CSK4 (invivoGen, SanDiegp, CA, USA) was used as a positive control, and rat anti-human TLR2 IgG antibodies (invivoGen) diluted in serum-free RPMI-1640 were added for the experiments that examined the blocking of TLR2 binding.

IL-8 measurement by ELISA

The concentration of IL-8 secreted into the culture medium from stimulated-BEAS-2B was assessed using human CXCL8/IL-8 DuoSet ELISA (R&D Systems, Minneapolis, MN, USA). Briefly, the capture antibody anti-human IL-8 was coated onto a 96-well microplate and incubated overnight, and then the plates were washed and incubated with PBS containing 1% bovine serum albumin (BSA) for at least 1 hour. Various concentrations of human IL-8 and cultured supernatants were added and incubated at room temperature for 2 hours. The detection antibody was added and incubated at room temperature for 2 hours. Streptavidin-HRP was added and incubated at room temperature for 20 min. The substrate solution was then added and the reaction was stopped by 2N sulfuric acid. The absorbance was recorded at 450 and 570 nm. The final value was calculated from the values at 450 minus the values at 570 nm.

Basophil degranulation assay

RBL-SX38 cells that express the rat high-affinity IgE receptor Fcε-RI and have additionally been transfected with a functional human Fcε-RI were maintained in MEM 1 (MEM supplemented

with 10% heat-inactivated FCS, glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin solution). For the degranulation assay, the RBL-SX38 cells were cultured in MEM containing 0.4 mg/mL geneticin (G418) until the cells reached 85%–95% confluence. They were then incubated in MEM 1 for another 36 hr before being harvested with 0.05% Trypsin/EDTA solution. The cells were re-suspended in MEM 1, plated on 200,000 cells/well of 48-well plate, and incubated overnight with serially diluted sera. All cells were washed with PIPES buffer (140 mM NaCl, 5 mM KCl, 0.6 mM $MgCl_2$, 1.0 mM $CaCl_2$, 5.5 mM glucose, 0.1% BSA and 10 mM PIPES, pH 7.4).

Various concentrations (1, 10, 100 and 1,000 ng/mL) of D20110 and S47W or diluted goat anti-human IgE antibody, as a positive control, were added at 50 µL in pre-warmed PIPES buffer to each well and the cells were incubated for 30 minutes at 37°C. For background degranulation, cells incubated with PIPES without serum and cells incubated with serum without allergen were included. Supernatants were collected and attached cells were lysed with 0.2% Triton X-100.

The activity of β-hexosaminidase in the medium and within the cells was determined by adding 0.1 mM 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide in 100 mM citrate, pH 4.5 and incubated for 30 minutes at 37°C. The reaction was stopped with 0.25 M glycine. The fluorescence was measured by using 380-nm excitation and 440-nm emission filters.

The degranulation levels were calculated from the following formula.

$$R (\%) = (Rsup - blank)/(Rsup - blank + Rppt - blank) \times 100$$

where R is the degranulation level; Rsup is released enzyme activity in the supernatant; Rppt is residual enzyme activity within the cell; blank is the buffer control.

Statistical analysis

Results were analyzed by 1-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test to determine the statistical significance using GraphPad Prism (GraphPad Software, La Jolla, CA, USA).

RESULTS

Recombinant D20110 and S47W were observed to be monomeric proteins when purified through the size-exclusion column. The analysis of their CD spectra showed that the composition of the secondary structure of the S47W was 5.7% alpha helix, 38.9% β-sheet, and 55.4% random coil compared to that of D20110 which was 4.4% α-helix, 58.9% β-sheet, and 36.6% random coil (**Fig. 1A**). The fluorescent of ANS bound to the S47W that had a λmax of 475 nm had a low fluorescent intensity of 13.01 arbitrary units (a.u.) compared to that of ANS bound to D20110 that had a λmax of 469 nm with a high fluorescent intensity 168.32 a.u. (**Fig. 1B**).

The results showed that the 1D8-monoclonal antibody bound to D20110 with an average $OD_{650\,nm}$ of 1.1, whereas it did not bind to the S47W $OD_{650\,nm}$ <0.05 (**Fig. 1C**). For the controls, the 1D8 bound to D20104 with an average $OD_{650\,nm}$ of 1.2, while it bound to D20101 with an average of 0.25 $OD_{650\,nm}$ (**Fig. 1C**).
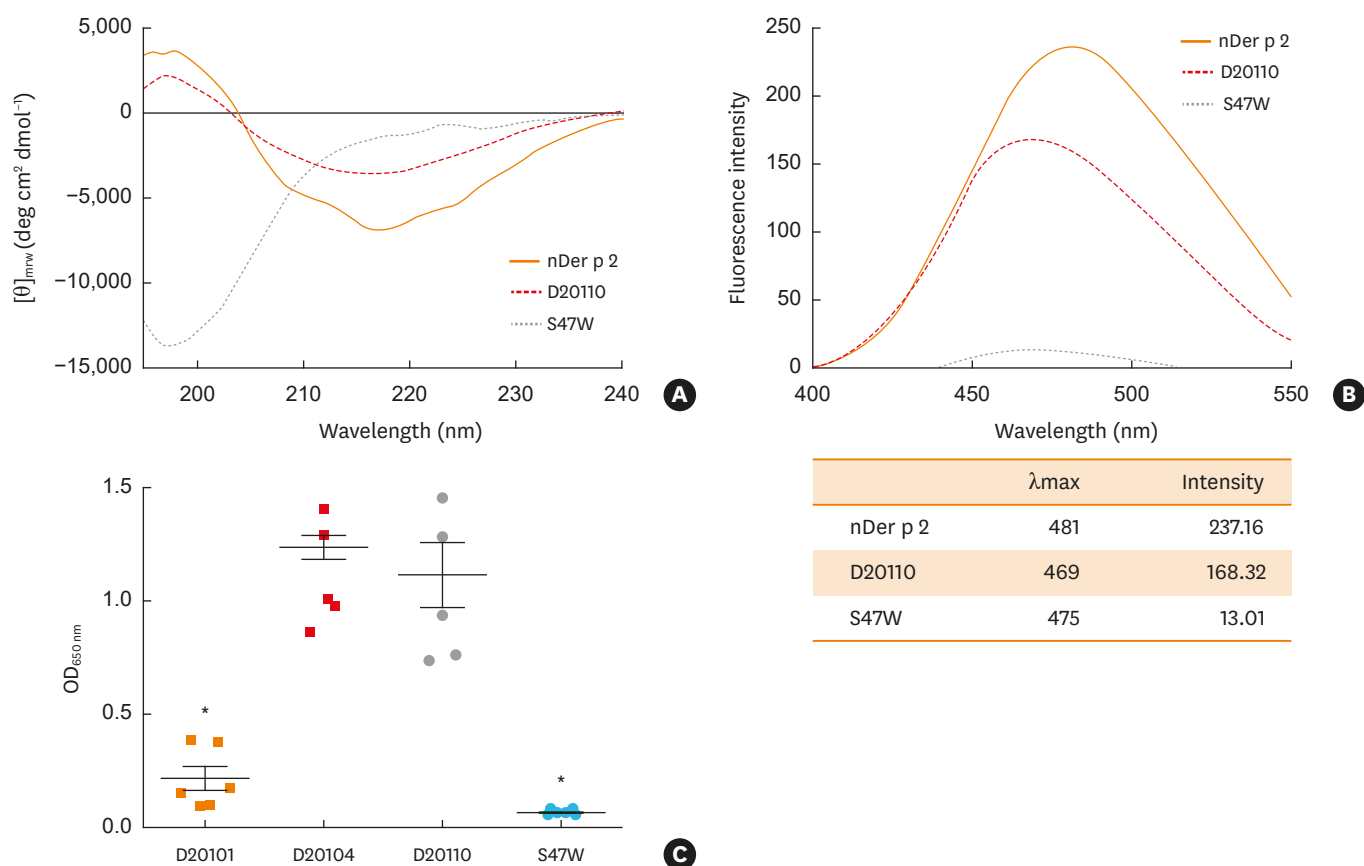
Fig. 1. (A) CD spectra of nDer p 2, D20110 and S47W. (B) Fluorescent spectra of ANS stained with (a) nDer p 2, (b) D20110, and (c) S47W. The fluorescent spectra were recorded from 400–550 nm, with excitation at 390 nm. The λmax of each protein and the respective intensity are showed in **Tables 1** and **2**. (C) Indirect binding of 1D8 mAb to Der p 2 isoforms: D20101, D20104, D20110 and S47W. Results represent mean ± SE of 5–6 experiments.
CD, circular dichroism; ANS, 8-Anilino-1-naphthalenesulfonic acid; SE, standard error.
*$P < 0.0001$.

The IgE assay showed that IgE of the Bangkok atopics bound to the coated D20110 with average OD_{650nm} value of 0.503 but bound to the coated S47W with average OD_{650nm} value of 0.12 (**Fig. 2A**). The results from IgE of the Perth atopics bound to coated D20110 with average 5,500 europium whereas IgE bound to the coated S47W with average 1,000 europium (**Fig. 2B**).

The ELISA assays showed that both D20110 and S47W inhibited IgE binding to D20110 when sera from the Bangkok atopics were examined, but that while D20110 inhibited the IgE binding with an IC_{50} of 20 ng/mL, the S47W only inhibited IgE binding with an IC_{50} of 1,330 ng/mL (**Fig. 2A**). This difference appeared more pronounced when the inhibition assays were conducted for IgE binding from the Perth atopics. The results showed that while D20110 inhibited IgE binding with an IC_{50} of 8.61 ng/mL, the S47W did not inhibit IgE binding at 100 ng/mL compared to the 30%–40% inhibition found for the Thai sera (**Fig. 2B**). It should be noted that all of the sera from Perth had over 10 IU/mL of IgE binding to natural Der p 2.

The basophil degranulation experiments were conducted with rat basophil RBL-SX38 primed with IgE from the sera of 4 HDM atopic subjects from Bangkok. The results showed that the background degranulation was 10%, while D20110 and the S47W, added at 1 μg/mL, induced 35% and 40% degranulation, respectively (**Fig. 3A**). In comparison, the anti-IgE antibody at 1 μg/mL induced 30% degranulation (**Fig. 3A**). The results of basophils primed with serum IgE
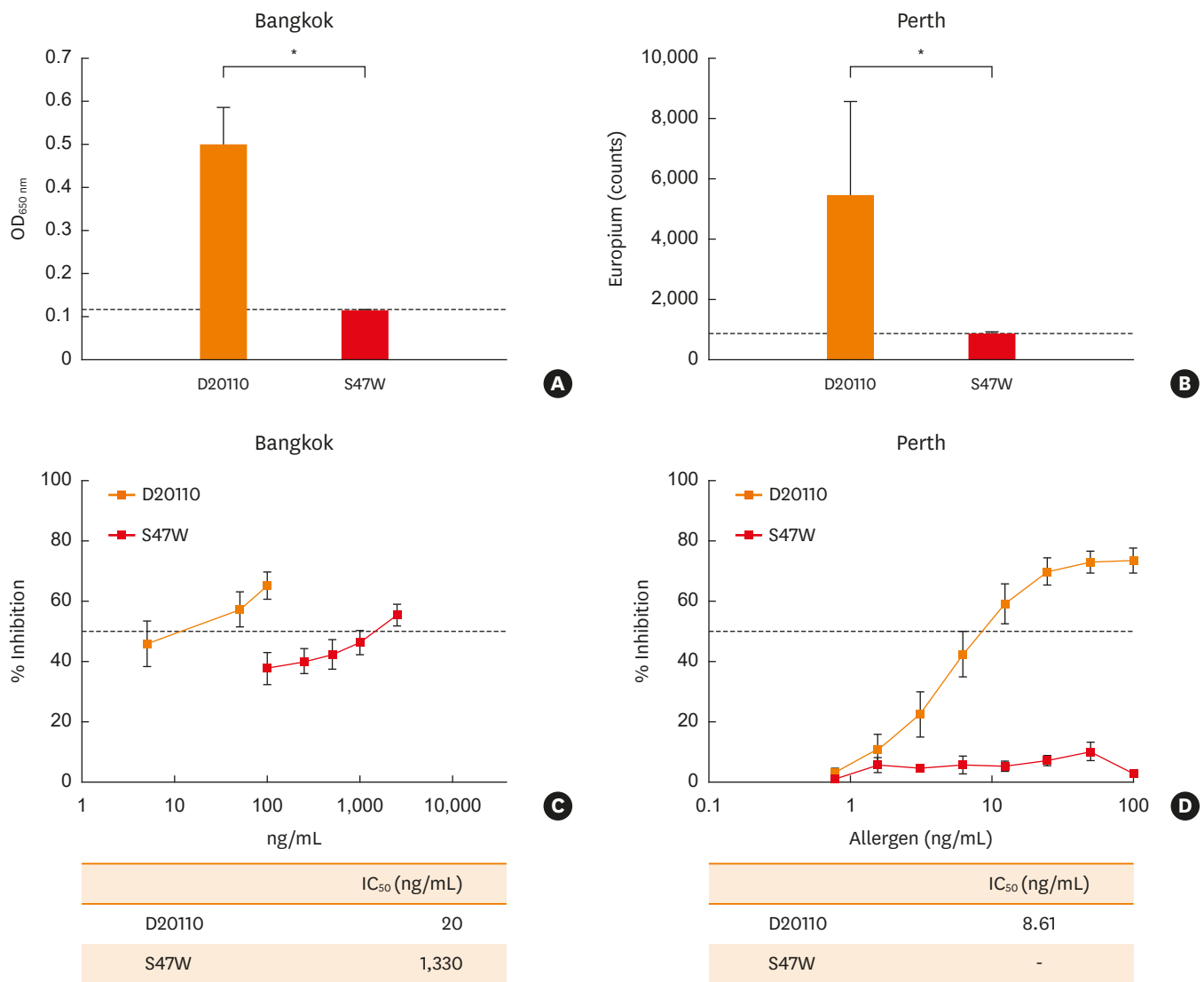
Fig. 2. Binding of specific IgE to D20110 and S47W. (A) Serum IgE of 8 Thai donors binding to D20110 and S47W. (B) Serum IgE of 6 Perth donors binding to D20110 and S47W. Results represent the mean ± SE. Dashed line indicates background. Inhibition of specific IgE binding using D20110 and S47W. (C) Serum IgE of 8 HDM-allergic Thai donors. (D) Serum IgE of 6 HDM-allergic Perth donors. Results represent the mean ± SE. The IC_{50} was showed in the inlet. Dotted line indicates 50% inhibition.
Ig, immunoglobulin; SE, standard error; HDM, house dust mite.

from 4-Bangkok atopics showed that 1 ng/mL of D20110 induced 17%–35% degranulation in 4 atopics (**Fig. 3B**), whereas 1 ng/mL of the S47W did not induce degranulation of basophil primed with serum IgE in 2 of 4 atopics (**Figs. 1**, **2**, and **3B**). For 1 atopic, S47W induced higher degranulation than D20110 (**Figs. 3B** and **4**) at higher concentrations. The sera that were negative at 1 ng/mL became positive (**Figs. 1**, **2**, and **3B**) and at the highest concentration, 1 µg/mL, S47W induced up to 43% degranulation which was higher than that induced by D20110 in 3 of 4 atopics (**Figs. 1-3**).

LAL assays showed that the preparations of purified D20110 and S47W had low levels (<0.001 EU/mg) of endotoxin contamination that would not cause inflammatory responses through the TLR4 of BEAS-2B cells. To determine if the S47W could induce inflammatory responses
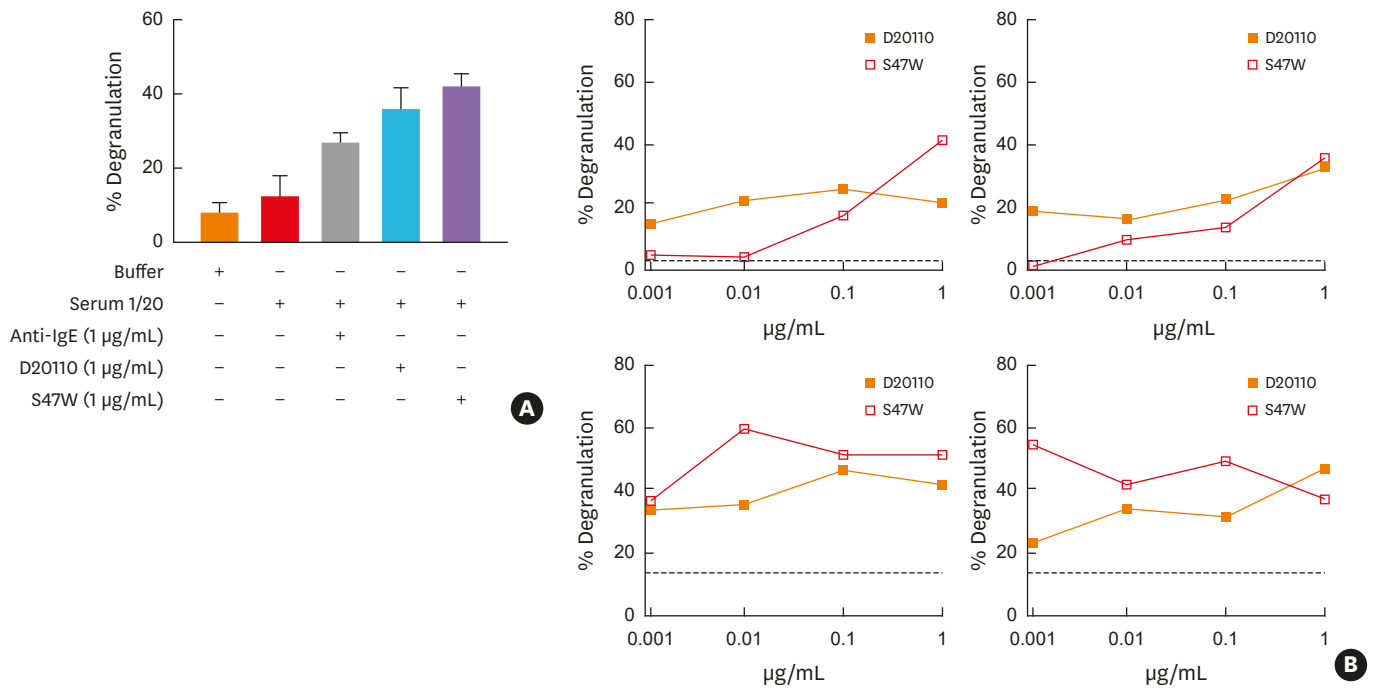
Fig. 3. Degranulation of rat RBL-SX38 basophils stimulated with D20110 and S47W. The percentage of degranulation was calculated from the released β-hexosaminidase and the total amount of β-hexosaminidase in the cells. (A) Degranulation experiments with 1 μg/mL allergen showing controls for the presence or absence of atopic serum and allergen and anti-IgE-mediated degranulation. Results show mean±SE using sera from 4 donors. (B) Degranulation of RBL-SX38 basophils primed with 1:20 dilutions of sera from 4 allergic Thai donors. Closed squares for D20110, open squares for S47W; dashed line background degranulation of cells incubated with sera, but not allergens.
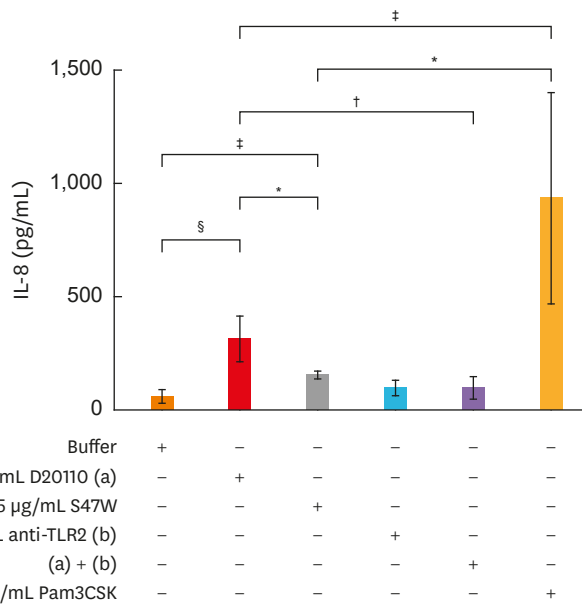Ig, immunoglobulin; SE, standard error.

Fig. 4. IL-8 was secreted from BEAS-2B cells after stimulation with D20110 and S47W. Data show mean ± SE of the 3–6 experiments.
IL, interleukin; SE, standard error.
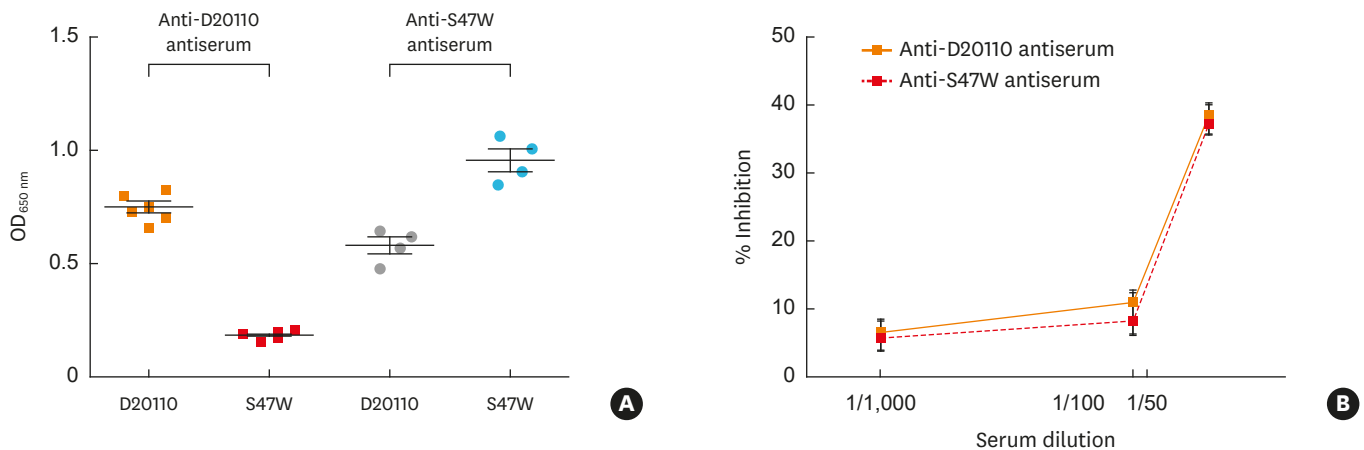*$P < 0.05$, †$P < 0.005$, ‡$P < 0.001$, §$P < 0.0001$.

Fig. 5. (A) Direct binding of mouse anti-D20110 antiserum and anti-S47W antiserum. Results represent mean ± SE of the 4–6 experiments. (B) Inhibition of serum IgE binding to Der p 2 by mouse antiserum against D20110 or -S47W. Results represent mean ± SE of D20110 antiserum blocking IgE from 8 individual sera and of -S47W antiserum blocking IgE from 8 individual sera.
SE, standard error; Ig, immunoglobulin.

through TLR2, D20110, and S47W were incubated with BEAS-2B cells. The results showed that, at 25 μg/mL, D20110 stimulated the release of 309.7 pg/mL IL-8, while the S47W stimulated the release of only 156.9 pg/mL (**Fig. 4**). The experimental TLR2 ligand Pam3CSK4 stimulated the release of 934.5 pg/mL IL-8. An anti-TLR2 IgG antibody reduced the IL-8 release from D20110-stimulated BEAS-2B cells to 100.8 pg/mL (**Fig. 4**).

The binding of the mouse D20110 antiserum and S47W antiserum- to D20110 and S47W coated on ELISA plates showed the binding of D20110 antiserum to S47W was 54% of its binding to D20110, while the binding of S47W antiserum to D20110 was up to 60% of its binding to the S47W (**Fig. 5A**). When tested for their ability to block human IgE binding to the wild type allergen (D20110), both the mouse antisera, made against D20110 and S47W, had similar activities (**Fig. 5B**). Both antibody preparations inhibited 40% of IgE binding to D20110 at 1:50 dilution (**Fig. 5B**).

DISCUSSION

This report describes a point mutation of Der p 2 that reduced its amount of β-structure, removed its ability to bind a hydrophobic ligand, ablated the epitope bound by the 1D8 monoclonal antibody, reduced IgE binding avidity by 100-fold and abrogated its ability to produce TLR2 mediated IL-8 release from epithelial cells.

The CD spectrum analysis showed that the amount of β-sheet was reduced from 59%[16] to 39%, by 34%. While the 39% represents the retention of considerable secondary structure, β-sheet formation is the major structural feature of Der p 2 and is required to form its flexible clamshell-like structure. A very significant change occurred because the fluorescence of the ANS-binding assay was almost completely ablated, proabably in the cavity. ANS binding to MD-2 has been shown to be restricted to the LPS-binding cavity[18] and studies with Der p 2 showed that it required folded protein.[16] Residue 47 is a poorly solvent accessible amino acid located in a loop that links the 2 β-sheets of Der p 2 via strand B (residues 34–42) of sheet 1 and strand C (residues 51–58) of sheet 2(**Fig. 6**).[3] This location is opposite to a proposed entrance of the hydrophobic cavity of Der p 2 where W92 is located (**Fig. 6**).[16] Another
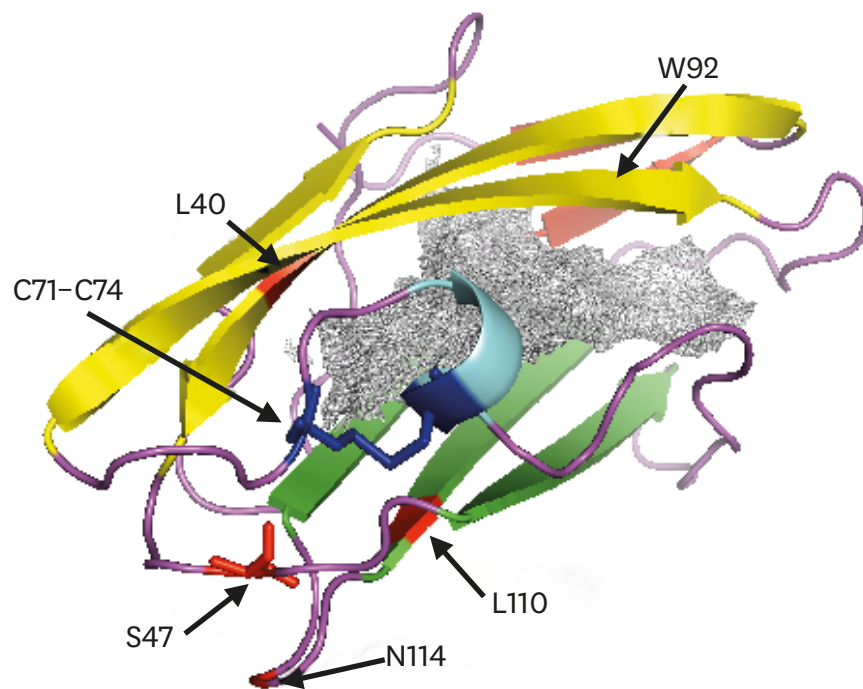
Fig. 6. Structural modelling of D20110 using the Der p 2 pdb file accession code 1KTJ and program PyMOL (Molecular Graphics System, DeLano Scientific, San Carlos,CA, USA). The ANS-accessible area of the cavity is shown as mesh; C71–C74 represents disulfide bond; L40, L110 and N114 are dominant polymorphic residues found in variants: D20103, D20104, D20109 and D20110. W92 is located at the entrance of the hydrophobic cavity. ANS, 8-Anilino-1-naphthalenesulfonic acid.

important function of the side chain of 47 is hydrogen bonding of the side chain of T49 and D113,[20,21] in the vicinity of which are C73–C78 disulfide bond indicating reduced flexibility in this area (**Fig. 6**).[3,21,22] Position 47 is one of the 4 common polymorphic positions found in Der p 2 in nature where it shows substitutions of T with S. The difference between these 2 residues is that T has methyl group in addition to a hydroxyl group in the side chain. The substitution with S would decrease the distance of hydrogen bonding of hydroxyl group and sidechain of T49 and D113 (**Fig. 6**), resulting in altered IgE binding as demonstrated by Hales *et al*.[13] In this work, W was chosen to substitute S as we wanted to replace the hydroxyl sidechain with a large aromatic indole ring which might disrupt the internal hydrogen network resulting in the observed change in conformation. Direct evidence that showed conformation change in this area was that the binding of the 1D8 mAb to the S47W was ablated. This monoclonal antibody has shown to only bind to the 20101 if the D114 of the 20101, on the loop immediately adjacent to the S47, is mutated to the N114 as found in many other variants.[12] The mechanism for the altered structure thus appears to change in the loop structure appears to be induced from a change in the loop structure in the region where the natural common polymorphisms are found. Moreover, this differs from other mutational strategies such as the removal of disulfide bonds,[6,7] fragmentation[4] and the alteration in surface exposed side chains.[8,9]

The comparison of IgE binding with sera from Perth and Bangkok probably shows that exposure to different variants of Der p 2 found in different environments can affect IgE binding to hypoallergens. HDM-producing D20110 and related variants (20103, 20104, and 20109) are common in Bangkok,[11] whereas mites producing D20110 have not been detected in Perth[10] and the variants found are skewed towards those more closely related to

the 20101. Perth individuals would accordingly have few no serum IgE antibodies induced by exposure to D20110.[23] As the direct IgE-binding results showed that the sera IgE of both Bangkok and Perth atopics bound only to coated D20110. The results suggest that the single mutation S47W may cause changes in IgE epitopes on Der p 2 resulting in no IgE binding. To confirm that this observation occurred due to altered structure, IgE binding to the S47W in solution was performed in IgE inhibition assays. Also, as IgE inhibition results showed, while IgE binding to D20110 was readily demonstrated in the sera from Perth residents, no inhibition was found with up to 100 ng/mL S47W. IgE binding to D20110 from the Thai sera was inhibited, depending on the individual by 30%–40% S47W at 100 ng/mL, although the IC_{50} was over 100-fold less than found for the wild type allergen. While the IgE results show remarkable differences in the binding affinity between the S47W and the wild type allergens, more extensive testing is required along with corroboration among other geographical regions taking account of the differences in Der p 2 allergen variants produced by mites in different regions. The basophil degranulation assays, however, showed that for 3 of the 4 Thais examined, the S47W at >10 ng/mL could trigger degranulation, similar to D20110. This observation could be attributed to cross-linking of the Der p 2 mutant by a mixture of weak and strong antibodies in the same repertoire as demonstrated by monoclonal antibody studies.[24] There were, however, differences in the profiles of degranulation for different individuals, so that it would be interesting to examine S47W-induced basophil degranulation with sera from patients in other regions. While the interactions between different epitopes might be behind the mechanism, it seems that even for modified allergens titration of the allergens, by skin testing would be required before therapy and that different mutations would perhaps be more suited to different individuals.

S47W had an attenuated interaction with epithelial cells as shown by its greatly reduced ability to induce an inflammatory response, IL-8 release, in BEAS-2B cells. Taking the results of others into account[19,20] this suggests that the interaction of Der p 2 with TLR2 required structured allergens. It is still unknown whether the proposed interaction between Der p 2 and TLR2 involves presentation of a lipid ligand to TLR2. Although it is reported that bacterial LPS bound to Der p 2 stimulated TLR4 in a similar manner to LPS bound to MD 2,[25] the role of a ligand in the interaction of Der p 2 with TLR2 has not been reported.

The mouse immunization experiments first demonstrated that antisera produced against the wildtype D20110 and the mutant S47W had very different specificities as shown by the different reactivities in the binding assays as illustrated in **Fig. 5A**. Despite this difference, however, the antiserum produced against the S47W not only had a considerable ability to block IgE binding to the wild type D20110, but also was the same as that of antiserum produced against D20110. Since the mice were first immunized with the Th1-type Freund's complete adjuvant, the blocking antibody is likely to be IgG.

In conclusion, the substitution of residue 47 of Der p 2 with W altered conformation of Der p 2, which reduced its ability to stimulate pro-inflammatory response from epithelial cells and to bind IgE, but, retained the molecule's ability to induce antisera with IgG-blocking activity.

ACKNOWLEDGMENTS

REFERENCES

1. Vrtala S, Huber H, Thomas WR. Recombinant house dust mite allergens. Methods 2014;66:67-74.
   PUBMED | **CROSSREF**

2. Marth K, Focke-Tejkl M, Lupinek C, Valenta R, Niederberger V. Allergen peptides, recombinant allergens and hypoallergens for allergen-specific immunotherapy. Curr Treat Options Allergy 2014;1:91-106.
   PUBMED | **CROSSREF**

3. Derewenda U, Li J, Derewenda Z, Dauter Z, Mueller GA, Rule GS, et al. The crystal structure of a major dust mite allergen Der p 2, and its biological implications. J Mol Biol 2002;318:189-97.
   PUBMED | **CROSSREF**

4. Chen KW, Blatt K, Thomas WR, Swoboda I, Valent P, Valenta R, et al. Hypoallergenic Der p 1/Der p 2 combination vaccines for immunotherapy of house dust mite allergy. J Allergy Clin Immunol 2012;130:435-43.e4.
   PUBMED | **CROSSREF**

5. Asturias JA, Ibarrola I, Arilla MC, Vidal C, Ferrer A, Gamboa PM, et al. Engineering of major house dust mite allergens Der p 1 and Der p 2 for allergen-specific immunotherapy. Clin Exp Allergy 2009;39:1088-98.
   PUBMED | **CROSSREF**

6. Smith AM, Chapman MD. Reduction in IgE binding to allergen variants generated by site-directed mutagenesis: contribution of disulfide bonds to the antigenic structure of the major house dust mite allergen Der p 2. Mol Immunol 1996;33:399-405.
   PUBMED | **CROSSREF**

7. Hakkaart GA, Aalberse RC, van Ree R. Epitope mapping of the house-dust-mite allergen Der p 2 by means of site-directed mutagenesis. Allergy 1998;53:165-72.
   PUBMED | **CROSSREF**

8. Smith AM, Chapman MD. Localization of antigenic sites on Der p 2 using oligonucleotide-directed mutagenesis targeted to predicted surface residues. Clin Exp Allergy 1997;27:593-9.
   PUBMED | **CROSSREF**

9. Reginald K, Chew FT. Conformational IgE epitope mapping of Der p 2 and the evaluations of two candidate hypoallergens for immunotherapy. Sci Rep 2018;8:3391.
   PUBMED | **CROSSREF**

10. Smith WA, Hales BJ, Jarnicki AG, Thomas WR. Allergens of wild house dust mites: environmental Der p 1 and Der p 2 sequence polymorphisms. J Allergy Clin Immunol 2001;107:985-92.
    PUBMED | **CROSSREF**

11. Piboonpocanun S, Malainual N, Jirapongsananuruk O, Vichyanond P, Thomas WR. Genetic polymorphisms of major house dust mite allergens. Clin Exp Allergy 2006;36:510-6.
    PUBMED | **CROSSREF**

12. Smith AM, Benjamin DC, Hozic N, Derewenda U, Smith WA, Thomas WR, et al. The molecular basis of antigenic cross-reactivity between the group 2 mite allergens. J Allergy Clin Immunol 2001;107:977-84.
    PUBMED | **CROSSREF**

13. Hales BJ, Hazell LA, Smith W, Thomas WR. Genetic variation of Der p 2 allergens: effects on T cell responses and immunoglobulin E binding. Clin Exp Allergy 2002;32:1461-7.
    PUBMED | **CROSSREF**

14. Park JW, Kim KS, Jin HS, Kim CW, Kang DB, Choi SY, et al. Der p 2 isoallergens have different allergenicity, and quantification with 2-site ELISA using monoclonal antibodies is influenced by the isoallergens. Clin Exp Allergy 2002;32:1042-7.
    PUBMED | **CROSSREF**

15. Jeong KY, Lee IY, Yong TS, Lee JH, Kim EJ, Lee JS, et al. Sequence polymorphisms of Der f 1, Der p 1, Der f 2 and Der p 2 from Korean house dust mite isolates. Exp Appl Acarol 2012;58:35-42.
    PUBMED | **CROSSREF**

16. Tanyaratsrisakul S, Malainual N, Jirapongsananuruk O, Smith WA, Thomas WR, Piboonpocanun S. Structural and IgE binding analyses of recombinant Der p 2 expressed from the hosts *Escherichia coli* and *Pichia pastoris*. Int Arch Allergy Immunol 2010;151:190-8.
    PUBMED | **CROSSREF**

17. Gray VE, Hause RJ, Fowler DM. Analysis of large-scale mutagenesis data yo assess the impact of single amino acid substitutions. Genetics 2017;207:53-61.
    PUBMED | CROSSREF

18. Manček-Keber M, Jerala R. Structural similarity between the hydrophobic fluorescent probe and lipid A as a ligand of MD-2. FASEB J 2006;20:1836-42.
    PUBMED | CROSSREF

19. Österlund C, Grönlund H, Polovic N, Sundström S, Gafvelin G, Bucht A. The non-proteolytic house dust mite allergen Der p 2 induce NF-κB and MAPK dependent activation of bronchial epithelial cells. Clin Exp Allergy 2009;39:1199-208.
    PUBMED | CROSSREF

20. Chiou YL, Lin CY. Der p2 activates airway smooth muscle cells in a TLR2/MyD88-dependent manner to induce an inflammatory response. J Cell Physiol 2009;220:311-8.
    PUBMED | CROSSREF

21. Mueller GA, Benjamin DC, Rule GS. Tertiary structure of the major house dust mite allergen Der p 2: sequential and structural homologies. Biochemistry 1998;37:12707-14.
    PUBMED | CROSSREF

22. Mueller GA, Smith AM, Chapman MD, Rule GS, Benjamin DC. Hydrogen exchange nuclear magnetic resonance spectroscopy mapping of antibody epitopes on the house dust mite allergen Der p 2. J Biol Chem 2001;276:9359-65.
    PUBMED | CROSSREF

23. Tanyaratsrisakul S, Jirapongsananuruk O, Kulwanich B, Hales BJ, Thomas WR, Piboonpocanun S. Effect of amino acid polymorphisms of house dust mite Der p 2 variants on allergic sensitization. Allergy Asthma Immunol Res 2016;8:55-62.
    PUBMED | CROSSREF

24. Christensen LH, Holm J, Lund G, Riise E, Lund K. Several distinct properties of the IgE repertoire determine effector cell degranulation in response to allergen challenge. J Allergy Clin Immunol 2008;122:298-304.
    PUBMED | CROSSREF

25. Trompette A, Divanovic S, Visintin A, Blanchard C, Hegde RS, Madan R, et al. Allergenicity resulting from functional mimicry of a Toll-like receptor complex protein. Nature 2009;457:585-8.
    PUBMED | CROSSREF